Pituitary Adenylate Cyclase 1 Receptor Internalization and Endosomal Signaling Mediate the Pituitary Adenylate Cyclase Activating Polypeptide-Induced Increase in Guinea Pig Cardiac Neuron Excitability

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After G-protein-coupled receptor activation and signaling at the plasma membrane, the receptor complex is often rapidly internalized via endocytic vesicles for trafficking into various intracellular compartments and pathways. The formation of signaling endosomes is recognized as a mechanism that produces sustained intracellular signals that may be distinct from those generated at the cell surface for cellular responses including growth, differentiation, and survival. Pituitary adenylate cyclase activating polypeptide (PACAP; *Adcyap1*) is a potent neurotransmitter/neurotrophic peptide and mediates its diverse cellular functions in part through internalization of its cognate G-protein-coupled PAC1 receptor (PAC1R; *Adcyap1r1*). In the present study, we examined whether PAC1R endocytosis participates in the regulation of neuronal excitability. Although PACAP increased excitability in 90% of guinea pig cardiac neurons, pretreatment with Pitstop 2 or dynasore to inhibit clathrin and dynamin I/II, respectively, suppressed the PACAP effect. Subsequent addition of inhibitor after the PACAP-induced increase in excitability developed gradually attenuated excitability with no changes in action potential properties. Likewise, the PACAP-induced increase in excitability was markedly decreased at ambient temperature. Receptor trafficking studies with GFP-PAC1 cell lines demonstrated the efficacy of Pitstop 2, dynasore, and low temperatures at suppressing PAC1R endocytosis. In contrast, brefeldin A pretreatments to disrupt Golgi vesicle trafficking did not blunt the PACAP effect, and PACAP/PAC1R signaling still increased neuronal cAMP production even with endocytic blockade. Our results demonstrate that PACAP/PAC1R complex endocytosis is a key step for the PACAP modulation of cardiac neuron excitability.

Introduction

G-protein-coupled receptor (GPCR) activation recruits a cascade of signaling events to modulate cellular responses. Following signaling initiation at the plasma membrane, GPCRs are rapidly phosphorylated by GPCR kinases for β -arrestin recruitment and internalization via clathrin-dependent endocytosis (Calebiro et al., 2010). Although the endocytic machinery is considered to be a mechanism for signal desensitization and to follow trafficking routes for receptor lysosomal degradation or recycling, the divergent sorting of GPCR complexes into signaling endosomes represents another mechanism that generates cellular signals that may be distinct from those produced at the cell surface (Jalink and Moolenaar, 2010). Unlike the rapid events at the plasma

membrane, signaling at the GPCR endosomes is capable of generating sustained adenylyl cyclase/cAMP, MAPK/ERK, and phosphoinositide 3-kinase (PI3K)/Akt activation, which appear to be central to antiapoptotic and proliferation responses. Therefore, these internalization events not only regulate cell surface receptor density, but also provide alternative signaling platforms that can diversify signaling events to coordinate neuronal response programs.

Pituitary adenylate cyclase activating polypeptide (PACAP; *Adcyap1*) is expressed in central and peripheral neurons and behaves as a neurotransmitter and neurotrophic peptide with critical roles in signaling, development, survival, proliferation, differentiation, and regeneration (Vaudry et al., 2009). We have demonstrated previously that PACAP is present in parasympathetic cholinergic preganglionic nerve terminals innervating guinea pig cardiac ganglia neurons (Braas et al., 1998; Calupca et al., 2000) and that neurally released or exogenous PACAP application depolarizes and increases cardiac neuron excitability via activation of the selective PAC1 receptor (PAC1R; *Adcyap1r1*) (Braas et al., 1998; Tompkins et al., 2006, 2007; Hoover et al., 2009). The increase in neuronal excitability persists with continuous PACAP exposure (Tompkins and Parsons, 2008; Tompkins et al., 2009; Merriam et al., 2012) and is suggested to be due in

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part to increased adenylyl cyclase/cAMP levels, which shift the voltage-dependent activation of the nonselective cationic conductance, I_h (Merriam et al., 2004; Tompkins et al., 2009). However, although the PACAP effect is seen in 90% of cardiac neurons, treatment with the cell-permeable cAMP analog 8-bromo-cAMP or the adenylyl cyclase activator forskolin enhances excitability in a significantly smaller fraction of the neurons (Tompkins and Parsons, 2008). Although earlier signaling pathway studies have excluded phospholipase C involvement, MAPK pathway activation does appear to participate in the PACAP/PAC1R-induced increase in excitability (Tompkins and Parsons, 2008). Because sustained adenylyl cyclase and MAPK activation are dependent on internalized receptor endosomal signaling in many GPCR systems, and because the PAC1R undergoes endocytosis upon ligand binding to activate neurotrophic signaling for cell survival (May et al., 2010), we investigated whether PAC1R internalization mediates the PACAP-induced change in excitability.

Our results show that the suppression of ligand-stimulated PAC1R endocytosis and membrane trafficking can blunt the PACAP-induced increase in neuronal excitability without altering cell surface signaling. The effect appears to be specific to endocytic mechanisms, because treatments to disrupt vesicular transport to and from the Golgi did not blunt the PACAP effect. These results demonstrate that PACAP/PAC1R complex internalization and endosomal signaling represent a critical mechanism supporting PACAP-induced changes in neuronal excitability.

Materials and Methods

Animals. All animal protocols were approved by the institutional animal care and use committees of the University of Vermont and Ithaca College and methods described in the NIH *Guide for the Care and Use of Laboratory Animals*. All electrophysiological experiments were completed *in vitro* using Hartley guinea pig atrial whole-mount preparations containing the intrinsic cardiac ganglia (either sex, 250–450 g). The guinea pigs were killed by isoflurane overdose and exsanguination; all efforts were made to minimize animal use and suffering. The heart was quickly removed and placed in cold standard Krebs' solution (in mM: 121 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 25 NaHCO₃, 1.2 NaH₂PO₄, 8 glucose, pH 7.4 maintained by 95% O₂/5% CO₂ aeration) for atrial whole-mount preparation and intracellular recording, as described below.

