Tachykinin-induced activation of non-specific cation conductance via NK_3 neurokinin receptors in guinea-pig intracardiac neurones

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1. Whole mount preparations from guinea-pig hearts were used to characterize the receptors and ionic mechanisms mediating the substance P (SP)-induced depolarization of parasympathetic postganglionic neurones of the cardiac ganglion.

2. Measurement of the amplitude of depolarization in response to superfusion of different tachykinin agonists (neurokinins A (NKA) and B (NKB), SP, and senktide) gave a rank-order potency of NKB = senktide > NKA > SP, indicating involvement of an NK_3 receptor. The use of the selective tachykinin receptor antagonists SR 140333, SR 48986, and SR 142801 demonstrated that only the NK_3 receptor antagonist SR 142801 inhibited the SP-induced depolarization.

3. The SP-induced depolarization was not inhibited by Ba^{2+}, TEA, or niflumic acid, or altered by reduced Cl^- solutions, but was attenuated in reduced Na^+ solutions. Single electrode voltage clamp studies demonstrated that the SP-induced inward current increased in amplitude at more negative potentials, had a reversal potential of approximately 0 mV, and was reduced in amplitude in reduced Na^+ solutions.

4. We conclude that the SP-induced depolarization in guinea-pig postganglionic parasympathetic neurones of the cardiac ganglion is due to NK_3-mediated activation of a non-selective cation conductance.

Parasympathetic ganglia, once thought to serve merely as relay stations, are now considered to be sites of integration from multiple synaptic, hormonal and immune-mediated inputs. Immunohistochemical studies demonstrate that many different neuropeptides could modulate the activity of postganglionic parasympathetic neurones. In the guinea-pig parasympathetic cardiac ganglia, several different neuropeptides have been localized in fibres surrounding intracardiac neurones (Gibbins, Furness, Costa, MacIntyre, Hillyard & Girgis, 1985; Morris, 1989; Hardwick, Mawe & Parsons, 1995). One such input is derived from primary afferent fibres which contain substance P (SP) and calcitonin gene-related peptide (CGRP) immunoreactivity (Gibbins *et al.* 1985; Urban & Papka, 1985). These processes could provide a direct sensory input to cardiac neurones for local reflex modulation of cardiac function. Previously, we showed that local application of SP depolarizes parasympathetic postganglionic neurones in a guinea-pig cardiac ganglion whole mount preparation (Hardwick *et al.* 1995). Furthermore, high frequency stimulation of interganglionic fibre bundles also produced a non-cholinergic calcium-dependent slow postsynaptic depolarization of these cells, similar to that produced by SP application.

SP is a member of the tachykinin family of peptides and can act via three known receptor subtypes: the neurokinin (NK) tachykinin receptors (NK_1, NK_2 and NK_3; Otsuka & Yoshioka, 1993). Based on our preliminary studies using tachykinin agonists, we suggested that both NK_2 and NK_3 tachykinin receptor subtypes may mediate SP-induced depolarizations in guinea-pig cardiac neurones (Hardwick *et al.* 1995). However, a complete pharmacological characterization of these receptors and determination of the ionic mechanisms responsible for the SP-induced depolarization was not investigated in these initial studies. Results from studies of SP-mediated depolarizations in other neurones have indicated that SP can initiate depolarization by several different ionic mechanisms, including a decrease in K^+ conductance, an increase in Cl^- conductance, and an increase in a non-selective cation conductance (Yamaguchi, Nakajima, Nakajima & Stanfield, 1990; Shen & North, 1992; Myers & Undem, 1993; Bertrand & Galligan, 1994; Inoue, Nakazawa, Inoue & Fujimori, 1995; Mawe, 1995; Akasu, Ishimatsu & Yamada, 1996).

The goals of this study were, first, to identify which tachykinin receptor subtype(s) mediates the SP-induced

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depolarization, and second, to establish the underlying ionic conductance(s) responsible for the depolarization in guinea-pig cardiac neurones. Our results show that the tachykinin-induced depolarization of guinea-pig parasympathetic postganglionic cardiac neurones is primarily due to an activation of NK_3 tachykinin receptors which initiates an increase in a non-selective cation conductance.

METHODS

Preparation

Adult guinea-pigs (250–300 g of either sex) were killed by stunning and exsanguination, a method approved by the University of Vermont Institutional Animal Care and Use Committee. The cardiac ganglion was dissected as described previously (Hardwick *et al.* 1995). Briefly, the heart was removed and placed into ice-cold standard Krebs solution of composition (mM): NaCl, 121; KCl, 5·9; $CaCl_2$, 2·5; $MgCl_2$, 1·2; $NaHCO_3$, 25; NaH_2PO_4, 1·2; glucose, 8; and aerated with 95% O_2–5% CO_2. For electrophysiological recordings, the parasympathetic neurones of the cardiac ganglion, which lie in the epicardium, primarily in the wall of the left atrium, were exposed by removing the overlying atrial muscle tissue and blood vessels. The preparation was then pinned out in a Sylgard-lined chamber (2·5 ml) and continuously superfused (6–10 ml min^{-1}) with oxygenated Krebs solution maintained at 35–37 °C. Changes were made in the composition of the bathing solution to determine the nature of the ionic conductance underlying the SP-induced depolarization. *N*-methyl-D-glucamine (NMG^+; Sigma) was used to reduce external Na^+ by replacing either half of the NaCl with NMG-Cl (61 mM NMG^+, 88 mM Na^+) or all of the NaCl with NMG-Cl (122 mM NMG^+, 26 mM Na^+). Sodium propionate (ICN Rare Chemicals, Costa Mesa, CA, USA) was used to reduce the external Cl^- concentration by replacing half of the NaCl with sodium propionate (67 mM propionate, 67 mM Cl^-). A few experiments were completed in a calcium-deficient solution ($CaCl_2$ was omitted) which also contained 0·3 μM tetrodotoxin (TTX; Sigma).

