Title: Activation of MEK/ERK signaling contributes to the PACAP-induced increase in guinea pig cardiac neuron excitability

Abbreviated Title: MEK/ERK signaling and cardiac neuron excitability

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ABSTRACT

Pituitary adenylate cyclase activating polypeptide (PACAP) peptides (Adcyap1) signaling at the selective PAC1 receptor (Adcyap1r1) participates in multiple homeostatic and stress-related responses, yet the cellular mechanisms underlying PACAP actions remain to be completely elucidated. PACAP/PAC1 receptor signaling increases excitability of neurons within the guinea pig cardiac ganglia and as these neurons are readily accessible, this neuronal system is particularly amenable to studying PACAP modulation of ionic conductances. The present study investigated how PACAP activation of MEK/ERK signaling contributed to the peptide-induced increase in cardiac neuron excitability. Treatment with the MEK inhibitor PD 98059 blocked PACAP-stimulated pERK and in parallel, suppressed the increase in cardiac neuron excitability. However, PD 98059 did not blunt the ability of PACAP to enhance two inward ionic currents, one flowing through hyperpolarization-activated nonselective cationic channels (I_h), and a second flowing through low voltage-activated calcium channels (I_T), which support the peptide-induced increase in excitability. Thus, a PACAP and MEK/ERK-sensitive, voltage-dependent conductance(s), in addition to I_h and I_T, modulate neuronal excitability. Despite prior work implicating PACAP down regulation of Kv4.2 in modulation of excitability in other cells, treatment with the Kv4.2 current blocker 4-aminopyridine did not replicate the PACAP-induced increase in excitability in cardiac neurons. However, cardiac neurons express the ERK target Na_v1.7 channel and treatment with the selective Na_v1.7 inhibitor PF-04856264 decreased the PACAP modulation of excitability. From these results, PACAP/PAC1 activation of MEK/ERK signaling may phosphorylate Na_v1.7, enhancing sodium currents near the threshold, an action contributing to repetitive firing of the cardiac neurons exposed to PACAP.
INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP) peptides (Adcyap1) are trophic and intercellular signaling molecules that are widely distributed within neural and endocrine tissues (1, 40). PACAP modulates synaptic transmission and plasticity adaptations via pre- and postsynaptic mechanisms and its effects play critical roles in central stress challenges, regulation of sensory and autonomic function, cognitive learning and protection from injury paradigms (6, 13, 14, 18, 22, 28, 32, 35, 40). In all, PACAP peptides have diverse roles in maintenance of physiological homeostasis. PACAP is highly expressed in the hypothalamus, hippocampus and related allocortex, limbic system and central sensory and autonomic nuclei. The actions of PACAP are mediated through several seven transmembrane G-protein coupled receptor subtypes including the PACAP-selective PAC1 receptor (Adcyap1r1) and PACAP/VIP VPAC receptors (Vipr1 and Vipr2; also VPAC1 and VPAC2, respectively) (1, 2, 17, 30, 40).

We have shown previously that PACAP is co-localized with acetylcholine in virtually all parasympathetic preganglionic terminals innervating neurons in the guinea pig cardiac ganglia (3, 4). Furthermore, both endogenously released and exogenously applied PACAP can increase cardiac neuron excitability exclusively through PAC1 receptor activation (3, 19, 26, 35). As these cells represent a responsive and readily accessible neuronal system compared to CNS nuclei for experimental manipulation, we have investigated cardiac ganglia neurons as a means to better understand PACAP/PAC1 receptor-mediated modulation of second messengers and ionic conductances that contribute to the regulation of neuronal excitability.
Our laboratory showed previously that PACAP/PAC1-mediated activation of adenyl cyclase and subsequent increase in cellular cAMP enhanced a hyperpolarization-induced nonselective cationic current \( I_h \), which contributed to the PACAP-induced increase in cardiac neuron excitability (27, 37). More recently, we have also reported that PACAP activation of the nickel-sensitive, low voltage-activated calcium current \( I_T \) was also a component of the heightened excitability (38).

In primary neurons and a stable HEKPAC1 receptor-expressing cell line, several studies have shown that PACAP can potently activate MEK/ERK signaling (23, 24). Among many different functions, the MEK/ERK signaling cascade has critical roles in synaptic plasticity and regulation of neuronal excitability (33) and prior studies have shown that ERK activation can modulate many different excitable cells through phosphorylation of a variety of voltage-dependent channels (9, 20, 31, 34). Accordingly, we have investigated whether PACAP stimulates MEK/ERK signaling in guinea pig cardiac neurons and whether this mechanism contributes to the PACAP-induced modulation of neuronal excitability. Our results indicate that PACAP increases cardiac neuron ERK phosphorylation (pERK) and that pretreatment of cardiac ganglia whole mounts with a MEK inhibitor significantly suppresses the PACAP-induced increase in neuronal excitability. However, the MEK inhibitor did not alter either the PACAP enhancement of \( I_h \), seen as rectification in hyperpolarizing voltage steps, or the hyperpolarization-induced rebound depolarization due to activation of \( I_T \). Our current studies demonstrate that in addition to an enhancement of \( I_h \) or \( I_T \), modulation of the voltage-dependent Nav1.7 sodium channel by MEK/ERK signaling contributes to the regulation of neuronal excitability by PACAP.
METHODS

Animals: All experiments were performed using cardiac ganglia whole mount preparations from Hartley guinea pigs (either sex, 250 - 350 g), following animal protocols approved by the Institutional Animal Care and Use Committees of the University of Vermont, the University of California, Los Angeles, and Ithaca College. Approved procedures also followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The guinea pigs were euthanized by isoflurane overdose and exsanguinations. Hearts were quickly removed and placed in cold 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).

