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Pretreatment with Nonselective Cationic Channel Inhibitors Blunts the PACAP-Induced Increase in Guinea Pig Cardiac Neuron Excitability

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Abstract

Calcium influx is required for the pituitary adenylyl cyclase activating polypeptide (PACAP)-induced increase in guinea pig cardiac neuron excitability, noted as a change from a phasic to multiple action potential firing pattern. Intracellular recordings indicated that pretreatment with the nonselective cationic channel inhibitors, 2-aminoethoxydiphenylborate (2-APB), 1-[β -(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl]-1H-imidazole HCl (SKF 96365), and flufenamic acid (FFA) reduced the 20-nM PACAP-induced excitability increase. Additional experiments tested whether 2-APB, FFA, and SKF 96365 could suppress the increase in excitability by PACAP once it had developed. The increased action potential firing remained following application of 2-APB but was diminished by FFA. SKF 96365 transiently depressed the PACAP-induced excitability increase. A decrease and recovery of action potential amplitude paralleled the excitability shift. Since semiquantitative PCR indicated that cardiac neurons express TRPC subunit transcripts, we hypothesize that PACAP activates calcium-permeable, nonselective cationic channels, which possibly are members of the TRPC family. Our results are consistent with calcium influx being required for the initiation of the PACAP-induced increase in excitability, but suggest that it may not be required to sustain the peptide effect. The present results also demonstrate that non-selective cationic channel inhibitors could have other actions, which might contribute to the inhibition of the PACAP-induced excitability increase.

Keywords

Parasympathetic neurons; Neuropeptide; TRPC channels; Semiquantitative PCR; Calcium influx

Introduction

The neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), is a pluripotent peptide that has marked trophic roles in development, cell survival, and regeneration as well as being a key signaling molecule modulating neuroendocrine and neuronal function (Vaudry et al. 2009). PACAP is present in neurons within the central and peripheral nervous systems (Vaudry et al. 2009). Our laboratory determined that PACAP is co-localized with acetylcholine in essentially all parasympathetic preganglionic nerve terminals innervating neurons in the guinea pig intrinsic cardiac nervous system (Braas et al.

1998; Calupca et al. 2000; Parsons et al. 2006). In addition, neurally evoked or exogenous application of PACAP depolarizes and increases excitability of the cardiac neurons via activation of the PACAP selective PAC1 receptor (Braas et al. 1998; Tompkins et al. 2007; Hoover et al. 2009).

Our previous results demonstrated that calcium influx, but not calcium release from caffeine/ryanodine-sensitive internal stores, is a requisite for the PACAP-induced increase in guinea pig cardiac neuron excitability (Tompkins et al. 2006). Previously, we did not identify the PACAP-activated calcium influx pathway. A PACAP activation of voltage-dependent calcium channels (VDCCs) is unlikely as PACAP suppresses whole cell barium currents flowing through VDCCs (Tompkins et al. 2006). In addition, bath application of PACAP significantly shortened the duration of calcium action potentials in neurons within whole mount cardiac ganglia preparations (Merriam and Parsons, unpublished observations), a result consistent with the whole cell recordings of barium currents from dissociated cardiac neurons.

PACAP has been suggested to activate nifedipine-sensitive calcium channels (Chatterjee et al. 1996). However, we have also shown that pretreatment with nifedipine does not block the PACAP-induced increase in excitability (Tompkins et al. 2006).

We hypothesized that the calcium influx pathway activated by PACAP might be a nonselective cationic channel, perhaps a member of the TRPC channel family (Clapham et al. 2001). TRPC channels are known to be activated by G-protein coupled receptors and can be calcium-permeable (Clapham et al. 2001; Strübing et al. 2001). Thus, in the present study, we used semiquantitative PCR to determine whether the guinea pig cardiac neurons contain transcripts for TRPC subunits. We also tested whether three different putative nonselective cationic channel blockers (2-amino-ethoxydiphenylborate (2-APB), 1-[β -[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl]-1H-imidazole HCl (SKF 96365), and flufenamic acid (FFA)) suppressed the PACAP-induced increase in excitability.

