Actions of substance P on membrane potential and ionic currents in guinea pig stellate ganglion neurons

ROBERT GILBERT, JENNIFER S. RYAN, MAGDA HORACKOVA, FRANK M. SMITH, AND MELANIE E. M. KELLY

Departments of Pharmacology, Physiology and Biophysics, and Anatomy and Neurobiology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

The purpose of the present study was to examine the effects of substance P (SP) on guinea pig SG neurons. SP is an undecapeptide that acts as a neurotransmitter and a neuromodulator in the central and peripheral nervous systems (27, 28). SP is found within the cell bodies and nerve fibers of a number of different mammalian autonomic ganglia, including the neurons of the enteric plexus (9, 27) and the neurons of the SG in situ (13, 20). Immunohistochemical studies have indicated that nerve terminals in the guinea pig SG may contain SP or other peptides, including calcitonin gene-related peptide (13, 17). These peptide-containing fibers are believed to originate from preganglionic sympathetic neurons of the spinal cord or from sensory neurons (13).

The actions of SP in the mammalian nervous system are typically associated with slow depolarization (26, 27, 37, 39, 41). In rat sympathetic SG neurons, SP-coupled signaling pathways appear to be involved in the modulation of Ca2+ channels (35), whereas in guinea pig submucosal and celiac neurons a decreased K+ conductance (the M current) is primarily responsible for the slow excitatory postsynaptic potential and depolarization produced by SP (37, 41). This depolarization also included a component that was due to SP activation of a nonspecific cation current (37). Although the different ion channels underlying the modulation of action potential (AP) threshold and excitability in mammalian SG neurons have not been resolved, SP was found to depolarize ganglion neurons through a decreased conductance, possibly a M + conductance (25). Our data provide evidence that SP depolarizes dissociated guinea pig SG neurons via a decrease in outwardly rectifying K+ current (I_K), primarily the Ca2+-sensitive component [I_K(Ca)]. We show that the peptide-signaling pathway involves a pertussis toxin (PTX)-insensitive G protein and that the inhibition of I_K(Ca) may be mediated in part through SP inhibition of voltage-dependent Ca2+ influx. Furthermore, we also demonstrate the occurrence of a transient K+ current (I_A) in guinea pig SG neurons that was not affected by SP. Although M current has been shown to be blocked by SP in other autonomic neurons (37), an M-type current was rarely observed in guinea pig SG neurons in this study and is therefore unlikely to contribute significantly to the observed reduction in K+ conductance.

MATERIALS AND METHODS

Ganglion dissection and cell culture. SG were isolated from adult guinea pigs (250–300 g) according to Horackova et al. (18). All procedures conformed to guidelines of the Canadian Council on Animal Care. Briefly, animals were anesthetized with halothane (Fluothane) and decapitated. The right and
left SG were exposed rostral to the first rib, removed, and placed in bubbled PBS solution. After removal of excess connective tissue, ganglia were dissociated by incubation at 37°C in 5 mg/ml collagenase and 1 mg/ml trypsin. The trypsin action was terminated after 50 min by the addition of 1 ml of newborn calf serum. Cells were then centrifuged (1,000 g) and resuspended in 2 ml of DMEM (GIBCO BRL, Burlington, ON, Canada) and gently triturated with a fire-polished Pasteur pipette. Suspensions of isolated SG somata were seeded in growth medium on collagen-coated (Boehringer Mannheim, Laval, PQ, Canada) glass coverslips and placed in a 37°C incubator with an atmosphere of 5% CO2-95% air. Growth medium consisted of DMEM containing 10% newborn calf serum, 1% penicillin-streptomycin, 10 ng/ml nerve growth factor, and 5 μg/ml cytosine 1-β-D-arabinofuranoside, used to inhibit the growth of nonneuronal cells (17). Neurons were maintained in primary culture for 24–72 h and identified by morphological and electrophysiological criteria (see RESULTS). For experiments examining G protein subtypes, pretreatment with PTX was accomplished by the addition of PTX (100 ng/ml) to the culture medium at the time of cell plating. SG neurons were exposed to PTX for at least 24 h before electrophysiological recording. Control SG neurons were plated from the same ganglion culture as the PTX-treated cells, and recordings of control and PTX-treated neurons were obtained on the same experimental day to ensure consistent experimental conditions.

Superfusion and solutions. Cultured SG neurons grown on glass coverslips were placed in a shallow recording chamber (2 ml) and superfused at 1–2 ml/min with standard recording solution containing (in mM) 140 NaCl, 5 KCl, 20 Na+-HEPES, 1 MgCl2, 2 CaCl2, and 10 glucose. In experiments examining Ca2+ currents, the external solution was (in mM) 150 tetraethylammonium chloride (TEA), 5 BaCl2, 10 HEPES, 0.8 MgCl2, and 10 glucose. In experiments examining K+ and Ca2+ currents, 1 μM TTX was present in the external solution, unless otherwise stated. Standard internal pipette solutions used in whole cell recordings contained (in mM) 140 KCl, 0.4 CaCl2, 1 MgCl2, 20 HEPES, 1 EGTA, 5 MgATP, and 0.3 NaGTP. For recording Ca2+ currents the internal pipette solution was composed of (in mM) 125 CsCl2, 4.5 MgCl2, 1 HEPES, 9 EGTA, 5 MgATP, and 0.3 MgGTP. All solutions were maintained at pH 7.3–7.4, and osmolarity of solutions was 324–329 mosM. Ca2+ concentration was calculated using a software program based on the algorithm of Goldstein (11).

Free Ca2+ in the standard pipette solution was estimated at 100 nM, and in the 125 mM CsCl pipette solution it was <20 nM.

