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Ca²⁺ Influx, But Not Ca²⁺ Release From Internal Stores, Is Required for the PACAP-Induced Increase in Excitability in Guinea Pig Intracardiac Neurons

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Tompkins, John D., Jean C. Hardwick, Sarah A. Locknar, Laura A. Merriam, and Rodney L. Parsons. Ca²⁺ influx, but not Ca²⁺ release from internal stores, is required for the PACAP-induced increase in excitability in guinea pig intracardiac neurons. *J Neurophysiol* 95: 2134–2142, 2006. First published December 21, 2005; doi:10.1152/jn.01077.2005. Mechanisms modulating the pituitary adenylate cyclase activating polypeptide (PACAP)-induced increase in excitability have been studied using dissociated guinea pig intrinsic cardiac neurons and intact ganglion preparations. Measurements of intracellular calcium (Ca²⁺) with the fluorescent Ca²⁺ indicator dye fluo-3 indicated that neither PACAP nor vasoactive intestinal polypeptide (VIP) at either 100 nM or 1 μM produced a discernible elevation of intracellular Ca²⁺ in dissociated intracardiac neurons. For neurons in ganglion whole mount preparations kept in control bath solution, local application of PACAP significantly increased excitability, as indicated by the number of action potentials generated by long depolarizing current pulses. However, in a Ca²⁺-deficient solution in which external Ca²⁺ was replaced by Mg²⁺ or when cells were bathed in control solution containing 200 μM Cd²⁺, PACAP did not enhance action potential firing. In contrast, in a Ca²⁺-deficient solution with Ca²⁺ replaced by strontium (Sr²⁺), PACAP increased excitability. PACAP increased excitability in cells treated with a combination of 20 μM ryanodine and 10 mM caffeine to interrupt release of Ca²⁺ from internal stores. Experiments using fluo-3 showed that ryanodine/caffeine pretreatment eliminated subsequent caffeine-induced Ca²⁺ release from intracellular stores, whereas exposure to the Ca²⁺-deficient solution did not. In dissociated intracardiac neurons voltage clamped with the perforated patch recording technique, 100 nM PACAP decreased the voltage-dependent barium current (*I*_{Ba}). These results show that, in the guinea pig intracardiac neurons, the PACAP-induced increase in excitability apparently requires Ca²⁺ influx through Cd²⁺-sensitive calcium permeable channels other than voltage-dependent Ca²⁺ channels, but not Ca²⁺ release from internal stores.

INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP), which is a member of the vasoactive intestinal polypeptide (VIP), secretin, and glucagon family of peptides (Arimura 1998; Vaudry et al. 2000), is co-localized with the acetylcholine synthesizing enzyme, choline acetyltransferase (ChAT), in the parasympathetic preganglionic fibers innervating the intrinsic cardiac neurons of guinea pigs (Calupca et al. 2000) and rats (Richardson et al. 2003). Braas et al. (1998) determined, using a combination of RT-PCR and immunocytochemical staining, that guinea pig intracardiac neurons express the PACAP-selective PAC₁ receptor. More recently, DeHaven and

Cuevas (2002) showed using RT-PCR that rat neonatal intracardiac neurons express both PAC₁ receptors and VPAC receptors. However, there are multiple isoforms of the PAC₁ receptor and the PAC₁ receptor isoforms expressed by adult guinea pig and neonatal rat intracardiac neurons are different (Braas et al. 1998; DeHaven and Cuevas 2004).

PACAP markedly increases excitability of both guinea pig and rat intracardiac neurons (Braas et al. 1998; DeHaven and Cuevas 2004). In guinea pig intracardiac neurons, PACAP was much more effective than VIP, suggesting that the increase in excitability apparently requires activation of only PAC₁ receptors (Braas et al. 1998). In contrast, DeHaven and Cuevas (2004) reported that the increase in excitability by PACAP in rat neonatal cardiac neurons required co-activation of PAC₁ and VPAC receptors. These authors also showed that activation of VPAC receptors expressed on rat neonatal intracardiac neurons mediated a VIP- or PACAP-induced rise in intracellular calcium ([Ca²⁺]_i) and proposed that the PACAP-induced increase in excitability in rat neonatal intracardiac neurons required Ca²⁺ release from internal stores.

Given that the cardiac neurons in adult guinea pigs and neonatal rats apparently express different combinations of receptors, these studies were undertaken to test whether Ca²⁺ release from internal stores also was required for the PACAP-induced increase in excitability in adult guinea pig intracardiac neurons. We determined that neither PACAP nor VIP elicits a rise in [Ca²⁺]_i in dissociated adult guinea pig intracardiac neurons. In addition, treatment with a combination of ryanodine and caffeine to inhibit the release of Ca²⁺ from internal stores does not eliminate the PACAP-induced increase in excitability, whereas removal of external Ca²⁺ or addition of cadmium (Cd²⁺) to the bath solution blunts the PACAP-induced increase in excitability. These observations indicate that a PACAP-induced Ca²⁺ influx through Cd²⁺-sensitive membrane channels, rather than Ca²⁺ release from internal stores, is required for the peptide-induced increase in excitability in adult guinea pig intracardiac neurons. Preliminary results of this study were presented at the Seventh International Symposium on VIP, PACAP, and Related Peptides (Hardwick et al. 2005).

METHODS

General methods

Experiments were performed in vitro on atrial whole mount preparations containing the intrinsic cardiac ganglia from Hartley guinea

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pigs (either sex; 250–350 g). Guinea pigs were killed by halothane or isoflurane overdose followed by exsanguination using animal protocols approved by the University of Vermont Institutional Animal Care and Use Committee and the Ithaca College Institutional Animal Care and Use Committee and methods described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their 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, and 8 glucose). The pH was maintained at 7.4 by aeration with 95% O₂-5% CO₂.

For experiments to quantify barium currents (I_{Ba}) through voltage-dependent calcium channels or to measure $[Ca^{2+}]_i$ transients with fluo-3 fluorescence, the neurons were dissociated from the cardiac ganglia whole mount preparation using techniques described previously (Merriam et al. 2004). After dissection of the cardiac ganglia, the tissue was dissociated by incubation at 37°C in 10 mg/ml collagenase A (Roche Molecular Biochemicals, Indianapolis, IN) for 35 min and incubated for another 35 min in the same enzyme solution to which 5 mg/ml trypsin XII-S (Sigma, St. Louis, MO) was added. After dissociation, the neurons were placed in Eagle minimal essential medium (HEPES modification) containing 2 mM CaCl₂ and supplemented with 10% fetal bovine serum, 0.1% bovine serum albumin, 1 mg/ml DNase I, 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamicin (All from Sigma, except DNase I from Worthington Biochemical, Lakewood, NJ). The cells were stored overnight in a 37°C incubator before recording.