Materials and Methods

Animals

Guinea Pigs—All electrophysiological experiments and laser capture of cardiac neurons for PCR were completed in vitro on atrial whole mount preparations containing the intrinsic cardiac ganglia from Hartley guinea pigs (either sex; 250–350 g). Guinea pigs were killed by isoflurane overdose followed by exsanguination. 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's solution (in millimolars: 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.

All animal protocols were approved by the University of Vermont Institutional Animal Care and Use Committee and methods described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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's solution also containing 10 mM HEPES buffer (Braas et al. 1998; Tompkins et al. 2006, 2007, 2009; Tompkins and Parsons 2008). The experiments were done with the bathing solution maintained between 32–35°C. 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; 60–120 M Ω). Resting membrane potential and action potentials were recorded from the impaled neurons using an Axoclamp-2A amplifier coupled with a Digidata 1322A data acquisition system and pCLAMP 8 software (Molecular Devices, Sunnyvale, CA). 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 the 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, 1,000 ms) were applied to characterize neuron excitability. 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 three classes of cardiac neurons. This reflects the PACAP-induced increase in excitability. In this study as in the past for statistical analysis, we have grouped the cardiac neurons in the different experimental conditions into just two firing patterns: “phasic” and “multiple firing”. Cells termed phasic fired four or fewer action potentials with increasing intensity of the 1,000 ms current pulses up to 0.5 nA. Multiple firing cells fired five or more spikes with the same increasing stimulus protocol. Multiple firing cells included bursting (fast accommodating cells) as well as tonic cells (cells with action potentials generated over the duration of the depolarization) if the number of action potentials produced was five or greater. For each cell, an excitability curve was constructed by plotting the number of action potentials generated by increasing stimulus intensities.

We also used 500 ms hyperpolarizing constant current pulses of increasing amplitude to assess the presence of rectification in the voltage response. This rectification commonly is used to demonstrate the activation of the inward current (I_h) flowing through hyperpolarization-activated nonselective cationic channels (Edwards et al. 1995; Merriam et al. 2004).

Laser Capture of Cardiac Neuron Clusters and Semiquantitative Polymerase Chain Reaction

Transcript levels for TRPC channels were determined either from extracts of cardiac ganglion whole mounts or from laser-captured cardiac ganglia. Cardiac ganglia whole mounts were dissected under RNase free conditions. For the laser capture technique, clusters of neurons (ganglia) were identified visually under $\times 10$ magnification within fresh (not fixed) whole mounts; the neurons were removed by laser dissection using a Zeiss P.A.L.M. microlaser dissection system (P.A.L.M. Microlaser Technologies, Thornwood, NY), catapulted into tubes, and immediately frozen on dry ice. Extracts were collected from a total of 71 ganglia from 10 cardiac ganglia whole mounts. The samples were consolidated and prepared for analysis of TRPC subunit transcript levels. RNA was extracted following manufacturer instructions with RNeasy Micro Kit (Qiagen, CA, USA). RNA quantity and quality were assessed on an Agilent 2100 bioanalyzer in the Vermont Cancer Center DNA Analysis Facility.

Whole mount extracts and laser-captured neuron (LCN) samples were subjected to Sybr Green melting curve analysis to assess the presence of a single amplification product with the same melting temperature as the positive control. With the TRPC subunit transcripts, when amplification for product was present, the melting point curve had a single peak. Each product was sequenced to confirm guinea pig gene specificity. Extracts of guinea pig brain served as a positive control. All samples were run on an ethidium bromide gel.

Amplification of the cDNA templates was performed using HotStart IT[®] SYBR[®] Green qPCR Master Mix (USB). The primers used to determine the presence of TRPC transcripts were those used previously to test for TRPC transcripts in extracts of guinea pig stellate ganglia (Merriam et al. 2010).

Chemicals

PACAP27 was used exclusively in this study and is referred to as PACAP throughout the text. All drugs were obtained from commercial sources: PACAP27 (American Peptide Co., Sunnyvale, CA); 2-APB, SKF 96365 (Calbiochem, EMD Biosciences; San Diego, CA), and FFA (Sigma-Aldrich, St. Louis, MO). All drugs were applied directly to the bath solution from frozen concentrated stocks prepared in either DMSO (2-APB, FFA) or water (PACAP, SKF 96365). The concentration of DMSO in the bath solution never exceeded 0.1 %.