Chemicals. All drugs and chemicals were obtained from Sigma Chemical (Mississauga, ON, Canada), except PTX, neurokinin A (NKA), and neurokinin B (NKB), which were obtained from Rose Scientific (Edmonton, AB, Canada) and Calbiochem (San Diego, CA).

Electrophysiological recording techniques. Membrane potential (Vm) and currents were recorded using standard whole cell patch-clamp recording procedures (12). Currents were recorded with an Axopatch 1-D amplifier (Axon Instruments, Foster City, CA), filtered with a four-pole low-pass Bessel filter (3 db at 1 kHz), and digitized at a sampling frequency of 5 kHz. Micropipettes were prepared from borosilicate glass (Sutter Instruments, Novato, CA) using a two-stage vertical electrode puller (model PP83, Narishige, Tokyo, Japan). Pipette resistances were 2–3 MΩ when filled with standard electrophysiological recording solutions. The tips of the electrodes were coated with beeswax to reduce capacitance. Voltage commands and acquisition of membrane current were accomplished using pCLAMP software (Axon Instruments).

Except where indicated, leak and capacitance subtraction were routinely employed. Series resistance compensation (90%) was used in all recordings. Liquid junction potentials (LJP) between the bath and patch-clamp electrode were measured experimentally and determined as the potential of the bathing solution with respect to the pipette solution (2). For whole cell recording, the Vm of the cell was then calculated as Vm = Vp – LJP, where Vp is corrected membrane potential and LJP is pipette potential. The LJP was 3 mV for the standard internal and external solutions used to record K+ currents and was not corrected for in the data shown. For solutions used to record Ca2+ currents, the LJP was 11 mV and Vm was corrected in the current-voltage (I-V) plots shown. Values for cell capacitance and series resistance were obtained from the amplifier. Series resistance was always <10 MΩ. Current and voltage signals were stored on computer disk as well as on videotape with the aid of a digital data converter (Medical Systems, Greensville, NY).

All experiments were conducted at room temperature (20–22°C). Values are means ± SE. Student’s unpaired t-test was used to compare differences between data groups unless otherwise noted. Differences between groups were considered significant when P < 0.05.

RESULTS

Membrane properties of SG neurons. Details of cell morphology, survival, and the neuropetide content of adult guinea pig SG neurons grown in culture for up to 3 mo have been described previously (18). The average resting Vm of cultured (1–3 days) SG neurons determined under current-clamp conditions was −49 ± 2 mV (n = 40). Cell capacitance averaged 42 ± 2 pF (n = 114). Input resistance was 577 ± 212 MΩ (n = 5, range 52–1,350 MΩ). These values are similar to mean values described for dissociated rat SCG neurons and rabbit celiac ganglion neurons (1).

Injection of depolarizing current under current-clamp conditions elicited a fast overshooting AP in SG neurons that showed adaptation (a slow decrease in frequency of AP elicited by maintained depolarization; Fig. 1A). Previous investigations of neurons from isolated intact rat and guinea pig SG and from cultured SG neurons have demonstrated that, in response to sustained depolarization, the majority of neurons fire phasically (show adaptation), whereas a smaller proportion show little or no adaptation and fire tonically (18, 25). Although we did not investigate firing properties in detail in isolated cultured guinea pig SG neurons, we found that ~80% of neurons (32 of 40) from which APs were recorded could best be described as phasic (Fig. 1A); the remaining 20% (8 of 40) exhibited tonic properties (data not shown).

The minimum current required to generate an AP in 22 neurons examined was 20 ± 2 pA. The average AP amplitude, measured from the resting Vm to the peak of the AP, was 79 ± 6 mV (n = 38); the peak afterhyperpolarization (AHP) amplitude was −14 ± 1 mV (n = 34); the AHP duration (measured at half-maximal AHP amplitude) was 42 ± 8 ms (n = 27), and the AP duration measured at resting Vm and at 0 mV was 20 ± 3 (n = 24) and 5 ± 0.3 ms (n = 36), respectively. These values are comparable to those reported for neurons in vitro rat SG and SCG preparations (1, 25). The amplitude of the

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.3 on June 10, 2017
APs generated by depolarizing current injection was diminished by exposure to 1 µM TTX (Fig. 1A, n = 4). For the cell shown in Fig. 1B, when 1 mM TEA was added to the superfuse the resting $V_m$ depolarized by 2 mV, the AP duration increased by 13%, and the AHP amplitude was reduced by 41%. Similar effects were observed in two other cells exposed to TEA and are consistent with block of voltage-dependent K$^+$ current.

Exposure to the Ca$^{2+}$ channel blocker Cd$^{2+}$ (0.2 mM) depolarized the $V_m$ (11 ± 5 mV) in four of seven neurons sampled, whereas in the remaining three neurons, Cd$^{2+}$ produced no effect or a slight hyperpolarization (1–5 mV). However, in all neurons examined, Cd$^{2+}$ consistently decreased the AHP amplitude and duration by 43 ± 13 and 25 ± 2% (n = 7), respectively (Fig. 1C), suggesting the contribution of a Ca$^{2+}$-activated K$^+$ current to AP repolarization.

