ACTIONS OF SUBSTANCE P ON RAT SPINAL DORSAL HORN NEURONES

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SUMMARY

1. The membrane actions of substance P (SP) and the effects on the Ca-dependent action potential of dorsal horn neurones have been investigated by means of intracellular recording techniques in the immature rat *in vitro* spinal cord slice preparation.

2. Bath application of SP (2×10^{-6} to 1×10^{-5} M) induced a biphasic membrane response consisting of an initial hyperpolarization followed by a depolarization in about one-third of the cells examined. Initial hyperpolarization was not observed when synaptic activity was blocked by perfusing the slice with a tetrodotoxin-containing or low Ca, high Mg Ringer solution. This result is consistent with a presynaptic action of SP mediated through excitation of inhibitory interneurones. This interpretation was supported by recording of repetitive spontaneous inhibitory post-synaptic potential (i.p.s.p.)-like hyperpolarizing potentials during the initial hyperpolarization.

3. When Co ions were used to block voltage-dependent Ca conductance and possible indirect presynaptic actions, SP induced only a small depolarization of membrane potential. It seems, therefore, that Ca conductance may have contributed to the depolarizing phase of the SP response, either through its mediation of synaptic transmission or through direct effects as a charge carrier for inward current.

4. When tetrodotoxin was used, the SP-induced increase in neuronal input resistance was not modified, although depolarization was slightly diminished. In contrast, in medium containing tetrodotoxin and tetraethylammonium, the SP-depolarizing response was enhanced and accompanied by a small decrease in input resistance and firing of Ca spikes. These results suggest that SP-induced depolarization might be a consequence of a reduction in a voltage-dependent K conductance allowing Na and/or Ca conductances to dominate.

5. SP modified the duration of Ca-dependent action potentials of dorsal horn neurones, the most consistent change being an initial dose-dependent and reversible decrease in the spike duration. The decrease in Ca spike duration was associated with a small reduction in the rate of rise and peak amplitude, and a significant parallel increase in dV/dt of the falling phase of the Ca spike.

6. Our data indicate that the initial decrease in Ca spike duration was not due to the depolarizing action of SP, although shunting of the membrane resistance, either

through presynaptic or post-synaptic mechanisms, has not been ruled out. Alternatively, these data are consistent with the possibility that SP shortens the duration of the Ca spike by decreasing a voltage-sensitive inward Ca current and/or augmenting an outward K current.

7. Although the direct test of our interpretation can be accomplished only with a voltage-clamp analysis, the nature of our preparation does not seem to allow this study at present because of a probable dendritic origin of Ca spikes and a very high input resistance of dorsal horn neurones.

INTRODUCTION

Experimental evidence supports the concept that substance P (SP) may function as a neurotransmitter and/or neuromodulator in synaptic transmission between primary afferent fibres and neurones within the spinal cord (Nicoll, Schenker & Leeman, 1980). Electrophysiological data derived largely from extracellular recording showed that SP-induced excitation of dorsal horn neurones has a slow onset and decay (Henry, Krnjević & Morris, 1975; Henry, 1976; Randić & Miletić, 1977), and intracellular recordings demonstrated that SP produces slow neuronal depolarization associated with an increase in input resistance (Krnjević, 1977; Murase & Randić, 1981; Murase, Nedeljkov & Randić, 1982) or no change (Sastry, 1979; Zieglgänsberger & Tulloch, 1979). In addition to the SP depolarization, an initial hyperpolarization associated with a decrease in membrane input resistance occurred in a smaller proportion of rat dorsal horn neurones (Murase et al. 1982).

Recent data obtained in cultured mouse and rat spinal cord neurones suggested that the post-synaptic excitatory action of SP was due to a decrease in a membrane K conductance (G_K) (Hösli, Hösli, Zehntner & Landolt, 1981; Nowak & Macdonald, 1982), possibly a voltage-dependent G_K, similar to the M-current in sympathetic ganglion neurones (Brown & Adams, 1980; Adams, Brown & Constanti, 1982). Muscarine-sensitive K conductance has recently been demonstrated in cultured mouse spinal cord neurones (Nowak & Macdonald, 1981). However, nothing is known about the mechanism of the initial hyperpolarizing SP response.

In our experiments the cellular mechanisms underlying the initial hyperpolarization and ionic mechanisms of the SP depolarizing response of immature rat spinal cord neurones in the superficial parts of the dorsal horn were examined. Action potentials in these spinal neurones are generated by voltage-dependent conductance increases to Na and Ca ions (Heyer, Macdonald, Bergey & Nelson, 1981; Murase & Randić, 1982, 1983) and two distinct types of Ca spikes are probably present in immature rat dorsal horn cells (Murase & Randić, 1982, 1983). We have chosen to examine the effects of SP on Ca action potential properties since the control of the Ca channel by neurotransmitters is potentially of great importance as a mechanism for explaining plasticity and modulation at central synapses. Certain chemical messengers (catecholamines, 5-hydroxytryptamine, γ-aminobutyric acid) and the putative peptide transmitters (somatostatin and enkephalin) modulate voltage-dependent Ca currents (Dunlap & Fischbach, 1978, 1981; Klein & Kandel, 1978; Mudge, Leeman & Fischbach, 1979; Werz & Macdonald, 1982). Preliminary results of our findings have already been communicated (Randić & Murase, 1983).

METHODS

Preparation

Experiments were performed on 10–15-day-old Sprague–Dawley rats. The animals were anaesthetized with ether and cooled by immersing the thorax and abdomen in an ice–water slurry. During the period of cooling, close attention was paid to respiration. In the majority of animals respiration continued for at least 10 min following the onset of cooling, by which time the skin temperature had fallen to 20–22 °C. The dissection was then started and a laminectomy performed to expose the lower-thoracic and lumbosacral spinal cord together with dorsal roots. Following laminectomy a segment of lumbosacral spinal cord about 1–1·5 cm long with attached dorsal rootlets, was quickly excised and immersed in aerated (95 % O_2 and 5 % CO_2) Ringer solution at approximately 24 °C. The composition of the solution was (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1·2; $CaCl_2$, 2·4; $MgSO_4$, 1·3; $NaHCO_3$, 26; glucose, 10; pH 7·4. After the removal of the pia mater on the lateral aspects of the spinal cord, the spinal segment was cut manually into 5 mm blocks, and one of the blocks fixed with cyanoacrylic glue (Borden, Inc.) to the bottom of a Plexiglas cutting chamber of an Oxford Vibratome. The bath of the Vibratome was filled with the aerated Ringer solution, maintained at 24 °C. The blade of the Vibratome was positioned 300 μm below the dorsal surface of the spinal cord, and the spinal segment sectioned to yield one horizontal 300 μm thick dorsal horn slice. The duration of the entire procedure from the removal of the spinal cord until the slice was made rarely exceeded 5 min. The slices were incubated in Ringer solution at 35 °C for about an hour. After incubation, a slice was transferred to the recording chamber where it was continuously perfused with oxygenated modified Ringer solution (NaCl, 127 mM; KCl, 1·9 mM; KH_2PO_4, 1·2 mM; $CaCl_2$, 2·4 mM; $MgSO_4$, 1·3 mM; $NaHCO_3$, 26 mM; glucose, 10 mM) at 33 $\pm$ 1 °C at a flow rate of about 2 ml/min. The recording chamber had a capacity of 0·5 ml. Variations in the composition of the perfusing solution are indicated in the Results.