Statistical Evaluation

Statistics were performed using GraphPad Prism statistical software (version 5.4; La Jolla, CA). Differences among conditions that produced either phasic or multiple firing responses were tested using the Fisher's exact test. The averaged number of action potentials produced during depolarizing step pulses was compared among conditions using either an unpaired *t* test, paired *t* test, or one-way ANOVA. Differences between means were considered statistically significant if $P < 0.05$.

Results

Cardiac Neurons Express Transcripts for Multiple TRPC Channel Subunits

G-protein receptors can activate TRPC channels, which form nonselective cationic channels, many of which are calcium-permeable (Clapham et al. 2001). Previously, we demonstrated that PACAP activation of the PAC1 receptor in cultured neonatal rat superior cervical ganglia (SCG) neurons lead to membrane depolarization (Beaudet et al. 2000). The depolarization was due at least in part to the activation of a nonselective cationic current. Also, it was shown that the rat sympathetic neurons expressed transcripts for members of the TRPC family. As PACAP can also depolarize guinea pig cardiac neurons (Braas et al. 1998; Tompkins et al. 2007; Hoover et al. 2009), we determined whether guinea pig cardiac neurons expressed transcripts for TRPC channel subunits. Initially, we analyzed extracts from cardiac ganglia whole mounts. Using semiquantitative PCR, we found that transcripts were present for TRPC 1, 3, 4, 5, and 6 subunits (Fig. 1a). Extracts of whole guinea pig brain were used as a positive control.

However, because the whole mount preparation also contains cardiac muscle, vascular smooth muscle, and connective tissue, we laser-captured clusters of neurons within individual ganglia and immediately froze them for subsequent semiquantitative PCR analysis. Transcripts for TRPC 1, 3, 4, and 5, but not TRPC 6, were evident in extracts of laser-captured cardiac ganglia neurons (Fig. 1b).

Pretreatment with Putative Nonselective Cationic Channel Inhibitors Suppresses the PACAP-Induced Increase in Excitability

In initial experiments, intracellular voltage recordings were made to quantify excitability of neurons in whole mount cardiac ganglia preparations before and following bath application of 20 nM PACAP. Ninety-three percent of the cardiac neurons in the absence of PACAP exhibited a phasic firing pattern (four or fewer action potentials) during 1,000 ms, suprathreshold depolarizing current pulses. In 7 % of the neurons, long depolarizing pulses elicited multiple firing (five or more action potentials). The presence of PACAP significantly increased cardiac neuron excitability. In these experiments, initial recordings

were made from a cell in control solution and then from the same cell during exposure to PACAP. Recordings were then made from other randomly selected neurons in the same whole mount preparation over a 60-min period with the bath solution containing 20 nM PACAP. During bath application of 20 nM PACAP over the 60-min period, 92 % of the cardiac neurons exhibited a multiple firing pattern (Fig. 2b). The shift in

The next experiments tested whether treatment with putative nonselective cationic channel blockers affected the ability of PACAP to increase cardiac neuron excitability. Three different drugs, FFA, 2-APB, and SKF 96365, were tested because none of these putative nonselective channel blockers is specific (Yan et al. 2009). Each drug was tested at both 10 and 30 μ M. Whole mount cardiac ganglia preparations were pretreated for at least 15 min with the blockers before 20 nM PACAP was added to the bath solution along with the blocker. Before PACAP was applied, there was no noticeable difference in action potential properties and excitability between control cells and cells pretreated with the blockers.

Pretreatment with all three drugs, at either concentration, significantly suppressed the ability of PACAP to increase excitability of the cardiac neurons. Figure 3a shows that fewer cardiac neurons exhibited a multiple firing pattern when exposed to PACAP in the presence of the blockers. 2-ABP and SKF 96365 produced a concentration-dependent inhibition, suppressing the PACAP effect more at 30 μ M than at 10 μ M. The suppression of the PACAP effect by FFA pretreatment was similar at 10 and 30 μ M. The suppression of the PACAP increase in excitability is also evident from the averaged excitability curves shown in Fig. 3b.

