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Review Article

Source and Action of Pituitary Adenylate Cyclase-Activating Polypeptide in Guinea Pig Intrinsic Cardiac Ganglia

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Abstract

The mammalian parasympathetic cardiac ganglia form a complex intrinsic cardiac nervous system presumed to contain multiple neuron types and are innervated by extrinsic fibers from multiple sources, each containing specific neurotransmitters and neuropeptides. In the guinea pig, the preganglionic parasympathetic cholinergic fibers contain the neuropeptide, pituitary adenylate cyclase-activating polypeptide (PACAP), and essentially all cardiac neurons express the PACAP selective PAC_1 receptor. Application of exogenous PACAP depolarizes and enhances the excitability of guinea pig cardiac neurons. The mechanism by which PACAP enhances excitability is not established. However, Ca^{2+} influx through PACAP-activated nonselective cation channels is required for the PACAP-induced increase in excitability of guinea pig cardiac neurons. In addition, a PACAP-induced shift in the voltage dependence of activation of the cyclic nucleotide-gated, hyperpolarization-activated current, I_h, most likely participates in the peptide-induced increase in neuronal excitability. The release of endogenous PACAP by repetitive stimulation of preganglionic fibers *in vitro* contributes to the generation of a slow excitatory postsynaptic potential and also can enhance cardiac neuron excitability. We hypothesize that PACAP released during ongoing vagal activity may regulate cardiac neuron excitability *in vivo*. [*Tzu Chi Med J* 2008;20(1):11–18]

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1. Introduction

For many years, the parasympathetic intracardiac ganglia were considered simple relay stations; however, it is now thought that the intracardiac ganglia form an integrative intrinsic cardiac nervous system (ICNS) intimately involved in local control of cardiac function [1–3]. The ICNS is suggested to contain multiple neuron types: postganglionic parasympathetic neurons, which provide an inhibitory drive to cardiac tissues; interneurons, whose axons stay within the intrinsic cardiac nervous system; and afferent neurons, whose axons project to higher cardiovascular centers [1,4–7]. In addition, small intensely fluorescent (SIF) cells are present, often in clusters, within the ganglia. The parasympathetic preganglionic axons originating from neurons in the cardioregulatory brainstem nuclei project to distinct intracardiac

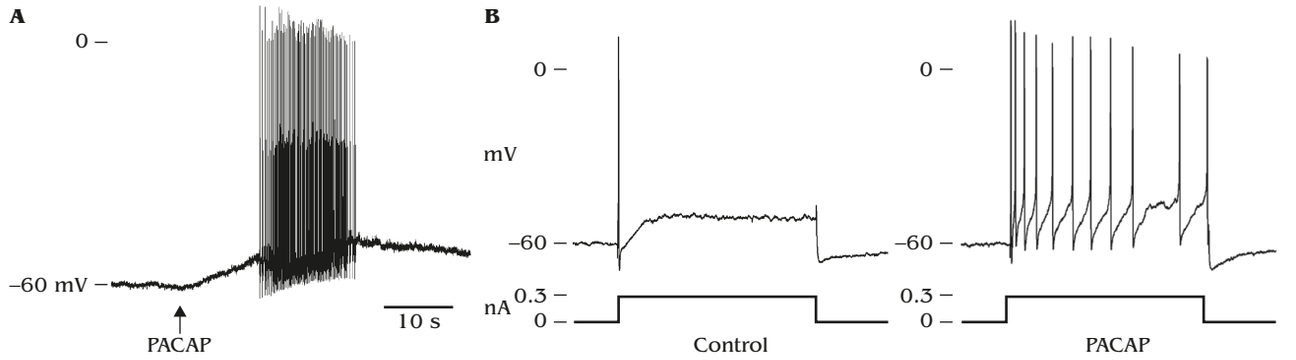

Fig. 1 — PACAP depolarizes and increases excitability of intracardiac neurons. (A) A local 0.5-second application of PACAP produces a slowly developing depolarization, which initiates a burst of action potentials. (B) A 1-second suprathreshold depolarizing current pulse produced one action potential prior to PACAP application. Following PACAP application and after the depolarization had subsided, the same 1-second current pulse initiated multiple action potentials, an indication that excitability had increased.

ganglia, each of which specifically regulate function of the S-A node, A-V node and left and right ventricular myocardium (6,8–13).