When $MgCl_2$ concentration (10^{-2} M) was increased or when $CoCl_2$ ($1-2 \times 10^{-3}$ M) was added to the bathing solution to eliminate synaptic activity, the NaCl concentration was adjusted to maintain osmolarity. The NaCl concentration was also adjusted when 4-aminopyridine ($1-2 \times 10^{-3}$ M) or tetraethylammonium chloride (TEA, 2×10^{-3} to 2×10^{-2} M, Kodak) was added to the bathing medium in order to block voltage-dependent K conductances (Armstrong, 1971; Yeh, Oxford, Wu & Narahashi, 1976). Fast voltage-dependent Na conductance was blocked with tetrodotoxin (TTX, $1-2 \times 10^{-6}$ M, Sankyo) (Narahashi, Moore & Scott, 1964). In a majority of experiments, TTX, rather than Co or Mg, was used in order to reduce spontaneous and synaptic activity. SP (Beckman, Peninsula Labs.) was applied by bath perfusion in known concentrations. It took about 25 s for SP to reach the slice in the chamber due to dead space between the slice and the reservoirs containing drug solution. Stock solution of SP (5×10^{-3} M) was made by dissolving the peptide in ammonium acetate/acetic acid buffer (pH 5·5), which also contained in some experiments 0·1 % bovine serum albumin. Aliquots (10 μl) of stock solution were pipetted into plastic tubes and frozen, until used in the experiment. The pH of all applied solutions was maintained between 7·2 and 7·4.

Intracellular recording technique

Intracellular recordings were performed with micropipettes filled with 3 M-K acetate having d.c. resistances of 100–120 MΩ. Stable intracellular recordings from single dorsal horn neurones could be maintained during multiple solution changes for as long as 5 h. Electrical properties of dorsal horn neurones were determined by means of a high-input impedance bridge amplifier (WP Instruments, M707) allowing current injections of the order of 0·05–1·0 nA through the recording electrode. Data were recorded on a Gould-Brush pen recorder (model 2200) or stored in the disks of a Nicollet digital oscilloscope (model 4094) until processed and printed out onto an X–Y chart recorder.

RESULTS

Effects of SP on membrane potential: initial hyperpolarization

Bath application of SP (2×10^{-6} to 1×10^{-5} M) induced a biphasic membrane response consisting of an initial hyperpolarization followed by a depolarization in

thirteen out of the forty-five dorsal horn neurones examined (Fig. 1 A). However, as shown previously (Murase et al. 1982), the rest of the cells ($n = 32$) responded to the peptide with a monophasic response consisting of a slow depolarization frequently accompanied by an increase in synaptic activity and action potential firing.

The amplitude of hyperpolarization was small (-1.7 ± 1.0 mV, mean $\pm$ S.D., $n = 13$); it ranged from -0.5 to -4.0 mV and lasted from 20 to 150 s (64.0 ± 35.0 s, $n = 13$). However, the magnitude of the SP hyperpolarizing response was not easily
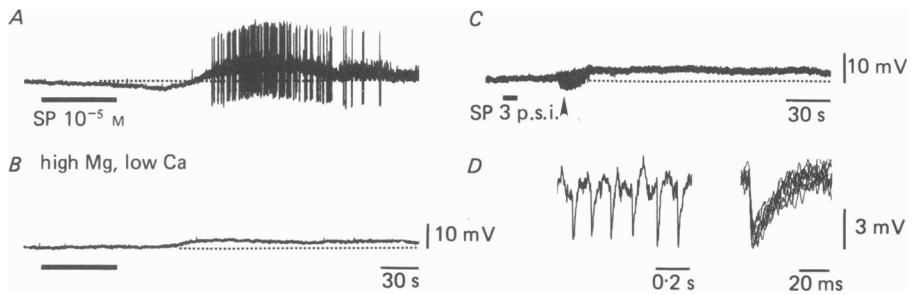

Fig. 1. Biphasic membrane potential changes and inhibitory post-synaptic potentials evoked by SP. In A, bath application of SP (10^{-5} M) induced a biphasic response consisting of an initial hyperpolarization followed by a depolarization and spike discharges in a dorsal horn neurone of an 11-day-old rat. Resting membrane potential was -64 mV. In B, the initial hyperpolarization was blocked by perfusing the slice with a low Ca (1.3×10^{-3} M), high Mg (10^{-2} M) Ringer solution. The resting membrane potential was -65 mV. In C, a pressure microinjection of SP (3 p.s.i., 2×10^{-2} M) evoked a burst of spontaneous hyperpolarizing events (arrow) in a different dorsal horn neurone of a 13-day-old rat. In D, higher gain and faster time scale records of the hyperpolarizing potentials are shown.

reproducible from trial to trial within a single cell or among different cells. Thus the variability of this phenomenon did not permit us to determine reliably dose–response dependence. The resting potential varied between -50 and -78 mV, with a mean value of -65.0 ± 9.0 mV (S.D.).

A typical biphasic response to SP in a dorsal horn neurone of a 10-day-old rat is illustrated in Fig. 1 A. Here, within 30 s following addition of SP (10^{-5} M), the neuronal membrane hyperpolarized by about 2.3 mV. This effect persisted for about 70 s. When the slice was perfused with a TTX-containing medium, or a low Ca (1.3 mM), high Mg (10 mM) Ringer solution (Fig. 1 B) in order to reduce or eliminate synaptic activity, the initial hyperpolarization was not observed. The latter finding suggested a possibility that the initial hyperpolarization was brought about by an indirect presynaptic action of SP. Thus SP may have excited presynaptic inhibitory interneurones, which in turn induced inhibitory synaptic events in other dorsal horn neurones.