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 was from American Peptide, brefeldin A was from Calbiochem/EMD Biosciences, Pitstop 2 (*N*-[5-(4-bromobenzylidene)-4-oxo-4,5-dihydro-1,3-thiazol-2-yl]naphthalene-1-sulfonamide) was from Abcam Biochemicals, and dynasore was from Sigma. All drugs were applied directly to the bath solution from frozen concentrated stocks prepared in either DMSO (brefeldin A, Pitstop 2, dynasore) or water (PACAP). The concentration of DMSO in the bath solution never exceeded 0.1%. Because dynasore is light sensitive, care was taken to minimize light exposure in these studies.

Intracellular recordings from neurons in whole-mount preparations. For intracellular recording, atrial whole-mount preparations were pinned in a Sylgard-lined chamber and superfused continuously (6–7 ml/min) with Krebs' solution containing 10 mM Na-HEPES buffer (Braas et al., 1998; Tompkins et al., 2006, 2007; Tompkins and Parsons, 2008). All experiments were performed with the bathing solution maintained at 32–35°C, except studies to assess the temperature sensitivity of PACAP-induced excitability, for which all solutions were kept at ambient room temperature (21–25°C). Individual intracardiac neurons were impaled under visual control using high impedance borosilicate microelectrodes (2 M KCl-filled; 60–120 MΩ). Membrane voltage was recorded from the impaled neurons using an Axoclamp-2A amplifier coupled with a Digi-data 1322A data acquisition system and pCLAMP 8 software (Molecular

Devices). When necessary, hyperpolarizing current was injected through the recording electrode to ensure that action potential generation was tested at the same potential throughout an experiment. With current applied, the resting membrane potential was maintained between –55 and –65 mV, values within the range of membrane potentials recorded from these cells.

Depolarizing current steps (0.1–0.5 nA for 1 s) were applied to characterize neuron excitability (excitability trial). The response of mammalian cardiac neurons to long depolarizing current pulses can be classified as a phasic, rapidly accommodating, or tonic-firing pattern (Adams and Cuevas, 2004). PACAP enhances action potential generation elicited by long depolarizing pulses in all 3 classes of cardiac neurons. This reflects the PACAP-induced increase in excitability.

For statistical analyses, the cardiac neuronal responses in the different experimental conditions were grouped into just two firing patterns, phasic firing and multiple firing. Phasic cells fired 4 or fewer action potentials with increasing intensity of the 1 s current pulses up to 0.5 nA. Multiple-firing cells generated 5 or more spikes with the same increasing stimulus protocol. Multiple-firing cells included bursting (rapidly accommodating cells) and tonic cells (cells with action potentials generated over the duration of the depolarization) as long as the number of action potentials produced was 5 or greater. Excitability curves were constructed by plotting the number of action potentials generated by increasing stimulus intensities.

The effect of inhibitors on the PACAP-induced shift in excitability was tested in two different recording protocols. In the first, the ability of inhibitor (brefeldin A, Pitstop 2, or dynasore) pretreatment to suppress the PACAP effect was assessed. In the second, PACAP was first applied to phasic control cells and when excitability was enhanced, the ability of either Pitstop 2 or dynasore to reverse the PACAP-induced increase in spike generation was examined. In these experiments, hyperpolarizing current pulses were also applied to measure input resistance and to assess the presence of rectification in the voltage response. The presence of rectification, commonly used to demonstrate the activation of the inward current flowing through hyperpolarization-activated nonselective cationic channels (Edwards et al., 1995; Cuevas et al., 1997; Merriam et al., 2004), was quantified using the following equation: steady-state hyperpolarization (mV)/initial hyperpolarization (mV). To determine action potential amplitude and properties of the afterhyperpolarization (AHP) that follows a spike, neurons were stimulated directly with 1 ms of suprathreshold depolarizing stimuli. The AHP amplitude represented the voltage change between the resting membrane potential and membrane potential at the peak of the AHP. To compare the time course of the AHP, AHP duration was measured at the voltage at which the amplitude decayed to one-third of the peak value.

To test neuronal excitability in general, either barium chloride (0.5–2 mM) was added to the bathing solution (Tompkins et al., 2009; Merriam et al., 2012) or bethanechol (1 mM) was applied locally (6–8 psi for 1 s) from a “puffer pipette” placed 50–100 μm from the cell (Girasole et al., 2010).

Primary sympathetic neuronal cultures and cAMP assays. Primary rat neonatal sympathetic superior cervical ganglion (SCG) neuronal cultures were used to quantify PACAP-induced cAMP production. The SCG neurons were prepared as described previously (Braas and May, 1999; Girard et al., 2002). In brief, 3-d-old rat pups were killed by decapitation and the SCGs quickly removed. The SCGs were then enzymatically dispersed to produce a pooled population of cells that were plated at a density of 1.5×10^4 neurons/cm² in collagen-coated 24-well plates. The cultures were treated with cytosine β-D-arabinofuranoside to eliminate non-neuronal cells and maintained in defined complete serum-free medium containing 50 ng/ml nerve growth factor. Assays for PACAP-induced cAMP production in the primary sympathetic cultures were performed exactly as described previously (Braas and May, 1999; Girard et al., 2002). In studies examining temperature effects, the cultures were incubated with PACAP27 (20 nM) for 30 min at 24°C or 34°C in defined serum-free medium containing the phosphodiesterase inhibitor RO20-1724 (50 μM; EMD Chemicals; *n* = 3–4 wells per group). For drug treatments, the cultures were pretreated with either Pitstop 2 or dynasore for 10 min before PACAP addition and incubation for 30 min at 34°C.

The cultures were extracted in absolute ethanol containing 100 μ M RO20–1724 and processed for cAMP immunoassay using the Biotrak nonacetylation protocol (GE Healthcare). All samples and standard curves were performed in duplicate.

