Substance P hyperpolarizes vagal sensory neurones of the ferret

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1. Intracellular recordings were made in intact and in acutely dissociated vagal afferent neurones (nodose ganglion cells) of the ferret to investigate the effects of substance P (SP).

2. In current-clamp recordings, SP (100 nM) applied by superfusion hyperpolarized the membrane potential ($7 \pm 0{\cdot}7$ mV; mean ± S.E.M.; $n = 105$) and decreased the input resistance in 80% of the neurones. With voltage-clamp recording, SP produced an outward current of $3 \pm 0{\cdot}2$ nA ($n = 10$).

3. The SP current was concentration dependent with an estimated EC_{50} of 68 nM. The SP-induced hyperpolarization or current was mimicked by the tachykinin receptor NK_1 agonist Ac-[Arg^6, Sar^9, $Met(O_2)^{11}$]SP(6–11) (ASM-SP; 100 nM; $n = 10$) and blocked by the NK_1 antagonist CP-96,345 (10 nM; $n = 6$), but not by the NK_2 antagonist SR48968 (100 nM; $n = 4$). No measurable change in membrane potential or input resistance was observed with application of either [β-Ala^8]neurokinin A or senktide, selective NK_2 and NK_3 receptor agonists, respectively (100 nM; $n = 3$ for each agonist).

4. The reversal potential (E_{rev}) for the SP outward current was $-85 \pm 2{\cdot}5$ mV ($n = 4$). The E_{rev} for the SP response shifted in a Nernstian manner with changes in extracellular potassium concentration. Alterations in extracellular sodium or chloride concentrations had no significant effect on the E_{rev} for the SP response ($n = 3$ for each ion).

5. Nominally Ca^{2+}-free external solution abolished the SP response. Removal of magnesium from the extracellular solution had no effect on the response.

6. Caesium (100 μM), barium (1 mM), tetraethylammonium (TEA; 5 mM), apamin (10 nM) and 4-aminopyridine (4-AP; 4 mM) each completely prevented the SP response ($n \geqslant 3$ for each).

7. These results indicate that SP, via an NK_1 receptor, can induce a Ca^{2+}-dependent outward potassium current which hyperpolarizes the resting membrane potential of vagal afferent somata.

Substance P (SP) is present in neurones of both the central and peripheral nervous system (reviewed by Otsuka & Yoshioka, 1993). In a subset of primary afferent neurones SP may function as a signal molecule following its release from central and peripheral terminals (Otsuka & Yoshioka, 1993). For example, noxious cutaneous stimuli elicit SP-mediated excitatory slow synaptic potentials in dorsal horn neurones of the spinal cord (De Koninck & Henry, 1991) and in neurones of the trigeminal nucleus (Henry, Sessle, Lucier & Hu, 1980). Released from peripheral afferent nerve endings during an axon reflex, SP functions in the development of neurogenic inflammation (Otsuka & Yoshioka, 1993). Most neurotransmitters, once secreted, can activate presynaptic autoreceptors to modulate the quantity of transmitter secretion (Starke, Gothert & Kilbinger, 1989). Thus, if SP functions as a neurotransmitter in primary afferent neurotransmission, distinct autoreceptors for this neuropeptide should exist in the membranes of these neurones. Indirect evidence for SP autoreceptors already exists. Some guinea-pig trigeminal ganglion neurones and bullfrog dorsal root ganglion neurones are depolarized by exogenous application of SP (Dray & Pinnock, 1982; Spigelman & Puil, 1990; Ishimatsu, 1994).

Many vagal primary afferent neurones (nodose and jugular ganglion neurones) of numerous species have been immunocytochemically shown to contain SP (Katz & Karten, 1980; Kummer, Fischer, Kurkowski & Heym, 1992; and see review by Otsuka & Yoshioka, 1993). No data, to our knowledge, exists assessing the presence of SP in primary afferent neurones of the ferret. However, preliminary immuno-

cytochemical results from our laboratory reveal the existence of this peptide in ferret nodose neurones. These SP-containing neurones probably use this peptide as a signal molecule because vagal stimulation results in the release of SP from peripheral nerve terminals in airway smooth muscle, oesophagus, heart and pylorus (Lidberg *et al.* 1982; Lundberg, Brodin & Saria, 1983; Saria, Martling, Yan, Theodorsson-Norheim, Gamse & Lundberg, 1988). Additionally, specific tachykinin receptor antagonists potently block contraction of isolated guinea-pig tracheal smooth muscle elicited by vagal stimulation (Ellis & Undem, 1992). Thus, vagal afferents probably utilize SP as a neurotransmitter and are good candidates for possessing autoreceptors for SP. Using intracellular recording techniques we have observed that the majority of nodose ganglion neurones of the ferret respond to exogenously applied SP. To date, all reports of SP action have shown it to have excitatory effects. Unexpectedly, nodose neurones were hyperpolarized by SP. In this study we describe physiological characteristics and pharmacological properties of this unusual SP response.