Although all three blockers inhibited the PACAP-induced increase in excitability, barium was able to increase excitability in preparations pretreated with these drugs. Figure 3c shows a recording in which excitability was tested in a neuron initially exposed to 30 μ M FFA and 20 nM PACAP and then after switching to a solution containing FFA, PACAP, and 1 mM BaCl₂. The neuron exhibited a phasic firing pattern in 30 μ M FFA and PACAP but shifted to a multiple firing pattern when barium was present. Depolarizing pulses elicited multiple firing when barium was added to the bath solution containing 2-APB or SKF 96365 as well (data not shown).

The Nonselective Cationic Channel Inhibitors Have Distinct Effects When Applied once the PACAP-Induced Increase in Excitability Is Established

Unfortunately, the drugs used in this study are not specific and can have other effects in addition to inhibiting nonselective cationic channels (Jan et al. 1999; Yan et al. 2009; Yau et al. 2010). Consequently, in additional experiments, we evaluated the effect of 2-APB, FFA, or SKF 96365 (all at 30 μ M) when these inhibitors were added following the initiation of the PACAP-induced increase in excitability. In this series of experiments, recordings were obtained from a phasic cell prior to and following addition of 20 nM PACAP. The PACAP-induced increase in excitability was allowed to develop over many minutes. Once a multiple firing pattern was consistently recorded, one of the nonselective cationic channel blockers was added to the PACAP-containing solution. Excitability was tested multiple times with both PACAP and the inhibitor present. Occasionally, the recording was lost just before switching from the solution containing only PACAP to one containing PACAP and the inhibitor. When this happened, recordings were initiated in another PACAP-treated cell that exhibited multiple firing and recordings obtained repeatedly in that cell prior to and following the addition of the inhibitor to the PACAP-containing solution. Results obtained under both recording conditions were identical.

One action of PACAP thought to contribute to the peptide-induced increase in excitability is an enhancement of the hyperpolarization-activated nonselective cationic conductance, I_h

(Merriam et al. 2004; Tompkins et al. 2009). As part of these experiments, we also evaluated whether any of these inhibitors affected the rectification noted in the hyperpolarization produced by long constant current pulses. This rectification is an index of the activation of I_h by hyperpolarization (Edwards et al. 1995; Merriam et al. 2004).

The effect of 30 μ M 2-ABP was tested first. When 2-ABP was added after the PACAP-induced increase in action potential generation was established, the multiple firing pattern and the shift in excitability curve remained (Fig. 4a). Similar results were obtained in a total of three cells.

We tested the effect of FFA next. In contrast to 2-APB, 30 μ M FFA quickly reduced the PACAP-induced increase in action potential generation ($n=3$ cells) (1a and 2a of Fig. 4b). However, the number of action potentials generated remained slightly elevated above that noted prior to the exposure to PACAP (3 of Fig. 4b). There was no noticeable change in the amplitude of action potentials generated during the depolarizing steps before and during exposure to FFA. Also, during exposure to either 2-APB or FFA, the rectification in the hyperpolarization elicited during long current pulses was unchanged indicating neither inhibitor affected I_h (2b of Figs. 4a and 2b of Fig. 4b).

In three neurons, SKF 96365 (30 μ M) produced a transient depression of excitability when applied once the PACAP effect had developed. The PACAP-induced increase in excitability decreased progressively during the exposure to PACAP and SKF 96365, reverting from a multiple firing pattern to phasic firing pattern. The amplitude of the action potential also decreased progressively when excitability was diminished in PACAP and SKF 96365 (Fig. 5). In two cells, the suppression of the PACAP effect gradually reversed so that the number of action potentials generated during the excitability trials progressively increased and the action potential amplitude increased in parallel with the increase in excitability (Fig. 5). In the third cell, the impairment was lost before recovery could occur. Although the action potential amplitude was diminished transiently by SKF 96365, there was no apparent effect on I_h as seen in the rectification evident in the hyperpolarizing responses to current injection (Fig. 5).