GFP-PAC1R transfection. The construction of the C-terminal GFP-tagged PAC1R construct was described previously (May et al., 2010). HEK 293 cells were transfected using TransIT-LT1 transfection reagent (Mirus Bio) and cultured in DMEM/F-12 containing 8% fetal bovine serum and 500 μ g/ml Geneticin for stable cell selection. Individual cell colonies were selected and expanded; as before, functional expression of the receptor was assessed by GFP fluorescence and PACAP-stimulated second messenger activation. For PAC1R endocytosis experiments, the cultures were pretreated with 15 μ M Pitstop 2 or 20 μ M dynasore in serum- and BSA-free medium for 10 min before PACAP addition (25 nM PACAP27 final concentration) and incubation for 20 min at 37°C. Studies examining temperature effects were performed similarly but at ambient temperature (21–25°C). Upon experiment termination, the cultures were fixed with 4% paraformaldehyde, washed, and imaged with a Leica DMRB inverted fluorescence microscope equipped with a GFP filter set.

Statistics. Statistics were performed using Prism statistical version 5.4 software (GraphPad). Except as otherwise indicated, data are presented as mean $\pm$ SEM. Differences among conditions that produced either phasic or multiple-firing responses were tested using the Fisher's exact test (see Figs. 2, 3, 4). Differences among means were compared with a two-tailed Student's *t* test (paired or unpaired). The average number of action potentials produced during depolarizing step pulses was compared among conditions using an unpaired *t* test (see Figs. 2, 3, 4). For Figures 5B and 6B, the data were normalized to values in PACAP alone, but paired *t* tests were performed on the actual values to determine significance. An unpaired *t* test was used to determine differences in cAMP generation with temperature or drug treatment (see Fig. 7). Differences between means were considered statistically significant at *p* < 0.05.

Results

Endocytosis inhibitors and low temperatures block PAC1R internalization

The initial experiments in this study used HEK293 cells to establish that Pitstop 2 and dynasore suppressed internalization of the PAC1R. Similar experiments demonstrated that receptor endocytosis was suppressed at room temperature. In HEK293 cells transfected with the GFP-PAC1R construct, the PAC1R was localized predominantly on the cell surface, as shown by the green fluorescence surrounding each cell in Figure 1A. A few GFP-PAC1R-containing vesicles were observed in the juxtanuclear Golgi region of the cell, suggesting that newly synthesized receptors were en route to the plasma membrane. The same cell surface expression pattern was observed whether the cells were held at 37°C or room temperature. When the transfected cultures were treated with 25 nM PACAP at 37°C, the GFP-PAC1Rs were rapidly internalized via endocytic vesicles (Fig. 1C); the disappearance of cell surface GFP-PAC1R fluorescence within 10–20 min of PACAP application was accompanied by the appearance of numerous punctate vesicles dispersed in the cytoplasm. These receptor localization and internalization patterns were comparable to previous PAC1R immunocytochemical staining and trafficking data in intact and cultured sympathetic neurons (Braas and May, 1999; May et al., 2010).

Several studies have shown that endocytic and trafficking mechanisms are blocked at reduced temperatures (Teng and Wilkinson, 2003; McCann et al., 2008). In good agreement, PACAP treatment of the GFP-PAC1R cells at room temperature (22–24°C) for 20 min failed to initiate significant endocytic events in the present study. As shown in Figure 1B, the GFP-PAC1R fluorescence remained on the cell surface at levels com-

Figure 1. Pitstop 2 and room temperature (RT) block PAC1R endocytosis. **A**, GFP-PAC1Rs were expressed predominantly on the cell surface of transfected, untreated control HEK 293 cells; few intracellular GFP-PAC1R-containing vesicles were evident at 37°C or room temperature (data not shown). **C**, The rapid internalization of GFP-PAC1Rs from the cell surface into numerous endocytic vesicles was evident after 25 nM PACAP addition (20 min). In contrast, maintaining the cultures at ambient room temperature (22°C; **B**) or pretreatment of cells with 20 μ M Pitstop 2 (10 min; **D**) followed by 25 nM PACAP addition (20 min) blocked GFP-PAC1R endocytosis. In both instances, the GFP-PAC1R fluorescence largely remained on the plasma membrane. Scale bar: (in **D**) **A–D**, 20 μ m.

parable to those in the untreated controls (Fig. 1, compare A, B) without apparent changes in nascent endocytic vesicle formation.

Pitstop 2 has been used as a potent cell-permeable inhibitor of clathrin-mediated endocytosis by competing for clathrin terminal domain binding with box motifs (von Kleist et al., 2011). GPCR internalization typically enters clathrin-mediated pathways, and when HEK GFP-PAC1R-expressing cells were pretreated with Pitstop 2 before PACAP incubations at 37°C, the receptors again were largely localized on the cell surface (Fig. 1D). However, inspection of the Pitstop 2/PACAP-treated cells revealed some endocytic PAC1R vesicle profiles under the plasma membrane apparently locked from trafficking even with prolonged incubation times. These observations appear comparable to the trapping of clathrin-coated vesicle intermediates attached to the plasma membrane (von Kleist et al., 2011). Dynasore, a dynamin I/II inhibitor that blocks vesicle scission from the plasma membrane, also blunted PACAP-stimulated GFP-PAC1R endocytosis at 37°C, thus showing a pattern nearly identical to that observed for Pitstop 2 (data not shown).