Drugs were applied either by superfusion or by local pressure ejection (Picospritzer; General Valve Corp., Fairfield, NJ, USA) through a small diameter (5–10 μm) pipette located 50–100 μm from the cell. When peptides were applied by pressure ejection, they were dissolved in Krebs solution from stock solution to give a final concentration of 100 μM. For superfusion, SP (Sigma) and NKA (Research Biochemicals Inc.) were initially dissolved in double-distilled H_2O to give a 1 mM stock solution, and then diluted to the appropriate concentration in Krebs solution. Bovine serum albumen (BSA, 1 mg ml^{-1}; Sigma) was added to the Krebs solution to reduce peptide adherence to the intervening tubing when SP or NKA was tested. NKB and senktide (Research Biochemicals International) solutions were prepared from a 1 mM stock solution in DMSO. Addition of BSA to the senktide solutions did not affect the senktide-induced depolarization (data not shown). Consequently, BSA was not routinely added to the superfusion solutions containing senktide. Non-peptide tachykinin receptor antagonists thought to be specific for the NK_1 (SR 140333), NK_2 (SR 48968), or NK_3 (SR 142801) receptor subtypes in guinea-pig tissues (Emons-Alt *et al.* 1992, 1993, 1995) were generously supplied by Dr Xavier Emons-Alt (Sanofi Recherche, Montpellier, France). These tachykinin receptor-specific antagonists were initially dissolved in DMSO to give a 10^{-2} M stock solution and then diluted with Krebs solution prior to use.

Electrophysiological recording

Electrophysiological recordings were obtained using an Axoclamp-2A amplifier from cells impaled with 2 M KCl-filled glass microelectrodes (40–60 MΩ). To measure membrane currents, recordings were made in discontinuous single electrode voltage clamp mode at a sampling frequency of 9–10 kHz with a 50% duty cycle. The head stage voltage was monitored throughout the recordings. To establish current–voltage (*I–V*) curves prior to and during tachykinin-receptor activation, we recorded membrane currents generated by voltage ramps from −100 to −30 mV using pCLAMP software (version 6.0.2; Axon Instruments) at a rate of 25 mV s^{-1} with a sampling rate of 500 Hz.

Curve fitting and statistical methods

The concentration–depolarization relationship for the individual tachykinin agonists was fitted assuming a single-site binding model using least-squares regression analysis, assuming a Hill coefficient of 1. From the curve fits generated by Sigma Plot 5.0, an EC_{50} value was estimated for each agonist. For analysis of statistical significance, either ANOVA or Student's paired *t* test was used with $P < 0·05$ considered significantly different. Data are shown as means ± S.E.M. Results from any experimental condition were duplicated in cells from three or more different animals.

RESULTS

The tachykinin-induced depolarization is mediated by NK_3 receptor activation

Application of SP (10^{-4} M) by local pressure ejection (for 1 s) consistently produced depolarization of the guinea-pig parasympathetic neurones, as was reported in our previous studies (Hardwick *et al.* 1995). All fifty-one cells that were examined for a response to SP application by local pressure ejection responded with a depolarization. This SP-induced depolarization had a mean amplitude of 8·4 ± 0·6 mV and a mean duration of 52·9 ± 12·3 s from a mean resting membrane potential of −50·3 ± 1·3 mV. The input resistance in these neurones normally ranges from 30 to 200 MΩ, with a mean of approximately 100 MΩ (Hardwick *et al.* 1995).

The ability of SP to depolarize the neurones was examined in calcium-deficient solution containing TTX to insure that the SP-induced depolarization resulted from direct action on the neurones, rather than inducing the release of another neurotransmitter from nearby nerve terminals. SP (10^{-4} M), applied by pressure ejection (for 1 s), elicited a depolarization of 8·0 ± 1·8 mV from a resting membrane potential of −48·3 ± 2·7 mV (number of cells, $n = 3$). The same cells were then superfused with calcium-deficient Krebs solution containing 4 mM Mg^{2+} and 0·3 μM TTX. In the presence of TTX and no added calcium, SP produced a depolarization with a mean amplitude of 8·2 ± 1·6 mV ($P > 0·05$).

Application of SP and other tachykinins by superfusion also produced depolarization of the cardiac neurones. NKB, NKA and SP, each at a concentration of 100 nM, were applied individually to different preparations by superfusion and the amplitude of the depolarization was determined. For all superfusion experiments, agonists at a concentration

of 100 nM or greater were applied only once to a preparation due to a tachykinin-induced desensitization of the responses. As shown in Fig. 1*A*, the three agonists each produced a depolarization, but the amplitude of the depolarization was agonist dependent. The depolarization produced by NKB (10·0 ± 0·7 mV, $n = 4$) was significantly greater than that produced by either NKA (6·0 ± 0·4 mV, $n = 4$) or SP (4·7 ± 0·9 mV, $n = 3$) at the same concentration. Superfusion with 100 nM senktide, a non-peptide NK_3-specific agonist, also produced a depolarization of the cardiac neurones (10·3 ± 0·9 mV, $n = 3$) similar in amplitude to that produced by NKB (Fig. 1*A*). The profile of agonist potency observed in these cells, NKB > NKA > SP, is suggestive of an NK_3 receptor (Otsuka & Yoshioka, 1993).