Chemicals: PACAP27 (referred to as PACAP throughout) was obtained from American Peptide Co., Sunnyvale, CA. The MEK inhibitor PD 98059 (2’-amino-3’-methoxyflavone) was obtained from Calbiochem, La Jolla, CA. The Kv4.2 inhibitor 4-amino pyridine (4-AP) was purchased from Sigma-Aldrich, St. Louis, MO and the Nav1.7 blocker PF-04856264 (3-cyano-4-(2-(1-methyl-1H-pyrazol-5-yl) phenoxy)-N-(thiazol-2-yl) benzenesulfonamide) from Alomone Labs, Jerusalem, Israel. The inhibitors PD 98059 and PF-04856264 were prepared as DMSO stocks, diluted and added directly to the bath solution. The final concentration of DMSO was ≤ 0.2%. 4-AP was added directly to the bathing solution for each experiment. When inhibitors were used, the preparations were exposed to drug containing solutions for at least 10 minutes prior to initiating data collection.

Immunocytochemistry and confocal imaging:
Following different experimental treatments, cardiac ganglia whole mounts were fixed in a 2% paraformaldehyde and 0.2% picric acid solution for 2 hours at 4°C, washed in blocking solution and treated with ice-cold methanol for 10 min before incubation overnight in 1:1,000 rabbit anti-phosphoERK1/2 (D13.14.4E, Cell Signaling Technology, Beverly, MA) for visualization with Cy3-conjugated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA). The guinea pig cardiac ganglia whole mounts were then mounted on glass slides, cover slipped and imaged with a Nikon/Yokogawa CSU W1 Spinning Disk confocal microscope using a Nikon Apo LWD 25X/1.10NA objective lens. Excitation was accomplished with a 100mW 561nm solid state laser, and emission collected from 590nm-650nm as 16-bit Nikon nd2 image files. 0.4 µm-step Z-series were taken on 5 random ganglia in each sample and regions of interest (ROI) generated for individual neurons at the axial midpoint adjacent to the nucleus and avoiding the cell membrane. Specifically, a single Z-slice was selected through the middle of the cell and an unique circular ROI was generated for each cell cytoplasm depending on cell size. Each ganglion generally contained 3-10 cells that met measurement criteria. All hardware settings were carefully maintained across samples and cross-checked by reviewing data header files. Data collection and analysis were all performed using Nikon Elements 4.30.10 (Build 1021). Data obtained from multiple neurons in each cardiac ganglia whole mount were averaged and the results from at least 3 preparations presented as mean and standard error for the group (Figure 1B).

Intracellular recordings from cardiac ganglia neurons: Intracellular recordings from cardiac neurons followed methods described previously (3, 19, 26, 35, 36). Cardiac ganglia preparations were superfused continuously (6-7 ml/min) with Krebs solution containing 10 mM NaHEPES (32 - 35°C) and individual neurons impaled using 2 M KCl-filled
microelectrodes (60-120 MΩ). Membrane voltage was recorded using an Axoclamp-2A amplifier coupled with a Digidata 1322A data acquisition system and pCLAMP 8 software (Axon Instruments, Foster City, CA). Depolarizing current steps (0.1-0.5 nA, 1 sec) were applied to characterize neuron excitability. PACAP enhances the number of action potentials elicited by long depolarizing current steps (Figure A1, B1). The degree of change in excitability is shown by plotting the number of action potentials generated at each stimulus intensity (Figure 1C). Hyperpolarizing current steps (500 msec) of increasing amplitude were used to test for (a) rectification in the current-induced hyperpolarization, which occurs when the hyperpolarization-activated current $I_h$ is initiated and (b) for a transient hyperpolarization-induced rebound depolarization, which is a characteristic feature of activation of the low voltage-activated calcium current, $I_T$. PACAP can enhance both the rectification in the hyperpolarizing step and the hyperpolarization-induced rebound depolarization (Figure 1 A2, B2).

Real time quantitative polymerase chain reaction (QPCR): Transcript levels were determined for KV4.2 and different voltage-dependent sodium channels (NaV1.2, NaV1.3, NaV1.6 and NaV1.7) from extracts of cardiac ganglia whole mounts, stellate ganglia, atrial muscle and brain, all of which were collected under RNase-free conditions (10). The primer pairs for determining the presence of guinea pig KV4.2 transcripts were: Forward 5’-TTCTACCGCACTGGGAAGCTC-3’ and Reverse 5’-CTCGTAACAGCAGTGCCGAT-3’. The primer sets for the different voltage-dependent sodium channels used in this study were taken from Sage et al (29).