Endocytosis inhibitors block PACAP-induced neuronal excitability

Parasympathetic cardiac neurons express PAC1Rs and, consistent with previous studies, 94% of the cells (15 of 16 neurons) shifted action potential generation from a phasic to a multiple-firing pattern after PACAP addition to the bath solution in the present study (Fig. 2A1,B). To determine whether PAC1R internalization might be required for the PACAP-induced increase in excitability, cardiac ganglia whole-mount preparations were pretreated with Pitstop 2 before initiating intracellular recordings. Pitstop 2 alone did not produce any noticeable change in action potential properties or excitability (data not shown). Unlike the increase in neuronal excitability observed after 20 nM PACAP exposure, pretreatment with Pitstop 2 (15 μ M for 7–20 min) blocked the PACAP-mediated responses. In 6 of 8 cell recordings,

Figure 2. Pretreatment with Pitstop 2 suppresses the PACAP-induced increase in excitability. **A**, Recordings from 2 different cells. **A1** illustrates the increase in excitability induced by 20 nM PACAP. **A2** shows that pretreatment with 15 μM Pitstop 2 blocks the increase in excitability induced by 20 nM PACAP. For all recordings, a 1 s, 0.4 nA depolarizing current step was used to initiate action potential activity. **B**, The percentage of cells exhibiting multiple firing when exposed to PACAP alone ($n = 16$) was significantly greater than when exposed to PACAP after pretreatment with 15 μM Pitstop 2 ($n = 8$; Fisher's exact test, $p < 0.0001$). **C**, Averaged excitability curves show that Pitstop 2 greatly suppressed the PACAP-induced increase in excitability. Asterisks indicate that the number of action potentials generated at each current step was significantly greater in PACAP ($n = 16$) than in PACAP and Pitstop 2 ($n = 8$; unpaired t test, $p < 0.0001$ for steps 0.2–0.5 nA).

the firing pattern after PACAP addition remained phasic throughout multiple trials. In the 2 remaining cells, a transient increase in excitability was noted after 4–6 min in PACAP; however, the shift in excitability was short-lived and by 10–12 min, the PACAP-induced action potential generation had reverted and remained in the original phasic pattern for the remaining duration of the excitability trials (16–17 min in PACAP). The transient increase in excitability in Pitstop 2–pretreated cells was unique to the drug treatment, because it was not observed after PACAP treatment alone. For comparison with PACAP alone, the data from excitability trials determined at the end of all 8 experiments were compiled. At this time point, all Pitstop 2–pretreated cells exhibited a phasic-firing pattern despite continuous PACAP exposure (Fig. 2A2,B). The averaged excitability curves generated by increasing stimulus strength were markedly depressed in cells exposed to PACAP after Pitstop 2 pretreatment (Fig. 2C).

Barium treatment was used to ensure that the Pitstop 2 block of the PACAP-induced increase in excitability was not due to a general suppression of excitability. At a time when PACAP had no effect on excitability in 15 μM Pitstop 2–pretreated neurons, the addition of barium could increase the number of action potentials elicited by depolarizing current pulses. For example, the maximum number of action potentials generated by a 0.4 nA depolarizing pulse for 1 s was 2 and 3 in Pitstop 2–pretreated cells exposed to PACAP for 15–16 min ($n = 2$). Four to 5 min after the addition of 1 mM BaCl₂ to the Pitstop 2 and PACAP solution, the number of action potentials generated by the same depolarizing pulse was 10 and 9, respectively.

We also investigated whether dynasore treatment would also blunt the PACAP-induced increase in excitability. Dynasore inhibits the dynamin I/II GTPase-mediated scission of endocytic vesicles from the plasma membrane (Herskovits et al., 1993; Macia et al.,

Figure 3. Pretreatment with dynasore suppresses the PACAP-induced increase in excitability. **A**, The percentage of cells exhibiting multiple firing when exposed to PACAP alone ($n = 13$) was significantly greater than when exposed to PACAP after pretreatment with 20 μM dynasore ($n = 13$; Fisher's exact test, $p < 0.0001$). **B**, Averaged excitability curves show that dynasore greatly suppressed the PACAP-induced increase in excitability. Asterisks indicate that the number of action potentials generated at each current step was significantly greater in PACAP ($n = 13$) than in PACAP and dynasore ($n = 13$; unpaired t test, $p = 0.0005$ for 0.1 nA; $p < 0.0001$ for 0.2 nA; $p = 0.0002$ for 0.3 nA; $p = 0.0001$ for 0.4 nA; $p = 0.0003$ for 0.5 nA). **C**, Recordings illustrated that bethanechol could increase excitability in a cell pretreated with dynasore and exposed to PACAP. Left trace shows the phasic-firing pattern generated by a 1 s, 0.4 nA depolarizing current pulse in a cell pretreated with dynasore and exposed to PACAP. After local puffer application of bethanechol, the same depolarizing current step elicited multiple firing.

2006) and thus provides a separate mechanistic means of assessing the role of endocytosis in the PACAP-induced increase in cardiac neuron excitability. In a separate set of untreated control cardiac whole-mount preparations, bath application of 20 nM PACAP induced a multiple-firing excitability pattern in 12 of 13 cells. Similar to the results with Pitstop 2, pretreatment of the ganglia whole-mount preparations with dynasore (20 μM for 5–20 min) blunted the PACAP-induced increase in excitability. In 12 of 13 cells pretreated with dynasore, subsequent PACAP application only generated a phasic-firing pattern over the 70 min PACAP and dynasore exposure period, demonstrating that the PACAP-induced excitability was significantly suppressed. The remaining neuron exhibited a multiple-firing pattern, although only 5 action potentials were generated in response to the largest depolarizing step. Both the percentage of neurons exhibiting a multiple-firing pattern and the averaged excitability curves were significantly decreased in the dynasore plus PACAP preparations compared with cells exposed to PACAP alone (Fig. 3A,B).

As with Pitstop 2, dynasore did not nonspecifically suppress neuronal excitability. First, in the absence of PACAP, cells in dynasore-treated whole-mount preparations were capable of generating multiple-firing patterns when tested with long depolarizing current pulses (data not shown). Second, local puffer application of bethanechol could elicit multiple-firing patterns in control cells and in neurons treated only with dynasore (data not shown) or dynasore plus PACAP (Fig. 3C). Therefore, the results of the Pitstop 2 and dynasore studies suggest that PAC1R endocytosis is a requisite step in recruiting mechanisms regulating neuronal excitability.

Brefeldin A pretreatment to block Golgi trafficking does not suppress PACAP-induced increases in excitability

Clathrin is also thought to play trafficking roles between endosome and Golgi compartments (Bonifacino and Traub, 2003; Hirst et al., 2009). Therefore, we investigated whether brefeldin

A, an antiviral antibiotic that interferes with endosomal budding (Drake et al., 2000) and trafficking to and from Golgi networks (Connors et al., 2008), affected the PACAP-induced increase in excitability. In 3 whole-mount cardiac ganglia preparations pretreated with brefeldin A (20 μ M for 7–20 min), 20 nM PACAP induced multiple-firing patterns in 5 of 6 cells. Therefore, brefeldin A had no apparent effects, suggesting that Golgi trafficking is not essential for the PACAP-induced increase in cardiac neuron excitability.