Actions of SP on $V_m$ and AHP in SG neurons. SP (500 nM) was applied to individual SG neurons by a single pressure ejection from a glass pipette with its tip positioned ~50 µm from the cell. Application of 500 nM SP produced a small depolarization (7 ± 3 mV) of the $V_m$ in seven of seven neurons tested, in which the mean resting $V_m$ was −50 ± 2 mV (Fig. 2Aa). Pressure application of standard external solution alone did not evoke a response in SG neurons. However, when neurons were current clamped to more depolarized potentials of −30 mV, a second SP application produced a repeatable depolarization of 16 ± 5 mV (n = 7; Fig. 2, Ab and Ac), which was associated with a 27 ± 8% (n = 7) decrease in membrane conductance. In four neurons current clamped at potentials of −30 mV, an initial pressure application of SP evoked a 25 ± 5 mV depolarization associated with a 42 ± 8% decrease in membrane conductance. When these same four neurons were superfused with 0.2 mM Cd$^{2+}$, the SP-evoked depolarization and conductance decrease were significantly less than values observed with SP alone (P < 0.01, Student's paired t-test), with SP now producing a mean depolarization of only 18 ± 5 mV accompanied by a 19 ± 9% decrease in conductance (Fig. 2Ad).

The effect of SP application on depolarization-elicited APs was examined in Fig. 2B. Pressure application of 500 nM SP decreased the AHP amplitude from −14.5 to −9 mV and decreased the AHP duration from 35 to 28 ms, with an increase in the AP duration (measured at the resting $V_m$) from 11 to 14 ms. In six other neurons, SP produced a mean decrease in AHP amplitude of 54 ± 15% and reduced the time to one-half AHP inactivation by 59 ± 13% (n = 6). Subsequent application of 500 nM SP in the presence of Cd$^{2+}$ in the neuron shown in Fig. 2C and three other neurons failed to significantly affect the AHP.

Thus the depolarizing actions of SP on $V_m$, which are associated with a decrease in the total membrane conductance and the inhibition of AHP in SG neurons, are consistent with inhibition of K$^+$ current. Because the actions of SP on $V_m$ and AHP were reduced in the presence of Cd$^{2+}$, which blocks voltage-dependent Ca$^{2+}$ influx, this further suggests that the actions of SP on SG neurons may include inhibition of $I_{K(Ca)}$.

Actions of SP on whole cell K$^+$ currents in SG neurons. Figure 3 illustrates representative whole cell currents recorded in guinea pig SG neurons using standard recording solutions. The protocol shown in Fig. 3 consisted of 500-ms depolarizations from holding
potentials ($V_h$) of $-100$ or $-35$ mV followed by test potential steps from $-40$ to $+60$ mV in 20-mV increments. In SG neurons, step depolarizations from a $V_h$ of $-100$ mV evoked a transient outward current followed by a sustained component (Fig. 3Aa). Depolarization of $V_h$ to $-35$ mV inactivated the transient outward current component, leaving the sustained component intact (Fig. 3Ab). Figure 3B shows the I-V relationship for the peak outward currents recorded at $V_h$ of $-100$ and $-35$ mV.

The $K^+$ selectivity of the transient outward current component was confirmed by examination of current tails, obtained by stepping the $V_m$ to potentials negative and positive to the $V_h$ after 10-ms step depolarizations to $+10$ mV (data not shown). Outward current tails reversed at a $V_m$ of $-71.0 \pm 5.0$ mV ($n = 14$, calculated equilibrium potential for outward $K^+$ current ($E_K$) = $-84$ mV) in standard recording solutions. The reversal potential of the tails shifted positively to $-48.0 \pm 7.0$ mV ($n = 3$, calculated $E_K = -41.0$ mV) and $-24.0 \pm 1.0$ mV ($n = 4$, calculated $E_K = -26.0$ mV) when the external $K^+$ concentration was increased to 20 and 50 mM, respectively. This shift of 47 mV per 10-fold change in extracellular $K^+$ concentration approaches the theoretical value of 58 mV predicted by the Nernst equation.

Figure 3C shows the conductance-voltage plot for the peak transient outward current, obtained by subtracting the whole cell currents activated by step potentials of $-100$ to $+60$ mV in 20-mV increments from $V_h$ of $-35$ mV from currents activated from $V_h$ of $-100$ mV. Figure 3C (inset) shows representative subtracted current traces elicited with step depolarizations to $-20$, $0$, and $+20$ mV. Conductance was calculated as follows

$$g = \frac{I_p(-100) - I_p(-35)}{(V - E_K)}$$

where $g$ is the transient outward $K^+$ conductance, $I_p(-100)$ is the peak outward current measured from a $V_h$ of $-100$ mV, and $I_p(-35)$ is the peak outward current measured from a $V_h$ of $-35$ mV. $V$ is the membrane potential, and $E_K$ is the equilibrium potential for outward $K^+$ currents measured in SG neurons (see above). The transient $K^+$ conductance activated at a $V_m$ of $-50$ mV, and the conductance increased with incremental depolarization to $0$ mV and then decreased. The de-
crease in conductance at potentials positive to 0 mV primarily reflects inactivation of the transient current (Fig. 3C, inset) but may also reflect a small reduction in $I_{K(Ca)}$, resulting from Ca$^{2+}$ channel inactivation at the depolarized $V_h$ of $-35$ mV (7, 30, 31).

In mammalian sympathetic neurons, the voltage-gated K$^+$ channels that mediate $I_A$ exhibit greater sensitivity to 4-aminopyridine (4-AP) than do other K$^+$ currents (3). Figure 4A shows representative current recordings in an SG neuron. The neuron was held at a $V_h$ of $-60$ mV, and 500-ms duration steps from potentials of $-100$ to $+20$ mV were applied in 20-mV increments. The outward current evoked by depolarization from a $V_h$ of $-60$ mV consisted of an initial transient component and a sustained component. Both components of the outward current were reduced by 4-AP. However, 4-AP was more selective for the transient current in guinea pig SG neurons, and 2 mM 4-AP essentially eliminated the transient outward current component over the $V_m$ range of $-50$ to $-20$ mV (those $V_m$ where the transient current activates in isolation from the sustained current). The I-V relationship shown in Fig. 4B was constructed from the peak current traces shown in Fig. 4A and demonstrates the reduction in transient K$^+$ current in the presence of 2 mM 4-AP.