Complementary DNA templates were assayed using HotStart-IT SYBR Green qPCR Master Mix (USB, Cleveland, OH, USA) and 300 nM of each primer in a final 25 μl reaction
volume. The amplified products were subjected to SYBR Green I melting analysis by ramping the temperature of the reaction samples from 60°C to 95°C. A single DNA melting profile was observed under these dissociation assay conditions demonstrating amplification of a single unique product free of primer dimers or other anomalous products (data not shown). Data were analyzed at the termination of each assay using sequence detection software (Sequence Detection Software, version 1.3.1; Applied Biosystems, Norwalk, CT, USA). In standard assays, default baseline settings were selected. The increase in SYBR Green I fluorescence intensity (ΔRn) was plotted as a function of cycle number and the threshold cycle was determined by the software as the amplification cycle at which the ΔRn first intersects the established baseline. At the end of the QPCR analysis, all samples were run on an ethidium bromide gel.

Statistics: Statistics were performed using GraphPad Prism statistical software (version 5.4; La Jolla, CA). Data are presented as mean ± SEM. Differences between means were either determined using an unpaired Students’ t-test or by one way ANOVA followed by Tukey post hoc analysis. Values were considered statistically significant at $P < 0.05$.

RESULTS

PACAP activates the MEK/ERK signaling cascade in guinea pig cardiac neurons

In the initial series of experiments, we confirmed that PACAP activates the MEK/ERK signaling cascade in cardiac neurons by quantifying changes in pERK immunoreactivity levels (pERK-IR) using confocal microscopy (7). In freshly dissected cardiac ganglia whole mount preparations maintained at 37°C for at least 20 minutes prior to fixation, basal levels of
pERK-IR were observed in cardiac neurons and in presumptive Schwann and satellite cells encircling axon bundles and neurons (Figure 2A1). Following a 20 minute PACAP exposure at 37°C, cardiac neuron cytosolic pERK-IR increased ~2-fold (Figure 2A2, B). As noted earlier, there also was a significant increase in nuclear pERK immunoreactivity following the 20 minute exposure to 25 nM PACAP at 37°C (7). Cardiac neuron cytoplasmic pERK immunoreactivity was also increased consistently following a 20 minute exposure to lower concentrations of PACAP (1-5 nM) or after a shorter exposure to PACAP (1-25 nM, 5 minutes) (data not shown).

Our recent studies with the HEKPAC1 receptor stable cell line demonstrated that PACAP/PAC1 receptor-mediated ERK activation involves PAC1 receptor internalization/endosomal and PLC/DAG/PKC signaling, both of which are suppressed at ambient temperatures (23). Similarly, the PACAP-induced increase of cytoplasmic pERK-IR was greatly suppressed when cardiac ganglia were exposed to 25 nM PACAP for 20 minutes at room temperature (~24°C; Figure 2A3, B), indicating that temperature-sensitive mechanisms contributed to the PACAP activation of MEK/ERK signaling in the cardiac neurons as well. As anticipated, a 15 minute pretreatment with the MEK inhibitor PD 98059 (50 µM) completely blocked the 25 nM PACAP activation of pERK (Figure 2A4, B).

As the number of cardiac neurons in individual cardiac ganglia whole mount preparations can vary by as much as an order of magnitude from a few hundred to ~1500 cardiac neurons (41), the immunocytochemical data could not be correlated directly with Western analyses of cardiac ganglia pERK levels. However, the high potency (nanomolar PACAP concentrations) and time course of the PACAP-induced pERK-IR levels in the
cardiac neurons agreed well with those observed previously in primary sympathetic neurons and the stable HEK PAC1 cell line by Western analyses (23, 24).

MEK inhibition suppresses the PACAP-induced increase in excitability without effecting hyperpolarization-induced rectification or rebound depolarization.

The next experiments evaluated whether pretreatment with the MEK inhibitor PD 98059 blunted the PACAP-induced increase in excitability. Initial intracellular recordings were obtained in control cells before and then during exposure to only PACAP (n = 9, 5 cells at 10 nM and 4 cells at 20 nM) (Figure 3). As shown in Figure 1 and in previous work (3, 19, 26, 35, 37, 38), cardiac neuron exposure to PACAP enhanced excitability as reflected by the marked increase in action potential generation initiated by depolarizing current steps. These experimental treatments were performed by bath application of PACAP and as the change in action potential generation was comparable with 10 or 20 nM PACAP, the data for cells exposed to either concentration were pooled to create an averaged excitability curve for the PACAP treatment group (Figure 3C).

To examine the role of PACAP-induced ERK activation in the increased neuronal excitability, the cardiac neurons in subsequent experiments were pretreated for at least 15 minutes with 50 µM PD 98059 before the addition of PACAP and 50 µM PD 98059 together. PD 98059 treatment by itself had no effect on cardiac neuron excitability compared to vehicle alone. However, PD 98059 pretreatment markedly blunted the ability of PACAP to increase excitability (Figure 3A1, B1). An averaged excitability curve was again created for the 10 nM and 20 nM PACAP-stimulated neurons pretreated with PD 98059 (Figure 3C; n = 6, 3 cells...
treated with 10 nM PACAP plus PD 98059 and 3 cells treated with 20 nM PACAP plus PD 98059). Note that the number of action potentials produced by increasing the stimulus intensity was significantly less in cells exposed to PD 98059 and PACAP, than in cells exposed to PACAP alone, indicative of a marked suppression of the PACAP-induced increase in excitability following inhibition of the MEK/ERK pathway.