This hypothesis is supported by our finding that spontaneous hyperpolarizing potentials resembling inhibitory post-synaptic potentials (i.p.s.p.s) were recorded in seventeen cells following application of SP (Fig. 1 C and D) and in seven cells i.p.s.p.s were associated with initial hyperpolarization. Amplitude of i.p.s.p.s varied between -0.5 and -6.0 mV, with a mean value of -2.9 ± 1.7 mV ($n = 12$). The i.p.s.p.s had a mean rise time of 7.8 ± 2.3 ms ($n = 8$), while on average 30.1 ± 8.4 ms ($n = 8$) were needed for the membrane potential to decline to 37 % of the maximum amplitude.

Furthermore, we have observed in several cells that spontaneous activity is usually depressed during the initial hyperpolarization, and that membrane input resistance decreased (mean $5.2 \pm 3.9 \%$, $n = 6$). Reduction of i.p.s.p. amplitude could be produced with soma hyperpolarization. Since the hyperpolarizing response was only present in about 29 % of all neurones examined, and because the response is relatively small in amplitude, an accurate determination of the reversal potential was not attempted.
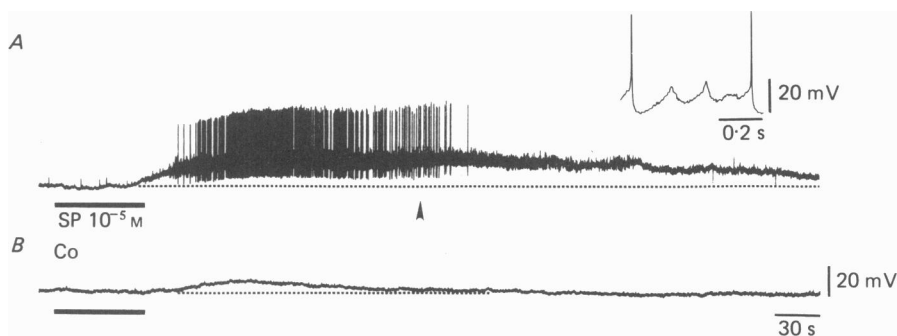

Fig. 2. A typical SP-induced depolarization of a dorsal horn neurone in a 14-day-old rat and the effect of Co on this response. *A*, bath application of SP (10^{-5} M) for 1 min produced a slow depolarization accompanied by an increase in synaptic activity and firing of action potentials (action potentials were truncated by the limited frequency response of the pen recorder). The inset showing slow depolarizing potentials and Na spikes was taken at the arrow in *A*. *B*, addition of Co (10^{-3} M) to the bathing medium substantially reduced the level and duration of the SP-induced depolarization. Resting membrane potential, -82 mV.

Depolarization and firing of Ca-dependent spikes

In confirmation of previous observations (Murase *et al.* 1982; Nowak & Macdonald, 1982), bath application of SP produced a slow reversible depolarization in the immature spinal dorsal horn neurones, accompanied by an increase in synaptic activity and firing of action potentials (Figs. 2*A* and 3*A*). However, addition of CoCl$_2$ (10^{-3} M) to the bathing medium substantially reduced the level (Table 1) and duration of depolarization (Fig. 2*B*) following SP application. It seems, therefore, that Ca conductance (G_{Ca}) may have contributed to the depolarizing phase of the SP response, either through its mediation of synaptic transmission or through the direct effects as a charge carrier for inward current. It is known that perfusion of slices with Co-containing solution reduces or eliminates synaptic transmission. In addition, Co blocks voltage-dependent G_{Ca} in rat dorsal horn neurones (Murase & Randić, 1982) and other neurones in the central nervous system. However, our result obtained with Co ions does not rule out participation of voltage-sensitive Na and K components in the slow SP-elicited depolarization (Nowak & Macdonald, 1982).

TTX- and TEA-containing solutions

As illustrated in Fig. 3, when the fast voltage-sensitive Na and K conductances were blocked by perfusion of the spinal cord slices with TTX (10^{-6} M) and/or TEA (2×10^{-3} to 2×10^{-2} M) a slow depolarizing response to SP could still be evoked although it was modified in its amplitude depending on the kind of solution used
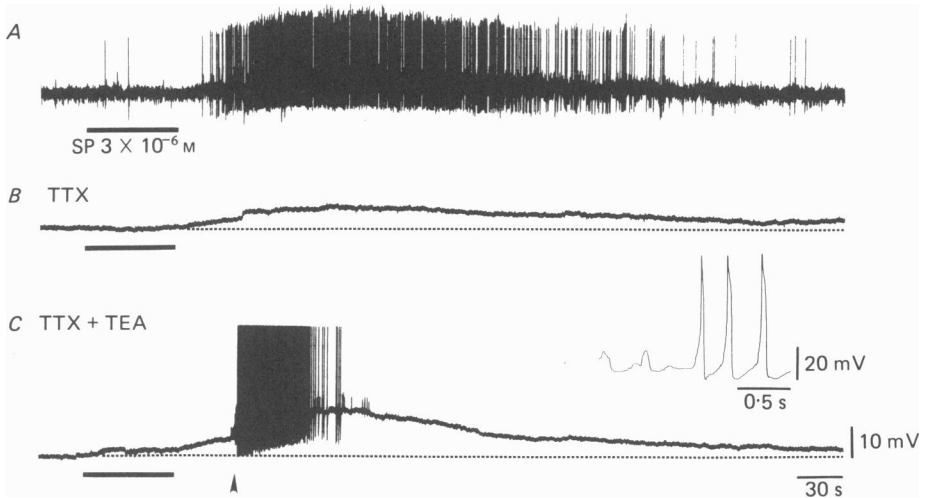
Fig. 3. Responses to SP of a dorsal horn neurone of a 10-day-old rat in solutions containing different ionic blockers. *A*, bath application of SP (3×10^{-6} M) induced a bursting firing pattern in a previously silent cell kept in normal Ringer solution. *B*, in the presence of TTX (10^{-6} M), Na-dependent action potentials were eliminated while the SP-induced depolarization remained. *C*, in media containing TTX (10^{-6} M) and TEA (2×10^{-2} M) the SP depolarizing response was significantly increased, and firing of TTX-resistant spikes was evoked. As illustrated in the inset (taken at the arrow in *C*) the TTX-resistant spikes were of two distinct amplitudes. The resting membrane potentials in *A*, *B* and *C* were -54, -56 and -53 mV, respectively.

TABLE 1.

(A) SP-induced depolarization in the normal Ringer solution. The values are expressed as mean $\pm$ S.D.