METHODS

Preparation of tissue

Nodose ganglia were isolated from adult male ferrets (1–2 kg) purchased from Triple F Farms (Sayre, PA, USA). After the animals were killed by CO_2 asphyxiation followed by exsanguination, the ganglia were removed and placed in 4 °C Locke solution of the following composition (mM): 136 NaCl, 5·6 KCl, 14·3 $NaHCO_3$, 1·2 NaH_2PO_4, 2·2 $CaCl_2$, 1·2 $MgCl_2$, 10 dextrose, equilibrated continuously with 95% O_2–5% CO_2 (pH 7·2–7·4). Ganglia were dissociated for use either on the day of dissection or stored in Locke solution (4 °C) overnight for dissociation the following morning. Storage did not measurably affect the electrophysiological or pharmacological characteristics of the dissociated cells. Neurones were enzymatically dissociated from desheathed ganglia using the procedure described by Christian, Togo, Naper, Koschorke, Taylor & Weinreich (1993). Briefly, the ganglion was desheathed, cut into three to four pieces, and placed in Ca^{2+}-free, Mg^{2+}-free Hanks' balanced salt solution (CMFH) of the following composition (mM): 138 NaCl, 5·0 KCl, 4·0 $NaHCO_3$, 0·3Na_2HPO_4, 0·3 KH_2PO_4, 5 dextrose and 0·03 Phenol Red. All incubations were done at 37 °C. The pieces of tissue were incubated for 10 min in 10 ml CMFH containing papain (100 μl; 0·1 mg ml^{-1}; Boehringer Mannheim) which was activated by L-cysteine (0·2 mg ml^{-1}; Sigma). The tissue was then washed twice with CMFH and incubated for 15 min in 4 ml CMFH containing dispase (grade II; 2 mg ml^{-1}; Boehringer Mannheim) and collagenase Type 1A (1 mg ml^{-1}; Sigma). During incubation the tissue was triturated with a fire-polished Pasteur pipette at 10 and 15 min. After washing with Leibovitz L-15 medium (Gibco) containing 10% fetal bovine serum (FBS, v/v; JRH Biosciences, Lexena, KS, USA), cells were resuspended in 0·5 ml L-15–10% FBS per coverslip. A twenty-four-well culture plate was prepared by placing a 15 mm poly-D-lysine-coated coverslip and 0·5 ml L-15–10% FBS in the bottom of each well. After resuspension, 0·5 ml of the cell suspension was added to each well. Neurones were incubated for at least 3 h at 37 °C before use. Recordings were performed on neurones within 48 h of plating.

Electrophysiological studies

Intracellular microelectrodes were fabricated from aluminosilicate capillary glass (1·0 mm o.d., 0·68 mm i.d.; Sutter Instruments, San Francisco, CA, USA) on a Brown–Flaming puller (Sutter) and backfilled with a solution of 3 M KCl (20–40 MΩ). The microelectrode was connected to an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA, USA). The discontinuous current injection mode (switching frequency, 5–6 kHz) of the amplifier was used for both current-clamp and voltage-clamp applications; the headstage voltage was monitored continuously. Current and voltage outputs were viewed on-line with an oscilloscope and chart recorder and digitized with a Neurocorder (Neurodata Instruments, New York, NY, USA) for storage on videocassette tapes for off-line analysis. Data acquisition and voltage-clamp protocols were controlled using pCLAMP5 software (Axon Instruments). Neurones were superfused with oxygenated Locke solution (3–4 ml min^{-1}) at room temperature (20–22 °C). The superfusate level was lowered to approximately 50 μm above the surface of the neurones to minimize electrode stray capacitance. A neurone was judged acceptable for study if its resting membrane potential (<-50 mV) and action potential overshoot (above 0 mV) remained stable for 5 min after impalement. In experiments where input resistance was assessed, current ($-0·1$ to $-1·0$ nA) or voltage (-5 or -10 mV) transients were applied throughout the experiment at 2·6 s intervals. For intracellular measurement in nodose neurons from intact ganglia, ferret ganglia were prepared as previously described for rabbit and guinea-pig nodose neurones (Leal-Cardoso, Koschorke, Taylor & Weinreich, 1993; Undem & Weinreich, 1993).

Preparation and delivery of solutions

For experiments using low extracellular sodium, NaCl (136 mM) was replaced by *N*-methyl-D-glucamine (136 mM; Sigma), resulting in a final sodium concentration of 15·1 mM. For experiments using low extracellular chloride, NaCl (136 mM) was replaced with NaCl (45 mM) and sodium isethionate (91 mM; Sigma), resulting in a final chloride concentration of 57·4 mM. The pH of both these solutions was adjusted to 7·4. To minimize a shift in the junction potential during experiments in low-chloride solutions, the bath was grounded through a 3 M KCl–agar bridge.

On the day of use, 4-aminopyridine (4-AP) was solubilized from powder. All other solutions were prepared daily for experiments from stock aliquots which were stored at −20 °C. Reservoirs containing solutions of various drugs or Locke solution with modified ionic content were connected to the inflow line of the recording chamber by three-way valves which could rapidly divert the source of superfusion from the main reservoir. This means of drug delivery introduced a 15 s delay from the activation of the valve to arrival of the drug solution in the chamber.

Apamin, 4-AP and SP were obtained from Sigma. CP-96,345 and CP-96,344 were provided by Dr Jim Heym, Pfizer Inc.

Data analysis

Data are expressed as means ± S.E.M. Statistical significance was assessed using Student's two-tailed, unpaired t test at the $P < 0·05$ level of significance. Figure construction and fitting of mathematical functions to data were accomplished with SigmaPlot software (Jandel Scientific, San Rafael, CA, USA). Cell diameters were computed by averaging the minimum and maximum diameters of the neuronal profiles viewed at ×400 magnification with Nomarski optics.

A semi-logarithmic plot of the concentration–response relationship was iteratively fitted using the four parameter logistic equation:

$$Y = \frac{(\max - \min)}{(1 + x/EC_{50})^b} - \min,$$

where Y is the response to x concentration of SP, max and min are the maximum and minimum responses, EC_{50} is the SP concentration at half-maximum response and b is the Hill coefficient using the Levenberg–Marquardt non-linear least-squares algorithm (Sigmaplot, Jandel Scientific).

RESULTS

General characteristics of ferret nodose neurones

Acutely dissociated ferret nodose neurones appeared spherical or ovoid in shape, with an average diameter of $54 \pm 0{\cdot}7$ μm ($n = 107$; range, 33–77 μm). Some dissociated cells revealed small tufted processes reminiscent of periglomerular and pericellular arborizations associated with some nodose neurones (Cajal, 1904; Lieberman, 1976). The mean resting membrane potential (V_m) of these neurones was $-61 \pm 0{\cdot}6$ mV ($n = 225$; range, −37 to −88 mV) and their input resistance (R_i) was $21 \pm 1{\cdot}1$ MΩ ($n = 225$; range, 2–120 MΩ). The relatively low mean R_i of these neurones did not appear to arise from injury associated with microelectrode impalement or trauma associated with the dissociation technique. Stable V_m and R_i values could typically be achieved for > 2 h, and impalement by higher resistance microelectrodes (> 80 MΩ with 3 M KCl) revealed similar V_m and R_i values (V_m, $-62 \pm 2{\cdot}2$ mV; range, −55 to −68 mV; R_i, $22 \pm 4{\cdot}1$ MΩ; range, 20–31 MΩ; $n = 5$ for both). In addition, the values recorded in the isolated neurones were not significantly different than those observed in neurones from intact nodose ganglia (V_m, $-70 \pm 0{\cdot}7$ mV; range, −53 to −84 mV; R_i, $24 \pm 1{\cdot}3$ MΩ; range, 10–60 MΩ; $n = 93$ for both).

