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Substance P decreases a potassium conductance of spinal cord neurons in cell culture

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Substance P (SP) produced membrane depolarization and decreased membrane conductance of mouse spinal cord neurons in primary dissociated cell culture. SP-responses were abolished by intracellular tetraethylammonium suggesting that SP decreased potassium conductance. Reversal of SP-responses was not observed with membrane hyperpolarization suggesting that SP reduced a voltage-dependent potassium conductance that was activated by membrane depolarization.

The undecapeptide substance P (SP) has a widespread, but specific, regional distribution in the central nervous system (CNS)[6,28,32], peripheral nervous system (PNS)[15–17], and gut[31,35]. The peptide has been investigated most extensively in the spinal cord where it is a neurotransmitter candidate for small diameter dorsal root primary afferent fibers[15–17,27,36,42]. SP-like immunoreactivity has been localized to a subpopulation of small diameter dorsal root ganglion (DRG) neurons[7,15–17] and to nerve fibers, synaptic terminals and synaptic vesicles in laminae I–III of the dorsal horn of the spinal cord[3,7–9,18,37,38]. Dorsal root and dorsal horn concentrations of SP were greater than ventral root and ventral horn concentrations[3,7,16,17,42], and transection or ligation of dorsal roots reduced the SP content of spinal cord[3,7,16,17,23,42]. Furthermore, calcium-dependent release of SP from small diameter, but not large diameter, primary afferents has been demonstrated in vivo[19]. A role for SP in transmitting noxious stimuli is particularly likely since SP stimulated dorsal horn neurons receiving nociceptive input but not those receiving mechanoreceptive input [13,39,41], and depletion of SP from primary afferent terminals by capsaicin treatment increased pain threshold in rats[44]. Electrophysiological studies in both CNS[12,13,23–26,33,39,41,45] and PNS[10,11,21,22] have shown that SP was excitatory, and when applied to spinal cord neurons, SP depolarized membrane potential and increased firing rate[24,30,41,43,45]. Thus, there is considerable evidence to support the hypothesis that SP is an excitatory neurotransmitter released from small diameter primary afferent fibers mediating noxious stimuli.

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The mechanism of action of SP, however, remains uncertain. Depolarization evoked by SP which was slow in onset and of long duration has been associated with an increase[10,30], a decrease[11,21,22,24] or no change[41,45] in membrane conductance. The membrane conductance increase was suggested to be due to an increase in sodium conductance (g_{Na})[30] while decreased potassium conductance (g_K)[21,22,24] or chloride conductance (g_{Cl})[24] was suggested to occur when membrane conductance was decreased.

We have investigated the slow excitatory postsynaptic SP-responses using intracellular recordings from mouse spinal cord neurons grown in primary dissociated cell culture and report that SP decreased a membrane potassium conductance and depolarized membrane potential.

Cultures were prepared from spinal cords and dorsal root ganglia dissected from 12–14-day-old mouse embryos[40]. Tissue was mechanically disrupted, plated on collagen-coated 35 mm dishes, and then grown and maintained in 90% Eagles minimal essential medium and 10% heat activated horse serum at 35 °C for 5–8 weeks prior to study. Multipolar spinal cord neurons bathed in Dulbecco's phosphate buffered saline (PBS) or Tris·HCl buffered saline (TBS) were penetrated with one or two 4 M KAc- or one 3 M KCl-filled glass micropipettes (25–50 M Ω) under direct visual guidance on the heated (temperature 35–37 °C), modified stage of an inverted phase microscope. Polarization of neuronal membrane potential (with steady currents) and measurement of membrane conductance (with 40–60 msec hyperpolarizing constant current pulses) were made with a single recording micropipette using a modified bridge circuit (WPI M707). During two electrode recordings, one micropipette was used to pass current and the other to record membrane voltage independent of the bridge circuit. The surface of the neuron under study was bathed with SP by miniperfusion of peptide solutions ejected from blunt glass micropipettes (tip diameter 2–10 μm) using regulated pressure pulses. Different concentrations of SP (Sigma, St. Louis) were made by diluting frozen aliquots of prepared stock solution (1 mM SP in 0.001 M ammonium acetate–acetic acid, pH 4.7) with buffered recording medium. Final pH in the miniperfusion pipettes was 7.2–7.4. The SP analog, eledoisin-related peptide (ERP; Sigma, St. Louis), was used in some experiments and produced responses similar to those evoked by SP[27]. Dithiothreitol (6 μM DTT; Sigma, St. Louis) and bovine serum albumin (0.1% BSA) were added to peptide stock solutions. DTT (6 μM) was included in all miniperfusion solutions including control solutions. Glassware was pretreated with 0.1% BSA prior to serial dilution of peptide solutions.