In addition to enhancing action potential generation by depolarizing steps, PACAP also can change the characteristics of the response to hyperpolarizing constant-current steps. The cardiac neurons exhibit rectification in hyperpolarizing steps, which is characteristic of the activation of an inward $I_h$ current flowing through cAMP-modulated hyperpolarization-activated nonselective cationic channels (8, 27). PACAP commonly enhances the rectification, consistent with a PACAP stimulation of cAMP generation and a cAMP-induced positive shift in the voltage dependence of $I_h$ activation (Figure 1) (27). Also in many cardiac neurons, following termination of the constant current-induced hyperpolarization, a post-hyperpolarization-induced rebound depolarization is recorded. This rebound depolarization, which is due to a large extent to the activation of nickel-sensitive low voltage-activated calcium currents also can be enhanced by PACAP (Figure 1) (38). In the present study, we tested whether pretreatment with the MEK inhibitor PD 98059 blocked either the PACAP enhancement of $I_h$ or the hyperpolarization-induced rebound depolarization. Although pretreatment with 50 µM PD 98059 significantly suppressed the PACAP-induced increase in excitability (Figure 3A1, B1), the presence of the MEK inhibitor did not alter the PACAP-induced enhancement of the rectification seen in hyperpolarizing voltage steps or the hyperpolarization-induced rebound depolarization (Figure 3A2, B2).
For these studies, the responses to hyperpolarizing current steps were evaluated in 9 cells pretreated with 50 µM PD 98059 before PACAP (10 or 20 nM) addition to the inhibitor superfusion solution. Prior to PACAP addition to the bath, 3 of the 9 cells treated with the MEK inhibitor exhibited no obvious or only slight rectification in the hyperpolarizing steps around -100 mV and exhibited a hyperpolarization-induced rebound depolarization that elicited 1 action potential, whereas during PACAP exposure, even in the presence of PD 98059, the rectification was augmented and the hyperpolarization-induced rebound depolarization increased sufficiently such that 2-4 action potentials were elicited (as an example see Figure 3A2, B2). In the same paradigm, 3 other cells in the MEK inhibitor alone exhibited slight rectification and a subthreshold hyperpolarization-induced rebound depolarization, but upon PACAP addition to the PD 98059 perfusate, the rectification was enhanced and accompanied by a modest, if any, increase in the hyperpolarization-induced rebound depolarization. In the case of the seventh cell in the MEK inhibitor, which exhibited a hyperpolarization-induced rebound depolarization that elicited 1 action potential, PACAP exposure increased the rectification without affecting the hyperpolarization-induced rebound depolarization (1 action potential was still elicited). For the remaining 2 PD 98059-treated cells, there was no noticeable rectification or hyperpolarization-induced rebound depolarization prior to and during PACAP exposure.

The variability in extent of rectification and hyperpolarization-induced rebound depolarization amplitude observed in MEK inhibitor-treated cells and during subsequent PACAP exposure was quite similar to that recently reported for control cells prior to and during PACAP exposure (38). Hence, from these observations, the inhibition of MEK/ERK signaling did not suppress the ability of PACAP to enhance the rectification, indicative of Iₜ. 
activation, or the hyperpolarization-induced rebound depolarization, a characteristic property of \( I_T \) activation.

Cardiac neurons express \( K_v4.2 \) transcripts, but block of \( I_A \) currents does not mimic the PACAP-induced increase in excitability

As neuronal treatment with the MEK inhibitor did not noticeably affect PACAP enhancement of \( I_h \) or \( I_T \), PACAP must modulate a different, MEK/ERK-sensitive membrane conductance that contributes to the PACAP-induced increase in excitability. PACAP has been shown to increase excitability of hippocampal neurons by decreasing surface expression of \( K_v4.2 \), which decrease the voltage dependent potassium current \( I_A \) (12). As for brain and atrial tissues, semiquantitative PCR analysis of cardiac ganglia whole mount extracts suggested that the cardiac neurons express transcripts for \( K_v4.2 \) (Figure 4A). However, when we treated cardiac ganglia preparations with 1 mM 4-AP to examine whether \( I_A \) current suppression could recapitulate the PACAP-induced increase in excitability, 4-AP failed to elicit any significant effect (Figure 4B). Recordings were obtained from 10 cells in 6 whole mount cardiac ganglia prior to 4-AP treatment and then from 13 cells from the same preparations following a 15 minute pretreatment with 4-AP. These results suggested that suppression of an \( I_A \) current by PACAP did not contribute to the peptide modulation of cardiac neuron excitability (Figure 4B).

