Actions of Vasoactive Intestinal Peptide and Neuropeptide Y on the Pacemaker Current in Canine Purkinje Fibers

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We have investigated the actions of vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) on the pacemaker current (I_p) in canine Purkinje fibers. On voltage pulses to the middle of the I_p activation range, VIP reversibly increases I_p, whereas NPY reversibly decreases I_p. A three-pulse voltage protocol suggests that VIP shifts I_p activation in the positive direction and that NPY shifts I_p activation in the negative direction on the voltage axis without changing maximal I_p conductance. These effects of VIP and NPY on I_p are exerted through their specific peptide receptors, since the effects are blocked by VIP and NPY receptor antagonists. VIP and NPY are colocalized in cardiac parasympathetic and sympathetic nerve endings, respectively, and can be released preferentially on high and long-lasting nerve stimulation. Given this colocalization and frequency-dependent release, these results suggest a role for these neuropeptides in controlling cardiac I_p, and consequently heart rate. (Circ Res. 1994;74:157-162.)

Key Words • neuropeptide Y • vasoactive intestinal peptide • pacemaker current • Purkinje fiber

The actions of the autonomic transmitters on the pacemaker current (I_p) have been studied in some detail.1 β-Adrenergic stimulation increases I_p in both the sinus node and Purkinje fibers.2-4 This alteration in I_p contributes to the increase in cardiac rate induced by sympathetic stimulation. Acetylcholine reduces I_p in sinus node cells5 and reverses the action of β-agonists on I_p in Purkinje fibers.6 This action of acetylcholine on I_p contributes to the vagal-induced reduction in cardiac rate. Therefore, normal cardiac rate and rhythm are determined by a balance of sympathetic and parasympathetic stimulation.

In addition to the classic neurotransmitters, there also exist small biologically active peptides in both the central and peripheral nervous systems.7 Accumulating evidence supports the existence of some of these peptides in nerve endings of the cardiovascular system and suggests that they might play an important transmitter role for cardiac function. However, little is known about the influence of these peptides on ionic currents in the heart. In the present study, we report for the first time the effect of two such peptides on cardiac I_p in Purkinje fibers. These two peptides, vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY), are colocalized with neurotransmitters in the nerve endings of the heart8 and have their specific receptors in cardiac tissue.9,10

Although the substantial physiological effects of VIP and NPY have interested many investigators, information regarding the action of these peptides on cardiac ionic currents is largely lacking. The purpose of the present study was to determine whether VIP and NPY have any effect on I_p in canine Purkinje fibers and, consequently, to evaluate the potential role of these peptides in the regulation of cardiac ventricular rate.

Materials and Methods

The two-microelectrode voltage-clamp technique was applied to canine Purkinje fibers of narrow radius (<0.15 mm) and short length (<1.5 mm). The methods for recovering the fibers and the electronic setup were as reported previously by Cohen and Mulrine.11 The control Tyrode's solution contained (mmol/L) NaCl, 140; KCl, 8; CaCl_2, 2; MgCl_2, 1; BaCl_2, 4; MnCl_2, 2; dextrose, 8; and HEPES 5 (buffered to a pH of 7.4). BaCl_2 was added to minimize background K⁺ permeability and the associated [K⁺] fluctuations. NPY (Sigma Chemical Co, St Louis, Mo), NPY fragment 18-36 (Sigma), VIP (Sigma), and [4Cl-d-Phe⁶,Leu⁷]VIP (Sigma) were mixed fresh for each experiment and added to the control solution at the concentrations indicated. The data were collected at temperatures between 33.5°C and 36°C and were constant to 0.5°C within each experiment. The voltage-clamp steps were delivered from a programmable pulse generator. I_p was displayed on an oscilloscope and recorded on an FM tape recorder at 4.76 cm·s⁻¹. The data were also filtered at 50 Hz and photographed by an oscilloscope camera (C-5A, Tektronix). I_p, the time-dependent current recorded in response to a hyperpolarizing voltage-clamp step, was digitized and stored on disk and analyzed with a Northstar Horizon computer. Percentage change in I_p amplitude is reported as mean±SD. Canine Purkinje fibers do not survive prolonged extreme hyperpolarizations well. Continuous application of hyperpolarizing voltage-clamp pulses can result in changes in holding current (see Fig 1, panel IB, and Fig 4C). However, unless these changes in holding current are very large, they should not affect the time-dependent current amplitude that is the subject of the present study. The amplitude of I_p during the course of the experiment was plotted by using the SIGMA-Plot program. As in our previous studies on canine Purkinje fibers,6 a three-pulse protocol was used to obtain information on the voltage dependence of I_p activation.

