Full Paper

Tachykinin Agonists Modulate Cholinergic Neurotransmission at Guinea-Pig Intracardiac Ganglia

Lili Zhang¹, John C. Hancock¹, and Donald B. Hoover^{1,*}

¹Department of Pharmacology, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614-1708, USA

Received April 27, 2005; Accepted September 7, 2005

Abstract. Effects of substance P (SP) and selective tachykinin agonists on neurotransmission at guinea-pig intracardiac ganglia were studied in vitro. Voltage responses of neurons to superfused tachykinins and nerve stimulation were measured using intracellular microelectrodes. Predominant effects of SP (1 μ M) were to cause slow depolarization and enable synaptic transmission at low intensities of nerve stimulation. Augmented response to nerve stimulation occurred with 29 of 40 intracardiac neurons (approx. 73%). SP inhibited synaptic transmission at 23% of intracardiac neurons but also caused slow depolarization. Activation of NK₃ receptors with 100 nM [MePhe⁷]neurokinin B caused slow depolarization, enhanced the response of many intracardiac neurons to low intensity nerve stimulation or local application of acetylcholine, and triggered action potentials independent of other stimuli in 6 of 42 neurons. The NK₁ agonist [Sar⁹,Met(O₂)₁₁]SP had similar actions but was less effective and did not trigger action potentials independently. Neither selective agonist inhibited cholinergic neurotransmission. We conclude that SP can function as a positive or negative neuromodulator at intracardiac ganglion cells, which could be either efferent neurons or interneurons. Potentiation occurs primarily through NK₃ receptors and may enable neuronal responses with less preganglionic nerve activity. Inhibition of neurotransmission by SP is most likely explained by the known blocking action of this peptide at ganglionic nicotine receptors.

Keywords: intracardiac ganglia, cholinergic neuron, ganglionic neurotransmission, neuromodulation, tachykinin

Introduction

Many studies over the last several decades have established that the heart contains an extensive network of ganglia and nerve fibers that comprise the intrinsic cardiac nervous system (1–3). Cholinergic efferent neurons are major components of this system and are recognized for their inhibitory influence on chronotropic, dromotropic, and inotropic functions of the heart. There is also evidence that intracardiac ganglia contain interneurons, sensory neurons and noradrenergic efferent neurons, which adds to the anatomical and functional complexity of the system (1, 2, 4–7). All of these neuronal types (i.e., efferent, sensory, and interneuron) have been identified in guinea-pig intracardiac

ganglia based on electrophysiological and morphological criteria (6, 7). Most neurons in guinea-pig intracardiac ganglia can be labeled with antibodies to choline acetyltransferase, which is a marker of the cholinergic phenotype (3, 4, 8, 9). However, many studies have shown that markers for one or more additional neurotransmitters (e.g., nitric oxide and neuropeptide Y) are present in subpopulations of these neurons (3, 4, 10). Accordingly, the cholinergic phenotype may not dominate in some cells. Preganglionic vagal efferent neurons, however, are an important source of excitatory cholinergic input to the intracardiac ganglia, and a majority of neurons in guinea-pig intracardiac ganglia are surrounded by cholinergic nerve fibers (3, 11). Intracardiac neurons also receive and integrate input from many other nerve fibers that utilize distinct neurotransmitters such as substance P (SP) (1 –

*Corresponding author. FAX: +1-423-439-8773
E-mail: hoover@mail.etsu.edu

3, 10, 12–14).

Studies from our laboratory have shown that SP can cause bradycardia when administered to isolated guinea-pig hearts (15, 16) or anesthetized vagotomized guinea pigs (17). These negative chronotropic responses are blocked by atropine and amplified by pretreatment with a cholinesterase inhibitor, indicating that they are mediated by intracardiac cholinergic neurons. This conclusion is supported by work of other investigators who demonstrated that SP acts directly on intracardiac neurons to cause slow depolarization and increase neuronal excitability (18–20). Since this effect occurs with virtually all intracardiac neurons in whole mount preparations of guinea-pig atrial ganglia, it is likely that efferent neurons and interneurons are sensitive to SP. Moreover, local application of the peptide, by pressure ejection from a micropipette, causes some intracardiac neurons to fire bursts of action potentials that persist for several seconds (18–22). This direct stimulant effect of SP on intracardiac neurons provides an explanation for the negative chronotropic response that SP evokes in isolated hearts and anesthetized guinea pigs.

It has been proposed that SP affects intracardiac neurons primarily through activation of the NK₃ subtype of the tachykinin receptor (19, 20). This conclusion was based on the relative sensitivity of intracardiac neurons to local microejection of selective agonists and the observation that NK₃-receptor blockade with SR 142801 caused a substantial reduction in the amplitude of slow depolarization produced by bath application of SP. It remains possible, however, that NK₁ and NK₂ tachykinin receptors are present in the intracardiac ganglia because we have detected specific binding sites for [¹²⁵I]Bolton-Hunter labeled SP and [¹²⁵I]neurokinin A at this location in guinea pig heart by in vitro receptor autoradiography (15, 23). Furthermore, some degree of bradycardia was evoked in isolated guinea-pig hearts by bolus administration of a selective agonist for each subtype of tachykinin receptor (24).