Low temperature blocks both endocytosis and PACAP-induced increase in excitability

To further establish that the results with Pitstop 2 and dynasore were not the result of unidentified pharmacological actions, we investigated the effect of temperature blocking of endocytosis on the PACAP-induced increase in excitability. Reducing ambient temperature has been used previously as a mechanism to block synaptic vesicle endocytosis (Teng and Wilkinson, 2003) and acetylcholine receptor $\alpha 7$ subunit internalization (McCann et al., 2008). PAC1R endocytosis was significantly depressed in cells exposed to PACAP at room temperature (Fig. 1), so the effects of temperature on the PACAP-induced excitability at 22°C (ambient room temperature) and 34°C were compared.

At room temperature, the PACAP-induced increase in excitability that had been seen consistently at warmer temperatures (34°C) was no longer evident (Fig. 4A2,B). All 10 cells tested at room temperature before PACAP exposure exhibited a phasic-firing pattern. During PACAP exposure at room temperature, only 3 of 23 cells (13%) exhibited a multiple-firing pattern in response to the long depolarizing current pulses. The averaged excitability curves obtained at room temperature for untreated cells and PACAP-treated cells were not significantly different (Fig. 4C), nor were the curves different from untreated control data obtained at 34°C. The temperature effects did not reflect a general suppression in cell excitability, because 2 mM BaCl₂ enhanced action potential generation initiated by depolarizing current steps (Fig. 4A3). The temperature dependence of the PACAP-mediated increase in excitability is consistent with the pharmacological block of endocytosis and suppression of the PACAP effect on excitability.

Pitstop 2 and dynasore can reverse the PACAP-induced increase in excitability

To evaluate whether continuous PAC1R endocytosis and signaling are necessary events for the effects of PACAP on excitability, Pitstop 2 or dynasore was introduced into the bath solutions after the PACAP-induced increase in excitability was established. In this experimental paradigm, recordings were first obtained from a phasic cell before and after the addition of 20 nM PACAP. The PACAP-induced increase in excitability was allowed to develop over many minutes and, once a multiple-firing pattern was consistently recorded, either Pitstop 2 (15 μ M) or dynasore (20 μ M) was added to the PACAP-containing solution. Excitability was subsequently retested over a prolonged period with PACAP and either of the two inhibitors present.

In experiments with Pitstop 2, the drug was added after PACAP had increased excitability in six cells. In five cells, action potential generation gradually switched from a multiple to a phasic-firing pattern after Pitstop 2 addition to the PACAP solution. The time required for action potential generation to switch from multiple firing to phasic firing in these 5 cells varied from ~6 min to ~16 min (Fig. 5A). In the sixth cell, the impalement was lost after 9 min in Pitstop 2. At this time, the number of

Figure 4. The PACAP-induced increase in excitability is temperature sensitive. **A**, Recordings from different cells showed that 20 nM PACAP increased excitability when the bath solution was 33°C (**A1**), but not when the bath temperature was 22°C (**A2**). The recording in **A3** demonstrated that the addition of 1 mM BaCl₂ (Ba²⁺) increased excitability at 22°C. In all three recordings, the cells exhibited a phasic-firing pattern before the addition of PACAP (**A1** and **A2**) or barium (**A3**). The firing pattern shifted to multiple firing in **A1** and **A3**, but not in **A2**. The amplitude of the 1 s depolarizing current pulse was 0.3 nA in each experiment. **B**, The percentage of cells exhibiting multiple firing in 20 nM PACAP was significantly greater when the temperature was 32–34°C ($n = 13$ cells) than when the bath temperature was 21–25°C ($n = 23$ cells; Fisher's exact test, $p < 0.0001$). At 32–34°C: control ($n = 28$) versus PACAP ($n = 13$), significantly different, $p < 0.0001$; at 21–25°C: control ($n = 10$) versus PACAP ($n = 23$), not significant, $p = 0.5363$. **C**, Averaged excitability curves generated in the cells maintained at either 33–34°C or 21–25°C before and during exposure to 20 nM PACAP. The number of action potentials generated at each current step was significantly greater (indicated by asterisks) at the warmer temperature in the presence of PACAP ($n = 13$) compared with control at warm temperature ($n = 28$; unpaired t test, $p = 0.0011$ at 0.1 nA; $p = 0.0002$ at 0.2 nA; $p = 0.0004$ at 0.3 nA; $p = 0.0002$ at 0.4 nA; $p = 0.0005$ at 0.5 nA), and to PACAP at room temperature ($n = 23$; unpaired t test, $p = 0.0029$ at 0.1 nA; $p = 0.0003$ at 0.2 nA; $p = 0.0005$ at 0.3 nA; $p = 0.0003$ at 0.4 nA; $p = 0.0005$ at 0.5 nA). The number of action potentials generated at each current step was not different between control ($n = 10$) and PACAP ($n = 23$) at room temperature ($p = 0.3502$ at 0.1 nA; $p = 0.4040$ at 0.2 nA; $p = 0.7132$ at 0.3 nA; $p = 0.4495$ at 0.4 nA; $p = 0.6642$ at 0.5 nA).

action potentials generated by a 1 s, 0.4 nA depolarizing step had declined from 20 in PACAP alone to 16 action potentials after 9 min in Pitstop 2. We also tested in one cell whether the suppression by Pitstop 2 could be reversed by barium. In this example, the firing pattern was phasic (2 action potentials elicited by a 1 s, 0.5 nA depolarizing step) in PACAP and Pitstop 2, but 3 min after the addition of 1 mM BaCl₂, the firing pattern became multiple firing (6 action potentials elicited by a 1 s, 0.5 nA depolarizing step).