PACAP/PAC1 receptor signaling regulates voltage-dependent Nav1.7 sodium channel in cardiac neuron excitability
Given that 4-AP did not recapitulate the PACAP-induced increase in excitability, we next focused on sodium channels as potential targets of MEK/ERK modulation in the cardiac neurons. MEK/ERK signaling can affect voltage-gated sodium channel activation (31) and using PCR, we examined the expression of sodium channel transcripts in cardiac ganglia whole mount and sympathetic stellate ganglia extracts. From semiquantitative analyses, the cardiac ganglia preparations expressed Na\textsubscript{V}1.3 and Na\textsubscript{V}1.7 transcripts; the expression of Na\textsubscript{V}1.2 or Na\textsubscript{V}1.6 mRNA was not evident in these samples (Figure 5A). By contrast, the control stellate ganglia tissues contained transcripts for all four sodium channel α subunits (Figure 5A). In good agreement, QPCR analyses confirmed that transcripts for Na\textsubscript{V}1.3 and Na\textsubscript{V}1.7, but not those for Na\textsubscript{V}1.2 or Na\textsubscript{V}1.6, were present in cardiac ganglia extracts, whereas all of these transcripts were present in stellate ganglia. Nav1.7 transcripts were not detected in atrial muscle extracts to mitigate channel expression in the small amount of contaminating non-neural tissue in the cardiac ganglia samples (data not shown).

To test the possibility that PACAP-enhanced currents flow through Na\textsubscript{V}1.7 channels, we examined whether a selective Na\textsubscript{V}1.7 channel inhibitor PF-04856264 (25) could blunt PACAP modulation of neuronal excitability. In the first experiments, the effect of pretreatment with 100 nM PF-04856264 on the PACAP increased excitability was determined in 4 cells from two cardiac ganglia whole mounts. In both ganglia preparations, recordings were made from 1 cell exposed to 100 nM PF-04856264 for at least 10 minutes and then from the same cell after switching to a bath solution containing the Na\textsubscript{V}1.7 inhibitor plus 20 nM PACAP. Excitability was tested multiple times and then recordings were obtained from another cell in the same ganglia preparation during continued exposure to 100 nM PF-04856264 and 20 nM PACAP. A similar recording protocol was followed in 2 additional
whole mount preparations with the concentration of PF-04856264 raised to 500 nM. The suppression of the PACAP-induced increase in excitability was the same with either 100 (4 cells) or 500 nM PF-04856264 (4 cells). Consequently, the data from the 8 PF-04856264-treated cells was combined to generate an averaged excitability curve (Figure 5B). Following pretreatment with PF-04856264, the number of action potentials elicited by depolarizing pulses was significantly less than that noted for 5 other cells exposed to PACAP alone (Figure 5B). Thus, we conclude that PF-04856264 markedly attenuated the PACAP-induced increase in cardiac neuron excitability, an observation implicating PACAP/PAC1 receptor-mediated signaling in the regulation of Nav1.7. Also, over the course of these experiments, it was apparent that exposure to 100 or 500 nM PF-04856264 did not have any obvious effect on action potential characteristics.

DISCUSSION

The key results of the present study are that (1) PACAP activates MEK/ERK signaling in the guinea pig cardiac neurons at concentrations that increased neuronal excitability and (2) that inhibition of the MEK/ERK signaling cascade significantly blunted the PACAP-induced increase in cardiac neuron excitability without impairing PACAP activation of I_h and I_T, two currents shown to participate in the enhancement of neuronal excitability. These results implicated PACAP/PAC1 receptor regulation of an additional current in modulating neuronal excitability, which might be the voltage-gated potassium current I_A or the voltage-dependent sodium current Nav1.7, both of which are known to be modulated by MEK/ERK signaling. Our current studies implicate the latter to participate in PACAP regulation of cardiac neuron excitability.
Previously, we determined that the PACAP-induced increase in excitability was significantly suppressed at room temperature (26). PACAP activation of MEK/ERK signaling in the cardiac neurons, as was shown previously for the HEKPAC1 receptor cells (23), also was generated by temperature-sensitive mechanisms. Thus, a diminished PACAP activation of MEK/ERK signaling at room temperature likely is one factor contributing to the temperature dependence of the peptide-induced increase in cardiac neuron excitability. In the HEKPAC1 cells, the increase in pERK following PACAP stimulation reflected the activities of two mechanisms downstream of PAC1 receptor activation, engagement of PLC/DAG/PKC signaling and endosomal signaling after receptor vesicular internalization (23). In a recent report, we demonstrate that PAC1 internalization/endosomal signaling and AC/cAMP/PKA signaling contribute to the activation of MEK/ERK signaling in the cardiac neurons (7). In contrast to the HEKPAC1 cells, activation of the PLC/DAG/PKC signaling does not contribute to the PACAP generation of pERK in the cardiac neurons. As both PAC1 receptor internalization and PKA activation are temperature-sensitive (5, 26), it is not surprising that PACAP-induced pERK was suppressed at room temperature.