Previous work from this laboratory demonstrated that SP can augment the fast excitatory response of most intracardiac neurons to microejection of acetylcholine (ACh) in whole mount preparations of guinea-pig atrial ganglionated nerve plexus (22). There were, however, a small number of intracardiac neurons at which SP attenuated ACh evoked responses. Our primary goal in the present study was to determine if SP could also modulate physiological responses evoked by endogenous ACh. For these experiments, cholinergic neurotransmission was activated by stimulation of nerve bundles in whole mount preparations and responses of single neurons within ganglia were recorded using an intracellular microelectrode. We also used selective

NK₁- and NK₃-receptor agonists ([Sar⁹,Met(O₂)₁₁]SP and [MePhe⁷]neurokinin B, respectively) to investigate the neuromodulatory influence of these tachykinin receptor subtypes at the intracardiac ganglia.

Materials and Methods

Experimental preparation

Male Hartley guinea pigs (250–350 g, n = 59) were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital. The heart was rapidly excised and placed into a Krebs buffer (composition: 121 mM NaCl, 25 mM NaHCO₃, 1.2 mM NaH₂PO₄, 5.9 mM KCl, 8 mM dextrose, 1.2 mM MgCl₂, 2.5 mM CaCl₂; equilibrated with 95% O₂–5% CO₂; pH 7.4; 8–10°C). The atria were removed, pinned to the Sylgard floor of a custom recording chamber (2.0 ml), and superfused (5 ml/min) continuously with oxygenated buffer at 10°C. Overlying atrial and vascular tissue were removed by dissection from the endocardial surface to reveal the ganglionated nerve plexus located in epicardial connective tissue and fat (4, 8–10). After completing the dissection, the preparation was allowed to equilibrate for about 1.5 h while the temperature was gradually increased. Conventional intracellular microelectrode recordings were made from intracardiac neurons with the temperature of the superfusate maintained at 34°C by a thermostatically controlled heater (Warner Instruments, Hamden, CT, USA).

Microelectrode recording

Intracellular recordings from neurons were made using high-impedance (70–150 MΩ) borosilicate glass micropipettes filled with filtered 3 M KCl. Transmembrane potentials were recorded in a current clamp configuration using an Axoclamp-2B amplifier (Axon Instruments, Union City, CA, USA). After intracardiac neurons were impaled, they were allowed to stabilize for approx. 3 min before membrane properties were measured. Brief intracellular current pulses, triggered by a Grass S88 stimulator, were given through the recording electrode to characterize neurons (data not shown, see 22). Intracellular current and potential signals were visualized using both analog and digital Tektronix oscilloscopes. Data were acquired and analyzed using a computer equipped with a Digidata 1200 analog-to-digital converter and the pCLAMP software suite version 8.0 (Axon Instruments).

Nerve stimulation

Stimulus trains, triggered by a Grass S88 stimulator, were given through a concentric bipolar electrode (Frederick Hare, Bowdoinham, ME, USA) placed on a

Fig. 1. Low magnification fluorescence photomicrograph of atrial ganglionated nerve plexus showing ganglia (arrows), nerve bundles, and typical sites for placement of stimulating electrodes (concentric circles). Cholinergic neurons and nerve fibers in a whole mount preparation were immunolabeled by routine methods using an antibody to choline acetyltransferase. Two sets of concentric circles on one of the bundles indicate sites where the stimulating electrode (200- μ m diameter) would be placed if recording from a neuron in the ganglion indicated by a large arrow. Scale bar = 200 μ m.

nerve bundle near the impaled neuron (Fig. 1). Our stimulation protocol is illustrated in Fig. 2. An estimate of the stimulation current required to reach threshold (I_t) was obtained by starting with low amplitude rectangular pulses and gradually increasing the current until an action potential was evoked from the recorded neurons or by starting with a higher amplitude pulse and decreasing the current until the stimulation no longer evoked an action potential. Both procedures were performed several times to refine our estimate of I_t . Subthreshold and suprathreshold current levels were set at values slightly below and above the I_t , respectively. Stimulus trains consisted of five pulses at the lower current followed by two pulses at the higher current (Fig. 2: A and B). The lower current pulses did not trigger action potentials or fast excitatory postsynaptic potentials (EPSPs, Fig. 2B). With the higher stimulation current, at least one of the two stimulation pulses in each set evoked an action potential (Fig. 2: B and C). This protocol was designed to detect both potentiation and inhibition of ganglionic neurotransmission by the tachykinins. Nerve stimuli were applied prior to, during, and after exposure to tachykinin agonists, which were added to the superfusion buffer. Stimulus current and intracellular potential signals were visualized and recorded as already described. The duration of each stimulation pulse was 2 ms in experiments with SP but

Fig. 2. Nerve stimulation protocol and representative control responses evoked from impaled intracardiac neurons. Subthreshold (Sub) and suprathreshold (Supra) stimulation current levels were determined as described in Materials and Methods. A: Diagram depicting stimulation protocol. Trains of 5 subthreshold and 2 suprathreshold square pulses (2-ms duration) were delivered throughout the experiment at 10- to 15-s intervals. B: Typical responses to nerve stimulation are shown in this panel. Subthreshold pulses usually produced a stimulation artifact in the impaled neuron but did not cause a synaptically evoked response. Suprathreshold stimuli produced a stimulation artifact, and at least one of the two pulses evoked a single action potential. Only the upper portion of stimulation current traces are shown. Threshold current (I_t) was approx. 3.0 μ A in this neuron. C: Infrequently, suprathreshold stimulation trains evoked an EPSP and an action potential as shown in this panel.

was reduced to 0.2 ms in experiments with selective tachykinin agonists so response latency could be measured more accurately.

Materials

Acetylcholine chloride, atropine sulfate, and hexamethonium chloride were purchased from Sigma (St. Louis, MO, USA). SP, [Sar⁹,Met(O₂)₁₁]SP, and [MePhe⁷]neurokinin B ([MePhe⁷]NKB) were obtained from Phoenix Pharmaceuticals (Mountain View, CA, USA).