Neurons within the ICNS can be innervated by extrinsic nerve fibers containing a variety of neurotransmitters/neuromodulators. In addition to the classical preganglionic parasympathetic cholinergic innervation, individual cardiac ganglia can be innervated by sympathetic postganglionic fibers, and afferent fibers derived from both the spinal dorsal root ganglia and the vagal sensory ganglia (2). The specific neuropeptide or neuromodulator expressed in the different extrinsic nerve fibers can be species-specific.

Over the past 12 years, our laboratory has used a guinea pig atrial whole mount preparation containing the intrinsic cardiac ganglia to investigate the histochemical organization and neuropeptide actions within a mammalian ICNS. The guinea pig intracardiac ganglia preparation was chosen as a model system because in this species, the intrinsic cardiac ganglia form a network of interconnected clusters of neurons on the dorsal surface of the atria, allowing immunohistochemical localization of bioactive substances within nerve fibers and cells, and intracellular recording from intact, innervated cardiac neurons (4,14–25). The neurons can also be dissociated for whole cell voltage clamp recordings (22,24).

As part of our analysis of the chemical coding of the guinea pig ICNS, we determined that fibers immunoreactive for pituitary adenylate cyclase-activating polypeptide (PACAP) innervate virtually every neuron within the guinea pig intrinsic cardiac ganglia (14,15,22). Using immunocytochemistry, Braas et al (14) also found that the intrinsic cardiac neurons express membrane-associated PAC_1 receptor, the PACAP selective receptor. RT-PCR confirmed that the cardiac ganglia express PAC_1 receptor transcripts with the predominant form expressed being the very short variant with neither HIP nor HOP cassettes (14). In a subsequent study using cardiac ganglia preparations that were double-labeled with antiserum against choline acetyltransferase and antiserum against PACAP, Calupca et al (15) showed that, in the guinea pig ICNS, PACAP is present in the cholinergic preganglionic parasympathetic terminals innervating the cardiac neurons. More recently, Richardson et al (26) demonstrated that PACAP is also present in the preganglionic parasympathetic fibers innervating rat intracardiac neurons.

PACAP has been shown to be a potent cardiovascular regulatory neuropeptide through actions both on cardiac tissues and on the intracardiac neurons in multiple species (27–30). In the guinea pig, application of PACAP can elicit bradycardia through stimulation of acetylcholine release from intracardiac neurons (27,29). Evidence supporting a direct action of PACAP on guinea pig intracardiac neurons was demonstrated by Braas et al (14). Using intracellular recordings from individual cardiac neurons in whole mount ganglia preparations, they showed that brief local applications of PACAP depolarized the intracardiac neurons, with the depolarization often initiating a burst of action potentials (Fig. 1A). They also demonstrated that PACAP produced a concentration-dependent increase in cardiac neuron excitability as determined by the number of action potentials produced by a long suprathreshold depolarizing current pulse. Most cardiac neurons in the intact ganglia whole mount are phasic such that they fire only 1–2 spikes regardless of stimulus intensity (Figs. 1B and 2A). However, following PACAP application, the neurons become tonic-like, generating multiple action potentials during an identical depolarizing current pulse (Figs. 1B and 2A). The increase in excitability