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Results

Our basic findings on VIP and NPY are illustrated in Fig 1. We first examined the effect of VIP on I, in Purkinje fibers. We used VIP at a concentration of $2 \times 10^{-5}$ mol/L (200 nmol/L). In general, VIP has been shown to exert a positive chronotropic effect within the wide range of $10^{-11}$ to $10^{-6}$ mol/L in intact heart of anesthetized dogs. In response to a hyperpolarizing voltage step from $-60$ to $-100$ mV, VIP at $200$ nmol/L increases the amplitude of $I_{\alpha}$ (Fig 1, panel IA). This is similar to the effect of the $\beta$-adrenergic agonist isoproterenol on $I_{\alpha}$, when we removed VIP from the bathing Tyrode’s solution, $I_{\alpha}$ returned to control levels (panel IB). Panel IC provides the time course of the changes in $I_{\alpha}$ amplitude during the experimental protocol. The same results demonstrating that VIP can increase $I_{\alpha}$ were obtained in six of seven experiments, where VIP increased $I_{\alpha}$ by an average of $35 \pm 17\%$ (n=6).

We also performed experiments to examine the effects of NPY on $I_{\alpha}$. NPY exerts a maximum effect between $10^{-7}$ and $10^{-5}$ mol/L in isolated rat or guinea pig ventricular myocytes. In our experiments, NPY was applied at a concentration of $2 \times 10^{-7}$ mol/L (200 nmol/L). Contrary to the increase in $I_{\alpha}$ caused by VIP, NPY at $200$ nmol/L decreases $I_{\alpha}$ (Fig 1, panel IIA), and on removal of NPY, $I_{\alpha}$ recovers to baseline levels (panel IIB). The time course of this experiment is plotted in panel IIC. Similar results were obtained in a total of five experiments with $200$ nmol/L NPY, where NPY decreased $I_{\alpha}$ by an average of $32 \pm 4\%$ (n=5).

The two peptides VIP and NPY have opposite effects on $I_{\alpha}$. Although the modulation of $I_{\alpha}$ has previously been shown to occur by altering voltage dependence, the increases in $I_{\alpha}$ by VIP could be due to an increase in $I_{\alpha}$ conductance. Similarly, the reduction of $I_{\alpha}$ by NPY could be due to a decrease in maximal $I_{\alpha}$ conductance or an $I_{\alpha}$ channel blockade by this peptide. We further investigated the voltage-dependent activation of $I_{\alpha}$ and its alteration by VIP and NPY. Because of slow relaxations of $I_{\alpha}$ in Purkinje fibers, we used a three-pulse protocol instead of constructing full activation curves. In this protocol, we applied a first hyperpolarizing pulse near the middle of the $I_{\alpha}$ activation range and a second hyperpolarizing pulse toward the top of the activation curve. These two hyperpolarizing pulses were followed by a depolarizing pulse to rapidly deactivate $I_{\alpha}$ before the next cycle of pulses was applied. If VIP increases the pacemaker conductance, $I_{\alpha}$ in response to both voltage steps would be increased. Our results with $200$ nmol/L VIP are shown in Fig 2A. In response to the first hyperpolarizing pulse, the time-dependent current $I_{\alpha}$ is increased; however, in response to the second pulse, $I_{\alpha}$ is reduced by VIP. This result clearly indicates that VIP is not increasing $I_{\alpha}$ conductance but instead shifting the $I_{\alpha}$ activation curve in the positive direction on the voltage axis. Similar results were obtained in two additional Purkinje fibers with the same protocol. The results of the same three-pulse protocol with NPY are shown in Fig 2B. NPY (200 nmol/L) reduces the amplitude of $I_{\alpha}$ in response to the
first pulse and increases $I_v$ in response to the second voltage step. This result argues against a reduction in maximal $I_v$ conductance or an $I_v$ channel blockade by NPY, both of which would decrease the amplitude of $I_v$ in response to both hyperpolarizing steps. Instead, it suggests that NPY shifts $I_v$ in the negative direction on the voltage axis. Similar results were obtained in two additional three-pulse experiments with NPY.