Treatment with PD 98059 to inhibit MEK, a regulatory kinase upstream of ERK, blunted the ability of PACAP to enhance excitability, demonstrating the involvement of the MEK/ERK signaling cascade in the modulation of excitability. However, inhibition of MEK/ERK signaling had no consistent effect on the PACAP enhancement of rectification in hyperpolarizing steps or the hyperpolarization-induced rebound depolarization. These observations are notable in suggesting PACAP modulation of at least one other conductance, in addition to I_h or I_T, contributes to the PACAP action on excitability. MEK/ERK signaling can modulate A-type potassium channels, high voltage-activated calcium channels and
voltage-dependent sodium channels (9, 20, 31, 34). PACAP suppresses high voltage-activated calcium currents suggesting enhancement of these currents did not contribute to the increased excitability in PACAP (36). Consequently, we evaluated the possible involvement of a PACAP modulation of $I_A$, a current shown previously to enhance hippocampal neuron and olfactory neuroepithelia activities through channel downregulation by PACAP (12, 15). Supporting a possible involvement, transcripts for K$_{v}$4.2 were present in extracts of cardiac ganglia whole mounts. However, exposure to 4-AP to inhibit $I_A$ currents did not noticeably increase cardiac neuron excitability, a result consistent with prior studies (11). Thus, suppression of $I_A$ did not mimic the PACAP modulation of cardiac neuron excitability, an observation suggesting that suppression of $I_A$ likely did not contribute to the PACAP modulation of cardiac neuron excitability.

Consequently, we focused on sodium channel $\alpha$ subunits as the potential target of the PACAP enhanced MEK/ERK signaling in the cardiac neurons. Prior studies have reported ERK activation modulates Na$_{v}$1.2 and Na$_{v}$1.7 in PC12 cells (34) and Na$_{v}$1.7 in DRG neurons (31). Our QPCR analysis indicated that the parasympathetic cardiac neurons, like myenteric neurons, express transcripts for Na$_{v}$1.3 and Na$_{v}$1.7, but not for Na$_{v}$1.2 or Na$_{v}$1.6 (29). Transcripts for Na$_{v}$1.2 and Na$_{v}$1.6 were detected in extracts of guinea pig stellate ganglia, suggesting that all of the primers used in this study worked in guinea pig. Na$_{v}$1.7 is a threshold voltage-activated sodium channel, which can amplify weak stimuli; thus it can modulate firing properties of DRG neurons (31). Inhibition of MEK/ERK signaling decreases excitability of DRG neurons and in HEK cells expressing Na$_{v}$1.7 induces a depolarizing shift in the voltage-dependence of activation and fast inactivation, an effect which would decrease excitability (31). Thus, we evaluated whether a recently described selective Na$_{v}$1.7 blocker
could suppress the PACAP-induced increase in excitability (25). Pretreatment with PF-04856264 suppressed the PACAP-induced increase in excitability; an observation consistent with an enhancement by PACAP of currents through Na\textsubscript{v}1.7 channels contributing to the peptide effect on excitability. Furthermore, the extent of the suppression of the PACAP-enhanced excitability by PF-04856264 was comparable to that produced by the MEK inhibitor PD 98059. However, of note, the suppression of the PACAP-enhanced excitability occurred without obvious effects on action potential properties, which is consistent with currents through Na\textsubscript{v}1.3 channels, which are insensitive to PF-04856264, being the primary inward current responsible for the depolarizing phase of action potentials in these cells. The specificity and potency of PF-04856264 is species dependent (25). However, the effect of PF-04856264 in the current studies implicates inhibition of guinea pig cardiac neuron Na\textsubscript{v}1.7 channels. Among sodium channel subtypes, the amino acid residues Y1537, W1538 and D1586 in Domain 4 S1 - S4 transmembrane segment confer PF-04856264 selectivity for Na\textsubscript{v}1.7 over Na\textsubscript{v}1.3 (25). The guinea pig Na\textsubscript{v}1.7 contains Y1537 and W1538 in the corresponding region but differs at D1586; these sequence variations are also found in mouse and canine channels and predictive of PF-04856264 preference for Na\textsubscript{v}1.7 but with a small decrease in potency. Further, there are multiple S/TP motifs in the L1 loop region of the human Na\textsubscript{v}1.7 (T531, S535, S608 and S712) for ERK phosphorylation to gate channel properties (31). The exact same four S/TP motifs are present in the guinea pig Na\textsubscript{v}1.7 L1 sequence and hence can be targeted by ERK upon receptor-mediated activation. Even with the potential limitation from inhibitor potency, it is hypothesized that a PACAP activation of MEK/ERK signaling phosphorylates Na\textsubscript{v}1.7, which causes a hyperpolarizing shift in the voltage dependence of activation. This in turn could increase sodium currents near the
threshold; thus, potentially contributing to the development of repetitive action potential firing noted in PACAP.

We have hypothesized based on recent studies that PF-04856264 is a selective inhibitor of Na\textsubscript{v}1.7 with limited effects on other sodium channel types (25). However, as drugs can have multiple targets, this conclusion remains tentative until the possibility that other types of ion channels contributing to the PACAP-induced increase in excitability in guinea pig neurons also are not altered by this compound.

Previously, we demonstrated that the PKA inhibitor H89 also suppressed the PACAP-induced increase in excitability, an observation suggesting a role of PKA-induced phosphorylation in the peptide effect (39). Recently, we extended the analysis of these earlier experiments to determine whether H89 (1-5 µM) treatment affected the characteristics of the response to hyperpolarizing current steps prior to and during exposure to 10 nM PACAP.