Spinal cord neurons were spontaneously active in PBS and responded to SP with slowly developing, reversible excitation (Fig. $1A_1$) and bursting that remained for 10 sec–3 min after SP application. SP was applied for only 1 sec with at least two minute intervals between applications to insure recovery from preceding responses. Some recordings were made in PBS with 10 mM Mg^{2+} or in TBS with 3 μM tetrodotoxin (TTX) to reduce or eliminate synaptic activity and thus allow a clearer recording of postsynaptic responses. In PBS with elevated Mg^{2+}, most large multipolar neurons responded to SP application with slowly developing (0.75–1.5 sec latency) reversible depolarization accompanied by up to a 45% decrease of conductance (Fig. $1A_2$).

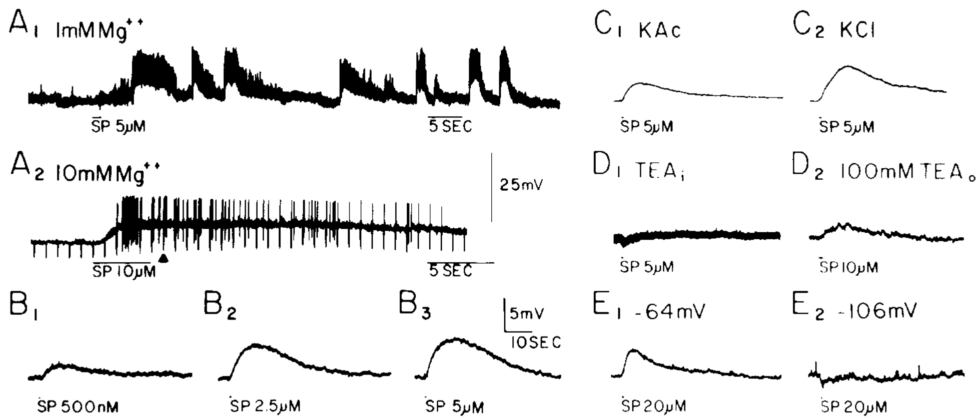

Fig. 1. Substance P (SP) excited cultured mouse spinal cord neurons. A_1: miniperfusion of SP for 1 sec produced slow, reversible depolarization and bursts of action potentials lasting 1.5 min. Resting membrane potential (RMP) was —48 mV. A_2: when 10 mM magnesium was added to the recording medium, neurons exhibited less spontaneous activity and responded to SP by slowly and reversibly depolarizing (0.75 sec latency) while membrane conductance was decreased. Hyperpolarizing constant current pulses applied prior to and during SP application increased in amplitude (compare the pulse at the triangle to the control pulses) indicating that membrane resistance increased. RMP was —59 mV. A_1 and A_2 were recorded in phosphate buffered saline. Action potentials were truncated by the limited frequency response of the pen recorder. B: SP-responses were dose-dependent. Recordings were made in recording medium containing 3 μM TTX which eliminated spontaneous activity without eliminating the postsynaptic response to SP. RMP was —62 mV. C, D_1: successive recordings from the same neuron with 4 M KAc-(C_1), 3 M KCl-(C_2) and 4 M TEA-Cl-(D_1) filled micropipettes showed that the SP-response during KAc recording (C_1, RMP = —66 mV) was smaller than the response during KCl recording (C_2, RMP = —64 mV) but both responses were depolarizing. However, the SP-response during TEA-Cl recording (initially 4 mV at RMP = —64 mV) was eliminated following intracellular iontophoretic injection of TEA (D_1, RMP = —47 mV). Membrane potential was depolarized by TEA but following repolarization to the initial RMP the response did not recover (not shown). The small hyperpolarization observed in D_1 (0.5 mV) was a pressure artifact. D_2: in 100 mM TEA-Cl containing recording medium SP-responses were reduced but not eliminated. E: SP-responses were not reversed at hyperpolarized membrane potentials. SP produced depolarizing responses at RMP (—64 mV) and at depolarized membrane potentials (E_1) the responses were abolished at hyperpolarized membrane potentials (—106 mV) (E_2). Responses B–E were recorded in Tris·HCl buffered saline containing 3 μM TTX and were filtered (15 Hz —3db $\pm$ 10%, except D_1).