To further characterize the receptor pathway mediating the actions of VIP and NPY, we performed experiments on $I_v$ to test the interaction between these peptides and their receptor antagonists. We used newly synthesized [4Cl-d-Phe$^6$Leu$^7$]VIP, which has been reported to be a competitive antagonist for the VIP receptor in pancreatic preparations.18 We began by adding 200 nmol/L VIP to the control Tyrode’s solution; as in Fig 1, panel IA, VIP increased $I_v$ (Fig 3A). We then applied 200 nmol/L [4Cl-d-Phe$^6$Leu$^7$]VIP to the VIP-containing solutions that returned $I_v$ to roughly control levels (Fig 3B). After washout of the VIP receptor antagonist, VIP alone again increased the amplitude of $I_v$ (Fig 3C). Fig 3D provides the time course of these effects on $I_v$. Similar results with [4Cl-d-Phe$^6$Leu$^7$]VIP were obtained in a total of four experiments, where the antagonist reversed, on average, 98±4% of the VIP effect.

We performed similar experiments with the NPY fragment 18-36, which is a competitive antagonist of NPY in rat cardiac ventricular membranes.19 Using the same approach as in Fig 3, we first demonstrated that NPY alone decreased $I_v$ (Fig 4A). Addition of NPY fragment 18-36 inhibited this NPY action (Fig 4B), and removal of the NPY antagonist from the NPY-containing solution recovered the negative effects of NPY on $I_v$ (Fig 4C). Fig 4D provides the time course of the changes in $I_v$ during this experimental protocol. Similar results with NPY fragment 18-36 were obtained in a total of three experiments, where the antagonist reversed, on average, 96±7% of the decrease in $I_v$ elicited by NPY. Our results with the receptor antagonists for VIP and NPY suggest that the actions of these two peptides on the cardiac $I_v$ are mediated through their unique peptide receptors.

**Discussion**

$I_v$ is a hyperpolarization-activated inward current20 carried by both Na$^+$ and K$^+$ ions.21 It has now become clear that $I_v$ is a significant contributor to cardiac pacing in the sinus node and particularly in Purkinje fibers. The experiments described in the present study were undertaken in an attempt to define the regulatory role of two peptides, VIP and NPY, on cardiac pacemaker activity.

**VIP and NPY May Play a Role in Regulating Cardiac Pacing**

VIP, a 28-amino acid polypeptide, was originally isolated from porcine gut22 and has been recognized as a cotransmitter present in parasympathetic ganglia of the mammalian heart.23 In canine cardiac tissues, immunoreactive VIP has been identified in coronary arteries, sinus node, atioventricular node, atria, and ventricles.24,25 A positive inotropic effect of VIP was initially observed in a canine heart preparation by Said et al.26 Intravenous infusion of VIP is associated with coronary vasodilation and cardiac output elevation in dogs26,27 and humans.28 Furthermore, VIP exerts a positive chronotropic action on the heart. VIP accelerates heart rate and facilitates atioventricular nodal
conduction in both in vitro and in vivo canine preparations. Our present results have demonstrated that VIP increases $I_r$ in cardiac Purkinje fibers (Fig 1, panels IA through IC). This finding is consistent with the positive chronotropic effects of VIP.

NPY is composed of 37 amino acids and was first purified from porcine brain. High levels of NPY have been identified in the postganglionic sympathetic nerves of the cardiovascular system. In isolated perfused rabbit heart, NPY causes coronary vasospasm, decreases coronary blood flow, and inhibits cardiac muscle contraction. NPY also exerts a negative inotropic effect on rat ventricular myocytes and reduces $Ca^{2+}$ current in guinea pig ventricular cells. The effects of NPY on heart rate depend on experimental conditions (see Reference 33 for review). NPY decreases heart rate in intact anesthetized cats and dogs, although these effects are not found in all previous studies. In isolated perfused heart preparations, a small increase or no change in heart rate has also been reported.