Intracellular recordings from neurons in whole mount preparations

For intracellular recordings, atrial whole mount preparations were pinned in a 2.5-ml Sylgard-lined chamber and superfused continuously (2–3 ml/min) with Krebs solution also containing 10 mM HEPES buffer (33–35°C) (Braas et al. 1998; Hardwick et al. 1995, 1997). A Ca²⁺-deficient solution was made simply by replacing Ca²⁺ with Mg²⁺. In other experiments, strontium (Sr²⁺) replaced calcium or 200 μM Cd²⁺ was added to the Ca²⁺-containing solution. Cardiac ganglia were visualized with an inverted microscope equipped with Hoffman optics, and individual intracardiac neurons were impaled using high-impedance borosilicate microelectrodes (2 M KCl-filled; 60–100 MΩ). Active and passive membrane properties were recorded from the impaled neurons using an Axoclamp-2A amplifier coupled with a Digidata 1322A data acquisition system and pCLAMP 8 (Axon Instruments, Foster City, CA). In some cells, hyperpolarizing current was injected through the recording electrode to ensure that action potential generation was tested at the same potential before and after PACAP application. When current was applied, the resting membrane potential was maintained between –55 and –65 mV, values within the range of membrane potentials recorded from these cells (Braas et al. 1998; Hardwick et al. 1995, 1997; Merriam et al. 2004; Zhang et al. 2001).

Because PACAP27 was determined previously to be more effective than PACAP38 on guinea pig intracardiac neurons (Braas et al. 1998, 2004), it was used exclusively in this study and is referred to as PACAP throughout the text. PACAP was applied by local pressure application (6 psi; Picospritzer, General Valve, Fairfield, NJ) through ~5 μm-diam “puffer” pipettes containing 50 μM PACAP and positioned 50–100 μm from the neuron. Depolarizing current steps (0.1–0.3 nA, 500 or 1,000 ms) were given to characterize changes in neuron excitability before and after PACAP application. Excitability curves were constructed by plotting the number of action potentials generated by increasing stimulus intensities. When both 500- and 1000-ms steps were used to test excitability in a given experimental condition, the number of action potentials generated during the first 500 ms were used to construct the excitability curve.

Recordings of I_{Ba} in dissociated intracardiac neurons

The perforated patch configuration of the whole cell patch-clamp technique (Horn and Marty 1988) was used to record I_{Ba} from isolated neurons. The recording solution contained 120 mM *N*-methyl-D-glucamine, 25 mM tetraethylammonium chloride, 10 mM HEPES, 1.2 mM MgCl₂, 3 mM BaCl₂, 8 mM glucose, and 0.3 μM TTX. The pH was adjusted to 7.3 with HCl. The pipette solution contained 140 mM Cs aspartate, 30 mM CsCl, 5 mM MgCl₂, and 10 mM HEPES. The pH was adjusted to 7.2 with CsOH. For some cells, the Cs aspartate was omitted, and 175 mM CsCl was used. The results were identical with either pipette solution. Patch pipettes were backfilled with 0.2 mg/ml amphotericin B (Sigma, St. Louis, MO).

Bath solutions were applied by a gravity flow system (5–8 ml/min) to a 0.5-ml bath chamber. The temperature was maintained at 33–36°C by an in-line solution heater controlled by a single channel heater controller (Warner Instruments, Hamden, CT). PACAP (100 nM) was added to the bath solution and applied to isolated neurons by gravity flow.

Voltage step protocols were generated and currents were recorded with an Axopatch 1-C amplifier in combination with a Digidata 1322A and pCLAMP 9 acquisition system using sample rates ranging from 5 to 50 kHz. Currents were filtered at 2 kHz and were leak subtracted using a P/5 subpulse protocol. The methods followed protocols used previously by our laboratory to record I_{Ba} in mudpuppy intracardiac neurons (Merriam and Parsons 1995). Reported voltages were not corrected for series resistance (except the *I-V* plot in Fig. 6B) or junction potential errors, but only data from cells with uncompensated series resistance of 20 MΩ or less were included in the data analysis.

[Ca²⁺]_i measurements

Changes in $[Ca^{2+}]_i$ were determined from variations in the fluorescence intensity of fluo-3 (Locknar et al. 2004). Cells were loaded with 5 μM fluo-3-AM and 5 μM pluronic F-127. Both the loading and AM-ester cleavage steps were performed at room temperature. The cells were plated on cover slips and continuously perfused at a flow rate of 1–1.5 ml/min. Because the dissociated cells occasionally fire action potentials spontaneously, 0.3 μM TTX was included in the bath solution during Ca²⁺ imaging. Images were acquired on two types of microscopes, a DeltaVision Restoration Microscopy system and a Noran Oz confocal microscope. Most of the Ca²⁺ imaging experiments were done on the DeltaVision Restoration Microscopy system, although similar results were obtained with both microscopes. Using the DeltaVision microscopy system, different sample rates were tried, varying from 1 to 0.33 Hz. There was no difference in the results obtained at different sample rates. Filter sets appropriate for FITC were used and the microscope stage was surrounded by an incubator (Solent Scientific, Segensworth, UK) to maintain the temperature at 37 ± 1°C. For the few experiments done using the Noran Oz confocal microscope (Middleton, WI), the sample rate was 0.33 Hz with 488-nm laser excitation and an emission filter appropriate for FITC. Temperature of the bath was maintained at 33 ± 1°C with an in-line heater (Warner Instruments). The small difference in temperature (33 vs. 37°C) did not affect the results obtained with the two instruments.

Regions of interest corresponding to the cytoplasm of the neurons were selected from the raw image files and average brightness over time plots were generated. Files generated by the DeltaVision microscope were corrected for variations in lamp intensity (recorded by a diode at the same time as image acquisition). The data sets were corrected for dye bleaching using a single or double exponential decay algorithm and normalized to this decay curve to give the fluo-3 fluorescence ratio (F/F_0). Because the F/F_0 responses varied from cell to cell (single vs. multiple peaks), we integrated the area under all peaks. The F/F_0 integral reflects the total amount of Ca²⁺ released from internal stores and is referred to as an increase in $[Ca^{2+}]_i$.

throughout the text. All corrections and integrations were performed with Microcal Origin 7.0 (Northampton, MA).