Figure 4C shows current recordings obtained from a representative SG neuron in the presence and absence of the Ca$^{2+}$ channel blocker Cd$^{2+}$. The neuron was stepped for 500 ms from a $V_h$ of $-100$ mV to potentials of $+140$ mV in 20-mV increments. Depolarization of $V_m$ in SG neurons elicited sustained outward K$^+$ current, the amplitude of which showed some decline at potentials positive to $+80$ mV. The I-V relationship (Fig. 4D) obtained for the current traces in Fig. 4C demonstrates that in the presence of 0.2 mM Cd$^{2+}$, the sustained outward current was reduced at all $V_m$ positive to $-30$ mV. The Cd$^{2+}$ difference current was obtained by subtracting currents recorded in the presence of Cd$^{2+}$ from control currents. The Cd$^{2+}$-sensitive current activated around $-30$ mV and increased with depolarization to $+75$ mV, which approaches the calculated equilibrium potential for Ca$^{2+}$ current (+125 mV) for these neurons under the recording conditions used, then the current declined. In 21 neurons tested, 0.2 mM Cd$^{2+}$ reduced the outward current by 35 ± 6 and 46 ± 5% at 0 and $+60$ mV, respectively. In four other neurons exposed to nominally Ca$^{2+}$-free extracellular solution, the outward K$^+$ current was reduced by 41 ± 11% at $+60$ mV, thus indicating that $30$–$50\%$ of $I_K$ activated at depolarized potentials in SG neurons may be due to $I_{K(Ca)}$.

Actions of SP on $I_K$ in SG neurons. Figure 5 shows current recordings from an SG neuron before and during a 30-s pressure application of SP measured at two different $V_h$. The current records in Fig. 5A demonstrate the lack of sensitivity of $I_K$ to SP. Current records were obtained from a neuron at a $V_h$ of $-100$ mV, and the $V_m$ was stepped in 20-mV increments from $-100$ to $+60$ mV. Control recordings indicate that transient and sustained $I_K$ are activated by depolarization in this neuron (Fig. 5Aa, top traces). Application of 500 nM SP reduced the sustained component of $I_K$ but did not appear to affect the transient component of outward conductance (Fig. 5Aa, bottom traces). The I-V
relationship for the peak $I_K$ recorded in the presence and absence of 500 nM SP is shown in Fig. 5B. At $V_m$ where the transient component of $I_K$ was activated in relative isolation from the sustained component ($-50$ to $-30$ mV), SP had little effect. Depolarizing $V_h$ to $-30$ mV in the same neuron produced a voltage-dependent inactivation of the transient component of $I_K$, yet left the sustained $I_K$ relatively unaffected (Fig. 5Ab, top traces). Application of 500 nM SP reduced $I_K$ (Fig. 5Ab, bottom traces). The I-V relationship shown in Fig. 5C for current measured at the end of the voltage pulse demonstrates that SP now reduced $I_K$ over the range of potentials ($-30$ to $+60$ mV) at which sustained $I_K$ would be activated. For the cell shown in Fig. 5, the inhibition of $I_K$, measured at $+60$ mV, by SP was greater at $V_h$ of $-100$ mV (31%) than at $V_h$ of $-30$ mV (20%) and may reflect some inactivation of Ca$^{2+}$ and K+ channels at depolarized potentials (1, 30, 42).

Figure 6A shows a dose-response curve for inhibition of $I_K$ at $+60$ mV by SP. $I_K$ was measured at the end of a 500-ms voltage pulse to $+60$ mV in cells held at $V_h$ of $-60$ mV. SP at $5$ nM had little effect on $I_K$, with maximal inhibition obtained at $500$ nM SP. $I_K$ was reduced $29 \pm 5$ and $33 \pm 4\% (n = 31)$ by $500$ nM SP at 0 and $+60$ mV, respectively.

SP inhibits $I_K$ through an NK$_1$ tachykinin receptor. The receptors for SP belong to the tachykinin receptor family and include receptors classified as NK$_1$, NK$_2$, and NK$_3$. The preferred endogenous ligands for these receptor types are SP, NKA (substance K), and NKB, respectively (22). We investigated which receptor type might be involved in SP’s inhibition of $I_K$ in SG neurons by using the nonpeptide NK$_1$ selective antagonist CGP-49823 (33). Figure 6B summarizes the effects of CGP-49823 (0.25 and 1 µM) on the response to 500 nM SP. In the absence of the antagonist, SP reduced $I_K$ by 44 ± 8% (n = 11, see also Fig. 6A). However, when neurons were exposed to 1 µM CGP-49823, the inhibition of $I_K$ by SP was reduced to 3 ± 1.5% (n = 5). At 0.25 µM, CGP-49823 did not significantly affect SP’s ability to inhibit $I_K$ (24 ± 12%, n = 3, P > 0.05).