Depolarizing current steps elicited an action potential overshooting 0 mV. The average spike amplitude was $96 \pm 1{\cdot}5$ mV ($n = 157$; range, 50–130 mV) and the average overshoot above 0 mV was $32 \pm 1{\cdot}1$ mV ($n = 169$; range, 5–60 mV). The presence of an overshooting action potential (> 5 mV) was used as the primary criterion for selection of healthy neurones (see Methods).

Substance P responses in acutely isolated and intact nodose neurones

The effect of SP was examined on 123 acutely dissociated neurones (Fig. 1). Bath application of 100 nM SP for 45 s produced a reversible membrane hyperpolarization and an associated decrease in R_i (Fig. 1*A*) in 105 neurones; the remaining cells were unresponsive to SP at this concentration. The mean response was $-7 \pm 0{\cdot}7$ mV (range, −1 to −17 mV) with $-6 \pm 1{\cdot}3$ MΩ (range, 0 to −35 MΩ) change in R_i. In ten neurones voltage clamped to −60 mV, 100 nM SP produced an outward current of $3 \pm 0{\cdot}2$ nA (range, 2–4·4 nA) with an associated increase in conductance ($125 \pm 14{\cdot}8$ pS; range, 75–200 pS; Fig. 1*B*). The 15 s time lag associated with the superfusion system (see Methods) was not sufficient to explain the 30 s lag between the beginning of the SP pulse and the onset of the responses. The basis for the delayed response was not pursued at this time.

An analogous SP-induced hyperpolarization of the membrane potential was confirmed in neurones in intact ferret nodose ganglia. Bath application of SP (100 nM) produced a mean hyperpolarization of $5 \pm 0{\cdot}8$ mV (range,

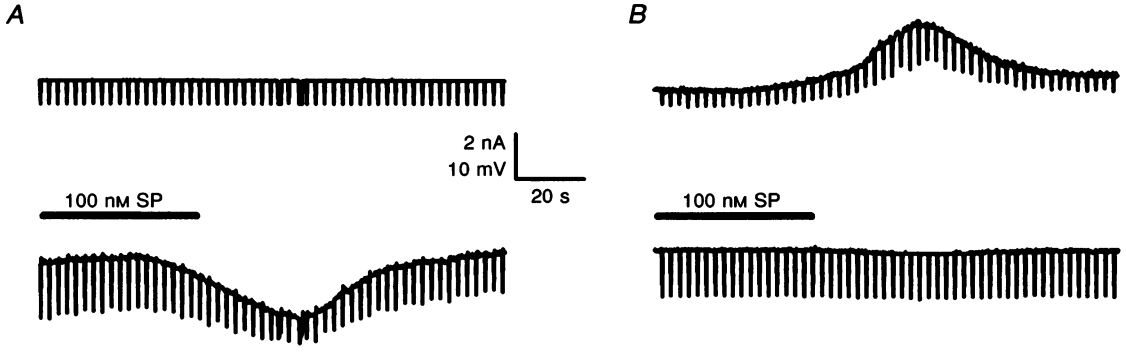

Figure 1. Effect of substance P (SP) on membrane properties in an acutely isolated adult nodose neurone

Current (upper traces) and voltage records (lower traces) from current- (*A*) or voltage-clamped (*B*) SP responses in the same neurone. Negative deflections in these and all subsequent traces are produced by hyperpolarizing transmembrane current or voltage steps (−1 nA or −10 mV, 160 ms duration, 260 ms interstep interval) to estimate membrane resistance or membrane conductance. *A*, reversible membrane hyperpolarization and associated decrease in input resistance elicited by superfusion of 100 nM SP for 45 s (horizontal bar above trace). Resting membrane potential was −60 mV. *B*, voltage-clamp recording in the same neurone showing an outward current and associated conductance increase elicited by superfusion of 100 nM SP for 45 s (holding potential, −50 mV).

3–8 mV) and a 11 ± 1·7 MΩ (range, 10–15 MΩ) decrease in R_i in six of six neurones tested from four ganglia. The remainder of this work deals exclusively with isolated adult nodose neurones.

Concentration–response relationship to substance P

The concentration–response relationship for the SP current was examined by voltage clamping V_m to −60 mV and measuring the peak amplitude of the SP-induced outward current (Fig. 2). In preliminary experiments we observed that repeated applications of SP elicited desensitized responses; following three 45 s pulses of 100 nM SP, spaced 5 min apart, the amplitude of subsequent SP currents was diminished by 32 ± 7·4% of the initial SP application ($n = 6$; range, 10–60%). To eliminate this problem, only one concentration of SP was applied to each cell. Normalization of responses was not necessary because of the reproducibility of the SP responses from cell to cell at −60 mV (see Fig. 2*A*). SP at concentrations ranging from 1 nM to 1 μM produced a concentration-dependent increase in outward current (Fig. 2*A*). A semi-logarithmic plot of current amplitude *versus* SP concentration revealed a sigmoidal relationship that varied over two orders of magnitude and was saturated at high concentrations; features diagnostic of receptor-mediated interactions. The EC_{50} was estimated to be 68 nM with a Hill coefficient of 1·2 (see Methods). Representative responses at each of the tested concentrations are illustrated in Fig. 2*B*.