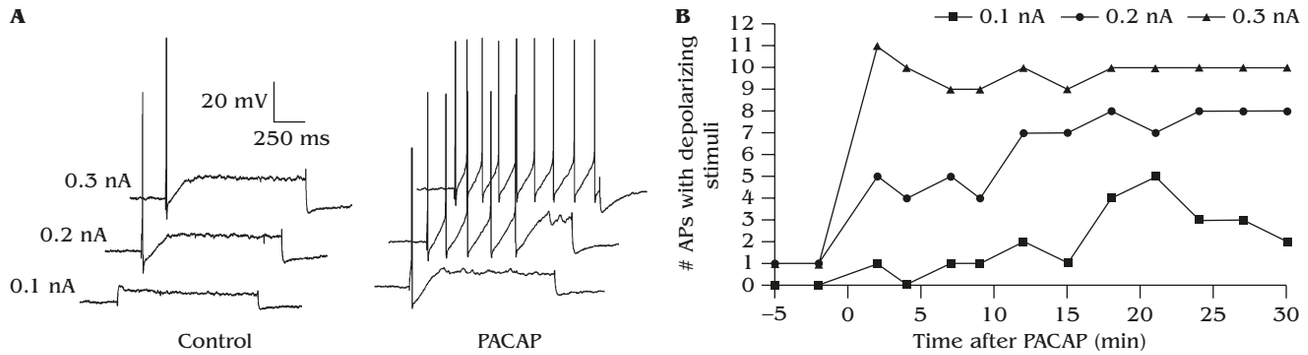

Fig. 2 — PACAP-induced increase in excitability can last for many minutes. (A) The response elicited by three 1-second depolarizing current pulses of increasing strength changes after a 0.5-second local application of PACAP. Prior to PACAP (control), no action potential was elicited by the first current pulse, whereas with the two subsequent pulses, one action potential was produced. Following PACAP (PACAP), one action potential was elicited during the first pulse and multiple action potentials were generated with the two stronger stimuli. (B) The plot shows that the number of action potentials generated by the three current pulses remained elevated for many minutes after the 0.5-second PACAP application.

produced following brief local "puffer" application can be quite long lasting, remaining evident in some cells for tens of minutes (Fig. 2B).

2. PACAP is released by stimulation of the preganglionic parasympathetic fibers innervating the intracardiac neurons

Although PACAP was identified in the parasympathetic preganglionic terminals and effects of exogenous PACAP on cardiac function have been demonstrated in multiple studies, until recently it was not known whether PACAP would be released during stimulation of preganglionic parasympathetic fibers. If PACAP has a physiological role in cardiac regulation, it is important to demonstrate release of the peptide during neural stimulation. In a recent study using intracellular recordings from neurons in the whole mount ganglia preparation, Tompkins et al [23] recorded both fast excitatory postsynaptic potentials (fEPSPs) and slow excitatory postsynaptic potentials (sEPSPs) following repetitive stimulation of interganglionic connectives containing preganglionic parasympathetic fibers [17,31]. Following termination of the sEPSP, cardiac neuron excitability was often enhanced [23]. A similar increase in neuronal excitability could also be elicited when the preparations were treated with hexamethonium and atropine to eliminate cholinergic receptor activation (Fig. 3A). Thus, both the sEPSP and increase in cardiac neuron excitability could be elicited by release of a transmitter other than acetylcholine. As indicated above, in the guinea pig, virtually every intracardiac neuron is innervated by PACAP-IR fibers [14–16,22]. However, in the guinea pig, substance P-IR fibers also innervate some, but not all cardiac ganglia [2], and substance P can depolarize the guinea pig cardiac neurons through activation of NK_3 tachykinin receptors [32]. Tompkins et al [23] confirmed that the intracardiac neurons could be depolarized by application of either PACAP or substance P. However, only PACAP caused an increase in neuronal excitability determined from the number of action potentials generated by long suprathreshold depolarizing current pulses [23]. It was also determined that the amplitude of the sEPSP and the percentage of neurons that exhibited an increase in excitability following repetitive intraganglionic nerve bundle stimulation was significantly reduced when the PAC_1 receptor antagonist PACAP6-38 (500 nM) was present in the bath solution (Fig. 3B). These experiments demonstrated that PACAP released from preganglionic neurons contributes to the generation of sEPSPs and is responsible for the increase in excitability noted following sEPSPs. However, the results did not exclude a possible contribution of substance P released from afferent nerve fibers contributing to the sEPSP recorded in some cells, but did show that release of endogenous substance P would not initiate an increase in excitability [23].

3. Mechanisms contributing to the PACAP-induced increase in excitability

The recent results from Tompkins et al [23] suggest that endogenous PACAP released from parasympathetic preganglionic terminals during neural activity could regulate intracardiac neuron excitability. Thus, additional studies have focused on an elucidation of mechanisms that contribute to the PACAP-induced increase in excitability.