SP (M)	ΔV_m (mV)	V_m (mV)	n
2–3×10^{-6}	$3 \cdot 0 \pm 1 \cdot 8$	$-65 \cdot 0 \pm 7 \cdot 5$	7
10^{-5}	$5 \cdot 5 \pm 4 \cdot 8$	$-63 \cdot 5 \pm 10 \cdot 4$	14

(B) SP-induced depolarization in solutions containing ionic blockers (TTX, 5×10^{-7} to 2×10^{-6} M; TEA, 2×10^{-3} to 2×10^{-2} M; Co, 1–3×10^{-3} M)

Solution	% control	V_m (mV)	n
Normal	100	$-64 \cdot 5 \pm 8 \cdot 9$	24
TTX	91 ± 41	$-63 \cdot 1 \pm 9 \cdot 8$	9
TTX + TEA	170 ± 78	$-63 \cdot 2 \pm 11 \cdot 1$	19
TTX + TEA + Co	57 ± 32	$-62 \cdot 8 \pm 11 \cdot 3$	6

(Table 1). Thus a slightly smaller depolarization was seen following SP application in the presence of TTX (Table 1) while the SP depolarizing response was increased in media containing TTX and TEA (Fig. 3*C*, Table 1). While Na-dependent action potentials were eliminated in a bathing medium containing TTX (Fig. 3*B*), SP depolarization in recording solution containing TTX and TEA was in four out of seventeen cells accompanied by firing of TTX-resistant spikes (Fig. 3*C*). As illustrated, in particular, in the insets of Figs. 3*C* and 6*A*, the TTX-resistant spikes were of two distinct amplitudes.
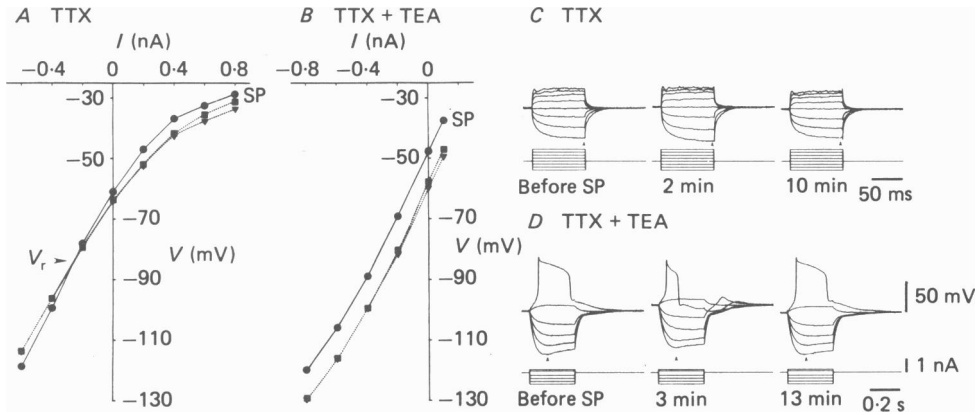
Fig. 4. Typical changes in current–voltage (I–V) relationships of two dorsal horn neurones elicited by SP in media containing TTX (A, 13-day-old rat) or TTX and TEA (B, 10-day-old rat). The I–V curves were reconstructed from the records shown in C and D respectively. A, an increase in the slope of a control I–V curve (▼) was observed 2 min after the introduction of SP (2×10^{-6} M) into a bathing medium containing 10^{-6} M-TTX (●). Almost complete recovery of the slope occurred 10 min after stopping SP perfusion (■). As indicated with an arrow, the reversal potential (V_r) for SP response was about -83 mV. B, in media containing TTX (10^{-6} M) and TEA (2×10^{-2} M), SP ($2 \cdot 5 \times 10^{-6}$ M) slightly decreased the slope (●, 3 min after SP) of a control I–V curve (▼). Recovery of the slope to the control level occurred 13 min after SP (■). C and D, lower traces show applied current pulses, upper traces voltage response. Intracellular responses to depolarizing (upward) and hyperpolarizing (downward) current pulses applied in progressive steps across the cell soma are illustrated. D, depolarizing pulse that evoked a Ca spike was applied last in the stimulation sequence in order to avoid the post-spike excitability changes. Note the decrease in Ca spike duration produced by SP (middle record) and a reversal of the after-depolarization to an after-hyperpolarization.

As shown in Fig. 4A–C the depolarization recorded in TTX-containing solution was associated with an increase in neuronal input resistance ($13 \cdot 3 \pm 1 \cdot 7\%$, $n = 4$), and the mean reversal potential determined from current–voltage curves was $-78 \cdot 0 \pm 6 \cdot 6$ mV ($n = 4$). Reduction of depolarization could be effected by soma hyperpolarization in several of the cells tested, but inversion of the response to a hyperpolarizing response could not be achieved even at a membrane potential of -104 mV (Fig. 5A). The latter result suggested that the depolarization may be due to a decrease in a voltage-dependent G_K.

In contrast, as evidenced from Fig. 4B and D, the current–voltage curve obtained in solution containing TTX and TEA showed that SP produced a decrease in input resistance and that the extrapolated reversal potential was much more positive than the resting membrane potential. In six cells the SP depolarization was associated with an average decrease in input resistance of $7 \cdot 6 \pm 6 \cdot 7\%$. The membrane hyperpolarization essentially did not modify the amplitude of SP-induced depolarization (Fig. 5B). These results suggest that the SP depolarization observed in neurones perfused with solution containing TTX and TEA is possibly due to an increase in G_{Na} and/or G_{Ca}. In support of the concept of participation of G_{Ca} in SP depolarization is our finding that the addition of Co either to the normal bathing medium (Fig. 2B) or that containing TTX and TEA (Fig. 6B, Table 1) produced a decrease in both amplitude
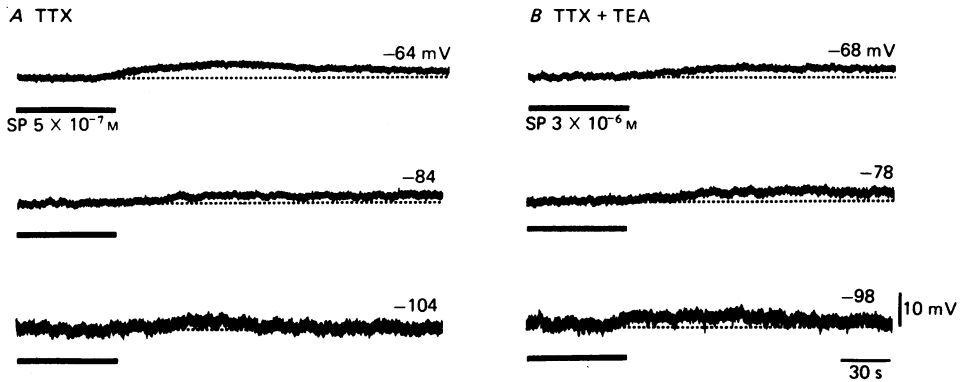
Fig. 5. The effects of soma hyperpolarization with a d.c. current on the amplitude of SP-induced depolarizations in two different dorsal horn neurones in media containing TTX (A) or TTX and TEA (B). A, in 10^{-6} M-TTX the soma hyperpolarization decreased the SP (5×10^{-7} M) response; 14-day-old rat. B, in solutions containing TTX (10^{-6} M) and TEA (2×10^{-2} M) the hyperpolarization of the cell body did not alter the SP (3×10^{-6} M) response; 13-day-old rat.
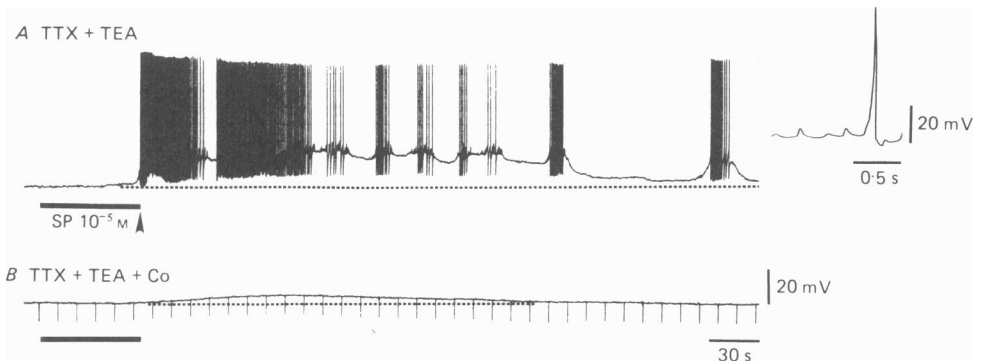