Further analysis of the agonist-induced depolarization was conducted to characterize fully which receptor subtype(s) mediated the depolarization in guinea-pig cardiac neurones. Concentration–depolarization curves for three different tachykinin receptor agonists were generated. SP and NKA have been localized in cardiac afferent nerve fibres by high pressure liquid chromatography (Hua, 1986). Both NKA and SP can activate all three receptor subtypes, but with differing degrees of affinity (i.e. NK_1: SP > NKA > NKB). However, NKB is not found in guinea-pig peripheral afferent fibres (Hua, 1986), and therefore, for the remainder of these experiments, we used senktide to confirm the presence of NK_3 receptors and to generate the dose–response curves. Preparations were superfused for 15 s with various concentrations (10^{-10} to 10^{-5} M) of SP, NKA, or senktide and the peak amplitudes of depolarization measured. To ensure that the maximum responses had been obtained with superfusions of 10^{-5} to 10^{-6} M, each agonist was applied at 10^{-4} M by local pressure ejection (1 s for senktide and NKA, 2 s for SP) and the responses compared. The results from this series of experiments, shown in Fig. 1*B*, demonstrate that senktide was the most potent agonist with an apparent EC_{50} of 38 nM, followed by NKA (EC_{50}, 235 nM) and SP was the least potent agonist with an apparent EC_{50} of 547 nM. This rank-order of potency (senktide > NKA > SP) is characteristic of the NK_3 receptor subtype (Otsuka & Yoshioka, 1993).

Experiments were conducted with subtype-specific non-peptide receptor antagonists to confirm further the involvement of NK_3 receptor subtypes in the tachykinin-induced depolarization. For these experiments, whole mount preparations were superfused with Krebs solution containing SR 140333 (NK_1 receptor antagonist; Emons-Alt *et al.* 1993), or SR 48968 (NK_2 receptor antagonist; Emons-Alt *et al.* 1992), or SR 142801 (NK_3 receptor antagonist; Emons-Alt *et al.* 1995), all at a concentration of 30 nM for 3–10 min prior to SP application. The preparation was then superfused for 15 s with the same solution containing 1 μM SP and the amplitude of the SP-induced depolarization determined. Incubation with the antagonists alone had no detectable

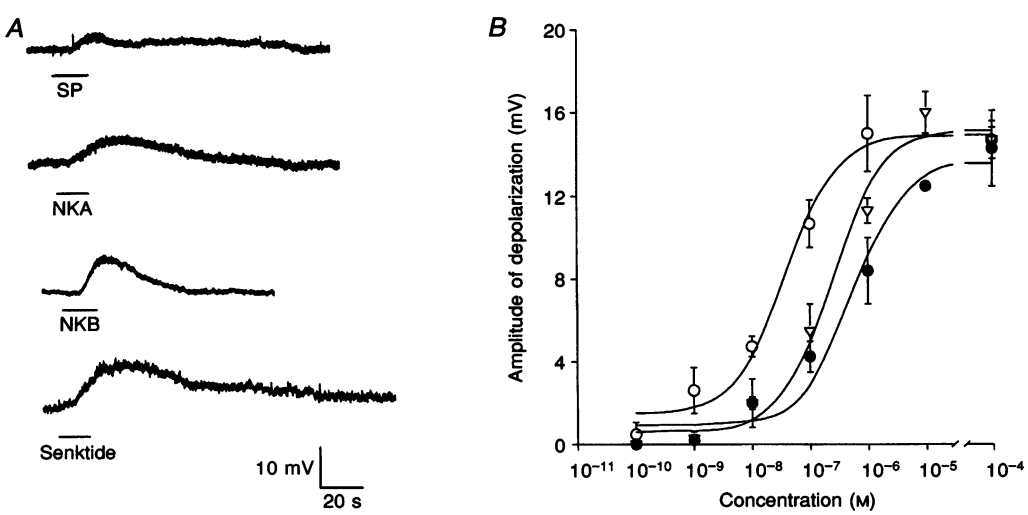

Figure 1. Agonist- and concentration-dependent depolarization of cardiac neurones

A, example recordings of the depolarization of individual parasympathetic postganglionic neurones in response to superfusion with 100 nM of NKB, NKA, SP, or senktide. The agonists were superfused over the preparation for 15 s (indicated by the bar). The resting membrane potentials were: −50, −48, −53 and −58 mV, respectively. *B*, the depolarization produced by a 15 s superfusion with senktide (○), SP (●) or NKA (▽) (ranging in concentration from 10^{-10} to 10^{-5} M), was determined in intracardiac neurones. To confirm maximum depolarization, the agonist was applied by local pressure ejection (10^{-4} M in the pipette, 1–2 s application). Symbols and bars represent means ± S.E.M. from 3 or more cells. The lines were generated from curve fits assuming a single-site binding model and a Hill coefficient of 1. The EC_{50} values estimated for senktide, SP and NKA were 38, 547 and 235 nM, respectively.

effect on the resting membrane potential or input resistance of the cardiac neurones (4 ± 5% change in input resistance in the presence of the inhibitors, $P > 0{\cdot}05$, $n = 3$). The response to superfusion with SP, summarized in Fig. 2, demonstrates that only the NK_3 receptor-specific antagonist SR 142801 significantly reduced the amplitude of the SP-induced depolarization. In previous studies using these non-peptide tachykinin receptor antagonists, the experimental tissues were equilibrated for tens of minutes before challenge with agonist (Patacchini, Bartho, Holzer & Maggi, 1995). Therefore, to ensure that the duration of exposure to SR 142801 in the present experiments was sufficient to achieve maximum inhibition of the NK_3 receptor subtype, we determined the extent of inhibition of a senktide-induced depolarization (superfusion with 1 μM senktide) after different exposure times in 30 nM SR 142801. As shown in the inset to Fig. 2, within 2 min, the senktide response was inhibited by greater than 60%. After a 12 min exposure approximately 85% inhibition was achieved and showed no further increase with incubations up to 2 h. Based on these data, we conclude that significant inhibition could be achieved within 10 min and that considerable inhibition would occur even earlier. These results indicate that the incubation times used in our experiments were sufficient for SR 142801 to inhibit NK_3 receptors effectively.