From this analysis, it appeared that H89 suppressed the PACAP enhancement of the hyperpolarization-induced rebound depolarization without any obvious effect on the development of rectification in the hyperpolarizing steps (data not shown). We recently suggested that activation of Ca\textsubscript{v}3.2 channels likely contributes to the development of the hyperpolarization-induced rebound depolarization based on its sensitivity to low concentrations of nickel (38). Activation of PKA phosphorylates T-type calcium channel α subunits (5, 21). Chemin et al (5) reported that at micromolar concentrations, H89 not only suppressed a dibutyryl cAMP-induced enhancement of T-type calcium currents, but also had direct inhibitory effects on T-type calcium currents in the absence of PKA enhancement. The observation that the hyperpolarization-induced rebound depolarization was either very small or absent in H89 treated cells and not enhanced by PACAP is consistent with our prior
conclusion that the hyperpolarization-induced rebound depolarization is generated primarily by activation of T-type calcium currents (38).

The present results indicate activation of the AC/cAMP/PKA signaling cascade in the cardiac neurons contributed to the PACAP-induced generation of pERK. Given this, a blockade of PKA/MEK/ERK by H89 could also contribute to the H89 suppression of the PACAP-induced increase in excitability.

From results obtained in previous and current studies, we have developed a schematic model to summarize potential mechanisms contributing to the PACAP-induced increase in excitability (Figure 5). First, interaction of PACAP with the PAC1 receptor leads to activation of adenylyl cyclase and generation of cAMP. The rise in cAMP shifts the voltage-dependence of activation of HCN channels, enhancing I_h. A rise in cAMP also activates PKA, enhancing T-type channel currents through protein phosphorylation and contributing to the activation of MEK/ERK signaling. Furthermore, internalization of the activated PAC1 receptor and the resulting formation of a signaling endosome provide a scaffold for recruitment of the MEK signaling cascade. Activation of MEK kinase signaling alters gating properties of another channel, likely NaV1.7, through protein phosphorylation. Although our data do not implicate involvement of a PACAP modulation of Kv4.2 expression as a mechanism contributing to the increase in cardiac neuron excitability, a PACAP activation of MEK/ERK signaling can decrease surface membrane density of Kv4.2 channels in other neurons; an action which would contribute to an enhanced excitability. Kv4.2 is often located in dendrites where it regulates synaptic efficiency (12). Cardiac neurons do not have dendrites and the excitatory synapses are located on the cell soma and axon hillock (8, 16). Thus, which mechanism(s)
PACAP utilizes to regulate excitability likely depends on the types of ion channels expressed in different neurons.

In conclusion, this study illustrates that the PAC1 receptor can potently modulate neuronal electrical properties through activation of multiple intracellular signaling cascades that modulate many different voltage-dependent ionic conductances.

References


37. Tompkins JD, Lawrence YT, Parsons RL. Enhancement of $I_h$, but not inhibition of $I_M$, is a key mechanism underlying the PACAP-induced increase in excitability of guinea pig intrinsic cardiac neurons. Am J Physiol Regul Integr Comp Physiol 297: R52-R59, 2009.


Figure legends

Figure 1. PACAP can enhance excitability, rectification and a hyperpolarization-induced rebound depolarization in guinea pig cardiac neurons. Panels A1, B1 show the 20 nM PACAP-induced shift from phasic to multiple action potential generation. Prior to PACAP application, a 1 second, 0.2 nA depolarizing constant current pulse elicited 1 action potential. During exposure to PACAP, the number of action potentials generated by this same depolarizing current pulse increased markedly. Panels A2, B2 show that PACAP also increased the rectification in the hyperpolarization elicited by a 500 second constant current pulse and likewise enhanced the hyperpolarization-induced rebound depolarization. C. An excitability curve showing the PACAP enhancement of action potentials generated by 1 second depolarizing current steps of increasing intensity. Open circles: number of action potential generated prior to PACAP; closed circles: number of action potentials elicited during exposure to PACAP.

Figure 2. PACAP stimulates pERK generation in guinea pig cardiac neurons. A: Confocal images (~1 µm optical sections) of cardiac ganglia neurons processed for pERK immunoreactivity and visualization using a Cy3 conjugated secondary antisera. A1: A control cardiac ganglia preparation maintained at 37ºC prior to fixation. Left arrow points to cardiac ganglion containing multiple neurons with only basal pERK-IR and the right arrow points to a nerve bundle surrounded by pERK-IR Schwann cells. A2: A cardiac ganglia preparation exposed to 25 nM PACAP at 37ºC for 20 minutes prior to fixation. The left arrow points to a nerve bundle surrounded by pERK-IR
Schwann cells and the right arrow points to a cardiac ganglion containing multiple neurons with increased pERK immunoreactivity. A3: A cardiac ganglia preparation exposed to 25 nM PACAP at 24°C for 20 minutes prior to fixation. Neuronal PACAP-stimulated pERK levels were attenuated consistent with blockade of endosomal signaling at room temperature. A4: A cardiac ganglia preparation pretreated with the MEK inhibitor PD 98059 (50 µM) for 15 minutes and then exposed to 25 nM PACAP and PD 98059 at 37°C for 20 minutes prior to fixation. MEK inhibition blocked PACAP-stimulated ERK activation. Calibration bar, 20 µm. B: Averaged fluorescence intensity/area for the different conditions shown in A. Results averaged from at least 3 cardiac ganglia whole mount preparations. Control at 37°C, 54 cells in 3 whole mount preparations; PACAP at 37°C, 61 cells in 4 whole mount preparations; PACAP at 24°C, 60 cells in 3 whole mount preparations; PD 98059 and PACAP at 37°C, 47 cells in 3 whole mount preparations. *, pERK-IR is significantly different from control. **, significantly different from PACAP treatment at 37°C, but not different from control preparations.