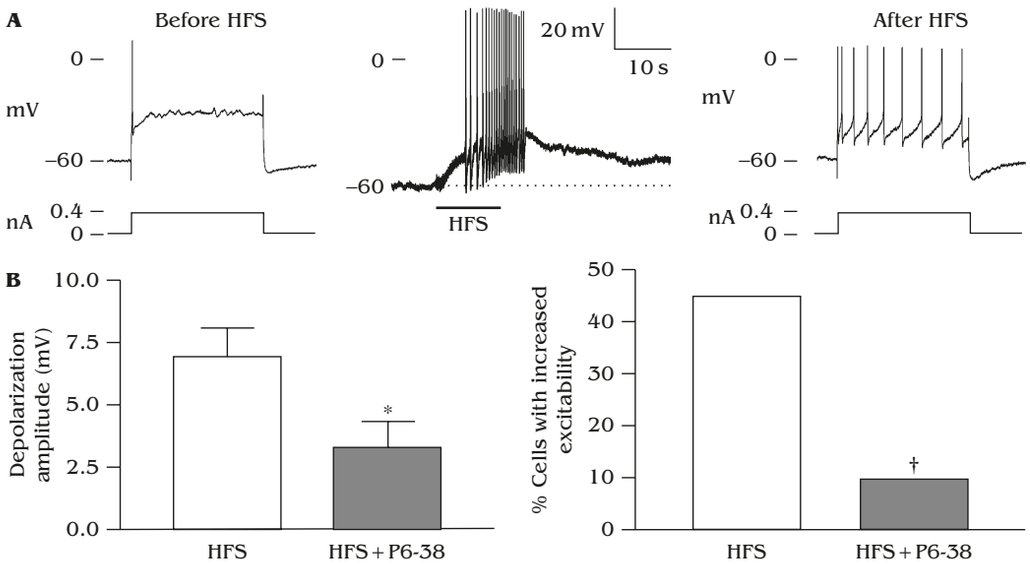

Fig. 3 — High frequency stimulation (HFS) of an interganglionic nerve connective can elicit a non-cholinergic-mediated sEPSP and an increase in cardiac neuron excitability. (A) In the presence of hexamethonium (1 mM) and atropine (1 μM), HFS elicited a sEPSP (the center recording), which in this cell was large enough to elicit a burst of action potentials. The recording on the left shows that prior to HFS, the neuron fired one spike when stimulated by a 1-second suprathreshold current pulse. The recording on the right shows that following HFS, and after the sEPSP had ended, the same depolarizing current pulse elicited multiple action potentials. (B) The bar graph shows that both the sEPSP amplitude and number of cells exhibiting HFS-induced increase in excitability was significantly reduced in preparations treated with the PAC_1 receptor antagonist PACAP6-38 (P6-38). *$p<0.05$, Student's t test; †$p<0.05$, χ^2 test.

3.1. Activation of PAC_1 receptors is sufficient for the PACAP-induced increase in excitability in guinea pig intracardiac neurons

Using intracellular recordings from cardiac neurons, PACAP, but not vasoactive intestinal peptide (VIP), significantly increased cardiac neuron excitability [14]. Furthermore, local application of the PAC_1 receptor selective agonist maxidilan can produce an increase in excitability similar to that produced by PACAP (Tompkins and Parsons, unpublished observations). Thus, in the case of the guinea pig intracardiac neurons, the PACAP-induced increase in excitability apparently only requires activation of PAC_1 receptors. In contrast, the PACAP-induced increase in excitability reported for rat neonatal intracardiac neurons requires a synergistic activation of both PAC_1 and VPAC receptors [33].

3.2. Ca^{2+} influx, but not Ca^{2+} release from internal stores, is required for the PACAP-induced increase in excitability in guinea pig intracardiac neurons

Other recent studies have investigated what ionic conductances contribute to the PACAP-induced increase in excitability. In a recent study, Tompkins et al [24] investigated a potential role of Ca^{2+} in the PACAP-induced increase in excitability. Using intracellular recordings, it was found that the PACAP-induced increase in excitability was eliminated when Ca^{2+} was replaced by Mg^{2+} in the extracellular solution (Figs. 4A and 4C). In contrast, when strontium was substituted for Ca^{2+} in the bath solution, the PACAP-induced increase in excitability remained. Addition of 200 μM cadmium to the extracellular solution significantly suppressed the PACAP-induced increase in excitability (Fig. 4E). These results indicated that Ca^{2+} influx might be a key step in the PACAP-induced increase in excitability.