Preparation and storage of drugs

SP and [Sar⁹,Met(O₂)₁₁]SP were dissolved in sterile saline while [MePhe⁷]NKB was dissolved in dimethyl sulfoxide. Aliquots of stock solutions were stored at -80°C and discarded after one use. Atropine and hexamethonium were prepared fresh for each experiment.

Drug administration

It is known that ACh can activate muscarinic receptors on intracardiac neurons to produce slow depolarization and/or slow hyperpolarization of the cell membrane (25). Therefore, atropine (1 μM) was added to the superfusion buffer to prevent these slow responses. SP, [Sar⁹,Met(O₂)₁₁]SP, or [MePhe⁷]NKB was added to the superfusion buffer and administered by bath application. The selective agonists were used at 100 nM and SP at 1 μM. Hexamethonium (100 μM) was added to the superfusion buffer at the end of each experiment to verify that responses to nerve stimulation were synaptically evoked and mediated by nicotinic receptors.

Influence of selective agonists on response to local application of ACh

These experiments used a protocol identical to that used previously to determine the effect of SP on responses to local application of ACh (22). Briefly, ACh (10 mM) was administered by local pressure ejection from a glass micropipette (approx. 5 μm tip bore) using a Picospritzer (General Valve Corp., Fairfield, NJ, USA). For each neuron, we first determined a duration of ACh pulse that caused a reproducible fast depolarization below the threshold for firing. ACh pulses of this length of time (10 to 100 ms) were then applied repeatedly at 25- or 30-s intervals to evoke fast depolarization of consistent magnitude. Desensitization of nicotinic receptors did not occur under these conditions. While continuing such phasic applications of ACh, the preparation was superfused for 2 to 2.5 min with buffer containing 100 nM [Sar⁹,Met(O₂)₁₁]SP or [MePhe⁷]NKB. Applications of ACh continued during the washout period.

Data analysis and statistics

All data were obtained from neurons with stable resting membrane potentials more negative than -40 mV. For nerve stimulation experiments, it was also required that stimulation-evoked action potentials could be blocked by hexamethonium.

The influence of tachykinin agonists on cholinergic neurotransmission at intracardiac neurons was classified as potentiation, inhibition, or no effect. Classification was based on the change in response to nerve stimulation that occurred during a 10-min interval beginning at the

time tachykinin was added to the bath. Potentiation was defined by the observation of at least one evoked action potential in each of three consecutive low current stimulus trains or in a total of five such trains. Inhibition was defined by the failure of high current stimuli to evoke action potentials during at least three consecutive stimulus trains or a total of at least five such trains. All other cases are reported as no effect. Depolarization of membrane potential in each group was expressed as the mean ± S.E.M. (n = number of neurons). An unpaired or paired *t*-test (2 tailed) was used to compare values for different groups. *P* < 0.05 was considered significant.

Results

Hexamethonium blocks action potentials evoked by nerve stimulation

Nerve bundle stimulation evoked action potentials from impaled intracardiac neurons with a latency of 3.1 ± 0.2 ms (n = 32, range of 1.2 to 5.6 ms). Bath application of hexamethonium caused reversible inhibition of these evoked action potentials (Fig. 3). Blockade occurred approximately 2 min after beginning superfusion with 100 μM hexamethonium, and responses returned to normal within 5 min after stopping the antagonist. Increasing the stimulus current during exposure to hexamethonium failed to trigger action potentials but could trigger EPSPs (Fig. 3B). These findings demonstrate that stimulation-evoked action potentials are mediated by endogenous ACh and therefore result from synaptic transmission. Hexamethonium blocked responses to nerve stimulation in all neurons included in this study.

SP can augment or inhibit responses to nerve stimulation

The predominant influence of SP (1 μM) was to facilitate synaptic transmission at lower intensities of nerve stimulation (Table 1, Fig. 4). Prior to application of SP, these lower intensity stimuli did not evoke EPSPs or action potentials in any of the intracardiac neurons that were recorded (Fig. 4A and left panel of 4B). During exposure to SP, a majority of stimuli at the same intensity triggered a single action potential from the recorded neurons (Fig. 4A and middle panel of 4B). SP had only a minor influence on the response to stimulation at a higher current in the same neurons. One pulse in each pair of stimuli at the higher current occasionally failed to trigger an action potential under control conditions, whereas both stimuli consistently evoked a single action potential during treatment with SP (Fig. 4B, left and middle panels). Multiple action potentials occurred

Fig. 3. Inhibition of evoked responses by hexamethonium. A: Superfusion with 100 μ M hexamethonium, an antagonist of nicotinic receptors in autonomic ganglia, caused reversible inhibition of action potentials evoked by nerve stimulation. Increasing the nerve stimulus current from 4.4 μ A (Control) to 8.0 μ A still could not evoke an action potential during hexamethonium blockade. However, EPSPs could be evoked when the stimulus strength was increased during exposure to the nicotinic antagonist. B: Selected portions from the same recording are shown at an expanded time scale to demonstrate details of the responses. Dotted lines cut off lower portion ($<3.5 \mu$ A) of stimulus traces.

Table 1. Effects of substance P on neurotransmission at guinea-pig intracardiac neurons

Effect	Number of neurons	% of total neurons tested (n = 40)
Potentiation	29	72.5
Inhibition	9	22.5
None	2	5

in 6 out of 40 intracardiac neurons (15%) during superfusion with SP. For half of these neurons, the onset of firing did not coincide with nerve stimulation. In all cases (n = 40), the responses of intracardiac neurons to nerve stimulation returned to the control pattern after removal of SP (Fig. 4A and right panel of 4B).