Increased neuronal activity was also observed, which included: increased rate of firing of action potentials; brief, high amplitude, depolarizations (possibly postsynaptic potentials; PSPs) that sometimes brought the membrane to spike threshold; and paroxysmal depolarizing events (PDEs) (depolarizations with bursts of action potentials). Action potentials, PSPs and PDEs were not evoked when SP was applied to neurons in TTX-containing medium (Fig. 1B), but neurons still responded to SP with a slow depolarization and decreased membrane conductance suggesting that this was a postsynaptic response. SP-responses in TTX-containing medium were dose-dependent (Fig. 1B) but all responsive neurons were not equally sensitive with threshold concentrations ranging from 0.5 to 5.0 μM.

Investigation of the ionic basis for the conductance decreases produced by SP were made by manipulating intracellular chloride and extracellular potassium ($[K^+]_o$)

concentrations and by using specific ionic channel blockers. Since SP reduced membrane conductance and depolarized membrane potential, it was probable that SP reduced either g_K or g_{Cl}. Recording with 3 M KCl-containing micropipettes (which changed the chloride equilibrium potential (E_{Cl}) from about —60 mV to between —10 and —20 mV[4]) did not change the polarity of the response as would be predicted if SP decreased g_{Cl}. SP-responses evoked during intracellular recording with KAc-containing micropipettes (Fig. 1C$_1$) were increased in amplitude by intracellular injection of chloride ions (Fig. 1C$_2$). This would be expected if the change of E_{Cl} to a more depolarized potential had increased the net driving force for depolarization following a reduction in resting g_K. Thus the results obtained using KCl-recordings support the hypothesis that SP decreased g_K rather than g_{Cl}. This hypothesis was tested by using tetraethylammonium chloride (TEA-Cl) to block g_K[1,2]. Intracellular recordings were made with 4 M TEA-Cl containing micropipettes either during single electrode recordings, or as the second intracellular electrode after first recording with a KAc-filled micropipette. When TEA was iontophoretically injected into the neuron with depolarizing pulses, the membrane was depolarized, input resistance increased and SP-responses were reduced or eliminated (compare Figs. 1C$_1$ and C$_2$ with D$_1$). No SP-responses were observed from TEA-containing neurons when they were repolarized to the membrane potential recorded prior to TEA injection. Bathing neurons in medium containing 100 mM TEA-Cl (substituted for NaCl) also reduced SP responses (Fig. 1D$_2$) but was not as effective as intracellular TEA and did not produce membrane depolarization despite increasing input resistance. Thus, reduction of g_K eliminated SP-responses suggesting that SP decreased g_K. Decreasing sodium concentration in the bathing medium (during TEA substitution or by replacing all but 5 mM sodium with choline) did not abolish SP- or ERP-responses suggesting that the depolarizing responses were not due solely to a change in g_{Na}. Adding manganese or cobalt chloride (5 mM) to the recording medium to reduce calcium conductance did not alter SP-responses (not illustrated) suggesting that SP-responses were not due to alterations in calcium conductance.

The ionic basis of SP-responses was further studied by recording SP-responses at membrane potentials above and below resting membrane potential. SP-responses increased in amplitude with membrane depolarization and decreased with membrane hyperpolarization. Unequivocal reversal of the polarity of SP-responses (from depolarizing to hyperpolarizing) with membrane hyperpolarization was not obtained. For example, a depolarizing SP-response evoked at the resting membrane potential of —64 mV (Fig. 1E$_1$) should have had a reversal potential at about —90 mV if SP only reduced g_K; however, hyperpolarization to —106 mV (Fig. 1E$_2$) abolished, but did not invert, the response. Since we could not obtain unequivocal inversion of SP-responses, we determined extrapolated reversal potentials using the amplitude of SP-responses at membrane potentials above and below resting membrane potential ($\pm$ 15 mV). Extrapolated reversal potentials for SP and ERP were more negative than resting membrane potential (Fig. 2 compare circles and triangles) and changed as a function of $[K^+]_o$ (Fig. 2.). However, extrapolated reversal potentials only varied linearly with the log of $[K^+]_o$ (as would be predicted from the Nernst equation for potassium)