Pharmacology of the substance P response

Endogenous tachykinins (SP, neurokinin A (NKA) and neurokinin B) activate the three known tachykinin receptors, designated NK_1, NK_2 and NK_3 (Otsuka & Yoshioka, 1993). Activation of each of these receptor subtypes by SP has been previously associated with neuronal depolarization. To assess which tachykinin receptor subtype might subserve the hyperpolarizing response to SP, we applied agonists and antagonists selective for different tachykinin receptor subtypes. Agonists were applied at concentrations 100 times greater than their dissociation constant (K_d). Application of 100 nM ASM-SP, an NK_1 specific agonist, induced a membrane hyperpolarization (7 ± 0·5 mV; $n = 10$, Fig. 3*A*). In contrast, bath application of either 100 nM [β-Ala^8]neurokinin A or senktide, selective NK_2 and NK_3 receptor agonists, respectively, produced no measurable (<1 mV) membrane potential changes ($n = 3$ for each agonist).

CP-96,345 and SR48968 are specific non-peptide antagonists for the NK_1 (Snider *et al.* 1991) and NK_2 (Emonds-Alt *et al.* 1992) receptors, respectively. At the time of this study, no NK_3 specific antagonist was available. At concentrations as

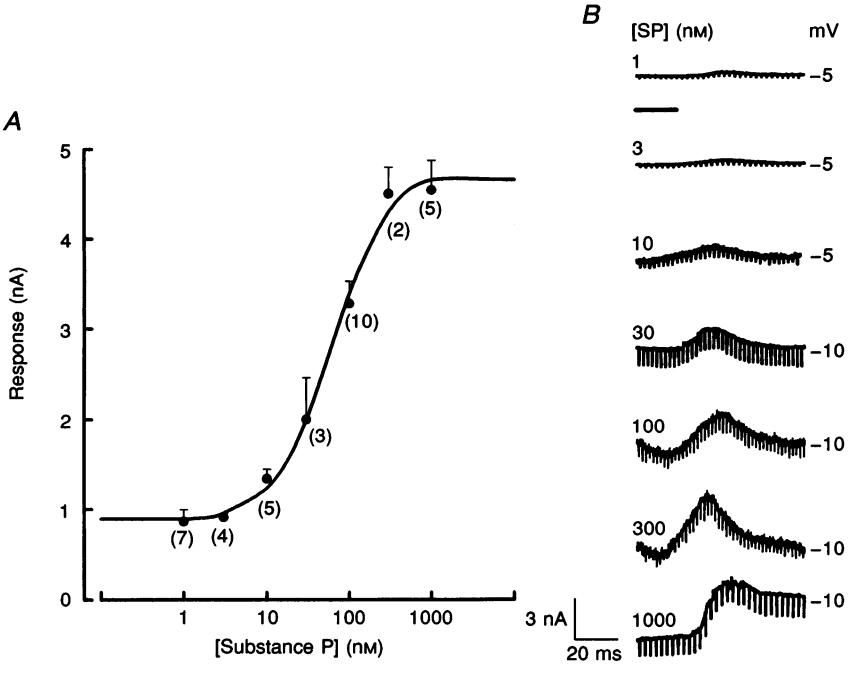

Figure 2. Relationship between substance P concentration and peak amplitude of SP-induced outward current

Each neurone was voltage clamped to −60 mV and treated with only one concentration of SP by superfusion for 45 s. *A*, semi-logarithmic plot of mean (± S.E.M.) concentration–response relationship (number of neurones in parentheses). Continuous line represents a logistic equation fitted to the data points (see Methods; correlation coefficient (r) = 0·934; EC_{50} = 68 nM; Hill coefficient = 1·2). *B*, representative current responses to increasing SP concentrations (45 s application bar). Each response was recorded in a different neurone. Downward current deflections were elicited by voltage steps of the amplitude given at the end of each trace.

low as 1 nM, CP-96,345 completely prevented the hyperpolarizing response to 100 nM SP (100% prevention in 6 of 6 neurones) or to 100 nM ASM-SP (100% prevention in 4 of 4 neurones; Fig. 3*B*). Because the effects of CP-96,345 may be a result of its interaction with calcium channels (Guard, Boyle, Tang, Watling, McKnight & Woodruff, 1993), control experiments were performed using CP-96,344, an enantiomer of CP-96,345 which is inactive at NK_1 receptors ($EC_{50} > 10$ μM; Guard *et al.* 1993) but has similar activity at calcium channels ($EC_{50} = 33$ nM; McLean *et al.* 1993). At 10 μM, CP-96,344 had little effect on the SP response ($8 \pm 3{\cdot}1$% reduction; $n = 3$; range, 0–12%). CP-99,994, another NK_1 specific antagonist ($EC_{50} = 250$ pM; McLean *et al.* 1993), but without the calcium channel blocking effects of CP-96,345 ($EC_{50} = 3$ μM; McLean *et al.* 1993) (see Discussion), also blocked the SP-induced hyperpolarization (1 μM, 100% prevention in 3 of 3 neurones). In the presence of 1 nM SR48968 the SP response was reduced by only $7 \pm 3{\cdot}9$% ($n = 4$; range, 0–14%). These results with receptor agonists and antagonists, as well as our estimated EC_{50} value of 68 nM for the hyperpolarizing response to SP, indicate that these SP receptors are tachykinin NK_1 receptors.

Ionic mechanism of substance P induced currents

To determine the ionic mechanisms involved in the SP response, reversal potentials (E_{rev}) for the SP-induced current were estimated under various ionic conditions using voltage ramps (from −130 or −120 to −60 or −50 mV for 6 s duration) recorded in the presence and in the absence of SP in the same neurone. The traces in Fig. 4*Ba* depict ramp currents recorded under control conditions and during the peak effect produced by SP (100 nM). The increased current flow during the ramps in the presence of SP reflects the increased membrane conductance produced by the peptide. In this neurone the control and SP ramps intersected at −85 mV (Fig. 4*Bb*). The mean E_{rev} for the SP response in four neurones was $-84 \pm 2{\cdot}5$ mV (range, −79 to −89 mV).