Fig. 6. The effects of Co on SP response of a previously silent dorsal horn neurone from a 12-day-old rat bathed in medium containing TTX (10^{-6} M) and TEA (2×10^{-2} M). A, SP (10^{-5} M) induced a depolarization and development of intermittent bursting of TTX-resistant spikes. Resting membrane potential was -57 mV. The records shown in the inset (taken at the arrow and displayed at a higher gain) illustrate three distinct amplitudes of TTX-resistant spikes. In B, addition of Co (10^{-3} M) to bathing medium containing TTX and TEA produced a marked decrease in both amplitude and duration of SP-induced depolarization. TTX-resistant spikes are completely blocked by Co, the result indicating Ca dependency of the bursts.

and duration of the SP depolarization. The latter was possibly in part due to an increase in G_{Ca}.

SP and generation of bursting activity

In the normal bathing medium we observed that about one-quarter of immature rat dorsal horn neurones showed spontaneous activity, predominantly of a bursting firing pattern. The remaining cells were silent. SP evoked alterations in firing mode, with development of burst generation in the silent cells (Fig. 1A).

Furthermore, after a single SP application some of the silent and non-bursting neurones perfused with medium containing TTX and TEA became capable of prolonged depolarizations and burst generation (Fig. 6*A*). The SP-induced mode of burst firing could last for hours. Besides large-amplitude spike pontentials, individual SP-evoked bursts contained a number of small spike-like potentials (Fig. 6*A*, inset).

Since the slow oscillations in the membrane potential which influence the total duration of the bursts are probably Ca-dependent, and since Ca entry is known to activate intracellular metabolic events which have a long time-course (Phillis, 1977), we have examined the nature and duration of SP-induced changes in resting and active membrane properties in slices perfused with Co. It is clear from Fig. 6*B* that when Co, a Ca current blocker, is added to the bath, the rhythmic firing behaviour of this cell is blocked. As noted further in Fig. 6*B*, although a small SP depolarization still occurred, its time course was significantly reduced compared with the duration of changes in the membrane properties following SP application in normal medium.

Ca-dependent action potentials and after-potentials

When the Na and K conductances are blocked by perfusion of the spinal cord slices with TTX (5×10^{-7} to 2×10^{-6} M) and TEA (2×10^{-3} to 2×10^{-2} M) depolarizing current pulses applied across the cell soma of a dorsal horn neurone elicit a high-threshold Ca spike (Murase & Randić, 1982, 1983). The spike duration varied from cell to cell presumably depending upon the degree of blockade of K conductances by TEA. Thus the duration of Ca spikes recorded in dorsal horn neurones bathed in a solution containing $2-10 \times 10^{-3}$ M-TEA was in the range of 5–15 ms (Fig. 7*C*). When a higher concentration of TEA (2×10^{-2} M) was used, the spike duration ranged from about 15 to 400 ms (occasionally up to 12 s), and the repolarization phase of action potentials was marked by a distinct plateau (Fig. 7*A*).

The Ca spikes of short duration (< 15 ms) were frequently followed by after-hyperpolarization (Fig. 7*C*). When the Ca spike duration was longer (> 15 ms), the spike was followed by a prolonged after-depolarization (Fig. 7*A*).

The effects of membrane polarization on Ca-dependent action potentials of a dorsal horn neurone in a 14-day-old rat are shown in Fig. 7*A*. Membrane depolarization resulted in a marked increase in spike duration and a decrease in after-depolarization (Fig. 7*A*2). Hyperpolarization in this cell only slightly reduced spike duration, but the amplitude of after-depolarization was increased (Fig. 7*A*3–4).

The effects of SP on Ca spike

Bath application of SP (10^{-5} M) modified the duration of Ca-dependent action potentials in a complex manner in fourteen out of twenty-two immature rat dorsal horn neurones examined. The remaining eight neurones showed no modification of the Ca spike duration despite the presence of membrane depolarization.