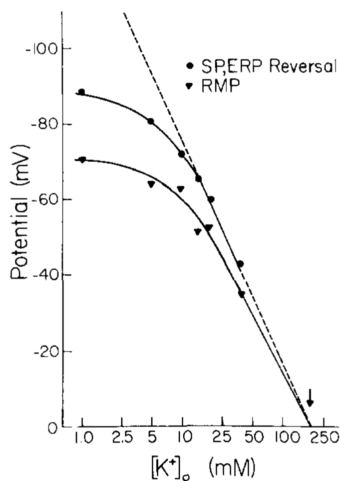

Fig. 2. Extrapolated reversal potentials of SP and ERP responses varied with $[K^+]_0$. Extrapolated reversal potentials (RPs; circles) were determined using two intracellular microelectrodes, one to record membrane potential and the other to pass current. RPs were hyperpolarized with respect to RMP (triangles). RPs and RMPs varied linearly with log $[K^+]_0$ as predicted by the Nernst potential for potassium (dashed line) in 10, 15, 20 and 40 mM $[K^+]_0$ with a slope of 61 mV per decade change in $[K^+]_0$, but were more depolarized than predicted in 1 and 5 mM $[K^+]_0$. Eledoisin-related peptide (ERP), a structural analog of SP, was used in some experiments and gave similar results. All recordings were made in Tris-buffered saline containing 3 μM TTX and 1 or 5 mM Ca^{2+} (altering $[Ca^{2+}]_0$ did not change RPs). Standard error of the mean was no greater than $\pm$ 5.0 mV for any point.

in 10, 15, 20 and 40 mM $[K^+]_0$ (—61 mV/decade $[K^+]_0$ change; dashed line Fig. 2); they were more depolarized than predicted in 1 and 5 mM $[K^+]_0$. Such a deviation fiom the Nernst potential for potassium might occur if SP decreased g_{Na} as well as g_K. However, since SP-responses could not be inverted by membrane hyperpolarization, it was more likely that SP reduced a voltage-dependent g_K which was decreased by membrane hyperpolarization and increased by membrane depolarization. At least two voltage-dependent potassium conductances have been described in neurons: (1) the voltage-dependent potassium conductance involved in repolarization of sodium–potassium action potentials (g_{Kv})[14]; and (2) a muscarine-sensitive potassium conductance (g_{Km})[5]. While both g_{Kv} and g_{Km} were activated by depolarization, g_{Km} was activated at more negative membrane potentials than was g_{Kv}, suggesting that, if present in spinal cord neurons, g_{Km} might be the conductance reduced by SP.

In addition to reducing a potassium conductance, SP produced bursting and appeared to evoke transmitter release. The depolarizing response, but not the synaptic potentials or bursting, remained when synaptic transmission was blocked by TTX suggesting that SP had both pre- and postsynaptic actions.

The slow postsynaptic depolarizing SP-responses from spinal cord neurons in cell culture were similar to those reported in cat ventral horn neurons[24] and guinea pig myenteric neurons[11,21,22] where membrane conductance also decreased and support the findings in myenteric neurons that SP decreased a g_K[21,22]. SP has also been reported to depolarize neurons with no change[41,45] or a slight increase in conductance[10,30]. The reason for the disparity in results could be due to differences in either

technique or preparation. The rapidly desensitizing depolarization accompanied by increased conductance recorded from mouse spinal cord neurons[43] in cell culture was not observed in our experiments.

Thus, we have demonstrated that SP depolarized and excited spinal cord neurons in cell culture by decreasing a membrane potassium conductance and that the SP-responses were voltage-dependent. If SP released from primary afferent terminals in vivo had a similar action, the resultant excitatory PSPs should increase with postsynaptic depolarization and decrease with hyperpolarization. Furthermore, if the postsynaptic neuron was hyperpolarized 10–15 mV by inhibitory synaptic input, synaptically released SP would have much less excitatory action. Therefore, excitation produced by synaptically released SP would be strongly modified by concurrent synaptic input to the same postsynaptic neuron.

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