A hyperpolarizing SP response with an accompanying increase in membrane conductance and a E_{rev} value near −85 mV suggested that potassium ions may be major charge carriers in this response. To test this hypothesis further, the E_{rev} of the SP current was measured in Locke solutions containing varied potassium concentrations. Superfusing with Locke solution containing double the normal potassium (11·2 mM) produced a E_{rev} of $-70 \pm 2{\cdot}2$ mV ($n = 3$; range, −67 to −74 mV; Fig. 4*A*). This 15 mV shift in the E_{rev} was close to the 17 mV shift predicted by the Nernst equation. In Locke solution containing one-third normal potassium (2 mM), the E_{rev} values averaged $-106 \pm 3{\cdot}0$ mV ($n = 4$; range, −98 to −112 mV; Fig. 4*C*), close to the predicted E_{rev} of −111 mV. As depicted in Fig. 4*D*, the relationship between the extracellular potassium concentration and E_{rev} was linear and closely approximated that predicted by the Nernst equation. These results support the hypothesis that the SP-induced outward currents are carried largely, if not entirely, by potassium ions.

To gauge the possible contribution of chloride ions to the SP response, the E_{rev} of the SP response was measured in normal Locke solution and in Locke solution containing one-third of the normal chloride concentration. There was no significant change ($P < 0{\cdot}05$) in the E_{rev} in Locke

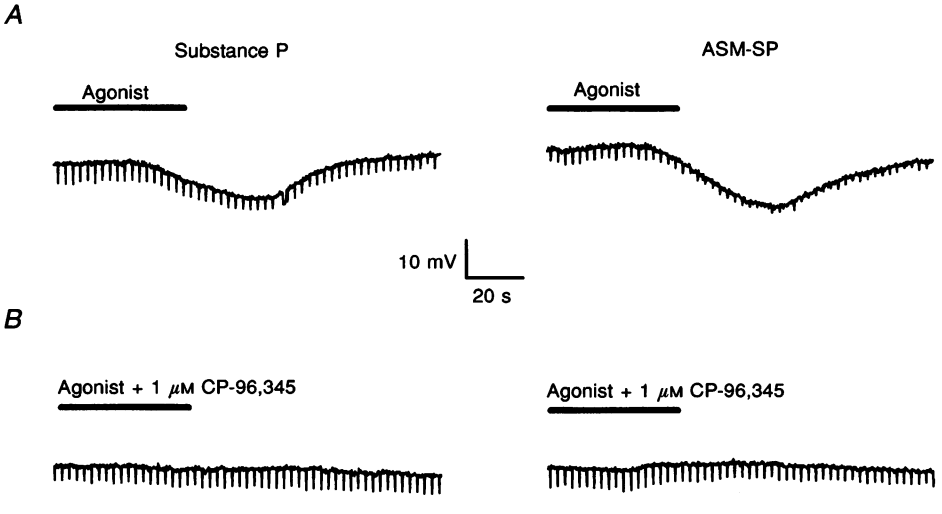

Figure 3. Effect of tachykinin receptor antagonists on substance P- and ASM-SP-induced hyperpolarization recorded in the same current-clamped neurone

A, membrane hyperpolarization resulting from superfusion of SP (left) or ASM-SP (right) for 45 s (horizontal bar). *B*, in the presence of 1 μM CP-96,345, a NK_1 receptor antagonist, 100 nM SP (left) or 100 nM ASM-SP (right) showed no significant membrane hyperpolarization. CP-96,345 was applied for 2 min prior to introduction of agonist. Resting membrane potential was −63 mV; −500 pA constant-current steps (160 ms in duration) were delivered at 0·4 Hz. Horizontal bar depicts the 45 s period of agonist application.

solution with lowered chloride compared with that measured in the control solution (−83 ± 3·3 mV; $n = 3$; range, −78 to −89 mV). Experiments where external sodium was reduced to 15·5 mM also failed to reveal any measurable change in E_{rev} for the SP responses (−86 ± 3·0 mV; $n = 3$; range, −80 to −90 mV). Similarly, the presence of 1 μM tetrodotoxin in the perfusate did not measurably affect the SP response ($n = 5$). These data further support the hypothesis that the SP response is produced predominantly by a potassium current.

Effects of potassium channel blockers

The results obtained with ion substitution indicate that the SP-induced outward current is carried primarily by potassium. To support this contention and to determine the