Augmentation of synaptic transmission by SP could be replicated over a long interval in the same neuron. This feature was identified in three neurons that had very

Fig. 4. Potentiation of cholinergic neurotransmission at intracardiac neuron by substance P (SP). A: The upper trace shows a continuous transmembrane potential recording from an intracardiac neuron during lower and higher current stimulation of a nerve bundle that provides preganglionic parasympathetic input to the impaled cell. Bath application of 1 μ M SP caused slow depolarization of the neuron and increased the number of action potentials evoked by nerve stimulation. B: Selected portions from the same recording are shown at an expanded time scale to demonstrate details of the responses. Before exposure to SP (Control), subthreshold stimuli (4.5 μ A) did not produce any synaptically evoked responses, while one or both of the higher current stimuli (4.8 μ A) evoked a single action potential. During exposure to SP, most of the lower current stimuli now evoked an action potential. Furthermore, both higher current stimuli evoked a single action potential in the presence of SP. Responses to nerve stimulation returned to the control level after wash-out of SP. Stimulation current traces are shown in the lower panels of A and B. Dotted lines cut off the lower portion ($<3.0 \mu$ A) of current traces.

stable impalements, which allowed recordings to be maintained for greater than one hour. For these cells, superfusion with SP was stopped after determining that the peptide potentiated neurotransmission at the lower of two stimulation intensities (standard protocol), and the cells were allowed to stabilize in the superfusion buffer solution for 30 min. The cells were then stimulated with only subthreshold pulse trains and superfused again with SP (1 μ M). In each of these experiments, SP augmented the response to nerve stimulation (data not shown).

SP had a distinct inhibitory influence on synaptic transmission in a smaller number of intracardiac neurons (Table 1, Fig. 5). Prior to the application of SP, supra-

Fig. 5. Inhibitory effect of SP on cholinergic neurotransmission at intracardiac neuron. A: The upper trace shows a continuous transmembrane potential recording from an intracardiac neuron during lower and higher current stimulation of a nerve bundle that provides preganglionic parasympathetic input to the impaled cell. All of the higher current stimuli triggered action potentials prior to treatment with SP. During the time indicated by the bar, a small depolarization occurred, the amplitude of evoked action potentials was reduced, and some of the suprathreshold stimuli failed to evoke an action potential (not seen due to time scale). Shortly after that time, both stimuli in the suprathreshold trains failed to evoke an action potential. B: Selected portions from the same recording are shown at an expanded time scale to demonstrate details of the responses. Before exposure to SP (Control), subthreshold stimuli ($3.2 \mu\text{A}$) did not produce any synaptically evoked responses, while both of the suprathreshold stimuli ($3.7 \mu\text{A}$) evoked a single action potential. Synaptically evoked action potentials were inhibited for several min after exposure to SP (middle panel). Responses to nerve stimulation returned to the control level after wash-out of SP. Stimulation current traces are shown below each recording. Dotted lines cut off the lower portion ($<2.5 \mu\text{A}$) of current traces.

threshold stimulus trains evoked action potentials in these neurons. Synaptically evoked action potentials were abolished in these cells for 1 to 5 min following superfusion with SP (Fig. 5).

Bath application of SP caused a slow membrane depolarization in all neurons tested ($5.6 \pm 0.5 \text{ mV}$, $n = 40$). There was no significant difference in the magnitude of this response between neurons where synaptic transmission was augmented or inhibited by SP ($5.8 \pm 0.7 \text{ mV}$, $n = 29$ versus $4.7 \pm 0.7 \text{ mV}$, $n = 9$; $P = 0.41$).

Fig. 6. Effects of NK_1 and NK_3 tachykinin receptor agonists on intracardiac neurons. Bath application of 100 nM $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ (NK_1 agonist) and $[\text{MePhe}^7] \text{NKB}$ (NK_3 agonist) each caused slow depolarization of intracardiac neurons. The NK_3 agonists also caused some intracardiac neurons to fire multiple action potentials.

NK_1 and NK_3 agonists induced slow depolarization of intracardiac neurons

Membrane voltage responses to separate bath applications of 100 nM $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ and $[\text{MePhe}^7] \text{NKB}$ were measured alone and in conjunction with nerve stimulation or local applications of ACh. Both selective agonists caused slow membrane depolarization in all neurons tested (Fig. 6). The magnitude of this response was significantly greater for $[\text{MePhe}^7] \text{NKB}$ than $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ whether tested in separate intracardiac neurons ($6.0 \pm 0.4 \text{ mV}$, $n = 42$ versus $3.3 \pm 0.4 \text{ mV}$, $n = 27$; $P < 0.0001$) or in the same cells ($3.9 \pm 0.4 \text{ mV}$ versus $1.8 \pm 0.5 \text{ mV}$, $n = 4$; $P = 0.042$). The effect of $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ on membrane potential was negligible when applied during blockade of voltage-gated calcium channels with $200 \mu\text{M}$ CdCl_2 ($0.7 \pm 0.7 \text{ mV}$, $n = 3$), while the depolarizing action of $[\text{MePhe}^7] \text{NKB}$ was unaffected by this treatment ($6.6 \pm 1.1 \text{ mV}$, $n = 7$). Application of the NK_3 -receptor agonist also evoked action potentials independent of nerve stimulation or pressure ejection of ACh in 6 of 42 intracardiac neurons (Fig. 6B). There were no cases where $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ alone triggered firing of intracardiac neurons ($n = 27$).