Figure 3. MEK inhibitor PD 98059 pretreatment suppresses the PACAP-induced increase in excitability without suppressing the rectification in constant current elicited hyperpolarizations or the hyperpolarization-induced rebound depolarization. A: MEK inhibitor PD 98059 treatment blunted the PACAP induced increase in neuronal excitability. A1: In inhibitor pretreated cells, prior to PACAP, a 1 second, 0.3 nA current step elicited 3 action potentials. B1: During exposure to PD 98059 and PACAP, 4 action potentials were elicited by the same stimulus. A2: In PD 98059, prior
to PACAP exposure, some rectification was evident in the hyperpolarization elicited by a 500 msec constant current step and at the termination of the hyperpolarization there was a hyperpolarization-induced rebound depolarization that was large enough to generate an action potential. B2: During exposure to PD 98059 and PACAP, the rectification in the hyperpolarization was more evident and the hyperpolarization-induced rebound depolarization elicited multiple action potentials. C: Averaged excitability curves generated by plotting the number of action potentials elicited by 1 second depolarizing constant current steps of increasing strength. Data shown for one group of cells exposed only to PACAP and for different cells exposed to PD 98059 and PACAP. Open squares and closed squares show the averaged excitability curve generated for 9 cells before PACAP and then during exposure to PACAP (5 cells, 10 nM; 4 cells, 20 nM). Open circles and closed circles show the averaged excitability curve generated for 6 cells exposed only to 50 µM PD 98059 and then the same cells during exposure to PD98059 and PACAP (3 cells, 10 nM; 3 cells, 20 nM). The asterisks indicate that the number of action potentials generated in cells exposed to PACAP and PD 98059 was significantly less than those exposed to PACAP alone at the same current step.

Figure 4. Guinea pig cardiac ganglia express Kv4.2 transcripts but inhibition of I_A with 4-AP does not simulate PACAP-induced excitability. A: Semiquantitative polymerase chain reaction (PCR) demonstrating Kv4.2 transcript expression in brain, atrial muscle and cardiac ganglia. NTC, no template control. B: Averaged excitability curves for 10 cells prior to (open squares) and for 13 cells during exposure to 1 mM 4-AP (closed
squares) treatment. These results demonstrate that 4-AP treatment to suppress \(K_{V4.2}\) currents had no effect on cardiac neuron excitability.

Figure 5. Cardiac ganglia neurons express select voltage dependent sodium channels and preferential \(NaV1.7\) blockade can attenuate PACAP-enhanced excitability. Semiquantitative PCR demonstrate \(NaV1.3\) and \(NaV1.7\) transcript expression in cardiac ganglia; \(NaV1.2\) and \(NaV1.6\) expression was not evident in these tissues. Stellate ganglia contained transcripts for all 4 sodium channel \(\alpha\) subunits. NTC, no template control. B: Cardiac neuron pretreatment with the \(NaV1.7\) blocker PF-04856264 suppressed the PACAP-induced increase in cardiac neuron excitability. Averaged excitability curve generated for 5 cells during exposure to 20 nM PACAP (closed squares) and for 8 cells pretreated with PF-04856264 and then to PF-04856264 plus 20 nM PACAP (4 cells at 100 nM PF and 4 cells at 500 nM) (closed circles). *, number of action potentials for PF-04856264 and PACAP is statistically different from PACAP alone at the indicated current steps.

Figure 6. Schematic of intracellular signaling cascades and ionic conductances potentially contributing to the PACAP-induced increase in cardiac neuron excitability. Interaction of PACAP with the \(PAC_1\) receptor (1) leads to activation of adenylyl cyclase and generation of cAMP (2). The rise in cAMP and interaction with HCN channels enhances \(I_h\) (3) and a rise in cAMP also activates PKA (4), enhancing T-type channel currents through protein phosphorylation (5) and possibly contributes to the activation of MEK/ERK signaling (6). Internalization of the PACAP/\(PAC_1\) receptor
complex and formation of a signaling endosome (7) provides a scaffold for
recruitment of the MEK/ERK signaling cascade (8) and activation of the MEK/ERK
kinase signaling potentially alters the voltage-dependent Na\textsuperscript{+} channel, Na\textsubscript{V}1.7, through
protein phosphorylation (9). For other cell types, a PACAP-induced decrease in
surface expression of K\textsubscript{V}4.2 (dashed line 9) could also contribute to the increased
excitability.
A. PD98059
B. PD98059 + PACAP

C. Graph showing the number of action potentials vs. current step (nA) for Control, PACAP, PD98059, and PD98059 + PACAP conditions.