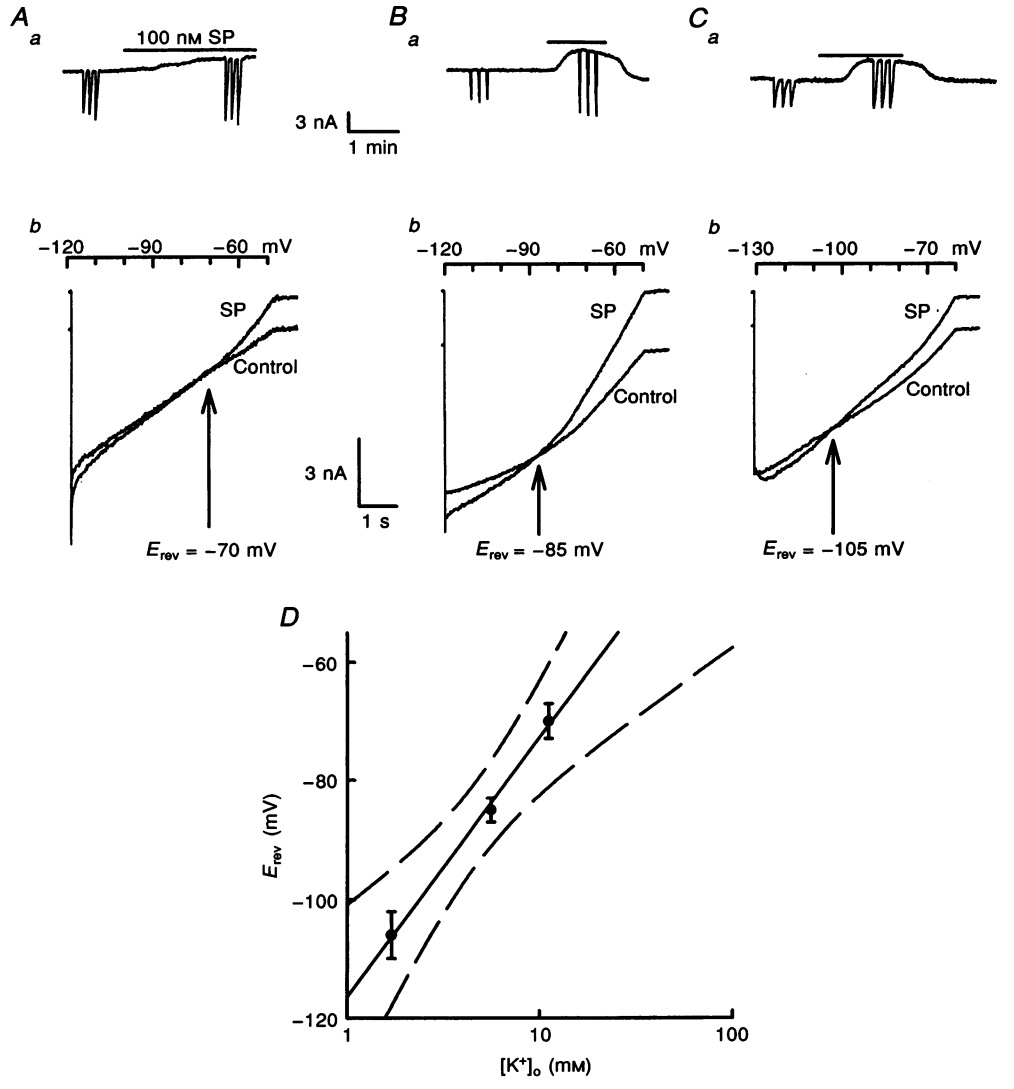

Figure 4. Effects of altering extracellular potassium concentration on the reversal potential value of the substance P-mediated current

A, *B* and *C* represent three neurones in Locke solution containing 11·2 mM (*A*), 5·6 mM (*B*) and 2 mM (*C*) potassium. *A a*, *Ba* and *Ca* are membrane currents evoked by superfusion of 100 nM SP (horizontal bar). Negative deflections were produced by 6 s linear voltage ramps (11·7 mV min^{-1}) between *A* and *B*, −120 and −50 mV (holding potential, −50 mV), or *C*, −130 and −60 mV (holding potential, −60 mV). Voltage ramps were delivered before and during SP application (horizontal bars). *A b*, *Bb* and *Cb*, expanded current responses produced by voltage ramps before and during the steady-state peak outward current generated by superfusion of 100 nM SP. Indicated reversal potentials (E_{rev}) were measured as the potential at which the traces intersect. Each trace is the average of the 3 ramps shown above under the appropriate conditions. *A*, *B*, and *C* were recorded from three different neurones. *D*, semi-logarithmic plot of the relationship between the extracellular potassium concentration and the mean (± S.E.M.) reversal potential values for the SP-induced currents recorded in 3–4 neurones. The shift in the reversal potential value was linear ($r = 0·935$) and approximates that predicted by the Nernst equation (continuous line). Dashed lines are the 95% confidence limits.

type of potassium channel affected by SP, several known blockers of potassium channels were assessed for their effects on the SP-induced outward current. Interestingly, all reagents tested blocked the SP response. Caesium (100 μM), a non-specific blocker of several classes of potassium channels (Rudy 1988), completely prevented responses to 100 nM SP (100% block in 5 of 5 neurones). Barium (Hille, 1992) and TEA (Farley & Rudy 1988) have been reported to block large conductance calcium-activated potassium channels (maxi-K^+ channels). Both 1 mM barium (100% block in 3 of 3 neurones) and 5 mM TEA (100% block in 3 of 3 neurones) completely blocked the SP-induced hyperpolarization. The SP-induced hyperpolarization was also blocked by 10 nM apamin, a selective blocker of small-conductance calcium-activated potassium channels (Fig. 5*B*) and by 4 mM 4-AP, a blocker of A-current (Thompson 1982) (100% block in 3 of 3 neurones). The blocking effect of all potassium channel blockers on the SP response was reversible after switching to drug-free Locke solution. These data indicate that a potassium channel activated by SP is sensitive to block by disparate types of potassium channel blockers.

Effect of divalent cations

To test whether the outward potassium current produced by SP is influenced by influx of Ca^{2+}, the concentration of extracellular calcium ($[Ca^{2+}]_o$) was reduced. In nominally Ca^{2+}-free Locke solution, the response to SP was almost absent (98 $\pm$ 0·9% block; $n = 3$; range, 97–100%, Fig. 5*A*). Because the reduction in $[Ca^{2+}]_o$ may have affected membrane properties important for SP responses, additional experiments were conducted in which the extracellular Ca^{2+} was replaced with Mg^{2+} giving a final Mg^{2+} concentration of 3·4 mM. In this solution the SP response was also completely blocked (100% block in 3 of 3 neurones).

Spigelman & Puil (1990) observed that a depolarizing response to SP in trigeminal neurones was unaffected by lowering $[Ca^{2+}]_o$, but it was reduced by decreasing $[Mg^{2+}]_o$. In ferret nodose neurones, however, the hyperpolarizing SP response in a nominally Mg^{2+}-free Locke solution with normal Ca^{2+} was not measurably affected (<1 mV; $n = 3$; data not shown).