Fig. 7. Potentiation of cholinergic neurotransmission at intracardiac neurons by NK_1 and NK_3 agonists. Effect of $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ (A) and $[\text{MePhe}^7] \text{NKB}$ (B) were evaluated using the same nerve stimulation protocol as described for SP. Bath application of each peptide at 100 nM allowed the lower current stimuli to evoke action potentials. In paired experiments with both peptides evaluated using the same intracardiac neurons, the incidence and magnitude of this effect were greater with $[\text{MePhe}^7] \text{NKB}$. The amplitudes of stimulation pulses were 10 and 11 μA (A) and 16 and 20 μA (B). Dotted lines show cut off of current traces at 9.8 and 14 μA , respectively.

Selective tachykinin agonists can augment responses to nerve stimulation and local application of ACh

Effects of the NK_1 and NK_3 agonists (100 nM) on the response of intracardiac neurons to nerve bundle stimulation were evaluated by the same method as used for SP. Lower intensities of nerve stimulation, which cause no postsynaptic response under control conditions, evoked action potentials in some intracardiac neurons during bath application of $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ or $[\text{MePhe}^7] \text{NKB}$ (Fig. 7). This effect occurred with 8 of 17 neurons (47%) for $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ and 10 of 17 neurons (59%) for $[\text{MePhe}^7] \text{NKB}$. The percentage of low current stimuli that caused action potentials did not differ statistically between peptides if all data were considered ($37 \pm 8\%$ for NK_1 agonist versus $54 \pm 10\%$ for NK_3 agonist, $P = 0.22$). However, in four neurons where effects of both peptides were evaluated, more

Fig. 8. Potentiation of neuronal response to application of ACh by NK_1 and NK_3 agonists. Top traces show intracellular recordings from intracardiac neurons and bottom marks indicate times when ACh (10 mM) was given by local pressure ejection. The duration of ACh pulses was set to be subthreshold for action potential generation and was held constant within each experiment. Slow depolarization occurred with bath application of 100 nM $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ (A) or $[\text{MePhe}^7] \text{NKB}$ (B). Although each peptide exhibited the ability to augment the response to ACh, the incidence of this effect was greater with $[\text{MePhe}^7] \text{NKB}$.

stimuli evoked action potentials in the presence of $[\text{MePhe}^7] \text{NKB}$ compared to $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ ($82 \pm 2\%$ versus $18 \pm 3\%$, paired t -test; $P = 0.006$, $n = 4$). In contrast to SP, neither $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ nor $[\text{MePhe}^7] \text{NKB}$ inhibited stimulation-evoked action potentials.

To evaluate postsynaptic interactions of the selective tachykinin agonists with ACh more directly, these peptides were added to the superfusion buffer (100 nM) during repeated local administration of ACh. ACh evoked fast depolarization was potentiated in 4 of 11 intracardiac neurons (36%) by $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ and in 15 of 20 neurons (75%) by $[\text{MePhe}^7] \text{NKB}$ (Fig. 8). The incidence of potentiation was significantly higher for $[\text{MePhe}^7] \text{NKB}$ ($P = 0.036$, Chi-square test). Application

of ACh evoked multiple action potentials in 7 of 15 neurons where [MePhe⁷]NKB caused potentiation. The NK₃ agonist did not cause multiple action potential independent of ACh application in this group of neurons. The augmenting influence of [Sar⁹,Met(O₂)₁₁]SP was manifest as only a single action potential per ACh pulse. The interaction of ACh and the selective agonists was further quantified by determining the percentage of ACh applications that evoked an action potential or multiple action potentials during a 10-min interval starting at the time when superfusion with tachykinin agonist was initiated. Although more ACh pulses triggered action potentials with [MePhe⁷]NKB treatment compared to [Sar⁹,Met(O₂)₁₁]SP ($21.3 \pm 6.3\%$, $n = 15$ versus $10.0 \pm 3.5\%$, $n = 4$), this difference was not statistically significant ($P = 0.38$). Treatment with [Sar⁹,Met(O₂)₁₁]SP or [MePhe⁷]NKB did not inhibit the response of neurons to ACh.

Discussion

Previous reports have established that SP and selective tachykinin agonists can each affect the activity of canine intracardiac neurons when applied locally in anesthetized animals (26–28). The present study provides direct evidence that cholinergic neurotransmission within intracardiac ganglia of the guinea pig can be potentiated by treatment with the natural tachykinin SP or with each of two selective tachykinin agonists (i.e., [Sar⁹,Met(O₂)₁₁]SP and [MePhe⁷]NKB). Our results further demonstrate that SP can inhibit neurotransmission at a significant number of intracardiac neurons. Augmentation was manifest primarily as the generation of one action potential per nerve stimulus delivered at a current intensity that evoked no measurable response in the absence of tachykinins. Higher intensity stimuli, which evoked single action potentials under control conditions, generally did not cause multiple action potentials in the presence of tachykinins. Bath application of [MePhe⁷]NKB also enhanced the response of most intracardiac neurons to local application of ACh, as reported previously for SP (22), and evoked firing of multiple actions potentials in some neurons independent of nerve stimulation or application of ACh. Potentiation of responses to ACh occurred at a significantly lower incidence with [Sar⁹,Met(O₂)₁₁]SP, and this tachykinin did not cause action potentials independent of other stimuli. Neither [Sar⁹,Met(O₂)₁₁]SP nor [MePhe⁷]NKB inhibited synaptic neurotransmission at intracardiac ganglia, suggesting that the inhibitory effects of SP are not mediated by NK₁ or NK₃ receptors.