In general, it appears that the direction of a change in the Ca spike duration is highly dependent upon the initial control value. Thus, when the control Ca spike duration was approximately 15 ms, or more, the effect observed in about half of the cells examined was a biphasic modification of the spike duration consisting of an initial reversible decrease followed by an increase (Fig. 7*B*). However, when the spike duration was less than 15 ms a monophasic increase only was observed (Fig. 7*C*).
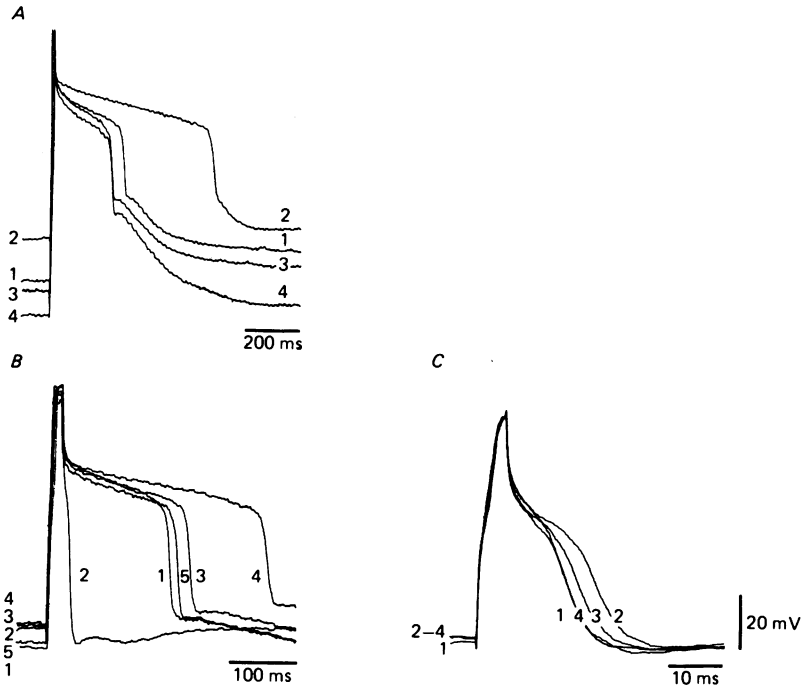
Fig. 7. The effects of membrane polarization and SP (10^{-5} M) on Ca spike duration in three different dorsal horn neurones bathed in a solution containing TTX (10^{-6} M) and TEA (2×10^{-2} M). Oscilloscope records of superimposed Ca-dependent action potentials are shown. Action potential duration was determined at half-maximal amplitude. A, 14-day-old rat; B, 13-day-old rat; C, 10-day-old rat. A, resting membrane potential was -66 mV (trace 1). Membrane depolarization (trace 2, membrane potential (V_m) = -50 mV) resulted in a marked increase in spike duration and a decrease in after-depolarization. Hyperpolarization in this cell (trace 3, $V_m = -70$ mV; trace 4, $V_m = -77$ mV) reduced the spike duration and increased the magnitude of after-depolarization. B, SP induced a biphasic change in the spike duration (1, before SP) consisting of an initial decrease (2, 1 min after SP) followed by an increase (3, 3 min after SP; 4, $5\frac{1}{2}$ min after SP). Almost full recovery of the spike duration occurred at about 20 min after stopping the SP application (trace 5). Stimulus pulse: 1 nA, 20 ms. $V_m = -51$ mV. C, when Ca spike duration was less than 15 ms, SP-elicited increase in the spike duration only was observed (1, before SP; 2, 2 min after SP; 3, 4 min after SP; 4, $6\frac{1}{2}$ min after SP). The spike broadening was accompanied by a reduction in the amplitude and duration of after-hyperpolarization. Stimulus pulse: 2 nA, 5 ms. $V_m = -50$ mV.

The SP-induced initial decrease in Ca spike duration was accompanied by a significant reduction in the magnitude of after-depolarization (Fig. 7B2). The SP-induced monophasic spike increase was usually associated with a decrease in the amplitude and duration of after-hyperpolarization (Fig. 7C).

The SP-elicited changes in Ca spike duration were not easily reproducible from trial to trial in a single cell; usually three consecutive applications of SP led to a reduction in the response. Thus the desensitization of this response did not permit us to determine reliably dose-dependence in a single cell. It is of interest that during the desensitization of the Ca spike the depolarizing response of SP remained.
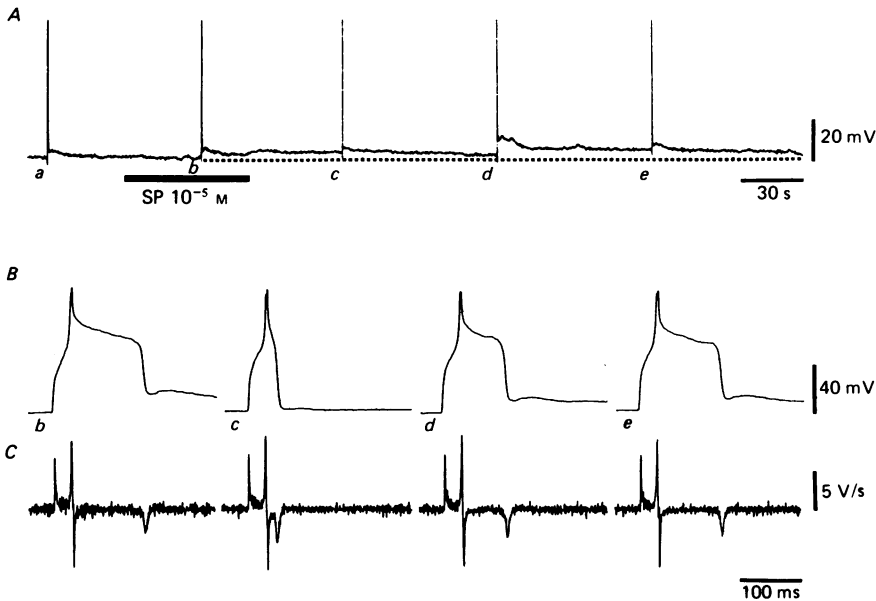
Fig. 8. *A* and *B*, the membrane of a dorsal horn neurone of a 13-day-old rat bathed in solution containing TTX (10^{-6} M) and TEA (2×10^{-2} M) was depolarized by SP (10^{-5} M). During the depolarizing response a Ca spike was evoked with depolarizing current pulses (1 nA, 30 ms) applied across the cell soma at regular intervals of 1 min and the differentiation was calculated (*C*). *B*, the same records of Ca spikes as in *A* taken at a higher gain. Since the shape of the Ca spike at *Ab* was identical to one recorded at *Aa*, *Aa* was omitted from *B* and *C*. In *Bc*, SP produced a reversible decrease in spike duration which was associated with an increase in d*V*/d*t* of the repolarization phase of the spike. Also, the after-depolarization was almost completely abolished and the shunting effect on the after-depolarization lasted more than 20 s (*Bc*). Note a late increase of slow after-depolarization (*Ad* and *Ae*).

To identify the component of the Ca spike responsible for the initial decrease in spike duration, the d*V*/d*t* of the Ca spike was utilized. As seen in Fig. 8*B* and *C*, in the cells showing an initial decrease in spike duration the d*V*/d*t* of the repolarizing phase of the spikes was significantly increased by SP ($167\% \pm 13$, $n = 5$). In contrast, only small reductions in d*V*/d*t* of the rising phase of Ca spikes ($94 \cdot 1\% \pm 7 \cdot 9$, $n = 5$) or in V_{max} of Ca spikes were observed.

The initial decrease in Ca spike duration was not due to the depolarizing action of SP for two reasons: (1) the effect is present when resting membrane potential was re-established by passing adequate d.c. current, and (2) consistent increase in spike duration occurred as the membrane potential was made more positive than the resting membrane potential (Fig. 7*A*2).