Using the same experimental regimen, dynasore also blunted the PACAP-induced excitability, but with more variability with respect to extent and time course of suppression of the PACAP-induced increase in excitability. In 5 cells, the change in excitability after the addition of dynasore was monitored for at least 20 min, whereas in the 6th cell, the impalement was lost 9 min after dynasore addition. In 1 of the 6 cells exposed to dynasore after PACAP had increased excitability, the dynasore effect was modest so that only a small decline in the PACAP-induced multiple firing occurred during the 20 min recording period (Fig. 6A).

Figure 5. Pitstop 2 progressively reverses the PACAP effect after the increase in excitability has developed. **A**, Results from 6 different cells in which 15 μ M Pitstop 2 was added after the PACAP-induced shift from a phasic- to a multiple-firing pattern had developed. Note that the number of action potentials elicited by the same stimulus (1 s, 0.5 nA for the cell indicated by the solid and open circles; 1 s, 0.4 nA for the other cells) declined until action potential generation reverted back to a phasic-firing pattern in 5 of the 6 cells. In the sixth cell, the number of action potentials declined progressively until the impalement was lost. Each solid symbol represents data from one cell recorded in PACAP alone, followed in time by the corresponding open symbol representing the data recorded from the same cell after Pitstop 2 was applied. **B**, Normalized data from the 5 cells that had returned to a phasic-firing pattern, which illustrated that the action potential amplitude (AP), AHP amplitude (AHP amp), AHP duration (AHP dur), the rectification occurring with hyperpolarizing steps (Rect), and effective membrane resistance (EMR) were not altered by Pitstop 2 at a time when the firing pattern was phasic ($n = 5$; paired t test, AP: $p = 0.2104$; AHP: $p = 0.2359$; AHP dur: $p = 0.0882$; Rect: $p = 0.4792$; EMR: $p = 0.6553$). The results presented in **B** are normalized to values obtained in the presence of PACAP before the addition of Pitstop 2.

In three other cells, the number of action potentials generated in the continued presence of PACAP and dynasore decreased progressively, but retained a multiple-firing pattern (>5 action potentials) throughout the recording period (9 and 20 min; Fig. 6A). In two other cells examined, there was a shift from a multiple to a phasic-firing pattern (Fig. 6A). In one of these cells, 500 μ M BaCl₂ was added to the PACAP and dynasore solution to ensure that excitability was not generally depressed in this cell. Within 5 min after the addition of barium, a shift from phasic (1 action potential elicited by a 1 s, 0.3 nA depolarizing pulse) back to multiple firing (5 action potentials elicited by a 1 s, 0.3 nA depolarizing pulse) occurred.

In previous studies of PACAP effects, we observed that the PACAP-induced increase in excitability was persistent and long-lasting. To confirm this observation for the present study, we recorded excitability trials in 3 cells continuously exposed to PACAP alone for >30 min. The protocol was identical to that used to generate the data summarized in Figures 5 and 6. The results indicated that once the PACAP-induced increase in excitability had peaked, the number of action potentials elicited by repeated stimuli remained constant for >20 min. In these 3 cells, the average maximum number of action potentials generated initially in PACAP by a 1 s, 0.4 nA step was 17.0 ± 2.5 . The number of action potentials generated in multiple excitability trials during continued PACAP exposure did not change, so after

Figure 6. Dynasore reduces the PACAP effect after the increase in excitability have developed. **A**, Results from 6 different cells exposed to 20 μ M dynasore after the PACAP-induced shift from a phasic- to a multiple-firing pattern had developed. Each solid symbol represents data from one cell recorded in PACAP alone, followed in time by the corresponding open symbol representing the data recorded from the same cell after dynasore was applied. Note that the number of action potentials elicited by the same stimulus (1 s, 0.4 nA) declined over time in dynasore. In two cells, action potential generation reverted back to a phasic-firing pattern. In three other cells, although action potential generation declined, the firing pattern remained multiple at the time the recording was terminated. In the remaining cell, the firing pattern changed to a lesser extent in dynasore. **B**, Normalized data from 5 cells illustrating that the action potential amplitude (AP), AHP amplitude (AHP amp), AHP duration (AHP dur), the rectification occurring with hyperpolarizing steps (Rect), and effective membrane resistance (EMR) was not altered by exposure to dynasore for 10–20 min ($n = 5$ paired t test, AP: $p = 0.5957$; AHP: $p = 0.1863$; AHP dur: $p = 0.0573$; Rect: $p = 0.3437$; EMR: $p = 0.8442$). The results presented in **B** are normalized to values obtained in the presence of PACAP before the addition of dynasore.

20 min, the number of action potentials generated by the same step was 16.7 ± 1.8 (paired t test; $p = 0.741801$). Therefore, in the presence of PACAP alone, excitability remained elevated for prolonged periods without noticeable decline.

We also evaluated in these experiments whether a Pitstop 2 or dynasore-induced change in action potential properties or rectification in hyperpolarizing steps could account for the reversal of the PACAP effect by these inhibitors. The membrane potential was maintained at ~ -60 mV electrotonically so that any differences noted would not be due to differences in resting membrane potential. Neither Pitstop 2 nor dynasore affected action potential amplitude, AHP amplitude or duration, or the input resistance in PACAP-treated cells (Figs. 5B, 6B). Furthermore, the rectification noted during 500 ms hyperpolarizations was not affected by Pitstop 2 or dynasore (Figs. 5B, 6B).