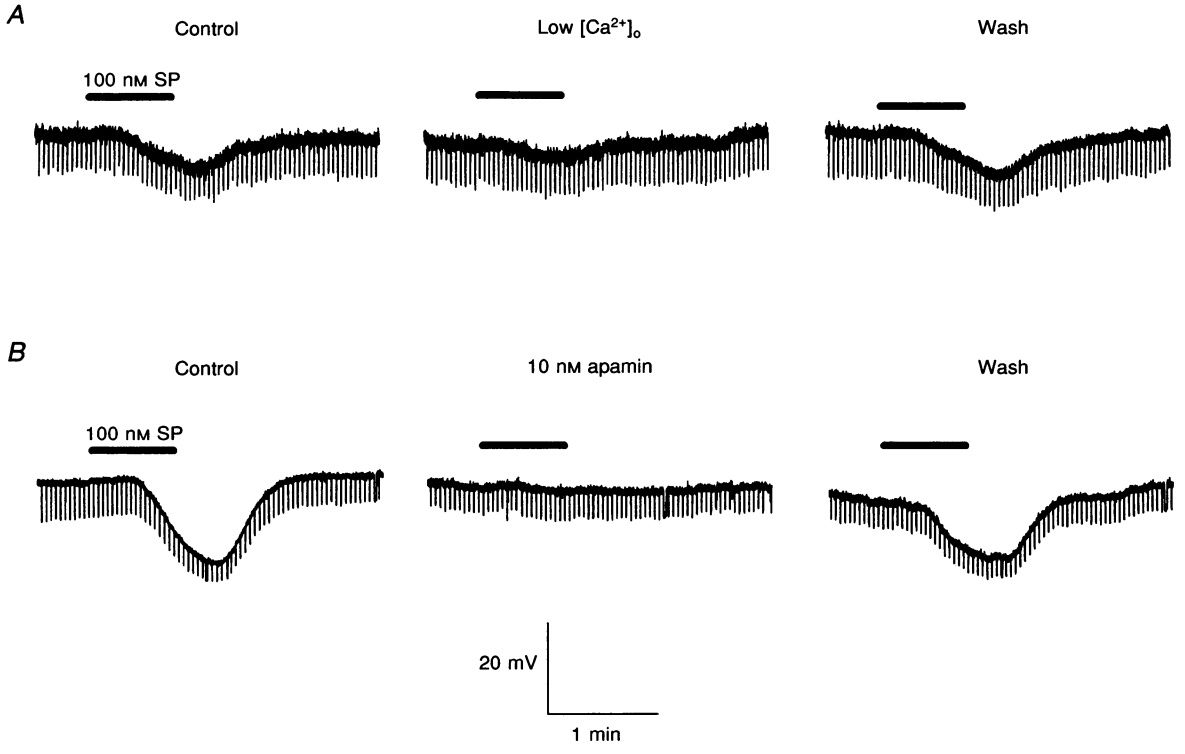

Figure 5. Block of substance P-induced hyperpolarization (*A*) in nominally zero extracellular calcium or (*B*) by apamin

In all panels, horizontal bar above traces indicates application of 100 nM SP for 45 s. *A*, responses to SP application in control Locke solution (2·2 mM Ca^{2+}), 3 min after superfusion with nominally zero calcium Locke solution and 5 min after return to control Locke solution. Resting membrane potential was −66 mV; constant current steps (−500 pA; 160 ms in duration) were delivered at 0·4 Hz. *B*, responses to SP application in control Locke solution, 3 min after superfusion with 10 nM apamin-containing Locke solution and 5 min after return to control Locke solution. Resting membrane potential was −73 mV; constant current steps (−1 nA; 160 ms in duration) were delivered at 0·4 Hz. *A* and *B* are from different neurones.

DISCUSSION

Substance P-induced hyperpolarization

The major observations of this work are firstly, that SP in nanomolar concentrations hyperpolarizes the membrane potential of ferret nodose neurones by activation of an outward calcium-dependent potassium current and secondly, that the response is mediated through a tachykinin NK_1 receptor subtype.

SP is widely accepted as a signal molecule mediating excitatory events in both the central and the peripheral nervous systems (reviewed by Otsuka & Yoshioka, 1993). Our results on the effects of SP on primary afferent neurones are in marked contrast with previous reports showing SP has exclusively depolarizing effects (Dray & Pinnock, 1982; Spigelman & Puil, 1990). One distinction between our results and previous work is the relatively high concentrations of SP that were used: 2–3 μM in guinea-pig trigeminal root ganglia (Spigelman & Puil 1990) and 10 μM in rat dorsal horn neurones (Murase & Randic 1984), compared with nanomolar concentrations used in the present work. We tested higher concentrations of SP but even at concentrations up to 5 μM, SP still produces a membrane hyperpolarization. Another distinction is our demonstration that the SP response in nodose neurones is produced by activation of a pharmacologically definable recognition site, namely, the tachykinin NK_1 receptor.

Although SP previously has not been reported to hyperpolarize neurones directly, Murase & Randic (1984) described a SP-induced hyperpolarization in rat dorsal horn neurone slice preparations that was mediated indirectly. They showed that the hyperpolarizing portion of a biphasic response to SP resulted from presynaptic action of SP on inhibitory interneurones. It is unlikely that this type of mechanism is responsible for the SP responses observed in the present work for the following reasons. First, the SP response was monophasic. Second, nodose neurones are believed to be asynaptic (Lieberman, 1976); the surface of these acutely isolated neurones were devoid of bouton-like structures when viewed with Nomarski optics at ×400 magnification. Third, the nodose neurones were plated sparsely (10–20 neurones per 15 mm round coverslip) and were continuously superfused at 3–4 ml min^{-1} minimizing the concentration of any mediators released from cells on the coverslip, as well as the possibility of these mediators affecting neighbouring neurones. Finally, the hyperpolarizing phase of the biphasic response reported by Murase & Randic (1984) was abolished by 1 μM tetrodotoxin; in contrast, our studies show the SP response was unaffected by the same treatment. Taken together these observations provide compelling evidence that the SP-induced hyperpolarizing response is due to the direct action of SP on the plasma membrane of ferret nodose neurones.