Drugs

All drugs were obtained from commercial sources: PACAP27 (American Peptide, Sunnyvale, CA); ryanodine and TTX (Calbiochem, La Jolla, CA); pluronic F-127 and fluo-3-AM (Molecular Probes, Eugene, OR); and caffeine (Sigma Chemical). A ryanodine stock solution was made up each day in dimethylsulfoxide (DMSO). Fluo3-AM and pluronic F-127 were diluted each day from frozen aliquots of concentrated solutions dissolved in DMSO.

Statistical evaluation

Paired and unpaired Student's *t*-test were used to evaluate differences between means as appropriate. Differences were considered statistically significant if $P < 0.05$.

RESULTS

PACAP does not initiate Ca^{2+} release from Ca^{2+} stores in dissociated guinea pig intracardiac neurons

In rat neonatal intracardiac neurons, both PACAP and VIP elevate $[Ca^{2+}]_i$ (DeHaven and Cuevas 2004). Furthermore, the peptide-stimulated rise in $[Ca^{2+}]_i$ in rat intracardiac neurons is blocked by VPAC receptor antagonists indicating that this effect requires activation of VPAC receptors rather than PAC₁ receptors (DeHaven and Cuevas 2004). We tested whether PACAP can initiate a rise in $[Ca^{2+}]_i$ in adult guinea pig intracardiac neurons using fluorescence measurements of the Ca^{2+} -sensitive dye fluo-3. PACAP (100 nM or 1 μ M) was applied for 1–3 min to dissociated neurons loaded with fluo-3. PACAP (13 cells at 100 nM, 4 cells at 1 μ M) produced no change in F/F_o (Fig. 1A). In additional experiments, we determined that VIP also did not elicit a rise in $[Ca^{2+}]_i$ (13 cells at 100 nM, 3 cells at 1 μ M; Fig. 1B).

To insure that the cells were adequately loaded with the Ca^{2+} -sensitive dye and changes in $[Ca^{2+}]_i$ could be observed, the same cells also were challenged with 10 mM caffeine to initiate Ca^{2+} release from internal stores (Locknar et al. 2004; Smith and Adams 1999). As is evident from inspection of Fig. 1, caffeine elicited a transient rise in $[Ca^{2+}]_i$ even though PACAP did not stimulate a rise in $[Ca^{2+}]_i$. In some cells, caffeine challenge resulted in a single rapid transient elevation of $[Ca^{2+}]_i$, whereas in other cells, multiple Ca^{2+} transients were seen.

PACAP increase in excitability is eliminated in Ca^{2+} -deficient solutions or in solutions containing Cd^{2+}

Previously, we showed that PACAP increases excitability in dissociated guinea pig intracardiac neurons (Merriam et al. 2004; Parsons et al. 2000). In addition, the results of the preceding experiments suggested that a PACAP-induced Ca^{2+} release from internal stores must not be required for this effect. Consequently, we initiated electrophysiological experiments using neurons in the cardiac ganglia whole mount preparation to establish whether Ca^{2+} influx, rather than Ca^{2+} release from internal stores, might be required for the PACAP-induced increase in excitability.

For these experiments, intracellular recordings from neurons in whole mount ganglia preparations were used to quantify the

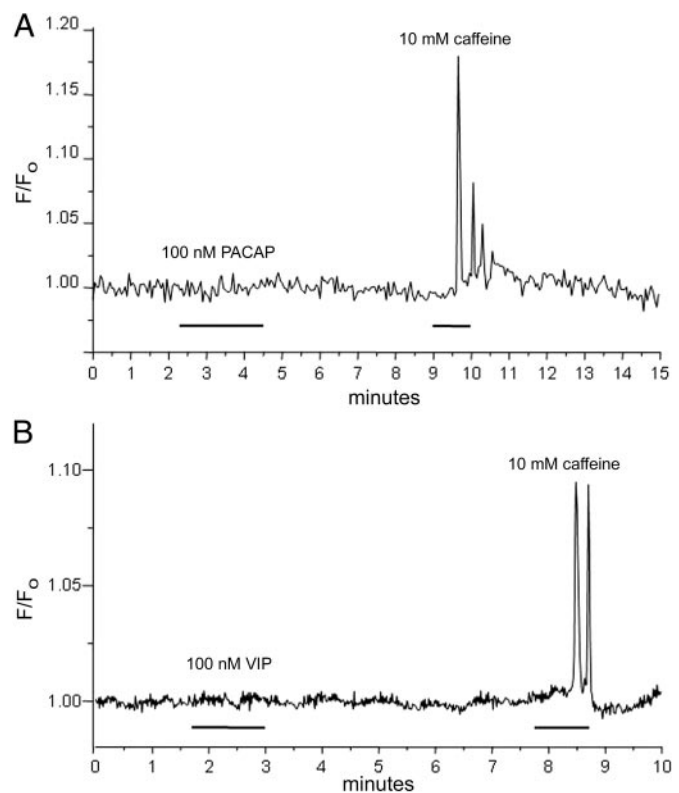


FIG. 1. No change in $[Ca^{2+}]_i$ is observed in response to either 100 nM pituitary adenylate cyclase activating polypeptide (PACAP) or 100 nM vasoactive intestinal polypeptide (VIP). *A*: fluo-3 fluorescence ratio as observed with the Noran Confocal microscope (acquisition rate, 0.33 Hz); 100 nM PACAP was applied during the 1st solid bar. Temperature was 33°C. *B*: fluo-3 fluorescence ratio as observed with the DeltaVision microscope (acquisition rate, 1 Hz); 100 nM VIP was applied during the 1st solid bar. Temperature was 37°C. In both *A* and *B*, 10 mM caffeine was also applied toward the end of the experiment (2nd solid bar) to verify dye loading and an ability to observe a change in $[Ca^{2+}]_i$.

change in membrane excitability initiated by puffer application of PACAP (Braas et al. 1998). After a 500-ms PACAP application, a variable depolarization (4–15 mV), which lasted up to a few minutes, was sometimes produced. Because the extent of the depolarization was variable, we did not quantify the PACAP-induced depolarization in this study. Rather, the emphasis of this study focused on mechanisms involved in the PACAP-induced enhancement of excitability. Excitability was tested once the peptide-induced depolarization subsided, by applying long duration, depolarizing current pulses of increasing intensity to elicit action potentials. An excitability curve was generated by plotting the number of action potentials initiated as a function of the current strength (Braas et al. 1998). The majority of the guinea pig intracardiac neurons in the whole mount preparation are phasic so that long depolarizing current pulses only elicit one to two action potentials regardless of current strength (Hardwick et al. 1995). However, PACAP can enhance excitability in phasic cells so that additional action potentials are generated by the depolarizing current pulses (Braas et al. 1998). Less than 10% of the intracardiac neurons in the whole mount ganglia preparation are tonic and fire multiple action potentials during a long current pulse (Hardwick et al. 1995). Although PACAP also enhanced excitability of tonic cells, the change in action potential generation in tonic cells was not included in the averaged data.