Discussion

The key observation of the present study is that the PACAP-induced increase in excitability is essentially eliminated by pretreatment with putative nonselective cationic channel blockers. Because these drugs can have multiple actions on neurons, we used three different blockers, FFA, 2-APB, and SKF96365. Although pretreatment with these blockers essentially eliminated the ability of PACAP to increase cardiac neuron excitability, barium was able to still increase excitability in cardiac neurons pretreated with any of these three drugs. Even though all three blockers can exhibit multiple actions on neurons, the common action shared by all three drugs is a block of nonselective cationic channels at concentrations used in the present study. Therefore, we propose that these results are consistent with PACAP activating calcium influx through nonselective cationic channels, an effect blocked by these drugs.

Exposure to FFA and SKF 96365 reduced the ongoing PACAP-induced increase in excitability. In contrast, application of 2-APB did not affect the PACAP-induced increase in excitability, once initiated. These results confirm that these drugs can have multiple actions, and interpretation of results obtained with them must be made with this in mind. With either 2-APB or FFA, there was no noticeable change in action potential amplitude or the rectification evident during hyperpolarizing steps. FFA has been reported to decrease excitability of pyramidal cells by slowing the recovery of sodium channels from inactivation

(Yau et al. 2010). Although the concentration of FFA used in the study by Yau et al. (2010) was considerably higher than the concentration of FFA used in this study, a similar effect could have contributed to the FFA-induced reduction of the increased excitability in PACAP. Alternatively, the decrease in PACAP effect could have resulted from other undefined actions.

Application of SKF 96365, after the PACAP-induced increase in excitability was initiated, diminished excitability so that action potential generation reverted from a multiple firing to a phasic firing pattern. This switch in firing pattern became evident after a few minutes, and a decrease in action potential amplitude accompanied the decrease in excitability. Both excitability and action potential amplitude recovered with time during continued exposure to both PACAP and SKF 96365. We have not attempted to determine the underlying cause for the transient effect of SKF 96365 on excitability and action potential amplitude. However, prior studies have indicated multiple effects of SKF 96365 on cellular calcium metabolism in addition to blocking nonselective cationic channels (Jan et al. 1999). Some of these actions are transitory. Whether these actions or more direct effects on voltage-gated channels contribute to the change in neuronal excitability remains to be determined.

Because SKF 96365 exerted complex actions on the PACAP-induced increase in excitability once established, interpretation of the results with SKF 96365 pretreatment must be drawn cautiously. Thus, even though the preparations were pretreated with SKF 96365 for much longer than 15 min in most experiments prior to application of PACAP, it is possible in some of the pretreatment experiments that a direct inhibitory effect of SKF 96365 on neuronal excitability contributed to the suppression of the PACAP effect. However, two observations diminish this reservation somewhat. First, action potential properties and neuronal excitability noted for SKF 96365 pretreated cells did not appear different from untreated cells at times when PACAP was applied. Second, barium increased action potential generation in the preparations pretreated with SKF 96365 at times when the PACAP effect was blocked.

A PACAP enhancement of I_h has been suggested to be one mechanism contributing to the peptide-induced increase in cardiac neuron excitability (Merriam et al. 2004; Tompkins et al. 2009). Pretreatment with I_h blockers significantly blunts the PACAP effect on excitability and eliminated the rectification occurring in the hyperpolarizations elicited by long constant current pulses (Tompkins et al. 2009). Because none of the nonselective cationic channel inhibitors used in this study altered the rectification produced by hyperpolarizing current steps (Figs. 4a, b and 5), we suggest that it is unlikely that the effects we observed in this study were related to an inhibition of I_h .