Single pressure pulses of ACh commonly evoked more than one action potential during exposure to SP

(22), but nerve stimulation in the presence of SP usually evoked only one action potential per stimulus. For that reason, the augmenting influence of SP was more apparent when a lower intensity of nerve stimulation was used. In our previous study, pressure pulses of 10 mM ACh were 10 to 100 ms in duration, while nerve stimulation in this study used 2-ms square pulses. Thus, the length of ACh delivery and the total amount of ACh exposure were larger with pressure ejection than nerve stimulation. It is likely that these factors contributed to the more frequent triggering of multiple action potentials by pressure ejection of ACh compared to nerve stimulation during bath application of SP. This view is supported by the results obtained when [MePhe⁷]NKB was employed in the present study. Local application of ACh in the presence of [MePhe⁷]NKB triggered multiple action potentials in 7 out of 20 neurons evaluated (35%), while nerve stimulation under the same condition did not evoke multiple action potentials.

The incidence of inhibitory modulation by 1 μ M SP was higher in the present study than observed when ACh was applied by pressure ejection (22). Synaptically evoked responses were inhibited in 22% of the intracardiac neurons studied (9 out of 40), while the same concentration of SP attenuated the response to exogenous ACh in only 1 out of 18 cells (approx. 6%). There are several possible explanations for this discrepancy. First, this study focused on ganglia located adjacent to a large nerve bundle, while in the previous project, we recorded from neurons throughout the atrial ganglionated nerve plexus. Accordingly, it is possible that the neuronal populations were not identical. However, it is considered more likely that differences in the method of stimulation are involved. It is possible that SP might attenuate ganglionic transmission by inhibition of ACh release from preganglionic nerve terminals, but we are not aware of any studies establishing a precedent for this mechanism. Furthermore, our previous study established that micromolar concentrations of SP could attenuate the response of intracardiac neurons to exogenous ACh (22). Thus, an alternative explanation for the present finding is that synaptically evoked responses may be more susceptible to inhibition at the postsynaptic site. Nerve stimulation releases limited amounts of ACh at discrete locations where rapid degradation by cholinesterase is likely to occur. This contrasts with pressure ejection, which bathes the neuron and its dendrites with ACh for the duration of the pressure pulse. Since responses evoked by nerve stimulation are clearly more physiological, the present findings suggest that inhibitory modulation by SP could be more prevalent and important than

predicted based on its ability to affect responses to exogenous ACh. Experiments with several other nicotinic receptors systems (29–32), including rat intracardiac ganglia (31), have provided convincing evidence that micromolar concentrations of SP inhibit responses to ACh by a mechanism that involves direct binding of the peptide to an allosteric site on the ganglion nicotinic receptor. This conclusion is supported by our results with $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ and $[\text{MePhe}^7] \text{NKB}$. Each of these peptides was used at a concentration sufficient to saturate their respective tachykinin receptor subtype (33), yet neither of these selective tachykinin agonists inhibited the response of intracardiac neurons to nerve stimulation or local application of ACh.

The safety factor for cholinergic neurotransmission appears to be high at intracardiac neurons that we studied since small increases in stimulation current evoked action potentials abruptly instead of first producing graded EPSPs (34). This finding is consistent with results reported for SAH cells of guinea-pig intracardiac ganglia (6) and for rat intracardiac neurons (35). The absence of graded EPSPs precluded the use of paired pulse experiments (e.g., 36) to determine if SP might augment the response to nerve stimulation by enhancing the release of ACh. We attempted to resolve this dilemma by evaluating the effects of NK_1 and NK_3 tachykinin agonists. Previous electrophysiological experiments and functional studies with the isolated guinea-pig heart provided evidence that the NK_3 subtype is the dominant tachykinin receptor on intracardiac neurons (19, 20, 24). In contrast, specific SP binding sites that we observed in intracardiac ganglia (15) might reflect the presence of presynaptic NK_1 receptors. Unfortunately, experiments with the selective agonists did not eliminate the possibility that tachykinins might affect release of ACh from preganglionic nerve terminals. In fact, both agonists augmented the response to nerve stimulation in the same manner as SP. Nevertheless, $[\text{MePhe}^7] \text{NKB}$ caused a greater incidence and extent of potentiation compared to $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ in paired experiments. The NK_3 agonist was also more effective in augmenting the response of intracardiac neurons to local application of ACh. These findings support the proposed role of NK_3 receptors in mediating direct effects of SP on intracardiac neurons (20).

The prominent role of NK_3 receptors in guinea-pig intracardiac ganglia may have a counterpart in the canine heart where there is autoradiographic and functional evidence that NK_3 receptors are present on intracardiac neurons (28). Local administration of senktide increased the activity of most *in situ* right atrial neurons in that study and, in some cases, also affected cardiac function. Such an effect might be important in the

human heart since SP-immunoreactive nerves are present in the intracardiac ganglia (37).

The small depolarization of intracardiac neurons produced by $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ in our study contrasts with a previous report that found no effect (19). This divergence could be due to different methods of peptide application, since $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ was applied by local pressure ejection ($100 \mu\text{M}$, 1 s) in the previous study and bath application for about 2 min was used in our experiments. It is possible that the small depolarization we observed with bath application of $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ might be an indirect effect due to release of another mediator from some site in the whole mount preparations (38, 39). In accord with this hypothesis, $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ did not cause significant depolarization when voltage-gated calcium channels were blocked by $200 \mu\text{M}$ CdCl_2 , whereas $[\text{MePhe}^7] \text{NKB}$ still evoked a large depolarization. In any event, our findings are consistent with the fact that $[\text{Sar}^9, \text{Met}(\text{O}_2)_{11}] \text{SP}$ can evoke a small bradycardia in the isolated guinea-pig heart preparation (24).