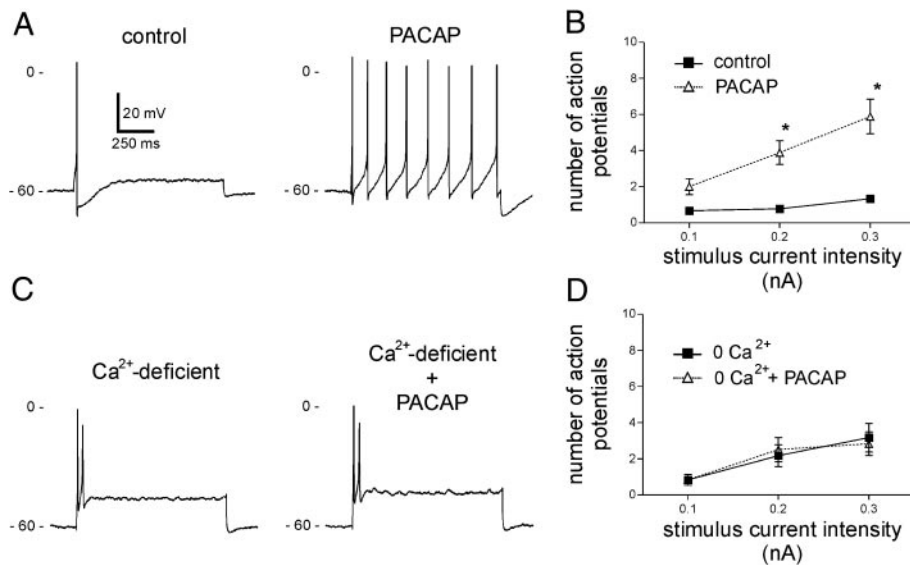


FIG. 2. PACAP enhancement of excitability in guinea pig intracardiac neurons is Ca²⁺ dependent. *A*: membrane potential recording from a phasic intracardiac neuron in response to a long duration depolarizing current pulse (0.3 nA, 1 s) before (*left*) and after PACAP (*right*). PACAP-induced increase in neuronal excitability is evident by increased spike number in response to depolarizing stimulus. *B*: excitability curve obtained before and after puff application of PACAP under control conditions (**P* < 0.05, *n* = 9 cells). *C*: in the Ca²⁺-deficient solution, neuronal excitability is slightly enhanced (*left*; record shows response to a 1-s, 0.2-nA depolarizing step). PACAP did not produce a change in excitability in the Ca²⁺-deficient solution (*right*; averaged results for 6 cells are shown in *D*). Scale bar same for all records.

PACAP enhances excitability in both phasic and tonic neonatal rat intracardiac neurons as well (DeHaven and Cuevas 2004).

Figure 2*A* shows the increase in excitability produced after PACAP application to a phasic cell, and the results from nine phasic cells are summarized in Fig. 2*B*. The PACAP-induced increase in excitability often was long lived, lasting many minutes after a single application. Subsequent applications of PACAP repeatedly enhanced excitability.

We next tested whether the PACAP-induced increase in excitability remained when preparations were bathed in a Ca²⁺-deficient solution in which Ca²⁺ was replaced by magnesium (Mg²⁺). The action potential duration was increased, and amplitude of the hyperpolarizing after potential (HAP) was decreased in the Ca²⁺-deficient solution (data not shown). Excitability was tested after the action potential configuration had changed. After the change in the action potential, depolarizing voltage steps typically elicited multiple spikes. PACAP did not produce a further increase in neuronal excitability. A representative recording is shown in Fig. 2*C* with averaged results for six cells shown in Fig. 2*D*. In these same cells, PACAP increased excitability after washout of the Ca²⁺-deficient solution with the control solution (data not shown).

Previously, Parsons et al. (2000) showed that the addition of 1 mM barium (Ba²⁺) enhanced the excitability of intracardiac neurons, presumably by inhibiting the voltage-dependent non-inactivating potassium conductance *I*_M (Brown 1988). We found that, although the PACAP-induced increase in excitability was eliminated in the Ca²⁺-deficient solution, exposure to 1 mM Ba²⁺ still increased action potential firing (data not shown). These observations indicated that cells maintained in the Ca²⁺-deficient solution remained capable of generating multiple action potentials.

In dissociated neonatal rat intracardiac neurons, removal of extracellular Ca²⁺ results in a rapid depletion of Ca²⁺ from internal stores (DeHaven and Cuevas 2004). Additional experiments were done to establish whether exposure to the Ca²⁺-deficient, Mg²⁺-substituted solution depleted intracellular Ca²⁺ stores in the dissociated guinea pig intracardiac neurons. Cells were loaded with fluo-3, and the increase in [Ca²⁺]_i produced by 10 and 20 mM caffeine was determined. The dissociated intracardiac neurons were maintained either in the

control Ca²⁺ containing solution or the Ca²⁺-deficient solution for ~10 min before application of caffeine. For neurons maintained in control solution, 10 and 20 mM caffeine elicited a comparable increase in [Ca²⁺]_i (Fig. 3). When intracardiac neurons were kept in the Ca²⁺-deficient solution, 10 mM caffeine often did not produce a measurable increase in the fluo-3 fluorescence ratio. In contrast, 20 mM caffeine consistently produced an increase in *F*/*F*₀, and the magnitude of the increase in [Ca²⁺]_i was identical to that produced by 20 mM caffeine in cells maintained in the control solution (Fig. 3).