Nonselective cationic channels, composed of members of the TRPC family, are possible calcium influx pathways, as these channels can be activated by G-protein coupled receptors (Clapham et al. 2001). Transcripts for multiple TRPC subunits were present in extracts from the cardiac neurons. We have used laser capture of the neurons from whole mount preparations to minimize contamination by other cell types in the preparation. Detectable levels of transcript were obtained for TRPC 1, 3, 4, and 5, but not TRPC 6. Recently, we found that transcripts for TRPC 1, 3, 4, 5, and 6 were present in extracts of the guinea pig sympathetic stellate ganglia (Merriam et al. 2010). In addition, Beaudet et al. (2000) found previously that transcripts for TRPC 1, 3, and 6 were present in extracts from neonatal cultured rat SCG neurons and rat adult SCG. Thus, the expression of TRPC subunits likely is both species and ganglion specific. Furthermore, the PAC1 receptor in rat sympathetic neurons is coupled to both adenylyl cyclase and phospholipase C whereas the PAC1 receptors on guinea pig cardiac neurons appear to be coupled solely to adenylyl cyclase (Braas and May 1999; Braas et al. 2004; Tompkins et al. 2008). Thus, if the activation of

TRPC channels is responsible for the PACAP-induced depolarization in rat SCG or guinea pig cardiac neurons, the channels activated and mechanism(s) leading to channel activation must be unique to each neuron type.

Previously, using commercially available antibodies directed against TRPC subunits, we noted that virtually all of the cardiac neurons in whole mount preparations were TRPC1 immunoreactive whereas <3 % of the cardiac neurons were immunoreactive for TRPC 3, 4, or 6 (Calupca et al. 2002). The same antibodies were used successfully to demonstrate TRPC subunit distribution in guinea pig enteric neurons (Liu et al. 2008). TRPC6 transcript levels were not detected in the extract from laser-captured cardiac neurons. This could have resulted because only a small subpopulation of cardiac neurons expresses TRPC6, and those neurons expressing TRPC6 might not have been among those neurons collected by the laser capture technique. It is also possible that the cumulative amount of TRPC6 transcript was less than that which our analysis required for detection. Furthermore, RNA derived from cardiac and smooth muscle cells likely contributed to the presence of TRPC6 transcript in the extract from atrial whole mount preparations. Commercial antibodies directed against TRPC5 were not available when our earlier studies were undertaken. Although a commercial antibody directed against TRPC5 is now available, it has given inconsistent results when tested in the guinea pig cardiac ganglia whole mount preparations (Young, Girard and Parsons, unpublished observations). Most commercial TRPC antibodies are directed against mouse or human antigens so that the interpretation of results using these antibodies with guinea pig tissue must be made cautiously. The combination of TRPC 1 and 4, or 5 is known to form a calcium-permeable channel gated by G-protein coupled receptors (Strübing et al. 2001). Given that TRPC 4 immunolabeling appeared to occur in a very small percentage of cardiac neurons, we speculate that a channel composed of TRPC 1 and 5 subunits could be the nonselective cationic, calcium-permeable channel gated by the PACAP/PAC1 receptor interaction. How this channel might be activated following the PACAP/PAC1 receptor interaction in guinea pig cardiac neurons remains to be determined.

Conclusion

We hypothesize that the PACAP/PAC1 receptor interaction activates calcium-permeable, nonselective cationic channels, potentially members of the TRPC family. The activation of TRPC channels allows calcium influx, which is a key step in initiating the PACAP-induced increase in excitability. We also suggest that calcium influx is not required to sustain the PACAP-induced increase in excitability once it is established. Support for the latter conclusion is the observation that 2-APB did not reverse an ongoing PACAP-induced increase in excitability. However, we realize that because two of the drugs, FFA and SKF 96365, did affect the ongoing PACAP increase in excitability, this latter interpretation remains tentative until more selective blockers become available.

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Fig. 1.

Multiple TRPC channel transcripts are expressed by guinea pig cardiac neurons. **a, b** PCR products viewed on an ethidium bromide gel after amplification of freshly dissected cardiac ganglia whole mounts (**a**) or laser capture cells (**b**) and brain cDNA. All reactions were processed on a 7500 ABI fast cyclor using a USB Sybr Green mix. All reactions were run at 53–58°C for 50 cycles. **a** Extracts obtained from cardiac ganglia whole mounts contained transcripts for TRPC 1, 3, 4, 5, and 6. **b** Extracts of laser-captured cardiac neurons had measureable levels of transcripts for TRPC 1, 3, 4, and 5, but not 6. (+) *RTLCN* cDNA from laser-captured neurons. (–) *RTLCN* RNA processing without reverse transcriptase as check for genomic contamination in LCN sample. *NTC* no template control is a negative control assessing the absence of primer dimers or other contaminations excitability occurred within a few minutes when recordings were made from the same cell before and after PACAP was added to the bath solution (Fig. 2a). Figure 2c shows that the averaged excitability curves for cells exposed to 20 nM PACAP are significantly different from those for untreated, control cells.