Characterization of the tachykinin-activated ionic conductance

SP can depolarize neurones by several different mechanisms, including: (i) inhibition of K^+ conductances (Yamaguchi *et al.* 1990; Shen & North, 1992; Koyano, Velimirovic, Grigg, Nakajima & Nakajima 1993; Shen & Surprenant, 1993; Vanner, Evans, Matsumoto & Surprenant 1993; Ishimatsu, 1994; Akusa *et al.* 1996); (ii) activation of a Cl^- conductance (Bertrand & Galligan, 1994; Mihara & Nishi, 1994); and (iii) activation of a non-specific cation conductance (Shen & North, 1992; Koyano *et al.* 1993; Myers & Undem, 1993; Inoue *et al.* 1995; Mawe, 1995). Experiments were done to test potential involvement of all three conductance changes in the SP-induced depolarization recorded from the cardiac neurones.

To examine the potential role of K^+ conductances in the SP-induced depolarization, we tested the ability of SP to depolarize cardiac neurones in the presence of either 5 mM tetraethylammonium (TEA), 2 mM Ba^{2+}, or a combination of the two. An example of the SP-induced responses in the presence of these K^+-conductance blockers is shown in Fig. 3. For each cell tested, the amplitude of the SP-induced depolarization in response to a brief local application of peptide was determined in Krebs solution (Table 1). The

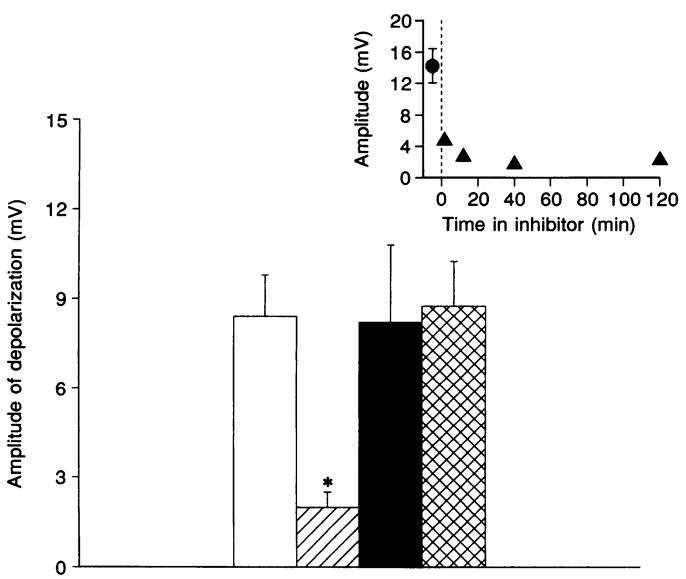

Figure 2. The SP-induced depolarization was preferentially inhibited by SR 142801

Whole mount preparations were superfused with Krebs solution (control, □) or 30 nM of SR 142801 (▨), SR 48968 (■) or SR 140333 (▩) for 3–10 min and then challenged with a 15 s superfusion of 1 μM SP and the SP-induced depolarization was determined. Columns and bars represent the means and S.E.M. from 3 or more different preparations. Only SR 142801 produced a significant reduction in the amplitude of the SP-induced depolarization ($P < 0{\cdot}01$, ANOVA). Inset, whole mount preparations were pretreated with 30 nM SR 142801 for times ranging from 2 to 120 min (onset of inhibitor application indicated by the dashed line) prior to superfusion with 1 μM senktide. The amplitude of the senktide response in the absence of inhibitors (●, mean ± standard error from 4 cells) was significantly greater than that recorded at all exposure times (▲). The inhibition at 12 min was ~85% and was similar to that evident with longer incubation times. Each point represents a recording made in a different cell.

bath solution was then changed to Krebs solution containing either 5 mM TEA or 2 mM Ba^{2+}. The addition of Ba^{2+} alone caused a 10–15 mV depolarization of the cells and an approximate 2-fold increase in input resistance (230 ± 16%, $n = 3$). Consequently, all cells had to be electrotonically maintained at the original resting membrane potential. Even with these measures SP application in the presence of either TEA alone or 2 mM Ba^{2+} alone still produced a depolarization (Table 1 and Fig. 3). Inclusion of both Ba^{2+} and TEA also had no significant effect on the amplitude of the SP-induced depolarization. In fact, the amplitude of the depolarization increased slightly in the presence of the K^+ channel blockers and was often associated with a burst of action potentials (see Figure 3). These results indicated that the SP-induced depolarization was not due to the inhibition of a Ba^{2+}- or TEA-sensitive K^+ conductance.