Discussion of our data ultimately requires consideration of the complexity of the intracardiac nervous system. A primary issue is the type of neuron studied since intracardiac ganglia contain efferent neurons, sensory neurons and interneurons. It is unlikely that we recorded from intrinsic sensory neurons since they are the least abundant type of neuron in guinea-pig intracardiac ganglia and receive little or no synaptic input (6, 7). Most of the neurons that were studied had a short latency for generation of an action potential after nerve stimulation, and all of these responses were blocked by hexamethonium. These observations suggest that many of the neurons were activated by monosynaptic input from cholinergic nerve fibers. This characteristic would be expected for efferent neurons but might occur also with interneurons, since most neurons in guinea-pig intracardiac ganglia are surrounded by cholinergic nerve fibers (3, 11). Accordingly, it remains possible that we recorded from some interneurons as well as efferent neurons. In a few cases, evoked action potentials occurred at longer latencies, which would be consistent with a multisynaptic mechanism. Such responses might involve interneurons in the same ganglion or local circuit neurons located in adjacent ganglia. We also observed that SP and $[\text{MePhe}^7] \text{NKB}$ evoked multiple action potentials in some neurons independent of nerve stimulation. Such responses have been attributed to the direct stimulation of impaled neurons by tachykinin and the resulting increase in excitability (3, 18–20, 40). Nevertheless, we cannot eliminate the possibility that multineuronal processes within the intracardiac nervous system make some contribution to these responses.

In summary, our results demonstrate that SP can modulate cholinergic neurotransmission within intracardiac ganglia of guinea pigs by two distinct actions. The predominant effect of SP is to increase the response of intracardiac neurons to low intensity preganglionic nerve stimulation. This action can be attributed primarily to the stimulation of postsynaptic NK₃ receptors. Potentiation can occur also through activation of NK₁ receptors but this effect is smaller and probably occurs by an indirect mechanism. SP has the opposite effect at a smaller but significant population of intracardiac neurons where it inhibits neurotransmission. This effect does not involve NK₁ or NK₃ receptors and probably occurs through binding of SP to nicotinic receptors, as reported by other investigators (29–32). These findings are likely to have pathophysiological implications since SP is present in sensory nerves that innervate the intracardiac ganglia (3, 12, 13, 21, 37).

Acknowledgment

This study was supported by National Heart, Lung, and Blood Institute Grant HL-54633.

References

- 1 Ardell JL. Structure and function of mammalian intrinsic cardiac neurons. In: Armour JA, Ardell JL, editors. *Neurocardiology*. New York: Oxford University Press; 1994. p. 95–114.
- 2 Ardell JL. Intrathoracic neuronal regulation of cardiac function. In: Armour JA, Ardell JL, editors. *Basic and clinical neurocardiology*. New York: Oxford University Press; 2004. p. 118–152.
- 3 Parsons RL. Mammalian cardiac ganglia as local integration centers: histochemical and electrophysiological evidence. In: Dun NJ, Machado BH, Pilowsky PM, editors. *Neural mechanisms of cardiovascular regulation*. Boston: Kluwer Academic Publishers; 2004. p. 335–356.
- 4 Horackova M, Armour JA, Byczko Z. Distribution of intrinsic cardiac neurons in whole-mount guinea pig atria identified by multiple neurochemical coding. A confocal microscope study. *Cell Tissue Res*. 1999;297:409–421.
- 5 Singh S, Johnson PI, Javed A, Gray TS, Lonchyna VA, Wurster RD. Monoamine- and histamine-synthesizing enzymes and neurotransmitters within neurons of adult human cardiac ganglia. *Circulation*. 1999;99:411–419.
- 6 Edwards FR, Hirst GDS, Klemm MF, Steele PA. Different types of ganglion cell in the cardiac plexus of guinea-pigs. *J Physiol*. 1995;486:453–471.
- 7 Klemm MF, Wallace DJ, Hirst GDS. Distribution of synaptic boutons around identified neurones lying in the cardiac plexus of the guinea-pig. *J Auton Nerv Syst*. 1997;66:201–207.
- 8 Mawe GM, Talmage EK, Lee KP, Parsons RL. Expression of choline acetyltransferase immunoreactivity in guinea pig cardiac ganglia. *Cell Tissue Res*. 1996;285:281–286.
- 9 Leger J, Croll RP, Smith FM. Regional distribution and extrinsic innervation of intrinsic cardiac neurons in the guinea pig. *J Comp Neurol*. 1999;407:303–317.
- 10 Steele PA, Gibbins IL, Morris JL, Mayer B. Multiple populations of neuropeptide-containing intrinsic neurons in the guinea-pig heart. *Neuroscience*. 1994;62:241–250.
- 11 Hoover DB, Ganote CE, Ferguson SM, Blakely RD, Parsons RL. Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high affinity choline transporters. *Cardiovasc Res*. 2004;62:112–121.
- 12 Weihe E, Reinecke M, Forssmann WG. Distribution of vasoactive intestinal polypeptide-like immunoreactivity in the mammalian heart. Interrelation with neurotensin- and substance P-like immunoreactive nerves. *Cell Tissue Res*. 1984;236:527–540.
- 13 Wharton J, Polak JM, McGregor GP, Bishop AE, Bloom SR. The distribution of substance P-like immunoreactive nerves in the guinea-pig heart. *Neuroscience*. 1981;6:2193–2204.
- 14 Smith FM. Extrinsic inputs to intrinsic neurons in the porcine heart in vitro. *Am J Physiol Regul Integr Comp Physiol*. 1999;276:R455–R467.
- 15 Hoover DB, Hancock JC. Distribution of substance P binding sites in guinea-pig heart and pharmacological effects of substance P. *J Auton Nerv Syst*. 1988;23:189–197.
- 16 Hoover DB. Effects of substance P on rate and perfusion pressure in the isolated guinea pig heart. *J Pharmacol Exp Ther*. 1990;252:179–184.
- 17 Tompkins JD, Hoover DB, Hancock JC. Substance P evokes bradycardia by stimulation of postganglionic cholinergic neurons. *Peptides*. 1999;20:623–628.
- 18 Konishi S, Okamoto T, Otsuka M. Substance P as a neurotransmitter released from peripheral branches of primary afferent neurones producing slow synaptic excitation in autonomic ganglion cells. In: Jordan CC, Oehme P, editors. *Substance P metabolism and biological actions*. London: Taylor & Francis; 1985. p. 121–136.
- 19 Hardwick JC, Mawe GM, Parsons RL. Evidence for afferent fiber innervation of parasympathetic neurons in the guinea-pig cardiac ganglion. *J Auton Nerv Syst*. 1995;53:166–174.
- 20 Hardwick JC, Mawe GM, Parsons RL. Tachykinin-induced activation of non-specific cation conductance via NK₃ neurokinin receptors in guinea-pig intracardiac neurones. *J Physiol*. 1997;504:65–74.
- 21 Hoover DB, Chang Y, Hancock JC, Zhang L. Actions of tachykinins within the heart and their relevance to cardiovascular disease. *Jpn J Pharmacol*. 2000;84:367–373.
- 22 Zhang L, Tompkins JD, Hancock JC, Hoover DB. Substance P modulates nicotinic responses of intracardiac neurons to acetylcholine in the guinea pig. *Am J Physiol Regul Integr Comp Physiol*. 2001;281:R1792–R1800.
- 23 Hoover DB, Chang Y, Hancock JC. Characterization of responses to neurokinin A in the isolated perfused guinea pig heart. *Am J Physiol Regul Integr Comp Physiol*. 1998;275:R1803–R1811.
- 24 Chang Y, Hoover DB, Hancock JC, Smith FM. Tachykinin receptor subtypes in the isolated guinea pig heart and their role in mediating responses to neurokinin A. *J Pharmacol Exp Ther*. 2000;294:147–154.
- 25 Allen TG, Burnstock G. M₁ and M₂ muscarinic receptors mediate excitation and inhibition of guinea-pig intracardiac neurones in culture. *J Physiol*. 1990;422:463–480.