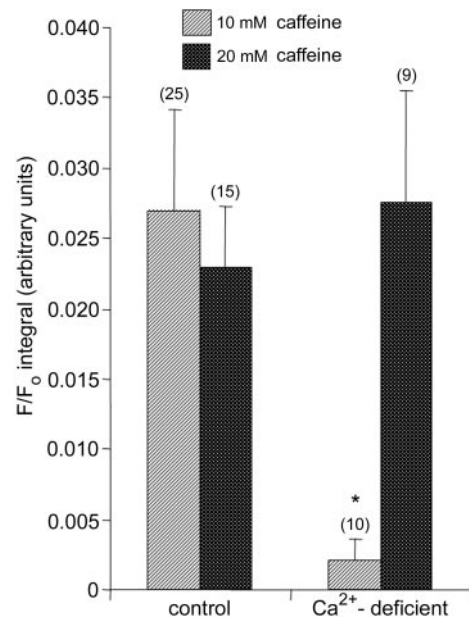


FIG. 3. Transient elevation of [Ca²⁺]_i in response to caffeine is concentration dependent in a Ca²⁺-deficient solution; 10 mM and 20 mM caffeine were applied to intracardiac neurons under control conditions and after pretreatment with a Ca²⁺-deficient solution. In Ca²⁺, 10 and 20 mM caffeine produced a similar change in fluo-3 fluorescence determined by integrating the increase in *F*/*F*₀ over time. In the Ca²⁺-deficient solution, response to 10 mM caffeine was significantly less than response to 20 mM caffeine. Bar graph shows means ± SE of integral of the increase in *F*/*F*₀, expressed in arbitrary units. Numbers in parentheses indicate the number of averaged cells. **P* < 0.05. All experiments were done using the DeltaVision deconvolution microscope with the temperature maintained at 37 ± 1°C.

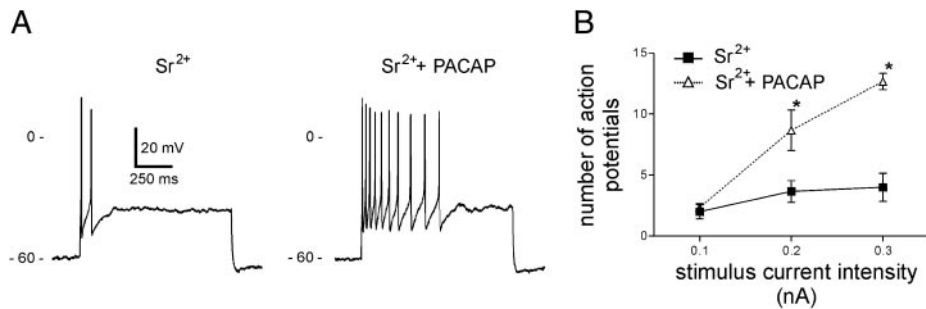


FIG. 4. Sr^{2+} can substitute for Ca^{2+} in mediating the PACAP-induced change in excitability. **A**: membrane potential recordings from an intracardiac neuron superfused with a Ca^{2+} -deficient Sr^{2+} substituted solution in response to a 1-s, 0.2-nA depolarizing current pulse before and after PACAP application. PACAP significantly increased excitability under these conditions. **B**: excitability curve shows averaged results ($*P < 0.05$, $n = 6$ cells).

In separate experiments, we tested whether omitting Ca^{2+} and replacing it with equimolar Sr^{2+} also inhibited the PACAP-induced increase in excitability. When preparations were bathed in the Ca^{2+} -deficient solution containing Sr^{2+} , the action potential duration was increased and HAP amplitude was decreased similar to, although not as great as, the effect produced by the Ca^{2+} -deficient solution containing Mg^{2+} (data not shown). After the change in the action potential waveform, a small increase in excitability was observed again, similar to the Ca^{2+} -deficient solution containing Mg^{2+} . Depolarizing steps often produced multiple (2–3) as opposed to single action potentials before PACAP application. PACAP further enhanced excitability in four of the six cells tested (example for 1 cell shown in Fig. 4A). In the two unresponsive cells, PACAP produced a change in excitability after washout with the control solution. The averaged results for all six cells are shown in Fig. 4B. Thus replacing Ca^{2+} with Sr^{2+} produced a similar effect on the action potential waveform but did not have the same effect on the PACAP-induced excitability as replacing Ca^{2+} with Mg^{2+} .

Cadmium blocks multiple Ca^{2+} influx pathways including voltage-dependent calcium channels (VDCCs). Treatment of intracardiac neurons with Cd^{2+} slows action potential repolarization and decreases the HAP (Adams and Cuevas 2004). We tested the effect of PACAP on excitability in the Cd^{2+} containing solution after the action potential had broadened and

HAP decreased. In five cells, pretreatment with 200 μM Cd^{2+} greatly diminished the PACAP-induced increase in excitability (Fig. 5, A and B).

PACAP inhibits barium current (I_{Ba})

The inability of PACAP to enhance excitability in cells exposed to the Ca^{2+} -deficient solution or Cd^{2+} -containing solution suggested that Ca^{2+} influx through a PACAP-activated Ca^{2+} permeable membrane channel was necessary for the PACAP-induced increase in excitability. Because Cd^{2+} is an efficient blocker of VDCCs in autonomic neurons, we postulated that PACAP may enhance Ca^{2+} influx through the VDCCs expressed in intracardiac neurons. Consequently, we completed perforated patch whole cell voltage-clamp experiments on dissociated intracardiac neurons to determine whether PACAP enhanced currents flowing through VDCCs. Barium was used as the charge carrier (I_{Ba}) to measure macroscopic current flow in these neurons. To elicit I_{Ba} , cells were held at -80 mV, and 200-ms depolarizing voltage steps were applied before and during superfusion with 100 nM PACAP. In addition, the I - V relationship was determined before and during PACAP application. As shown in Fig. 6, PACAP reversibly inhibited peak I_{Ba} . In six cells, 100 nM PACAP decreased peak I_{Ba} by $46 \pm 7\%$, showing that PACAP inhibits macroscopic I_{Ba} through VDCCs.