The possibility of involvement of a Cl^- conductance in the tachykinin-induced response was tested both by the inclusion of a Cl^- channel blocker and by alteration of the Cl^- equilibrium potential (Bertrand & Galligan, 1994; Mihara & Nishi, 1994). Addition of 100 μM niflumic acid, a Cl^- channel inhibitor (Bertrand & Galligan, 1994), to the circulating Krebs solution had no significant effect on the amplitude of the SP-induced depolarization (6·0 ± 1·5 mV *versus* 4·3 ± 0·6 mV control; $n = 3$). In addition, a 50% reduction of the extracellular Cl^- concentration (Cl^- replaced by the less permeant anion propionate) also did not affect the SP-induced depolarization. The SP-induced depolarization was 7·5 ± 2·4 mV ($n = 3$) in standard Krebs solution, whereas following replacement of 50% of the Cl^- by propionate in the Krebs solution the SP-induced

Table 1. SP-induced depolarization in the presence of Ba^{2+} and TEA

Conditions	Amplitude (mV)	n
Control	8·2 ± 0·9	8
2 mM Ba^{2+}	11·2 ± 1·8	6
5 mM TEA	11·5 ± 1·5	4
TEA + Ba^{2+}	14·7 ± 3·5	3

SP-induced depolarizations were recorded from cells at the resting membrane potential (−50·5 ± 3·3, $n = 8$) in standard Krebs solution and in Krebs solution containing either 2 mM Ba^{2+}, 5 mM TEA, or 2 mM $Ba^{2+}$and 5 mM TEA. Neither Ba^{2+} nor TEA treatment decreased the amplitude of the SP-induced depolarization; rather, there was a slight increase in the peak amplitude of the SP response.

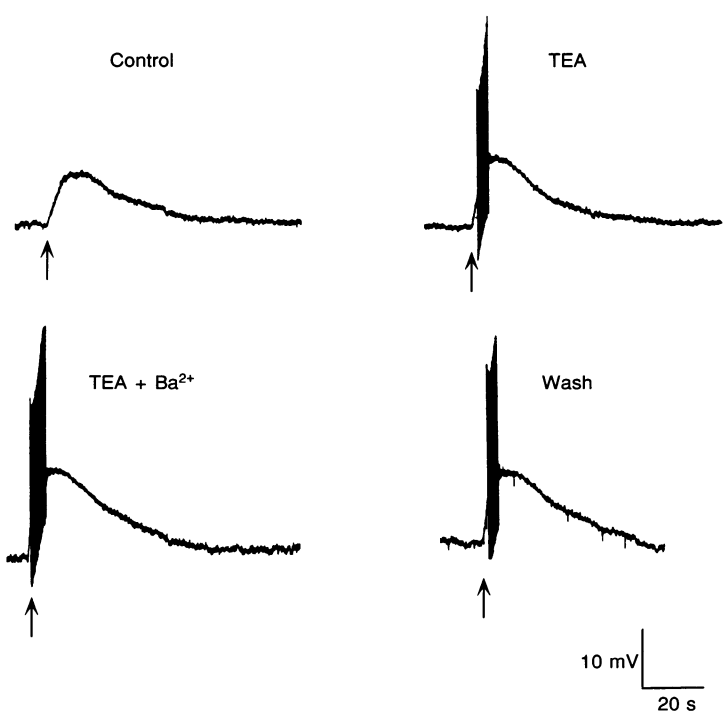

Figure 3. SP-induced depolarizations in the presence of Ba^{2+} and TEA

Example recordings of the depolarization caused by 1 s applications of SP (10^{-4} M, arrows) under different conditions: standard Krebs solution, in the presence of 5 mM TEA, in the presence of 5 mM TEA with 2 mM Ba^{2+}, and following a 5 min wash with standard Krebs solution. The resting membrane potential of this cell was maintained at −53 mV for all SP applications. The SP-induced depolarization was not inhibited by the K^+ blockers; rather, its amplitude increased slightly and was associated with a burst of action potentials.

depolarization was 5·0 ± 0·6 mV ($n = 3$) in these same cells. If the SP-induced depolarization had resulted, in part, from activation of a Cl^- conductance, then reduction of the extracellular Cl^- concentration should have augmented the SP-induced depolarization when measured at the resting membrane potential (Mihara & Nishi, 1994).

To examine the hypothesis that the depolarization was due to the activation of a non-selective cation conductance, the effect of reducing the extracellular Na^+ concentration on the amplitude of the SP response was tested. The extracellular Na^+ concentration was reduced by substitution of approximately 45% of the Na^+ ions with NMG^+. Reducing the external Na^+ concentration resulted in a 50 ± 6% reduction in the amplitude of the SP-induced depolarization (8·3 ± 1·2 mV in Krebs solution *versus* 4·2 ± 0·3 mV in the reduced Na^+ solution) at the resting membrane potential (−49 ± 10 mV, $n = 3$).

Further evidence for activation of a non-specific cation conductance was obtained by determining the effect of hyperpolarization on the SP-induced depolarization in standard Krebs solution. The amplitude of the depolarization in response to a 1 s application of SP was 8·3 ± 1·2 mV ($n = 3$) at the resting membrane potential (−49 ± 10 mV). The membrane potential of the same cells was then electrotonically shifted to a more negative voltage (−77 ± 3 mV) and the SP response determined again. In all three cells, the amplitude of the SP-induced depolarization increased (14·3 ± 3·8 mV) at the hyperpolarized potential. The observations of (i) an increase in the amplitude of the SP-induced depolarization with hyperpolarization, and (ii) the decrease in amplitude when external Na^+ was reduced suggested that an increase in a non-selective cation conductance was primarily responsible for the SP-induced depolarization.