To establish whether PACAP might facilitate Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs), voltage-activated currents carried by barium (I_{Ba}) through VDCCs were measured in dissociated guinea pig cardiac neurons before and during PACAP application [24]. PACAP significantly decreased I_{Ba}, suggesting that PACAP most likely did not enhance Ca^{2+} influx through VDCCs. However, PACAP has been reported to facilitate Ca^{2+} currents flowing through the L-type VDCCs [34,35]. Given that the L-type VDCC contributes a minor component of the total voltage-activated Ca^{2+} current in intracardiac neurons, it was considered possible that PACAP could selectively enhance Ca^{2+} currents through the L-type channels while inhibiting Ca^{2+} currents through the N-type channels with the overall effect being a decrease in total

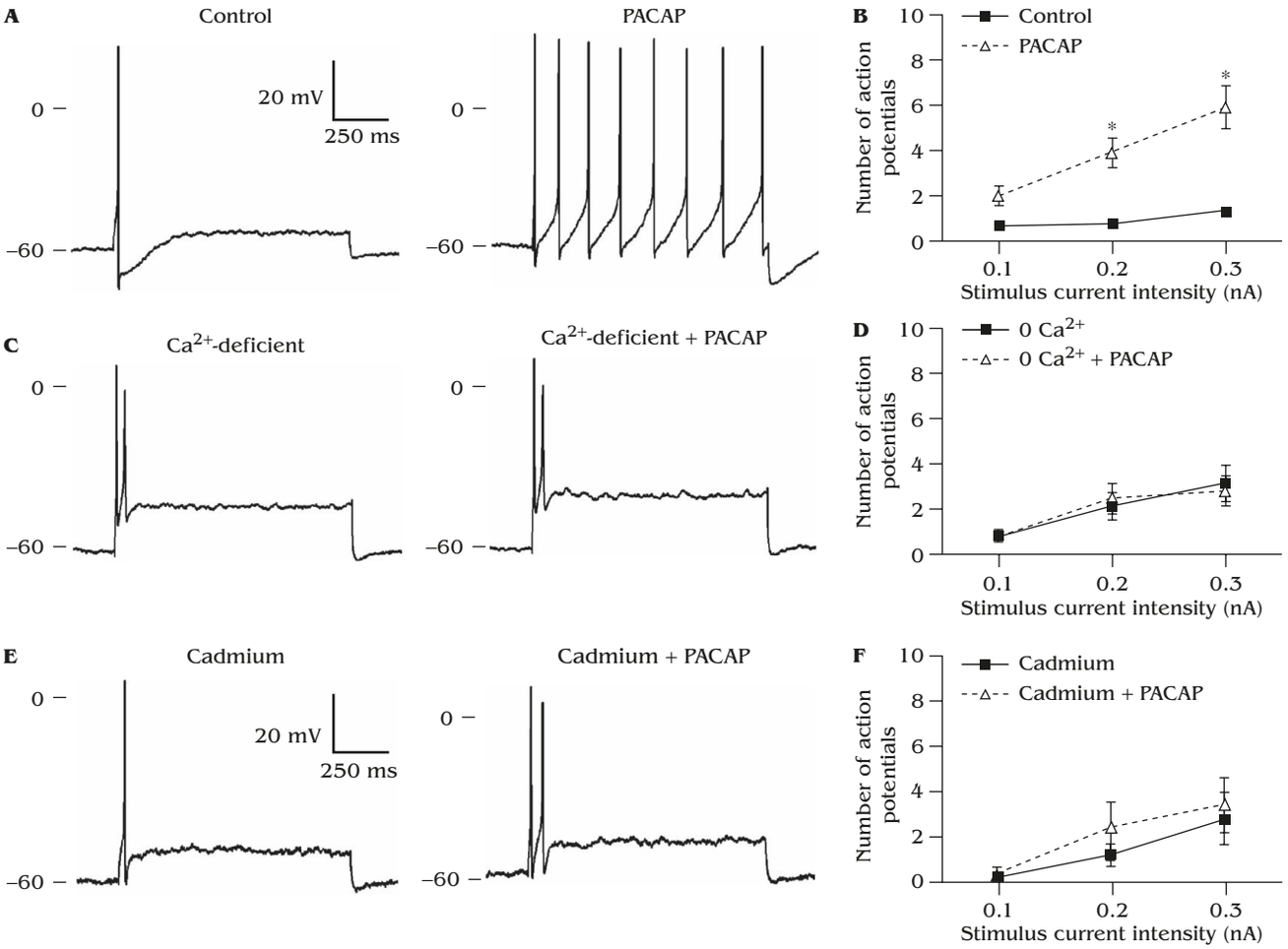

Fig. 4 — PACAP-induced increase in excitability was suppressed by substitution of Mg^{2+} for Ca^{2+} or addition of Cd^{2+} to the bath solution. (A) Membrane potential recording from a phasic intracardiac neuron in response to a long duration depolarizing current pulse (0.3 nA, 1 second) before (*left panel*) and after PACAP (*right panel*). The PACAP-induced increase in neuronal excitability is evident by the increased spike number in response to the depolarizing stimulus. (B) Excitability curve obtained prior to and following puffer application of PACAP under control conditions (*$p<0.05$, $n=9$ cells). (C) In a Ca^{2+}-deficient solution in which Mg^{2+} was substituted for Ca^{2+}, neuronal excitability was similar before (*left panel;* record illustrates response to a 1-second, 0.2 nA depolarizing step) and following PACAP application (*right panel*). (D) Averaged results for 6 cells. (E) Membrane potential recorded in response to a 1-second, 0.2-nA depolarizing current step in the same cell immediately before and after PACAP application in the presence of 200 μM Cd^{2+}. (F) The excitability curve summarized for data from 5 cells. Scale bar is the same for all records. Used with permission from the *Journal of Neurophysiology* [24].