Our results have shown that NPY reduces $I_r$ (Fig 1, panels IIA through IIC). This novel finding plus the increase in $I_r$ caused by VIP support the notion that these peptides, along with the classic neurotransmitters, play a role in regulation of the cardiac pacing rate.

Possible Mechanism of Peptide Action on $I_r$

As illustrated in Figs 3 and 4, the actions of VIP and NPY on $I_r$ are mediated by specific peptide receptors. This observation is consistent with the presence of VIP and NPY receptors in canine heart membrane preparations. Like $\beta$-adrenergic receptors, cardiac VIP receptors have been found to be coupled to a membrane-bound adenylate cyclase. In canine right atrial membranes, VIP stimulates cyclase activity with a potency greater than that of isoproterenol. Furthermore, most studies of the mechanism of the effects of VIP suggest that its action might be mediated by an elevation of intracellular cAMP levels. On the other hand, activation of the specific cardiac NPY receptor, named Y1, probably leads to an inhibition of adenylate cyclase and cAMP via a pertussis toxin-sensitive G protein. We have demonstrated previously in Purkinje fibers that elevation of cAMP by isoproterenol and reduction of cAMP by acetylcholine are the necessary prerequisites to $I_r$ regulation by neurotransmitters. Similarly, increases in cAMP increase $Ca^{2+}$ current and shift the activation of the delayed rectifier to more negative potentials (see Reference 1 for a detailed review of this area). Therefore, it is possible that the responses of $I_r$ to VIP and NPY could be mediated through the positive and negative alteration of adenylate cyclase and, consequently, the intracellular cAMP levels.

However, there is also evidence that both VIP and NPY can mobilize intracellular $Ca^{2+}$. These actions are independent of the cAMP-dependent pathway. Increases of $[Ca^{2+}]_i$ can shift $I_r$ activation to more positive voltages. Thus, increases in $[Ca^{2+}]_i$ shift $I_r$ in the opposite direction to explain the effects of NPY, but could contribute to the positive shift induced by VIP. Clearly, the second messenger pathways involved in the effect of VIP and NPY on cardiac $I_r$ will require further investigation.

Cooperation of Classic Neurotransmitters and Peptides in the Regulation of Cardiac Pacing

Traditionally, autonomic cardiac control has been attributed solely to adrenergic (sympathetic) and cholinergic (parasympathetic) neurotransmitters. We have demonstrated that two neuropeptides that are colocalized with classic neurotransmitters in the heart have specific effects on $I_r$. VIP increases and NPY reduces $I_r$. Both of these effects are due to shifts of $I_r$ activation on the voltage axis and are mediated through specific peptide receptors.

It is worth considering whether these novel peptide actions on $I_r$ might be of physiological significance. To answer this question, we first must describe how the neuropeptides are colocalized with neurotransmitters in the sympathetic and parasympathetic nerve endings. As already stated, VIP coexists with acetylcholine in parasympathetic nerve endings, whereas NPY is costored with catecholamines in sympathetic nerve terminals. However, these two types of messenger molecules are distinguished by different subcellular storage sites in nerve endings. The small clear vesicles contain exclusively the classic transmitter: catecholamine in sympathetic and acetylcholine in vagal terminals. The larger dense-core vesicles contain peptides plus classic transmitters. The release of the two types of vesicles can be differentially regulated by the frequency of nerve stimulation. Low-frequency stimulation causes release of small vesicles that mainly store classic neurotransmit-
ters, whereas higher-frequency stimulation induces the release of the larger vesicles containing both peptides and neurotransmitters. This differential regulation of transmitter release may have functional significance. In response to low or normal firing rates, there are small amounts of classic neurotransmitter released. Sympathetically stimulated Iν decreases, whereas vagal activation reduces Iβ to a relatively small degree. Therefore, the pacing rate changes only slightly. However, in response to higher or longer-lasting stimulation, a large amount of neurotransmitter is released. Strong and continuous sympathetic firing causing a large release of catecholamines could cause a tachyarrhythmia. On the other hand, extensive vagal activation, releasing high concentrations of acetylcholine might lead to cardiac standstill. NPY and VIP on Iβ, and potential effects on Iα, do not increase the force of contraction of the isolated canine Purkinje fibre. Circ Res. 1990;66:633-636.


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