Guinea-pig cardiac neurones have virtually no dendritic arborization (Edwards, Hirst, Klemm & Steele, 1995; Hardwick *et al.* 1995; Pauza, Skripkiene, Skripka, Pausziene & Stropus, 1997), allowing adequate voltage clamp of the cell soma during slow responses using single electrode voltage clamp. Consequently, we used single electrode voltage clamp recordings to characterize the SP-induced current in the guinea-pig intracardiac neurones and to test further whether SP increased a non-selective cation conductance. When cells were maintained at their resting membrane potential, SP elicited an inward current in all cells tested. Furthermore, although the peak amplitude of the SP-induced current varied considerably between individual cells (ranging from −100 to −624 pA; mean amplitude, −340 ± 185 pA, $n = 8$), the amplitude consistently decreased when cells were held at voltages more positive than the resting membrane potential and increased at more negative holding potentials (Fig. 4*A*). An example of the peak current amplitude *versus* the holding potential for the SP-induced responses from one cell is shown in Fig. 4*B*. The estimated reversal potential from a least-squares linear regression for this cell was −1·9 mV. Similar current–voltage relationships were determined in two other cells and the mean reversal potential was 2·0 ± 4·0 mV ($n = 3$).

Consistent with the results obtained with voltage recordings, reducing the extracellular Na^+concentration by partial

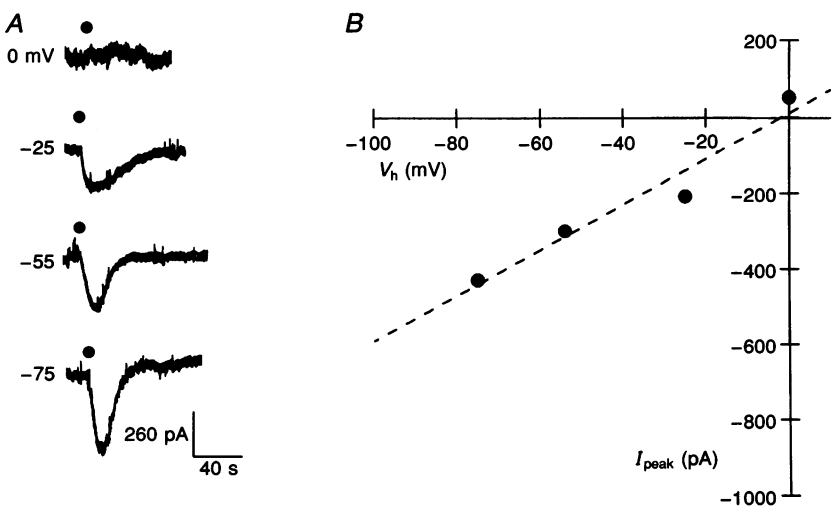

Figure 4. Voltage-dependent changes in the amplitude of the SP-induced inward currents in guinea-pig intracardiac neurones

A, traces show the current produced in response to a 1 s application of SP (100 μM, ●) in a cell voltage clamped to several different potentials (indicated to the left of each trace). SP applications were separated by at least 5 min to prevent decreases in amplitude due to desensitization. *B*, peak amplitude (I_{peak}) *versus* holding potential (V_h); the dashed line indicates a first-order linear regression fit of the data. The estimated reversal potential from the regression analysis was −1·9 mV.

substitution with NMG^+ (45% of Na^+ replaced with NMG^+) resulted in a 47 ± 11% ($n = 8$) reduction in the SP-induced inward current with cells held at the resting membrane potential (−44·6 ± 7·8 mV). In addition, when approximately 85% of the Na^+ in the Krebs solution was replaced with NMG^+, no measurable SP-induced current at the resting membrane potential was observed (3 preparations).

The current–voltage (I–V) curves measured before and at the peak of the SP-induced current by generating voltage ramps from −100 to −30 mV at a rate of 25 mV s^{-1} were compared. For these experiments, the cells were maintained in standard Krebs solution. Examples of the I–V curves obtained before and at the peak of the SP-induced current are shown in Fig. 5; the total current recorded is shown in the top panels (*Aa* and *Ba*) and subtraction of the control current from the current measured in the presence of SP is shown in the bottom panels (*Ab* and *Bb*). In all cells, application of SP caused a shift in the zero current value to a more positive membrane potential, consistent with the SP-induced depolarization. In approximately 80% of the cells (12 of 15), the slope of the I–V relationship in the presence of SP either remained similar to or was steeper than that of the control I–V relationship measured prior to SP application (Fig. 5*Aa*). Estimates of the SP-induced current also show an increase in current at negative voltages (Fig. 5*Ab*). In the remaining 20% of the cells (3 of 15), the control current and the current recorded in the presence of SP appeared to converge as the membrane potential was made progressively more negative (Fig. 5*Ba*). This is also observed in the subtracted current, where the SP-induced current appears to approach zero near the K^+ equilibrium potential (Fig. 5*Bb*). We suggest that in this small population of cells, SP may modulate other conductances in addition to activating a non-selective cation conductance. Because of the limited incidence of this I–V pattern, we have not attempted to analyse these responses further.