VDCC. If a PACAP-induced influx of Ca^{2+} required for the increase in excitability occurred through L-type channels, then treatment with an L-type VDCC blocker should suppress the PACAP-induced increase in excitability. However, when whole mount cardiac ganglia preparations were treated with 10 μM nifedipine to block L-type Ca^{2+} channels, PACAP still produced an increase in excitability [24].

DeHaven and Cuevas [33] determined for dissociated rat neonatal cardiac neurons that a PACAP-induced release of Ca^{2+} from ryanodine-sensitive intracellular stores was essential for the PACAP-induced increase in excitability. This rise in intracellular Ca^{2+} was mediated by activation of VPAC receptors. Therefore, Tompkins et al [24] tested whether PACAP also initiated a rise in intracellular Ca^{2+} in dissociated guinea pig intracardiac neurons. From measurements of global Ca^{2+} with the Ca^{2+}-sensitive dye fluo-3, it was determined that neither PACAP nor VIP produced a measurable rise in intracellular Ca^{2+}. In contrast, caffeine did cause a rise in intracellular Ca^{2+}, demonstrating that the cells were sufficiently loaded with dye and contained releasable intracellular Ca^{2+} stores. These observations strongly suggested that a PACAP-induced elevation of intracellular Ca^{2+} by release from internal stores was not involved in the PACAP-induced increase in excitability. This conclusion was confirmed from intracellular recordings

which demonstrated that the PACAP-induced increase in excitability remained after intracellular Ca^{2+} stores were depleted by pretreatment with thapsigargin and caffeine [24].

Based on the results of this study, Tompkins et al [24] hypothesized that for guinea pig intracardiac neurons, the PACAP-induced increase in cardiac neuron excitability requires Ca^{2+} influx, but not Ca^{2+} release from internal stores. Although the PACAP-activated conductance pathway allowing Ca^{2+} influx has not been identified as yet, it was postulated that PACAP activates a nonselective cation conductance, perhaps a transient receptor potential-like channel, which is permeable to Ca^{2+} as well as monovalent cations. It was further proposed that a local rise in Ca^{2+} within local domains on the inner side of the plasma membrane is a cofactor in the initiation of the increased excitability.