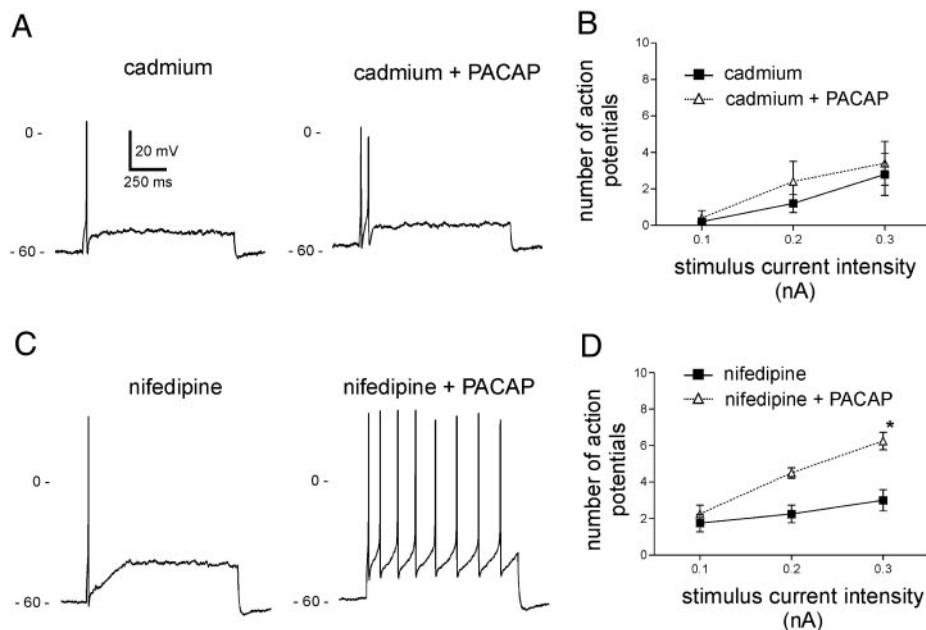


FIG. 5. PACAP-induced increase in excitability is blocked by the nonselective voltage-dependent calcium channel (VDCC) blocker Cd^{2+} but not by the selective L-type channel blocker nifedipine. **A**: membrane potential recorded in response to a 1-s, 0.2-nA depolarizing current step in the same cell immediately before and after PACAP application in the presence of 200 μM Cd^{2+} . **B**: excitability curve summarizes data for 5 cells. **C**: membrane potential recorded in response to a depolarizing current step (1 s, 0.2 nA) in the same cell (different from A) immediately before and after PACAP application in the presence of 10 μM nifedipine. **D**: excitability curve summarizes data for 4 cells ($*P < 0.05$).

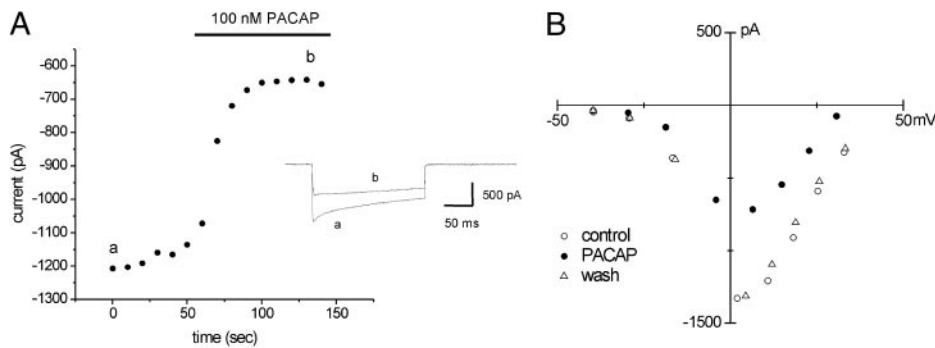


FIG. 6. PACAP inhibits currents through VDCCs. *A*: superfusion with 100 nM PACAP inhibits currents carried by barium (I_{Ba}) flowing through VDCCs. *Inset*: I_{Ba} elicited by a 200-ms depolarizing step from a holding potential of -80 to 0 mV before (*a*) and at the peak of the PACAP inhibition (*b*). *B*: I - V relationship for I_{Ba} before, after 2 min in PACAP, and after 5 min of wash with PACAP-free solution. Bath temperature was maintained at 33°C .

PACAP increase in excitability does not require L-type calcium channels

Intracardiac neurons express a number of different VDCCs with the predominant type expressed being the N-type (Adams and Cuevas 2004; Jeong and Wurster 1997). Previously, a number of investigators have reported that PACAP enhances Ca^{2+} currents through L-type VDCCs (Bhattacharya et al. 2004; Dziema and Obrietan 2002). Because current flow through the L-type VDCC contributes a minor component of the macroscopic I_{Ba} inward current, it was considered possible that a PACAP-induced augmentation of the current flowing through L-type channels would be masked by the inhibition of current flow through other VDCC types. Therefore we tested whether a PACAP-induced enhancement of the L-type current could be involved in the peptide-induced increase in excitability. If this was true, treatment with the specific L-type VDCC blocker nifedipine should eliminate the PACAP-induced increase in excitability. We found that, in four cells pretreated with $10 \mu\text{M}$ nifedipine, PACAP consistently increased excitability (Fig. 5, *B* and *C*). These observations indicated that the PACAP-induced excitability change did not require Ca^{2+} influx through L-type VDCCs.

Disruption of Ca^{2+} release from internal stores does not eliminate the PACAP-induced increase in excitability

Although fluo-3 imaging experiments indicated that PACAP did not elevate global Ca^{2+} levels, it was considered possible that the change in $[\text{Ca}^{2+}]_i$ was too localized for detection by our imaging methods. We undertook additional experiments to ensure that Ca^{2+} release from internal stores was not required for the PACAP-induced increase in excitability in adult guinea pig intracardiac neurons. DeHaven and Cuevas (2004) reported that treatment with ryanodine to block Ca^{2+} release from internal stores eliminated the PACAP-induced increase in excitability in the dissociated neonatal rat intracardiac neurons. Consequently, we tested the effect of ryanodine treatment on

the PACAP-induced excitability change in adult guinea pig intracardiac neurons in the whole mount preparation. In these experiments, whole mount cardiac ganglia preparations were pretreated with a $20 \mu\text{M}$ ryanodine/ 10 mM caffeine-containing solution for 5 min and, for most experiments, the preparations were returned to drug-free solution before testing the effect of PACAP on excitability. In a few experiments, the bathing solution contained ryanodine throughout the experiment. The results were similar with both conditions. In six cells following ryanodine/caffeine pretreatment, PACAP consistently increased excitability (Fig. 7), providing direct evidence that a peptide-induced release of Ca^{2+} from internal stores was not required for the increased excitability.