PAC1R-stimulated cAMP production is not dependent on endocytosis

Our results suggested that the PACAP-induced increase in excitability was dependent on PAC1R endocytosis and resultant downstream signaling. Given that increased adenylyl cyclase/cAMP signaling contributes to the PACAP effect and that adenylyl cyclase activity can be sustained in signaling endosomes (Calebiro et al., 2009; Ferrandon et al., 2009), we investigated whether the inhibitor- and temperature-dependent effects on

Figure 7. PACAP stimulates cAMP generation in dissociated rat neonatal SCG neurons after pretreatment with Pitstop 2 or dynasore and when cells are maintained at room temperature. **A**, PACAP increased cAMP production at 34°C after pretreatment with Pitstop 2 (10 min, 15 μ M) or dynasore (10 min, 20 μ M) and under control conditions. Values are expressed as the fold change of femtomoles/well determined at 34°C. Asterisks indicate that the generation of cAMP by PACAP at 34°C in inhibitor-pretreated cells was significantly greater than with cells exposed to inhibitor without PACAP (unpaired *t* test; *n* = 4 in all cases; control vs PACAP, *p* = 0.0025; dynasore vs PACAP + dynasore, *p* = 0.0144; Pitstop 2 vs PACAP + Pitstop 2, *p* = 0.0058). **B**, At 24°C, PACAP (*n* = 3) significantly increased the generation of cAMP compared with control (*n* = 3). **p* = 0.0004, unpaired *t* test. At 34°C, PACAP (*n* = 3) significantly increased the generation of cAMP compared with control (*n* = 3). ***p* = 0.0005, unpaired *t* test. The generation of cAMP was significantly greater at 24°C (*n* = 3) than at 34°C (*n* = 3) after a 30 min incubation in 20 nM PACAP. ****p* = 0.0315, unpaired *t* test. Values are expressed as the fold change of femtomoles/well determined at 34°C.

PACAP-stimulated excitability reflected diminished cAMP production either at the plasma membrane or endosomal compartments. For these studies, primary sympathetic neuronal cultures were used to simulate PAC1R signaling responsiveness in autonomic neurons (Braas and May, 1999). Incubation of cultures with PACAP alone for 30 min at 34°C stimulated cAMP levels ~4- to 8-fold (Fig. 7A). Neither Pitstop 2 nor dynasore had any effect on basal cAMP levels (Fig. 7A). In addition, pretreatment with Pitstop 2 or dynasore for 10 min to block endocytosis did not inhibit total PACAP-stimulated cAMP production (Fig. 7A). In fact, the endocytosis inhibitors appeared to enhance PACAP-stimulated cAMP levels by sustaining plasma membrane adenylyl cyclase activity from receptor sequestration. Low-temperature blocking of endocytosis also did not diminish culture cAMP production. Conversely, 30 min of PACAP stimulation at ambient temperature (24°C) significantly enhanced culture cAMP production by ~1.6-fold compared with PACAP-stimulated levels at 34°C (Fig. 7B). These results demonstrated that the diminished

PACAP effect after inhibitor- and temperature-mediated blocking of the PAC1R complex endocytosis was not a consequence of diminished adenylyl cyclase/cAMP signaling. Furthermore, these observations suggest that PAC1R-stimulated adenylyl cyclase/cAMP signaling was largely confined to the plasma membrane.

Discussion

The results of the present study demonstrated that the PACAP-induced increase in cardiac neuron excitability (Braas et al., 1998) was dependent on PAC1R vesicular endocytosis and signaling. The PACAP responses were essentially eliminated by pretreatment with the endocytosis inhibitors Pitstop 2 or dynasore, which block clathrin terminal domain binding and dynamin I/II GTPase activity, respectively, or by maintaining whole mounts at room temperature to halt vesicle internalization. All of these conditions dramatically suppressed PAC1R endocytosis, as shown in complementary studies using GFP-PAC1R cell lines. In contrast, pretreatment with brefeldin A to inhibit Golgi network budding and trafficking did not blunt the PACAP effect, demonstrating that plasma membrane endocytic mechanisms, rather than intracellular vesicular trafficking in general, were necessary to generate the response.

The internalization of GPCRs via clathrin-coated vesicles and the resultant formation of signaling endosomes provide a novel means to engage diverse intracellular signaling pathways that may be distinct from those activated at the plasma membrane (Calebiro et al., 2010; Scita and Di Fiore, 2010; McMahon and Boucrot, 2011). After ligand binding to GPCRs, the ensuing receptor conformational change and specific Ser/Thr phosphorylation by GPCR kinases result in β -arrestin recruitment for accessory protein scaffolding, vesicle formation, and endocytosis. PAC1Rs belong to the class B group of GPCR families that exhibit high-affinity binding for β -arrestins (Shenoy and Lefkowitz, 2003; Milligan, 2004). The resulting long GPCR/ β -arrestin association times may be particularly amenable to PAC1R internalization and robust signaling events. Upon cointernalization with GPCRs, β -arrestins have principal roles in vesicle clathrin heavy chain and AP2 adaptor protein assembly and can scaffold signaling proteins, including MAPKs, in the formation of signaling endosomes. The terminal domains of the clathrin triskelia bind to accessory protein clathrin box motifs for vesicle stabilization and recruitment of other endocytosis regulators, and the recently identified small-molecule compounds such as Pitstop 2 interfere with clathrin terminal domain binding and stall clathrin-mediated endocytosis (von Kleist et al., 2011). Dynasore blocks vesicular endocytosis via a different mode. Vesicular scission from the plasma membrane requires dynamin GTPase activity and dynasore preferentially inhibits dynamin I/II (Macia et al., 2006).

PAC1Rs are internalized upon PACAP activation (May et al., 2010), as demonstrated by the PACAP-stimulated GFP-PAC1R internalization *in vitro* (Fig. 1), an action of PACAP blocked by Pitstop 2 and dynasore. These endocytosis inhibitors also effectively suppressed the PACAP-induced increase in cardiac neuron excitability. This did not reflect a nonspecific effect on endosomal trafficking, because brefeldin A pretreatments to suppress routing to and from Golgi cisternal networks, which may also involve clathrin mechanisms, did not blunt the PACAP effect. The inhibitor results also did not reflect alterations in neuronal membrane properties or nonspecific suppression of cell excitability. With Pitstop 2 or dynasore, cardiac neuron action potential properties were not significantly changed and, further, the addition of barium or bethanechol after PACAP and Pitstop 2 or dynasore treat-

ment still increased excitability. Because Golgi trafficking was not necessary for the PACAP responses and neither drug appeared to depress plasma membrane signaling or excitability nonspecifically, the cellular trafficking and electrophysiological studies in aggregate implicate dynamin- and clathrin-dependent endocytosis of the PAC1R as a key step initiating the PACAP-induced change in excitability.