We also compared the effects of the agonists SP, NKA, and NKB on the inhibition of $I_K$ in guinea pig SG neurons. The potency order has been reported to be SP $>$ NKA $>$ NKB for the NK$_1$ receptor, NKA $>$ NKB $>$ SP for the NK$_2$ receptor, and NKB $>$ NKA $>$ SP for the NK$_3$ receptor (22, 35). Figure 6C shows the mean inhibition of $I_K$ produced by 500 nM SP, NKA, or NKB in 27 separate cells stepped to $+60$ mV for 500 ms from a $V_h$ of $-60$ mV. Whereas inhibition by SP was $44 \pm 8\% (n = 11)$, NKA and NKB reduced $I_K$ by only 6.0 ± 1.0% (n = 8) and 16.0 ± 4.0% (n = 8), respectively (P < 0.1 and P < 0.05 vs. control). These data indicate that SP effects on $I_K$ are mediated via NK$_1$ receptors, since the potency order for the inhibition of $I_K$ was SP $>$ NKA $>$ NKB.
Actions of SP on $\mathrm{I}_{\text{K(Ca)}}$ in SG neurons. The inhibition of $\mathrm{I}_{\text{K}}$ by SP was significantly diminished in neurons exposed to Cd$^{2+}$. Figure 7A shows current traces recorded before and after SP application in a neuron superfused with standard extracellular recording solution and subsequently with solution containing 0.2 mM Cd$^{2+}$. Depolarizing voltage steps to +60 mV were applied from a $V_h$ of −100 mV for 500 ms. In the

Fig. 6. SP inhibits $\mathrm{I}_{\text{K}}$ through interaction with NK1 tachykinin receptors. A: concentration-response curves for inhibition of $\mathrm{I}_{\text{K}}$ by SP. [SP], SP concentration. Each point is mean; error bars, SE; numbers in parentheses, number of cells tested. B: SP-induced inhibition of $\mathrm{I}_{\text{K}}$ is attenuated in presence of NK1-selective antagonist CGP-49823 (1 µM). Bars represent mean inhibition of $\mathrm{I}_{\text{K}}$ by SP in neurons incubated in absence and presence of 1.0 and 0.25 µM CGP-49823; error bars, SE. C: mean inhibition of $\mathrm{I}_{\text{K}}$ by 500 nM SP, neurokinin A (NKA), and neurokinin B (NKB); error bars, SE. NKA and NKB produced significantly less inhibition of $\mathrm{I}_{\text{K}}$ than SP: *P < 0.05, **P < 0.01 compared with SP. Currents in B and C were measured at end of 500-ms step depolarization to +60 mV from $V_h$ = −60 mV.
In the presence of Cd²⁺, the inhibitory actions of SP on Iₖ were reduced.

Figure 7B represents the mean inhibition of Iₖ by 500 nM SP in the presence of 0.2 mM Cd²⁺ and 10 μM ω-conotoxin. SG neurons were held at a Vₘ of -60 or -100 mV and stepped to 0 and +60 mV for 500 ms. Iₖ was measured at the end of the voltage pulse. Under control conditions, SP reduced Iₖ by 34 ± 6 and 37 ± 5.0% at 0 and +60 mV, respectively (n = 15); however, when the neurons were superfused with 0.2 mM Cd²⁺, Iₖ was reduced by only 8 ± 2 and 1.4 ± 0.9% (n = 8), respectively (P < 0.01 and P < 0.05 vs. control). Similarly, in the presence of ω-conotoxin, inhibition of Iₖ by SP at 0 and +60 mV was only 12 ± 6 and 3.0 ± 3.0% (n = 4).

Actions of SP on Iₖ are mediated by a PTX-insensitive G protein. The involvement of G proteins in the signaling pathway of SP is well established (14). To confirm that SP modulation of Iₖ is mediated through a G protein-coupled pathway in SG neurons, we compared cells dialyzed with a GTP-free pipette solution containing 2 mM guanosine 5'-O-(2-thiodiphosphate) (GDPβS), which is an antagonist of G protein activation, with results obtained using standard pipette solution (0.3 mM GTP) (16, 35). Neurons were dialyzed for at least 10 min before pressure application of 500 nM SP. Figure 8A summarizes the effects of GDPβS diaisy on the responsiveness of SG neurons to SP. SP inhibited Iₖ (measured at the end of a 500-ms voltage step to +60 mV from Vₘ of -60 mV) by only 9.0 ± 4.0% (n = 5) in neurons dialyzed with 2 mM GDPβS compared with 33 ± 4.0% (n = 31) in control cells dialyzed with GTP (Fig. 8A). These data confirm that the SP-mediated inhibition of Iₖ is via a G protein-coupled pathway. To identify the type of G protein involved in SP-mediated inhibition of Iₖ, we incubated SG neurons with PTX. PTX inactivates G proteins of the Gi, Gz, or Go class by catalyzing NAD-dependent ADP ribosylation of the α-subunit (24). Overnight (24-h) treatment with PTX (500 ng/ml) was ineffective in blocking SP inhibition of Iₖ (40 ± 8.0%, n = 5; Fig. 8B) compared with control untreated cells (43 ± 9%, n = 6). Thus we conclude that the G protein(s) involved in SP modulation of Iₖ in SG neurons is not of the Gi, Gz, or Go class.