DISCUSSION

SP-mediated inhibition

SP has depressant actions when applied iontophoretically near spinal dorsal horn neurones (Krnjević, 1977; Randić & Miletić, 1977; Sastry, 1979; Davies & Dray,

1980). An initial hyperpolarization associated with a decrease in membrane input resistance was observed in some immature rat dorsal horn neurones following SP application (Murase *et al.* 1982). A transient depression or even abolition of spontaneous activity during the initial hyperpolarization was seen in the present experiments. This apparent inhibition of dorsal horn neurones by SP might have been brought about either by a direct post-synaptic action or by an indirect presynaptic mechanism involving excitation of inhibitory interneurones.

Although direct evidence for presynaptic excitatory effects of SP on spinal dorsal horn inhibitory interneurones is lacking, our results suggest that the initial inhibitory effect of SP is produced by an indirect presynaptic action rather than direct post-synaptic actions. This hypothesis is supported by our findings that the initial hyperpolarization was not observed when synaptic activity was reduced or eliminated by perfusion of the slice with a TTX-containing, or low Ca, high Mg Ringer solution. In addition, spontaneous hyperpolarizing potentials resembling i.p.s.p.s were recorded in dorsal horn neurones following application of SP, and in several cells i.p.s.p.s were associated with hyperpolarization.

A presynaptic site of action for SP has also been suggested by other experimental data (Nicoll, 1976; Macdonald & Nowak, 1981; Randić, 1981; Randić, Carstens, Zimmermann & Klumpp, 1982). In addition, immunocytochemical studies of localization and synaptic relationships of SP in the rat spinal dorsal horn (Barber, Vaughn, Slemmon, Salvaterra, Roberts & Leeman, 1979) and trigeminal nucleus (Priestley, Somogyi & Cuello, 1982) suggest several possible targets for SP-containing terminals, including the large lamina 1 projection neurones and two types of lamina II interneurones, one of which possesses presynaptic dendrites.

Depolarization and firing of Ca-dependent spikes

To investigate further the ionic mechanisms of SP-induced depolarization we monitored changes in the magnitude of the depolarizing responses of dorsal horn neurones exposed to blockers of voltage-sensitive Na, K and/or Ca conductances (G_{Na}, G_K and G_{Ca}); we also investigated such changes as a function of membrane potential. The experiments utilizing the sequential blockade of G_{Na}, G_K and G_{Ca} in the same cell provide additional new data relevant to the nature of SP-induced depolarization. Thus, when voltage-sensitive Na and K conductances are blocked by perfusion of the spinal cord slices with TTX and TEA respectively, the SP depolarizing response of dorsal horn neurones was enhanced and in about one-quarter of all cells examined was accompanied by firing of TTX-resistant spikes. The current–voltage curve showed that SP produced a small decrease in neuronal input resistance and that the extrapolated reversal potential for SP response was much more positive than the resting membrane potential. In addition, the membrane hyperpolarization essentially did not modify the amplitude of SP-induced depolarization. These findings do not necessarily contradict the current concept that the principal ionic mechanism underlying SP-induced depolarization is a decrease in a voltage-dependent G_K in the post-synaptic cell (Hösli *et al.* 1981; Nowak & Macdonald, 1982). However, the fact that the SP reversal potential was more positive than the K equilibrium potential suggests that in addition to a decrease in G_K, SP may have increased positive ion conductances such as G_{Na} and/or G_{Ca}. The ratio of the changes

in these three conductances will ultimately determine the change of membrane resistance.

Furthermore, in support of the hypothesis of participation of G_{Ca} in prolonged SP depolarization is our finding that the addition of Co either to the normal bathing medium or to that containing TTX and TEA produced a significant decrease in both amplitude and duration of the SP-induced depolarization. This result suggests that Ca entry might be primarily responsible for the prolonged depolarizations associated with SP excitation.

We are fully aware that the present conclusions about specific ion conductance changes produced by SP have been inferred indirectly by measurements of membrane potential, input resistance, and by use of specific conductance blockers, and that only voltage-clamp analysis of SP actions in dorsal horn neurones, in addition to changes of Ca and Na levels in the extracellular environment, will allow us to test our hypothesis directly.

Modification of Ca spike duration by SP

In this paper we have demonstrated that SP modifies the duration of Ca-dependent action potentials of the immature rat dorsal horn neurones, the most consistent change observed being an initial dose-dependent and reversible decrease of spike duration.

In principle, the SP-induced decrease in Ca spike duration might be due to an effect on inward Ca current or outward K currents. Since we have observed a small decrease in the rate of rise and the peak amplitude of the Ca spike following SP application to dorsal horn neurones, and since the voltage-dependent conductance changes are probably occurring at dendritic sites, and the resting membrane of the cells is usually depolarized by SP, the possibility should be considered that SP shortens the duration of the spike by decreasing a voltage-sensitive inward Ca current. Employing voltage-clamp analysis Dunlap & Fischbach (1981) have shown that several neuro-transmitters including noradrenaline, γ-aminobutyric acid (GABA) and 5-hydroxytryptamine decrease the Ca conductance activated by depolarization of embryonic chick sensory neurones maintained in cell culture.

Our observation that in the cells showing an initial decrease in Ca spike duration the decrease was clearly paralleled by the increase in dV/dt of the falling phase of the Ca spike favours an alternative possibility, namely that the decrease may be due to an activation of an outward current(s). The latter interpretation is supported by two additional observations: (1) the after-depolarization was blocked, or even reversed into after-hyperpolarization during the initial decrease in Ca spike duration, and (2) the magnitude of the initial decrease is inversely proportional to the duration of the control spike. The direct test of our interpretations and identification of ionic current(s) involved are to be accomplished only with a voltage-clamp analysis.

Since the initial decrease in Ca spike duration of dorsal horn neurones was usually associated with SP-induced changes in membrane potential and resting membrane conductance, these effects of SP should be considered when discussing alternative mechanisms for explaining the decrease in Ca spike duration. Our data presented in the Results clearly indicate that the initial decrease in Ca spike duration was not due to the depolarizing action of SP. However, SP-modulation of voltage-sensitive ion

channels involving shunting of the membrane resistance, either through post-synaptic or even presynaptic mechanisms (perhaps through activation of inhibitory inter-neurones releasing GABA or enkephalin) has not been excluded.

REFERENCES

ADAMS, P. R., BROWN, D. A. & CONSTANTI, A. (1982). M-currents and other potassium currents in bullfrog sympathetic neurones. *J. Physiol.* **330**, 537–572.

ARMSTRONG, C. M. (1971). Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J. gen. Physiol.* **58**, 413–437.

BARBER, R. P., VAUGHN, J. E., SLEMMON, J. R., SALVATERRA, P. M., ROBERTS, E. & LEEMAN, S. E. (1979). The origin, distribution and synaptic relationships of substance P axons in rat spinal cord. *J. comp. Neurol.* **184**, 331–352.