- 26 Armour JA, Yuan B, Butler CK. Cardiac responses elicited by peptides administered to canine intrinsic cardiac neurons. *Peptides*. 1990;11:753–761.
- 27 Armour JA, Huang MH, Smith FM. Peptidergic modulation of in situ canine intrinsic cardiac neurons. *Peptides*. 1993;14:191–202.
- 28 Thompson GW, Hoover DB, Ardell JL, Armour JA. Canine intrinsic cardiac neurons involved in cardiac regulation possess NK₁, NK₂, and NK₃ receptors. *Am J Physiol Regul Integr Comp Physiol*. 1998;275:R1683–R1689.
- 29 Stafford GA, Oswald RE, Weiland GA. The β subunit of neuronal nicotinic acetylcholine receptors is a determinant of the affinity for substance P inhibition. *Mol Pharmacol*. 1994;45:758–762.
- 30 Lukas RJ, Eisenhour CM. Interactions between tachykinins and diverse, human nicotinic acetylcholine receptor subtypes. *Neurochem Res*. 1996;21:1245–1257.
- 31 Cuevas J, Adams DJ. Substance P preferentially inhibits large conductance nicotinic ACh receptor channels in rat intracardiac ganglion neurons. *J Neurophysiol*. 2000;84:1961–1970.
- 32 Andoh T, Itoh H, Watanabe I, Sasaki T, Higashi T. Mechanism of modulation of neuronal nicotinic receptors by substance P and OAG. *Am J Physiol Cell Physiol*. 2001;281:C1871–C1880.
- 33 Regoli D, Boudon A, Fauchere J-L. Receptors and antagonists for substance P and related peptides. *Pharmacol Rev*. 1994;46:551–599.
- 34 Akasu T, Nishimura T. Synaptic transmission and function of parasympathetic ganglia. *Prog Neurobiol*. 1995;45:459–522.
- 35 Selyanko AA, Skok VI. Synaptic transmission in rat cardiac neurones. *J Auton Nerv Syst*. 1992;39:191–200.
- 36 Kombian SB, Ananthalakshmi KVV, Parvathy SS, Matowe WC. Substance P depresses excitatory synaptic transmission in the nucleus accumbens through dopaminergic and purinergic mechanisms. *J Neurophysiol*. 2003;89:728–737.
- 37 Wharton J, Polak JM, Gordon L, Banner NR, Springall DR, Rose M, et al. Immunohistochemical demonstration of human cardiac innervation before and after transplantation. *Circ Res*. 1990;66:900–912.
- 38 Powers MJ, Peterson BA, Hardwick JC. Regulation of parasympathetic neurons by mast cells and histamine in the guinea pig heart. *Auton Neurosci*. 2001;87:37–45.
- 39 Jelson GS, DeMasi GM, Sager KL, Hardwick JC. Modulation of guinea pig intrinsic cardiac neurons by prostaglandins. *Am J Physiol Regul Integr Comp Physiol*. 2003;285:R682–R689.
- 40 Saria A, Ma RC, Dun NJ. Neurokinin A depolarizes neurons of the guinea pig inferior mesenteric ganglia. *Neurosci Lett*. 1985;60:145–150.