Pharmacology of the substance P response

The endogenous tachykinins SP, NKA and neurokinin B show preference for the NK_1, NK_2 and NK_3 tachykinin receptor subtypes, respectively (reviewed by Otsuka & Yoshioka, 1993). The SP response characterized in this study is mediated by a NK_1 receptor for the following reasons. It is mimicked by a NK_1 receptor specific agonist ASM-SP (100 nM) but not by agonists specific for the NK_2 or NK_3 receptors. Furthermore, a NK_1 specific antagonist CP-96,345 at 1 nM abolishes the response to either SP or ASM-SP. This latter observation must be interpreted with some caution because CP-96,345 is also known to block L-type calcium channels. The EC_{50} value (27 nM) for this effect of CP-96,345, however, is 100-fold higher than that needed to block NK_1 receptors (EC_{50} = 250 pM; McLean *et al.* 1993). This interpretation is supported by the lack of effect on the SP response of CP-96,344, an analogue of CP-96,345 which shares its calcium channel blocking effect (EC_{50} = 33 nM; McLean *et al.* 1993) without inhibiting NK_1 receptor activation (EC_{50} > 10 μM; Guard *et al.* 1993). At concentrations as low as 1 nM, CP-99,994, a high-affinity blocker of NK_1 receptors (EC_{50} = 250 pM; McLean *et al.* 1993) with low affinity for calcium channels (EC_{50} = 3 μM; McLean *et al.* 1993), also prevents the SP-induced hyperpolarization further implicating NK_1 receptor activation. Thus, our cumulative pharmacological data with tachykinin agonists and antagonists indicate that the hyperpolarizing response to SP is specifically mediated through an NK_1 subtype of tachykinin receptor. The concentration–response relationship for SP is a sigmoid log dose–response curve which saturated over two orders of magnitude with an EC_{50} value of 68 nM. EC_{50} values for SP effects in nervous tissue of ferret are currently not available. Comparison EC_{50} values for the effect of SP on non-neuronal tissue in ferret are 200 nM for tracheal albumin secretion (Webber, 1989) and 0·34 nM for airway macromolecular secretion (Gentry, 1991). Both of these studies compared the SP responses with that induced by NKA and found the EC_{50} for the NKA responses to be one to two orders of magnitude larger than that for SP. The EC_{50} values for SP reported in nervous tissue from other species ranged from 1 to 100 nM. Thus, the EC_{50} value for SP determined in nodose neurones is within the range reported for NK_1 receptors in ferret and in other species.

Ionic basis of the hyperpolarizing substance P response

The hyperpolarizing SP response (or the outward current) is associated with an increase in membrane conductance and has a E_{rev} near −85 mV indicating that potassium ions are the major charge carriers. The importance of potassium current to this response was documented by showing the shift of the SP E_{rev} values over a six-fold change in external potassium ion concentration was consistent with that predicted by the Nernst equation. Alterations in the external sodium or chloride concentrations did not

measurably affect the SP response or the E_{rev}, indicating that these latter ions do not substantively participate in this response. Thus, the SP-induced hyperpolarization appears to be mediated through activation of potassium channels. These channels seem pharmacologically unusual because they are blocked by a wide variety of potassium channel blockers (TEA, apamin, 4-AP, barium and caesium). The basis for this remains to be resolved. Our investigation of the ionic basis of the SP-induced hyperpolarization also suggested that this response is dependent upon $[Ca^{2+}]_o$; however, a possible role for intracellular Ca^{2+} stores cannot be discounted. Thus, it is produced by a calcium-dependent potassium conductance. Whether Ca^{2+} affects this potassium channel directly or indirectly requires further investigation.

Physiological relevance of the hyperpolarizing SP response

SP has been localized to primary afferent neurones of the dorsal root (Dalsgaard, *et al.* 1982; Lindh, Dalsgaard, Elfvin, Hokfelt & Cuello, 1983), trigeminal root (McCarthy & Lawson, 1989), jugular (Katz & Karten, 1980) and nodose ganglia (Katz & Karten, 1980; Helke & Hill, 1988; Kummer *et al.* 1992). Activation of these neurones elicits release of mediators, including tachykinins such as SP, from the sensory nerve terminals in the CNS and in the periphery (Saria *et al.* 1988). At peripheral endings released SP participates in neurogenic inflammation (Otsuka & Yoshioka, 1993) while at the central nerve terminals SP functions as an excitatory neurotransmitter (De Koninck & Henry, 1991). Our observations revealing SP-induced hyperpolarizing responses in many nodose neurones may imply that these NK_1 receptors function as autoreceptors in these neurones. Several lines of evidence are compatible with this possibility. First, ample evidence exists in guinea-pig (Kummer *et al.* 1992), rat (Helke & Hill, 1988), rabbit (Katz & Karten, 1980), monkey (Ling, Yick, Ng & Wong, 1992) and human (Lundberg *et al.* 1979) that some vagal afferents are immunopositive for SP although analogous data have yet to be described in ferret neurones. Second, visceral primary afferent neurons have been shown to release SP upon stimulation (Saria *et al.* 1988). Finally, field stimulation of guinea-pig airway smooth muscle elicited NK_2 receptor-mediated contractions that were potentiated by the NK_1 antagonist CP-96,345; an effect Renzetti, Shenvi & Buckner (1992) interpreted as possibly arising from activation of prejunctional NK_1 receptors.

Like many other neurotransmitters, impulse-released SP can also act presynaptically to modify its secretion. Nerve-terminal cell-surface receptors that are activated by mediators released by that neurone and serve to limit the amount of its mediator released are known as inhibitory autoreceptors. Autoreceptors are widespread throughout the peripheral nervous system and exist for a wide range of neurotransmitters including neuropeptides such as opioids (reviewed by Starke *et al.* 1989). These data, taken together, present the possibility that inhibitory autoreceptors for SP reside on vagal afferents. Whether a membrane hyperpolarization leads to an inhibition of SP release from these afferents remains unresolved. It is interesting to note in this regard that Ducreux & Puizillout (1995) observed that when nodose neurones are hyperpolarized, their action potential durations can decrease by about 40%, an effect which could profoundly influence neurosecretion.

A SP-induced hyperpolarization has not been previously reported which raises the possibility that this effect is species specific. This is unlikely because we have observed that SP (100 nM) acting through NK_1 receptors hyperpolarizes neurones of the superior vagal (jugular) ganglion of the rabbit (authors' unpublished observations). Interestingly, the density of SP immunoreactive neurones is significantly larger in this ganglion than in the nodose (Katz & Karten, 1980; Kummer, *et al.* 1992). In non-neuronal tissue, a SP-induced hyperpolarization has recently been reported in porcine endothelial cells (Sharma & Davis, 1994).

In summary, vagal afferents are known to contain and release SP. We have shown that SP, acting through an NK_1 receptor, activates a calcium-dependent potassium conductance which hyperpolarizes the nodose ganglion neurones. If these receptors also exist in nerve terminal membranes, they may subserve an inhibitory autoreceptor function.

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