The PACAP-induced change in excitability also was significantly suppressed when whole-mount preparations were kept at room temperature. Reducing ambient temperature has been used previously in different systems as a mechanism to block or slow endocytosis of synaptic vesicles or ligand-gated postsynaptic receptors (Teng and Wilkinson, 2003; McCann et al., 2008). The ability of low temperatures to retard clathrin-mediated endocytosis of the PAC1R in cardiac ganglia preparations was corroborated in the GFP-PAC1R cell line studies. The temperature-sensitive step was not due to reduced cAMP production, because PACAP still activated adenylyl cyclase and increased cellular cAMP levels at room or physiological temperature. In fact, the 20 nM PACAP concentration used in our electrophysiological studies appeared to have generated greater cAMP levels at room temperature than at 32–34°C, suggesting that PAC1R activation of adenylyl cyclase may be predominantly at the plasma membrane and that longer PAC1R dwell time at the cell surface from low-temperature blocking of endocytosis may sustain cyclase activity for greater second messenger production. Similarly, Pitstop 2 or dynasore pretreatment tended to enhance cAMP generation, although the differences were not statistically significant. Therefore, a diminished cAMP production did not contribute to the loss of PACAP effect on excitability at room temperature or by Pitstop 2 or dynasore.

One mechanism that can contribute to the PACAP-induced increase in excitability is an enhancement of I_h (Merriam et al., 2004; Tompkins et al., 2009). Rectification during hyperpolarizing steps is an index of the activation of I_h (Edwards et al., 1995; Merriam et al., 2004). Neither Pitstop 2 nor dynasore had any effect on the extent of the voltage rectification, indicating that these inhibitors did not alter I_h .

How PAC1R endocytosis supports the PACAP-induced increase in excitability remains to be established. However, GPCR receptor endocytosis and the resulting formation of signaling endosomes provides a singular means for GPCRs to recruit and activate distinct intracellular signaling cascades in spatial and temporal contexts important to the overall cellular response. For some GPCR systems, endosomal signaling appears to sustain adenylyl cyclase activity and cAMP production (Calebiro et al., 2009; Ferrandon et al., 2009); in others, GPCR endocytosis appears to be requisite for prolonged MAPK (Oakley et al., 1999; DeFea et al., 2000; Terrillon and Bouvier, 2004) and PI3K/Akt (García-Regalado et al., 2008; May et al., 2010) activation. Because intracellular cAMP and second messengers may not be readily diffusible in all cell types (Bacskai et al., 1993; Hempel et al., 1996), the delivery of signaling endosomes to specific intracellular sites may be key for local second messenger activation of regulatory substrates. Whether PAC1R endocytosis supports signaling diversification or sustains second messenger activation is still unclear. However, previous studies have shown that PACAP can sustain sympathetic neuronal culture ERK1/2 phosphorylation for >4 h (May et al., 2010). PACAP-induced increases in excitability can be suppressed markedly with MEK inhibitors (Tompkins and Parsons, 2008). Therefore, MEK/ERK activation from PAC1R endosomes may be a means of PAC1R-mediated change in neuronal excitability. To examine this possibility, the HEK PAC1R cell lines were used to determine whether inhibiting

receptor endocytosis blunts MEK/ERK activation. These ongoing experiments have demonstrated that PACAP-stimulated ERK phosphorylation, as determined by Western blot analysis, was significantly decreased under pharmacological and temperature treatment conditions that suppress PAC1R endocytosis (T. Buttolph, V. May, and R.L. Parsons, unpublished data). As in rat neonatal SCG neurons (Fig. 7), the PACAP-induced increase in HEK PAC1R cell cAMP production was not inhibited by restricting PAC1R endocytosis, suggesting that PAC1R-mediated stimulation of adenylyl cyclase signaling is predominantly a plasma membrane-associated event. These results are consistent with GPCR internalization mechanisms and support our hypothesis that many PACAP-activated signaling cascades require PAC1R endocytosis (May et al., 2010).

Interestingly, the ability of both Pitstop 2 and dynasore to suppress PACAP-induced increases in excitability was evident even if the inhibitors were applied after the neurons had already exhibited peptide-stimulated multiple-firing patterns. Our previous studies demonstrated that the effects of PACAP, even at nanomolar bath concentrations, can remain effective for >90 min (Tompkins and Parsons, 2008). Because the cycle of receptor internalization and reinsertion into the plasma membrane likely continues during sustained exposure to the peptide, a gradual block of the vesicular receptor-recycling process would contribute to the progressive reversal of the PACAP effect on excitability by both inhibitors. Therefore, the prolonged PAC1R-induced ERK activation times, coupled with endosome PAC1R recycling to the plasma membrane, might account for the time needed for Pitstop and dynasore to inhibit the ongoing PACAP effect (Figs. 5 and 6).

In summary, our results demonstrate that clathrin and dynamin inhibitors and low temperatures suppress PACAP effects on neuronal excitability. We suggest that these observations are consistent with the hypothesis that internalization of the PACAP/PAC1R to form a signaling endosome contributes to the varied actions of this neuropeptide. The formation of a signaling endosome would explain why PACAP is more effective than forskolin or the cell-permeable cAMP analog 8-bromo-cAMP in increasing cardiac neuron excitability (Tompkins and Parsons, 2008). All three (forskolin, cAMP analogs, and PACAP) would increase the intracellular concentrations of cAMP. However, the creation of a signaling endosome would provide a mechanism by which PACAP more effectively recruits other downstream transduction pathways, such as the MAPK pathway, that are required for the peptide's full effect (Tompkins and Parsons, 2008). Signaling endosomes have typically been shown to be important in mediating trophic responses including cell survival, proliferation, and differentiation. The current studies suggest that the panoply of responses can be more diverse and may include regulation of neuronal excitability. Furthermore, given that many early studies of metabotropic transmitter receptor signaling and actions were completed at room temperature, it is likely that significant actions mediated through these receptors were missed, especially if a comparable temperature dependence exists for these GPCRs.

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