Actions of SP on Ca²⁺ channel currents in SG neurons. The reduction of the inhibitory effect of SP on Iₖ in SG neurons pretreated with Cd²⁺ or the high-voltage-activated (HVA) N-type Ca²⁺ channel blocker ω-conotoxin (40) suggests that SP may act to inhibit Iₖ(Ca) in SG neurons via the reduction of voltage-dependent Ca²⁺ influx. SP has been reported to act on NK₁ receptors inhibiting N-type Ca²⁺ channels in dissociated rat SCG neurons (35). Therefore, we investigated the effects of SP on Ca²⁺ channel currents in SG neurons. Ion substitutions used to isolate Ca²⁺ channel currents are described in MATERIALS AND METHODS. In K⁺- and Na⁺-free solutions with external 5 mM Ba²⁺ as the current carrier, all K⁺ and Na⁺ currents were blocked and on membrane depolarization an inward
current ($I_{Ba}$) was observed. Figure 9A shows inward $I_{Ba}$ recorded after step depolarization to $+10$ mV from a $V_h$ of $-70$ mV. The inward current in this neuron was reduced by $>88\%$ by $0.2$ mM Cd$^{2+}$. Similar results were observed in all cells examined, with a mean reduction in $I_{Ba}$ amplitude measured at $0$ mV by Cd$^{2+}$ of $96.0 \pm 6.0\%$ ($n = 5$). Figure 9B shows $I_{Ba}$ recorded after a step depolarization to $0$ mV in the absence and presence of $10 \mu M$ $\omega$-conotoxin. In the presence of $\omega$-conotoxin, $>95\%$ ($97 \pm 1.5$, $n = 9$) of the inward current at $0$ mV was inhibited and showed no recovery during a 15-min postdrug recovery period. In five other cells, $I_{Ba}$ at $0$ mV was inhibited $96 \pm 3.0\%$ after a 15-min exposure to $\omega$-conotoxin. Subsequent application of $0.2$ mM Cd$^{2+}$ to these cells resulted in no further reduction in $I_{Ba}$ in four cells and an $11\%$ reduction in one cell, suggesting that current through $\omega$-conotoxin-sensitive Ca$^{2+}$ channels accounts for most of the Ca$^{2+}$ channel current in SG neurons under the conditions used in this study. Figure 9D shows the I-V plot for the $\omega$-conotoxin-sensitive current. Inward current, from a $V_h$ of $-90$ mV, activates at around $-30$ mV, with peak inward current occurring around a potential of $-10$ mV. However, voltage steps from $-90$ mV to $V_m$ positive to $+30$ mV elicited outward $\omega$-conotoxin-sensitive currents, which may represent Cs$^+$ moving out of the cell via $\omega$-conotoxin-sensitive Ca$^{2+}$ channels (7, 8).

Figure 9C shows $I_{Ba}$ recorded at $0$ mV in an SG neuron before and during application of $500$ nM SP. In this cell, SP decreased the amplitude of $I_{Ba}$ by $33\%$. Similar results were observed in eight cells examined with an average inhibition of $I_{Ba}$ by SP of $49 \pm 7.7\%$. The I-V plot in Fig. 9E represents the SP-sensitive inward current measured from peak inward currents recorded in the presence of $500$ nM SP. As shown for the $\omega$-conotoxin-sensitive current in Fig. 9B, the inward SP-sensitive current activates at $-30$ mV, with peak current around $0$ mV. These data indicate that $I_{Ba}$ in SG neurons is mediated by a pertussis toxin (PTX)-insensitive G protein. A: inclusion of guanosine 5'-O-(2-thiodiphosphate) (GDP\(\beta\)S) in pipette solution $0.3$ mM GTP) or with $2$ mM GDP\(\beta\)S (0 GTP); error bars, SE. In all experiments, $10$ min were allowed after break in and before recording to allow for sufficient guanine nucleotide dialysis. B: inhibition of $I_{K}$ by SP is PTX insensitive. Bars represent mean inhibition of steady-state $I_{K}$ at $+60$ mV by SP; error bars, SE. Cells were cultured overnight with $500$ ng/ml PTX. Current inhibition in control neurons was recorded with standard recording solutions.

**Fig. 8.** Inhibition of $I_{K}$ by SP is mediated by a pertussis toxin (PTX)-insensitive G protein; A: inclusion of guanosine 5'-O-(2-thiodiphosphate) (GDP\(\beta\)S) in pipette solution $0.3$ mM GTP) or with $2$ mM GDP\(\beta\)S (0 GTP); error bars, SE. In all experiments, $10$ min were allowed after break in and before recording to allow for sufficient guanine nucleotide dialysis. B: inhibition of $I_{K}$ by SP is PTX insensitive. Bars represent mean inhibition of steady-state $I_{K}$ at $+60$ mV by SP; error bars, SE. Cells were cultured overnight with $500$ ng/ml PTX. Current inhibition in control neurons was recorded with standard recording solutions.

*P < 0.05.
neurons is carried primarily via \( \omega \)-conotoxin-sensitive Ca\(^{2+} \) channels and that SP inhibition of Ca\(^{2+} \) current may account for the observed SP-induced decrease in \( I_{K(Ca)} \).

**DISCUSSION**

These results describe the cellular mechanisms by which the neuropeptide SP modulates ionic conductances in adult mammalian sympathetic neurons. We have used patch-clamp recording to investigate the membrane properties and whole cell K\(^+ \) and Ca\(^{2+} \) currents in guinea pig SG neurons. We have not investigated the voltage-dependent characteristics of the TTX-dependent Na\(^+ \) current that was observed in these SG neurons. Details of K\(^+ \) and Ca\(^{2+} \) currents recorded in SG neurons are discussed below and compared with ionic conductances described for sympathetic neurons of other vertebrate ganglia and species.

We also report that SP depolarized guinea pig SG neurons, decreased membrane conductance at potentials positive to resting \( V_{m} \), and inhibited the AP AHP. These actions of SP were reduced by the Ca\(^{2+} \) channel blocker Cd\(^{2+} \) and are consistent with inhibition of a Ca\(^{2+} \)-activated K\(^+ \) conductance. Voltage-clamp analysis of currents in SG neurons revealed that SP inhibited at least two ionic conductances: a Ca\(^{2+} \)-sensitive K\(^+ \) conductance and an inward Ca\(^{2+} \) conductance. Evidence for the modulation of these currents by SP and the results of an investigation of the molecular signaling pathways underlying the action of SP on \( I_{K(Ca)} \) are discussed.