Fluo-3 imaging experiments confirmed that treatment with $20 \mu\text{M}$ ryanodine and 10 mM caffeine prevented subsequent Ca^{2+} release by a second caffeine challenge. For these experiments, dissociated intracardiac neurons were challenged with $20 \mu\text{M}$ ryanodine and 10 mM caffeine for 2–5 min, and the cells were washed with drug-free solution for 13–23 min before challenging the cells a second time with 20 mM caffeine. We consistently observed a Ca^{2+} transient with the first caffeine challenge, but not with the second caffeine challenge even after 25 min of wash (Fig. 8).

DISCUSSION

This study tested whether a PACAP-induced Ca^{2+} release from internal stores was required for the peptide-induced increase in excitability in adult guinea pig intracardiac neurons. The study was prompted by a recent report that a PACAP-induced release of Ca^{2+} was required for a PACAP-induced increase in excitability in neonatal rat intracardiac neurons (DeHaven and Cuevas 2004). Our results suggest that, for adult guinea pig intracardiac neurons, a PACAP-induced Ca^{2+} influx rather than a peptide-induced Ca^{2+} release from internal stores is required for the peptide-induced increase in excitability. We base this conclusion on the following observations.

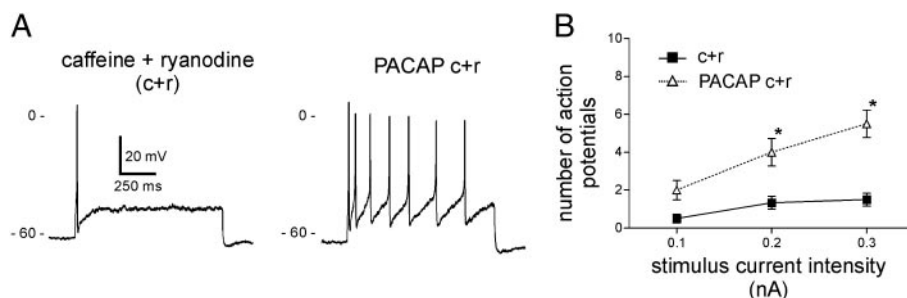


FIG. 7. PACAP increases excitability of guinea pig intracardiac neurons treated with $20 \mu\text{M}$ ryanodine and 10 mM caffeine. *A*: membrane potential recorded in response to a 1-s, 0.2-nA depolarizing current step in the same cell immediately before and after PACAP application. *B*: excitability curve summarizes data for 6 cells ($*P < 0.05$).

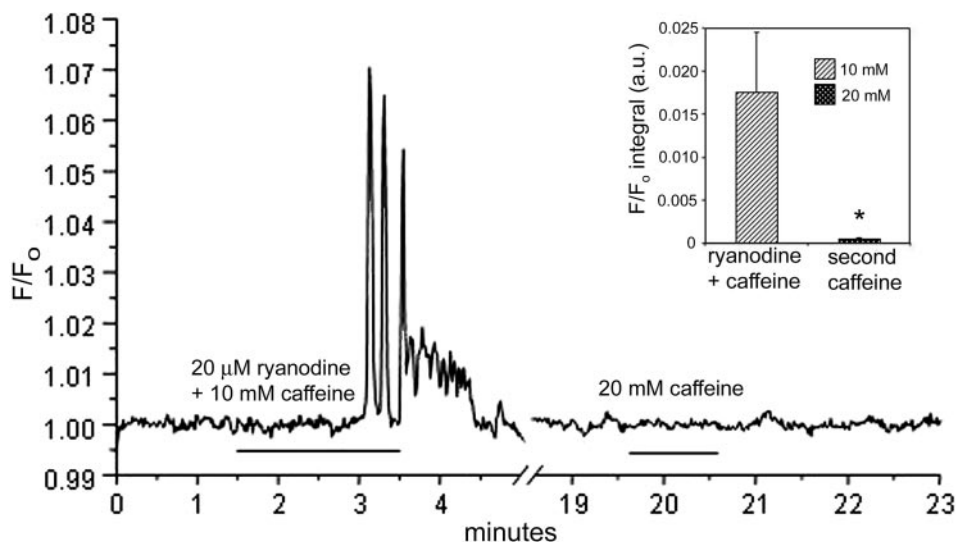


FIG. 8. Ryanodine + caffeine treatment blocks subsequent caffeine-induced Ca^{2+} transients. Trace of F/F_0 in response to simultaneous $20 \mu\text{M}$ ryanodine + 10 mM caffeine treatment (1st bar) followed by a wash period and a 2nd application of 20 mM caffeine (2nd bar). Inset: average F/F_0 integrals (expressed in arbitrary units) for the 1st and 2nd response of 8 cells ($*P < 0.05$). All experiments were done using the DeltaVision deconvolution microscope with the temperature maintained at $37 \pm 1^\circ\text{C}$.

First, neither PACAP nor VIP initiated Ca^{2+} release from internal stores. Second, the PACAP-induced increase in excitability was eliminated when cells were bathed in a Ca^{2+} -deficient solution or a solution containing Cd^{2+} . Third, ryanodine/caffeine pretreatment eliminated caffeine-induced Ca^{2+} release, but did not block the PACAP-induced increase in excitability.

DeHaven and Cuevas (2004) reported that exposure of dissociated neonatal rat intracardiac neurons to a Ca^{2+} -free solution rapidly depleted internal Ca^{2+} stores. We found in this study that for guinea pig neurons kept in the Ca^{2+} -deficient solution, the response to 10 mM caffeine was depressed, but that the response to 20 mM caffeine was similar to that of cells maintained in control solution containing Ca^{2+} . Thus we suggest that for adult guinea pig intracardiac neurons, exposure to the Ca^{2+} -deficient solution did not deplete internal Ca^{2+} stores, in the time frame that we tested. Rather, we postulate that exposure to a Ca^{2+} -deficient solution for short periods decreased the cytosolic Ca^{2+} concentration as reported for other autonomic neurons (Wanaverbecq et al. 2003). Thus with a decrease in the ambient Ca^{2+} surrounding ryanodine receptors, caffeine, at a concentration of 10 mM , was ineffective and the higher concentration was required to activate the ryanodine receptors.