3.3. *A PACAP-induced shift in the voltage dependence of activation of the hyperpolarization-activated current I_h may contribute to the peptide-induced increase in excitability*

PACAP could increase intracardiac neuron excitability by affecting a number of membrane ionic conductances. Previous studies have indicated that the increase in excitability is not simply due to the relatively small depolarization, a change in action potential configuration or inhibition of the voltage-dependent non-inactivating K^+ conductance, I_M [14,36]. Mammalian intracardiac neurons express the cyclic nucleotide-gated, hyperpolarization-activated nonselective cation conductance, I_h [4,37–39]. Because activation of the PAC_1 receptor commonly stimulates adenylyl cyclase and subsequent generation of cAMP, Merriam et al [40] investigated whether a PACAP-enhanced activation of I_h could contribute to the peptide-induced increase in excitability. To test this possibility, the effect of PACAP on hyperpolarization-activated currents was studied in dissociated cardiac neurons using the perforated patch configuration of the whole cell voltage clamp technique [40]. In initial experiments, using the current clamp recording mode, 100 nM PACAP increased the rectification of the hyperpolarization produced by 500-millisecond current pulses and increased the number of anodal break action potentials. Both results were indicative of a PACAP-enhanced I_h. In subsequent experiments, it was determined for the dissociated guinea pig intracardiac neurons that hyperpolarizing steps to voltages between −70 and −130 mV from a holding potential of −50 mV elicited cesium-sensitive, time-dependent inward currents reminiscent of I_h in other neurons. PACAP enhanced I_h and also increased the holding current, an observation consistent with a PACAP-induced depolarization. From measurements of tail currents, it was apparent that PACAP produced a significant positive shift in the voltage-dependence of activation. This effect was evident with concentrations of PACAP from 1 nM to 100 nM. In contrast to PACAP, VIP (100 nM) did not produce a significant change in I_h. To provide additional evidence that PACAP was indeed enhancing I_h, subsequent experiments determined that pretreatment with the I_h blocker ZD7288 eliminated the PACAP effect on the hyperpolarization-activated currents, but did not block the PACAP-induced change in holding current. To support the view that the PACAP effect on I_h was mediated by activation of adenylyl cyclase and generation of cAMP, additional experiments demonstrated that 10 μM forskolin, a potent activator of adenylyl cyclase, affected the hyperpolarization-activated currents in a manner similar to PACAP.

In sum, the results obtained by Merriam et al [40] demonstrate that a PACAP-induced change in I_h could contribute to the peptide-induced increase in guinea pig intracardiac neuronal excitability. Furthermore, given the difference in effectiveness between PACAP and VIP, we suggest that the PACAP effect on I_h in the cardiac neurons is mediated by PAC_1 receptor activation of adenylyl cyclase and generation of cAMP.

3.4. *Prolonged exposure to low concentrations of PACAP produces a sustained increase in excitability*

The recent results of Tompkins et al [24] provide strong evidence that PACAP can be released *in vitro* during repetitive stimulation of the vagal preganglionic parasympathetic fibers. As cardiac preganglionic neurons are tonically active *in vivo* with the frequency of action potentials dependent on respiratory cycle and environmental demands, we postulated that PACAP may be continually released *in vivo* during preganglionic activity. Under these conditions, it is hypothesized that low concentrations might be present within the cardiac ganglia *in vivo* and that exposure to PACAP may be a factor regulating intracardiac neuron excitability. Therefore, experiments were completed to test whether during prolonged exposure to low concentrations of PACAP, an increase in neuronal excitability was sustained. Intracellular recordings were obtained from neurons in the cardiac ganglia preparation bathed in 1 nM PACAP for up to 3 hours. During prolonged exposure to PACAP, two thirds of the intracardiac neurons exhibited a tonic-like firing pattern, determined from the response to long suprathreshold depolarizing current pulses [23]. This tonic-like firing pattern is

quite different from the phasic firing pattern seen in more than 90% of untreated intracardiac neurons maintained in an organ bath for comparable periods of time. We hypothesize that if endogenous PACAP is released constitutively from vagal terminals during preganglionic firing, the presence of a low level of PACAP could be a factor contributing to the regulation of cardiac neuron excitability.

4. Conclusion

Neurons within the ICNS exhibit diverse functional properties and can be innervated by extrinsic inputs from multiple sources, each fiber projection using specific neurotransmitters. In this review, results demonstrate the presence and illustrate actions of the neuropeptide PACAP within the guinea pig ICNS. We hypothesize that endogenous PACAP, which is released during ongoing action potential firing, may contribute *in vivo* to the regulation of intracardiac neuron excitability.

Acknowledgments

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