Fig. 2.

PACAP increases cardiac neuron excitability. **a** Recordings from a cell that show the increase in excitability during exposure to 20 nM PACAP. This cell exhibited a phasic firing pattern prior to the addition of PACAP (1) which shifted to a multiple firing (2) in the presence of PACAP. The amplitude of the 1,000 ms depolarizing current pulse was 0.3 nA. **b** The percentage of cells exhibiting multiple firing in 20 nM PACAP (13 cells) was significantly greater than occurring in untreated cells (13 cells). **c** Averaged excitability curves generated in the cells prior to and during exposure to 20 nM PACAP. The number of action potentials generated at each current step was significantly greater in PACAP-treated cells

Fig. 3.

Pretreatment with putative nonselective cationic channel blockers suppresses the PACAP-induced increase in excitability. **a** Micromolar concentrations of three different blockers, 2-APB, FFA, and SKF 96365 (SKF in figure), decrease the percentage of cells that exhibit multiple firing in 20 nM PACAP. **b** The presence of these blockers prevented the shift in the excitability curve normally recorded in PACAP-treated cells. The number of action potentials generated at current steps 0.2 nA or greater was significantly greater in PACAP-treated cells than in cells exposed to PACAP and each inhibitor at either concentration. **c** Cardiac neuron excitability can be increased by barium in a cell exposed to FFA and PACAP. Left hand recording obtained from a phasic firing cell bathed in 30 μ M FFA and 20 nM PACAP. Right hand trace obtained from the same cell after adding 1 mM BaCl₂ (Ba²⁺) to the bathing solution. In both recordings, action potential generation was initiated by a 1-s, 0.3 nA depolarizing current step

Fig. 4.

FFA, but not 2-APB, reduces the 20 nM PACAP-induced excitability change. **a** (1, 2) Recordings show that 30 μ M 2-APB (2a) does not change PACAP-induced action potential firing elicited by 1-s, 0.3 nA depolarizing current steps (1a) nor the rectification evident in the voltage change elicited by 500 ms, -0.4 nA hyperpolarizing current steps (1b, 2b). **a** (3) Averaged excitability curves from three cells demonstrate the PACAP-induced increase in excitability and that 2-APB does not affect the PACAP-induced increased excitability. **b** (1, 2) Recordings show that 30 μ M FFA (2a) decreases PACAP-induced action potential firing elicited by 1 s, 0.3 nA depolarizing current steps (1a) but has no effect on the rectification evident in the voltage change elicited by a 500-ms, -0.2 nA hyperpolarizing current step (1b) and a -0.15-nA hyperpolarizing current step (2b). **b** (3) Averaged excitability curves from three cells demonstrate the PACAP-induced increase in excitability and that FFA diminishes the PACAP effect. In both **a** (3) and **b** (3), results are plotted as mean $\pm$ SEM from three cells with the error bars commonly obscured by the *symbol*

Fig. 5.

SKF 96365 transiently suppresses the 20 nM PACAP-induced increase in excitability. **a** Record was obtained 6 min in PACAP alone, and **b–d** were obtained 6, 20, and 25 min after the addition of 30 μ M SKF 96365 to the bathing solution containing 20 nM PACAP. Note that in panel B1, there is an initial decrease in number and amplitude of action potentials, which were elicited by 1 s, 0.3 nA depolarizing current steps. With continued exposure to SKF 96365, both begin to recover. Records below the action potential traces (**b2–d2**) show that at similar time points there is no obvious alteration in I_h by SKF 96365 as seen by the rectification present in the hyperpolarizing response to 500 ms, -0.15 nA current steps