BROWN, D. A. & ADAMS, P. R. (1980). Muscarinic suppression of a novel voltage-sensitive K^+-current in a vertebrate neurone. *Nature, Lond.* **283**, 673–676.

DAVIES, J. & DRAY, A. (1980). Depression and facilitation of synaptic responses in cat dorsal horn by substance P administered into substantia gelatinosa. *Life Sci. Oxford* **27**, 2037–2042.

DUNLAP, K. & FISCHBACH, G. D. (1978). Neurotransmitters decrease the calcium component of sensory neurone action potentials. *Nature, Lond.* **276**, 837–839.

DUNLAP, K. & FISCHBACH, G. D. (1981). Neurotransmitters decrease the calcium conductance activated by depolarization of embryonic chick sensory neurones. *J. Physiol.* **317**, 519–535.

HENRY, J. L. (1976). Effects of substance P on functionally identified units in cat spinal cord. *Brain Res.* **114**, 439–451.

HENRY, J. L., KRNJEVIĆ, K. & MORRIS, M. E. (1975). Substance P and spinal neurones. *Can. J. Physiol. Pharmac.* **53**, 423–432.

HEYER, E. J., MACDONALD, R. L., BERGEY, G. K. & NELSON, P. G. (1981). Calcium-dependent action potentials in mouse spinal cord neurons in cell culture. *Brain Res.* **220**, 408–415.

HÖSLI, L, HÖSLI, E., ZEHNTNER, CH. & LANDOLT, H. (1981). Effects of substance P on neurones and glial cells in cultured rat spinal cord. *Neurosci. Lett.* **24**, 165–168.

KLEIN, M. & KANDEL, E. R. (1978). Presynaptic modulation of voltage-dependent Ca^{2+} current: mechanism for behavioral sensitization in *Aplysia californica*. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3512–3516.

KRNJEVIĆ, K. (1977). Effects of substance P on central neurones in cats. In *Substance P*, ed. VON EULER, U. S. & PERNOW, B., pp. 217–230. New York: Raven Press.

MACDONALD, R. L. & NOWAK, L. M. (1981). Substance P and somatostatin actions on spinal cord neurons in primary dissociated cell culture. In *Neurosecretion and Brain Peptides*, ed. MARTIN, J. B., REICHLIN, S. & BICK, K. L. pp. 159–173. New York: Raven Press.

MUDGE, A. W., LEEMAN, S. E. & FISCHBACH, G. D. (1979). Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. *Proc. natn. Acad. Sci. U.S.A.* **76**, 526–530.

MURASE, K., NEDELJKOV, V. & RANDIĆ, M. (1982). The actions of neuropeptides on dorsal horn neurons in the rat spinal cord slice preparation: an intracellular study. *Brain Res.* **234**, 170–176.

MURASE, K. & RANDIĆ, M. (1981). Intracellular recording from dorsal horn neurons in the rat spinal cord slice preparation. *Neurosci. Abstr.* **7**, 529.

MURASE, K. & RANDIĆ, M. (1982). Calcium-dependent action potentials of rat spinal dorsal horn neurons *in vitro*. *Neurosci. Abstr.* **8**, 124.

MURASE, K. & RANDIĆ, M. (1983). Electrophysiological properties of rat spinal dorsal horn neurones *in vitro*: calcium-dependent action potentials. *J. Physiol.* **334**, 141–153.

NARAHASHI, T., MOORE, J. W. & SCOTT, W. R. (1964). Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. gen. Physiol.* **47**, 965–974.

NICOLL, R. A. (1976). Promising peptides. In *Neurotransmitters, Hormones and Receptors: Novel Approaches*. Neuroscience Symposium vol. 1, ed. FERRENDELLI, J. A., McEWEN, B. S. & SNYDER, S. H., pp. 99–122. Bethesda: Society for Neuroscience.

NICOLL, R. A., SCHENKER, C. & LEEMAN, S. E. (1980). Substance P as a transmitter candidate. *A. Rev. Neurosci.* **3**, 227–268.

NOWAK, L. M. & MACDONALD, R. L. (1981). dl-Muscarine decreases a potassium conductance to depolarize mammalian spinal cord neurons in cell culture. *Neurosci. Abstr.* **7**, 725.

NOWAK, L. M. & MACDONALD, R. L. (1982). Substance P: ionic basis for depolarizing responses of mouse spinal cord neurons in cell culture. *J. Neurosci.* **2**, 1119–1128.

PHILLIS, J. W. (1977). The role of cyclic nucleotides in the CNS. *J. Neurol. Sci.* **4**, 151–195.

PRIESTLEY, J. V., SOMOGYI, P. & CUELLO, C. A. (1982). Immunocytochemical localization of substance P in the spinal trigeminal nucleus of the rat: a light and electron microscopic study. *J. comp. Neurol.* **211**, 31–49.

RANDIĆ, M. (1981). Presynaptic effects of γ-aminobutyric acid and substance P on intraspinal single cutaneous C- and A-fibers. In *Spinal Cord Sensation. Sensory Processing in the Dorsal Horn*, ed. BROWN, A. G. & RÉTHELYI, M., pp. 285–294. Edinburgh: Scottish Academic Press.

RANDIĆ, M., CARSTENS, E., ZIMMERMANN, M. & KLUMPP, D. (1982). Dual effects of substance P on the excitability of single cutaneous primary afferent C- and A-fibers in the cat spinal cord. *Brain Res.* **233**, 389–393.

RANDIĆ, M. & MILETIĆ, V. (1977). Effects of substance P in cat dorsal horn neurones activated by noxious stimuli. *Brain Res.* **128**, 164–169.

RANDIĆ, M. & MURASE, K. (1983). Actions of substance P on rat spinal dorsal horn neurones. *Proc. int. Union physiol. Sci.* (in the Press).

SASTRY, B. R. (1979). Substance P effects on spinal nociceptive neurones. *Life Sci. Oxford* **24**, 2169–2178.

WERZ, M. A. & MACDONALD, R. L. (1982). Opioid peptides decrease calcium-dependent action potential duration of mouse dorsal root ganglion neurons in cell culture. *Brain Res.* **239**, 315–321.

ZIEGLGÄNSBERGER, W. & TULLOCH, I. F. (1979). Effects of substance P on neurones in the dorsal horn of the spinal cord of the cat. *Brain Res.* **166**, 273–282.

YEH, J. Z., OXFORD, G. S., WU, C. H. & NARAHASHI, T. (1976). Interactions of aminopyridines with potassium channels of squid axon membranes. *Biophys. J.* **16**, 77–81.