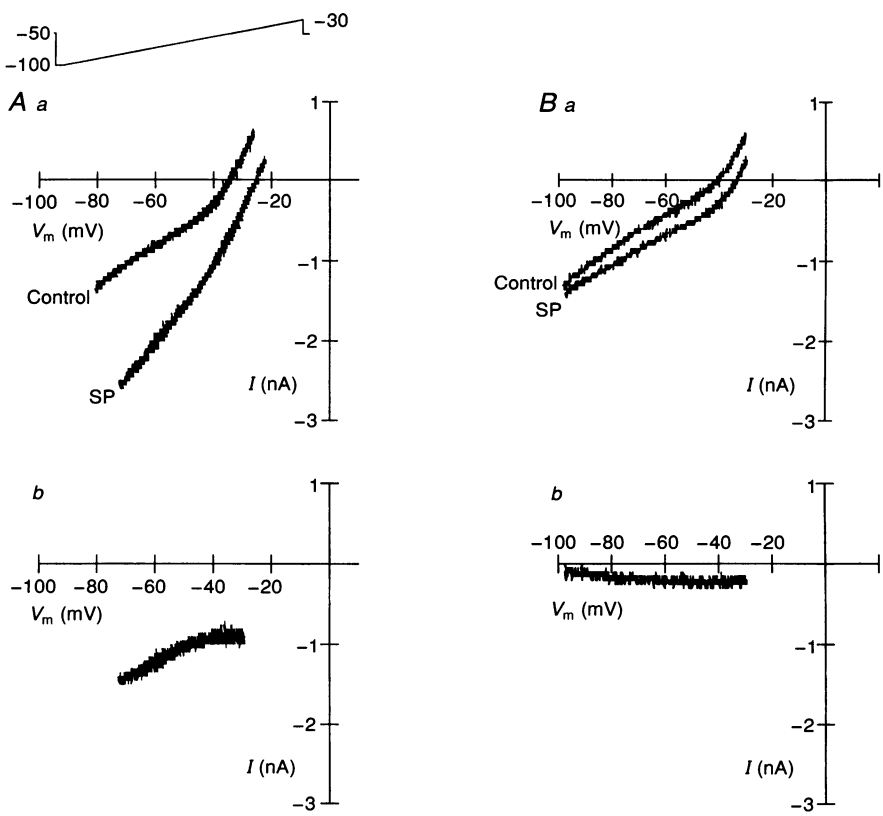

Figure 5. I–V relationships prior to and during the SP-induced current

I–V relationships were obtained prior to (Control) and at the peak of SP-induced current (SP; 1 s application by pressure ejection) in voltage clamped cells using voltage ramps from −100 to −30 mV at a rate of 25 mV s^{-1}. *A*, an example of the I–V relationship recorded in the majority of cells (12 of 15). In these cells, the total membrane current (*a*) immediately following the SP application increased in amplitude with increasing hyperpolarization. The SP-induced current (*b*) was determined by subtraction of the control current from the current recorded in the presence of SP. *B*, an example from the remaining minority of cells (3 of 15). In these cells, the total membrane current (*a*) after the SP application appeared to approach that of the control current at more negative membrane potentials. The SP-induced current (*b*) in this cell appeared to approach zero near the K^+ equilibrium potential.

DISCUSSION

Numerous morphological studies have provided strong evidence for peptidergic afferent input to the parasympathetic postganglionic neurones of the guinea-pig cardiac ganglion (Morris, 1989). Several of these studies have demonstrated tachykinin- and CGRP-immunoreactive fibres in close apposition to the cardiac neurones (Gibbins *et al.* 1985; Urban & Papka, 1985; Hardwick *et al.* 1995). However, very little physiological evidence for peptidergic synaptic input has been demonstrated. Recently, we showed that direct application of SP to the postganglionic neurones resulted in a depolarization of these cells (Hardwick *et al.* 1995). A similar depolarization could be elicited by high frequency stimulation of fibre bundles and was both non-cholinergic and calcium dependent. Using a whole mount preparation, we have been able to demonstrate that the tachykinin-induced depolarization of guinea-pig postganglionic parasympathetic cardiac neurones is due primarily to an increase in a non-selective cation conductance via activation of NK_3 receptors.

Tachykinins depolarize guinea-pig cardiac neurones via NK_3 receptors

In the peripheral nervous system (PNS), tachykinins have been shown to modulate the activity of postganglionic parasympathetic neurones (Myers & Undem, 1993; Hardwick *et al.* 1995; Mawe, 1995) as well as that of enteric neurones (Shen & Surprenant, 1993; Bertrand & Galligan, 1994; Mihara & Nishi, 1994) and sympathetic neurones (Vanner *et al.* 1993; Zhao *et al.* 1996). The tachykinin-induced modulation usually results in a membrane depolarization and/or an increase in neuronal excitability. In the peripheral nervous system, all three neurokinin receptor subtypes have been found to mediate tachykinin-induced responses. In most cases, the tachykinins responsible are either SP or NKA. NKB is rarely found in the peripheral nervous system, especially not in the guinea-pig (Hua, 1986; Otsuka & Yoshioka, 1993). NK_1 receptors are the most prevalent in the PNS, and can be found on neurones (Koyano *et al.* 1993; Shen & Surprenant, 1993; Akasu *et al.* 1996; Grady *et al.* 1996; Zhao, *et al.* 1996), smooth muscle (Grady *et al.* 1996), and other cells (for review see Otsuka & Yoshioka, 1993). NK_2 receptors are also found on smooth muscle, most notably in the bronchi, gut and urinary tract (Otsuka & Yoshioka, 1993; Grady *et al.* 1996). NK_3 receptors have been found in relatively few places in the PNS, but have been localized on enteric neurones (Bertrand & Galligan, 1994; Croci, Landi, Emons-Alt, Le Fur & Manara, 1995; Grady *et al.* 1996), on coeliac ganglion neurones (Zhao *et al.* 1996), as well as on the parasympathetic neurones of the bronchial ganglia (Myers & Undem, 1993) and gallbladder ganglion (Mawe, 1995).