Ca^{2+} influx seems to play an important role in mediating the PACAP-induced increase in excitability of the guinea pig intracardiac neurons. At $200 \mu\text{M}$, Cd^{2+} effectively blocks essentially all types of VDCCs in autonomic neurons (Adams and Cuevas 2004). Cd^{2+} blocked the PACAP-induced increase in excitability in the guinea pig intracardiac neurons. This observation suggested a role of Ca^{2+} influx through VDCCs. However, the results of our voltage-clamp studies showed that PACAP inhibited rather than enhanced macroscopic I_{Ba} . Thus as the N and P/Q channels are the predominant type of VDCCs, we concluded that PACAP does not enhance Ca^{2+} influx through these channels. We had thought it plausible that PACAP might enhance Ca^{2+} influx through L-type VDCCs, which contribute only a small component of the macroscopic current. However, nifedipine, a potent L-type VDCC blocker, did not affect the PACAP-induced excitability. Thus we concluded that PACAP did not enhance Ca^{2+} influx through any VDCCs. Rather, we hypothesize that PACAP activates a Ca^{2+} -

sensitive receptor operated Ca^{2+} influx pathway (Clapham 2003) and Ca^{2+} influx through this channel is required for the peptide-induced increase in excitability. We postulate further that the rise in $[\text{Ca}^{2+}]_i$ caused by Ca^{2+} influx must be restricted to areas close to the cell membrane because no PACAP-induced increase in global Ca^{2+} levels were noted in the fluo-3 studies.

Ca^{2+} has multiple cellular functions and for some of these actions, Sr^{2+} , but not Mg^{2+} , can substitute for Ca^{2+} . In this study, we found that after replacement of Ca^{2+} with Sr^{2+} , PACAP enhanced excitability in four of six cells. In the two cells in Sr^{2+} in which PACAP was ineffective, the peptide increased excitability when the preparation was returned to the Ca^{2+} -containing solution. Thus we suggest that unlike Mg^{2+} , which cannot substitute for Ca^{2+} in supporting the PACAP-induced increase in excitability, Sr^{2+} can, but it apparently is less effective than Ca^{2+} .

Merriam et al. (2004) have shown that PACAP, but not VIP, potently enhances the hyperpolarization-activated, cyclic nucleotide-gated conductance, I_h , which is known to regulate excitability in many neurons. A PACAP-enhanced I_h very likely contributes to the increased excitability in guinea pig intracardiac neurons. I_h is modulated by cAMP levels and PACAP activates adenylyl cyclase, leading to the generation of cAMP. Intracellular Ca^{2+} can promote the modulation of I_h by cAMP (Lüthi and McCormick 1999). Thus we propose that a PACAP-induced Ca^{2+} influx through receptor operated channels causes a local rise of $[\text{Ca}^{2+}]_i$ at the inner surface of the plasma membrane near I_h channels, and this local elevation of $[\text{Ca}^{2+}]_i$ may be a critical co-factor in the cAMP-induced shift in I_h voltage dependence.

After caffeine/ryanodine pretreatment, a second caffeine application did not elicit a rise in $[\text{Ca}^{2+}]_i$ even though the cells were washed with drug-free solution for $\sim 20 \text{ min}$. Caffeine sensitizes the ryanodine receptors to ambient intracellular Ca^{2+} causing internal Ca^{2+} stores to be dumped, whereas ryanodine at micromolar concentrations blocks the release channels (McPherson et al. 1991; Meissner 1994). The lack of a second response to caffeine suggests that either the internal Ca^{2+} stores that were dumped during the first application of caffeine/ryanodine exposure were not adequately refilled or that ryanodine blocked the release channels so that Ca^{2+} could not be

released even though internal stores had begun to refill. Thus the effect of the ryanodine/caffeine pretreatment seems to be irreversible on our time scale and eliminates subsequent release of Ca^{2+} from internal stores.

PACAP can exert its influence through three G protein-coupled receptors: the PAC_1 receptor and two VPAC receptors (Vaudry et al. 2000). The PAC_1 receptor is selective for PACAP, with VIP being $\sim 1,000$ -fold less effective. In contrast, the VPAC receptors are equally sensitive to VIP and PACAP. Braas et al. (1998) showed that adult guinea pig intracardiac neurons express the PAC_1 receptor. More recently, DeHaven and Cuevas (2002) reported that dissociated neonatal rat intracardiac neurons can express both PAC_1 and VPAC receptors. Based on the electrophysiological results, Braas et al. (1998) postulated that the PACAP-induced increase in excitability in adult guinea pig intracardiac neurons is mediated by PAC_1 receptors because PACAP was more effective than VIP. In addition, recent studies of other PACAP actions on guinea pig intracardiac neurons consistently suggest that these effects also are mediated through activation of PAC_1 receptors rather than VPAC receptors. For instance, PACAP decreases somatostatin expression (Braas et al. 2004) and enhances I_h (Merriam et al. 2004). VIP was considerably less effective than PACAP in both studies. In rat neonatal intracardiac neurons, both VIP and PACAP initiate a rise in $[\text{Ca}^{2+}]_i$, an effect mediated through VPAC receptors (DeHaven and Cuevas 2004). In this study, neither PACAP nor VIP elicited a rise in $[\text{Ca}^{2+}]_i$, an observation consistent with the absence of VPAC receptor expression in guinea pig intracardiac neurons. Consequently, we tentatively suggest that adult guinea pig intracardiac neurons do not express VPAC receptors.

In conclusion, PACAP increases excitability in adult guinea pig intracardiac neurons and neonatal rat intracardiac neurons (Braas et al. 1998; DeHaven and Cuevas 2004). In both species, the change in excitability occurs in both phasic and tonic type neurons. However, some mechanisms responsible for the increased excitability differ in the intracardiac neurons from these two species. In dissociated rat neonatal intracardiac neurons, a synergistic activation of PAC_1 receptors and of VPAC receptors coupled with a rise in $[\text{Ca}^{2+}]_i$ is required for the enhanced excitability (DeHaven and Cuevas 2004). In contrast, in adult guinea pig intracardiac neurons, the enhanced excitability requires activation of PAC_1 receptors alone, followed by Ca^{2+} influx, presumably through a receptor-operated channel, but does not require Ca^{2+} release from intracellular stores. Comparison of results from this study and those of DeHaven and Cuevas (2004) showed that the mechanisms of action of specific neuropeptides, such as PACAP, can be quite different in the same class of neurons from different species and/or different ages. Furthermore, comparison of results in these two studies raises the question of whether the differences in neuropeptide effect reflect differences between receptor expression in guinea pig and rat intracardiac neurons or differences between receptor expression in intracardiac neurons from neonatal versus adult animals. In either case, the results show that mechanisms responsible for comparable effects of a given peptide may be species- and/or age-dependent.

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