Voltage-dependent K\(^+ \) and Ca\(^{2+} \) currents in guinea pig SG neurons: comparison with other autonomic neurons. The outward current in guinea pig SG neurons was selective for K\(^+ \) and consisted of several distinct voltage-dependent K\(^+ \) conductances. These conductances were identified on the basis of voltage dependence and pharmacological blockade and consisted of a transient outward K\(^+ \) conductance, which resembled the A-type conductance described in mammalian autonomic neurons (3), and a delayed rectifier outward K\(^+ \) current consisting of Ca\(^{2+} \)-activated and Ca\(^{2+} \)-insensitive components.

In guinea pig SG neurons the transient outward K\(^+ \) current (\( I_A \)) demonstrated voltage-dependent activation and was significantly inactivated at potentials near the measured resting \( V_m \). This current typically activated within 1 ms of step depolarizations to \( V_m \) positive to −50 mV and reached a maximum amplitude within 10 ms. Peak conductance was followed by a rapid phase of current decay. The transient outward conductance was maximal after hyperpolarization and decreased sharply as the membrane voltage, before the depolarizing steps, became less negative. The voltage dependence and kinetics of the \( I_A \) in guinea pig SG neurons resembled the \( I_A \) described in other mammalian sympathetic neurons (1, 3, 6). Consistent with the pharmacological properties observed for \( I_A \) in neurons of other mammalian sympathetic ganglia, \( I_A \) in guinea pig SG neurons was also sensitive to 4-AP.

The predominant current expressed in all guinea pig SG neurons studied was a sustained outward K\(^+ \) current. This sustained outward current activated with a brief delay after the onset of membrane depolarization to potentials positive to −30 mV and persisted while the depolarization was maintained. The voltage dependence and kinetics of the sustained K\(^+ \) current in guinea pig SG neurons closely resembled delayed rectifier K\(^+ \) currents described in neurons from several different sympathetic ganglia, including the rat SCG (1, 3, 34) and guinea pig enteric ganglia (41). The delayed rectifier outward current in guinea pig SG neurons was reversibly decreased by the external application of 4-AP and abolished by internal dialysis with Cs\(^+ \). This is consistent with the pharmacological properties described for delayed rectifier K\(^+ \) currents in neurons of other mammalian autonomic ganglia (1, 41, 42). Recent voltage-clamp studies of mammalian sympathetic neurons (1, 19), together with data from other neuronal types (21, 42), have provided support for the existence of several classes of Ca\(^{2+} \)-dependent K\(^+ \) currents contributing to AP repolarization and spike AHP.

In the present study we observed a reduction of a portion of the sustained outward K\(^+ \) current after superfusion with Ca\(^{2+} \) channel blockers or nominally Ca\(^{2+} \)-free solution. Our data indicate that ~35–50% of the delayed rectifier K\(^+ \) current in guinea pig SG neurons can be attributed to the activation of Ca\(^{2+} \)-activated K\(^+ \) channels.

In addition to the \( I_{K(Ca)} \), we occasionally recorded a small noninactivating outward current in guinea pig SG neurons that exhibited voltage- and time-dependent properties similar to the M current described in several other sympathetic neurons, including the SCG neuron (5, 23), and guinea pig inferior mesenteric ganglia (10). Because of the infrequent occurrence (~15% of neurons) and the small amplitude of current relaxations (10 ± 5.6 pA, \( n = 15 \) with voltage steps from \( V_h = −30 \) to −70 mV) of M-like current in cultured guinea pig SG neurons, it is unlikely to make a significant contribution to the actions of SP described in this study. In support of this, the difficulty in studying the M current in sympathetic neurons has been reported previously in neurons where the M current is evident using intracellular recording techniques (41) but was not observed in the same cell type under whole cell patch-clamp recording techniques. This may reflect washout of the cytosolic constituents that are required for the regulation of the M current, a common unavoidable problem in the use of the whole cell patch-clamp configuration (12).

We have also identified an inward Ca\(^{2+} \) current in cultured SG neurons. Guinea pig SG neurons exhibited a voltage-dependent Ca\(^{2+} \) current that activated on step depolarization to voltages more positive than ±30 mV and was abolished by 0.2 mM Cd\(^{2+} \) or 10 µM \( \omega \)-conotoxin. This current resembles the descriptions of HVA, \( \omega \)-conotoxin-sensitive Ca\(^{2+} \) currents reported in a number of mammalian autonomic neurons, including the rat SCG neurons (30, 31, 34, 40) and the rat myenteric and guinea pig submucosal neurons (15, 37).
In mammalian sympathetic neurons the major component (85–90%) of whole cell inward Ca\(^{2+}\) current has been shown to be carried by dihydropyridine-insensitive, high-threshold N-type Ca\(^{2+}\) channels (30, 31, 34). The remainder of the conductance has been attributed to current carried through dihydropyridine-sensitive L-type Ca\(^{2+}\) channels. The block of >95% of the Ca\(^{2+}\) current by \(\omega\)-conotoxin in guinea pig SG neurons and the voltage dependence of the \(\omega\)-conotoxin-sensitive current indicate that the whole cell Ca\(^{2+}\) current observed in these neurons is due primarily to the activation of a high-threshold N-type Ca\(^{2+}\) conductance.

Actions of SP on voltage-dependent K\(^{+}\) and Ca\(^{2+}\) currents in guinea pig SG neurons. A variety of peptides including SP, somatostatin, luteinizing hormone-releasing hormone, angiotensin II, atrial natriuretic factor, and neuropeptide Y have been demonstrated to modulate neuronal ionic conductances (4, 15, 18, 26, 33, 35–37, 41). In light of the potential importance of such modulation to neuronal excitability, much attention has been placed on understanding the underlying mechanisms of the actions of these neuropeptides.