We have now demonstrated that NK_3 receptors are also present on the parasympathetic postganglionic neurones of the guinea-pig cardiac ganglion. The ability of SP to depolarize the neurones in the presence of TTX and zero calcium demonstrates that the tachykinin receptor is localized on the neurone itself. The receptor characterization is based on both the relative potencies of three different agonists, SP, NKA, and senktide, and the efficacies of receptor subtype-specific inhibitors. The rank order of EC_{50} values (senktide > NKA > SP) is consistent with the expected profile for NK_3 receptors. The results obtained using the subtype-specific antagonists reinforced this conclusion. Only SR 142801, the NK_3 receptor antagonist specific for guinea-pig tissues (Emons-Alt *et al.* 1995), significantly reduced the SP-induced depolarization, whereas, the NK_1 receptor antagonist (SR 140333) and NK_2 receptor antagonist (SR 48968) were ineffective. In our previous studies, we found that the NK_2 agonist [β-Ala^8]-neurokinin A(4–10) depolarized the guinea-pig cardiac neurones. Based on this observation, we suggested that the depolarization in some guinea-pig cardiac neurones may be mediated by activation of NK_2 tachykinin receptors. However, in the present study, the specific NK_2 receptor antagonist SR 48968 was not effective and furthermore the effective concentrations required for NKA-induced depolarizations were considerably higher than those normally required for an NK_2 receptor. These observations suggest that NK_2 receptor activation is probably not involved to any significant extent in the tachykinin-induced depolarization in guinea-pig cardiac neurones. Recently, Seabrook, Bowery & Hill (1995) reported that [β-Ala^8]-neurokinin A(4–10) at high concentrations, similar to those used in our previous study, can activate NK_3 as well as NK_2 receptors. Therefore, we conclude that the tachykinin-induced depolarization recorded in guinea-pig cardiac neurones is mediated primarily by activation of NK_3 receptors and that the depolarization produced by [β-Ala^8]-neurokinin A(4–10) most probably resulted from activation of NK_3 rather than NK_2 receptors.

The tachykinin-induced depolarization is primarily due to the increase in a non-selective cation conductance

Several different ionic conductances have previously been demonstrated to be modulated by NK_3 receptor activation. In both bronchial and gallbladder parasympathetic postganglionic neurones, NK_3 receptors are coupled to an increase in a non-selective cation conductance (Myers & Undem, 1993; Mawe, 1995). A similar ionic mechanism has been described in rat dorsal root ganglion neurones (Inoue *et al.* 1995). In guinea-pig myenteric neurones, Bertrand & Galligan (1994) found that the NK_3-mediated depolarization was due to both a decrease in a K^+ conductance and an increase in a Cl^- conductance. Thus, in our analysis of the ionic conductances underlying the tachykinin-induced depolarization in the guinea-pig cardiac ganglion, we examined several different potential mechanisms.

A SP-induced activation of a Cl^- conductance has been reported for both submucosal and myenteric neurones in the guinea-pig enteric system (Bertrand & Galligan, 1994; Mihara & Nishi, 1994). In the present study, we found no evidence that SP activated a Cl^- conductance in guinea-pig

cardiac neurones. Neither the presence of niflumic acid nor a reduction of the extracellular Cl^- concentration significantly altered the SP-induced depolarization.

Previously, in many types of neurones it has been found that the SP-induced depolarization results from inhibition of a K^+ conductance (Yamaguchi *et al.* 1990; Shen & North, 1992; Koyano *et al.* 1993; Shen & Surprenant, 1993; Vanner *et al.* 1993; Bertrand & Galligan, 1994; Ishimatsu, 1994; Akasu *et al.* 1996). We found in the present study that the SP-induced depolarization was not significantly altered by exposure to either 2 mM Ba^{2+} or 5 mM TEA. Consequently, we conclude that the SP-induced depolarization does not rely on inhibition of a Ba^{2+}-sensitive, or a TEA-sensitive K^+ conductance.

Our results are consistent with the view that the SP-induced depolarization is due to activation of a non-selective cation conductance. Reduction of the extracellular Na^+ concentration by partial replacement of Na^+ with NMG^+ consistently decreased the SP-induced depolarization in current clamped cells and decreased the SP-induced inward current in voltage clamped cells. Furthermore, the *I–V* relationship was shifted in the presence of SP in a manner consistent with an increase in non-selective cation conductance and a reversal potential of approximately 0 mV estimated from the measurements of SP-induced currents at different holding potentials corresponds with the expected value for a non-selective cation conductance. Consequently, in the majority of the neurones studied, in the presence of SP, total membrane current continued to increase as the holding potential was progressively made more negative. In a small percentage of neurones, the control *I–V* curve and the *I–V* curve recorded in the presence of SP remained parallel or appeared to converge with progressive hyperpolarization. We suggest that in these cells, inhibition of a K^+ conductance may have contributed to the SP-induced depolarization along with an activation of a non-selective cation conductance. Previously, we showed that the tachykinins increased excitability in a subpopulation of the guinea-pig cardiac neurones, an effect consistent with inhibition of a K^+ conductance such as the M current (Hardwick *et al.* 1995). Edwards *et al.* (1996) demonstrated that there are different populations of guinea-pig cardiac neurones based on their membrane conductance properties such as the presence of a hyperpolarization-activated current (I_H). This heterogeneity in membrane properties might also be a factor contributing to the observed differences in *I–V* relationships.

In conclusion, the results of the present study indicate that the tachykinin-induced depolarization of guinea-pig cardiac neurones is due to the activation of NK_3 tachykinin receptors. Furthermore, activation of the NK_3 receptors mediates an increase in non-selective cation conductance in these neurones. Therefore, the tachykinin-induced depolarization in the guinea-pig cardiac neurones, both receptor subtype and ionic mechanism, is similar to that produced by tachykinins in other guinea-pig parasympathetic postganglionic neurones such as those found in the gallbladder ganglia (Mawe, 1995) and in bronchial ganglia (Myers & Undem, 1993). The results of a recent study by Roccon, Marchionni & Nisato (1996) demonstrated a NK_3 receptor-mediated bradycardia in guinea-pigs which involved activation of parasympathetic neurones. This indicates that neuropeptides released from afferent fibres could be effective in local reflex modulation of cardiac function.

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