Under our recording conditions, SP reversibly depolarized \(V_m\) and decreased membrane conductance. These actions are consistent with inhibition of an outward K\(^{+}\) conductance by SP in SG neurons. In the presence of the Ca\(^{2+}\) channel blocker Cd\(^{2+}\), a reduction in SP's ability to elicit membrane depolarization and a conductance decrease were observed, suggesting that SP may exert its effects, in part, via inhibition of \(I_{K(Ca)}\). Our current-clamp studies also revealed a residual depolarization and small conductance decrease still elicited by SP in the presence of 0.2 mM Cd\(^{2+}\), which may reflect an incomplete block of \(I_{K(Ca)}\) by Cd\(^{2+}\) in those neurons tested and/or actions on K\(^{+}\) currents distinct from the \(I_{K(Ca)}\). For example, SP inhibition of a background (leak) \(K\) conductance has been described in other autonomic neurons, including guinea pig celiac and submucosal neurons (37, 39, 41).

Our subsequent voltage-clamp studies focused on \(I_{K(Ca)}\) and confirmed that the actions of SP on outward K\(^{+}\) current were primarily mediated via inhibition of \(I_{K(Ca)}\), since SP's inhibitory actions measured at 0 and +60 mV were reduced in the presence of 0.2 mM Cd\(^{2+}\) or the N-type Ca\(^{2+}\) channel blocker \(\omega\)-conotoxin (10 \(\mu\)M). There was a slightly greater reduction in SP's inhibitory action on \(I_{K(Ca)}\) at more positive potentials in the presence of Ca\(^{2+}\) channel inhibition, which may reflect alterations in the driving force for Ca\(^{2+}\) and decreased Ca\(^{2+}\) availability at these potentials (32). The Ca\(^{2+}\)-insensitive component of the sustained outward K\(^{+}\) current, evident in the presence of Cd\(^{2+}\), was not significantly affected by SP application.

The identity of the receptor believed to mediate SP-induced modulation of \(I_{K(Ca)}\) in guinea pig SG neurons was investigated. Three tachykinins, SP, NKA, and NKB, each possess a defined order of potency for the three known mammalian tachykinin receptors: NK\(_1\), NK\(_2\), and NK\(_3\) (22, 35). The rank order of potency for inhibition of \(I_K\) in this study was \(SP > NKA \geq NKB\), which demonstrates an NK\(_1\) subtype profile for tachykinin inhibition of \(I_{K(Ca)}\) by SP.

We have also examined the effects of SP on the Ca\(^{2+}\) channel current \(I_{Ca}\) in guinea pig SG neurons. Bath application of SP produced an inhibition of \(I_{Ca}\) similar to that described in frog and rat sympathetic neurons (4, 35). Because \(<5\%\) of the whole cell \(I_{Ca}\) in SG neurons is likely to be carried via L-type Ca\(^{2+}\) channels, we did not attempt to determine the effectiveness of SP on L-type currents in isolation. Our data indicate that SP inhibits \(\sim50\%\) of \(I_{Ca}\) in SG neurons, in which the majority of inward \(I_{Ca}\) represents activation of \(\omega\)-conotoxin-sensitive HVA Ca\(^{2+}\) channels. Although these findings suggest that the effects of SP on \(I_{K(Ca)}\) may represent a secondary consequence of the inhibition of Ca\(^{2+}\) influx, this does not exclude any additional direct effects of SP on K\(^{+}\) currents.

Transduction mechanisms underlying \(I_{K(Ca)}\) inhibition. A series of experiments were designed to further examine the potential signaling pathway underlying SP's actions on \(I_K\) in guinea pig SG neurons. We expected that the modulation of \(I_K\) by SP was mediated by G protein(s), and therefore we used GDP\(\beta S\) and PTX to test this hypothesis. In the presence of GDP\(\beta S\), a G protein blocker, SP's ability to inhibit \(I_K\) was significantly diminished, thereby indicating G protein involvement. We found no effect of PTX pretreatment on the ability of SP to inhibit \(I_K\) in SG neurons, indicating that the receptors responding to SP are not coupled to G proteins of the \(G_i\), \(G_o\), and \(G_z\) family. These findings are consistent with other reports of receptor-mediated SP actions that are PTX insensitive. These include the inhibition of N-type Ca\(^{2+}\) channels in rat SCG neurons and frog sympathetic neurons (4, 35) and the inhibition of inwardly rectifying K\(^{+}\) channels in the nucleus basalis of the rat forebrain (26), both of which occur through interaction with an NK\(_1\) tachykinin receptor.

In summary, this study presents the first description of ionic currents in mammalian SG neurons and demonstrates that SP, acting via NK\(_1\) receptors coupled to a PTX-insensitive G protein pathway, may increase excitability in mammalian SG neurons via inhibitory actions on K\(^{+}\) and Ca\(^{2+}\) currents.

We thank Dr. P. S. Pennefather for valuable discussions and suggestions, Z. Byczko for excellent technical assistance, and C. Jollimore for editing the manuscript.

This study was funded by Medical Research Council of Canada Grant MT-13484 to M. E. M. Kelly, MT-4128 to M. Horackova, and MT-11622 to F. M. Smith. F. M. Smith was a Heart and Stroke Foundation of Canada Research Scholar.

Address for reprint requests: M. E. M. Kelly, Dep. of Pharmacology, Sir Charles Tupper Medical Bldg., Dalhousie University, Halifax, NS, Canada B3H 4H7.

Received 31 March 1997; accepted in final form 16 December 1997.

REFERENCES
