Br. J. Pharmac. (1985), **84**, 663–673

The role of substance P as a neurotransmitter in the reflexes of slow time courses in the neonatal rat spinal cord

Hiroyuki Akagi[1], Shiro Konishi, Masanori Otsuka & Mitsuhiko Yanagisawa

Department of Pharmacology, Faculty of Medicine, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113, Japan

1 In order to reveal the spinal reflexes involving the transmitter action of substance P (SP), the effects of capsaicin and an SP antagonist on the isolated spinal cord of the neonatal rat were studied.

2 When a single shock stimulus was given to a dorsal root (L3–L5) or a sciatic nerve, depolarizing responses of various time courses were recorded extracellularly from both ipsi- and contra-lateral ventral roots of the corresponding segments. The reflex response recorded from the contralateral ventral root consisted of fast and slow components, which will be referred to as contralateral fast and slow ventral root potentials (v.r.ps). The latter contralateral slow v.r.p. had a time-to-peak of 2–5 s and lasted 10–30 s.

3 The threshold for the contralateral slow v.r.p. was about two times higher than that for the monosynaptic reflex, and it coincided with the threshold for activating the slow-conducting afferent fibres.

4 The contralateral slow v.r.p. was abolished after the spinal cord was treated with capsaicin (1 μM for 30 min) *in vitro*. The contralateral slow v.r.p. was absent in the spinal cord derived from 4 day-old rats that had received capsaicin (50 mg kg^{-1}, s.c.) on the 2nd day of life. The contralateral fast v.r.p. and other reflexes of fast time courses remained unaltered after treatment with capsaicin *in vitro* or *in vivo*.

5 Administration of an SP antagonist, [D-Arg^1, D-Pro^2, D-Trp^{7,9} Leu^{11}]-SP in concentrations of 5–16 μM depressed the contralateral slow v.r.p., but did not affect the monosynaptic reflex, the dorsal root potential and the contralateral fast v.r.p. [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-SP (5 μM) markedly depressed the SP-induced depolarizing response recorded from the ventral root whereas the responses to noradrenaline, 5-hydroxytryptamine, neurotensin and thyrotrophin releasing hormone (TRH) were unaffected by the SP antagonist. The response of the ventral root to acetylcholine was slightly depressed by the antagonist. The SP antagonist at 5–10 μM did not exert any agonist action on the motoneurones.

6 The present results in conjunction with those of previous studies support the hypothesis that SP released from certain primary afferent fibres acts as a neurotransmitter, producing in dorsal horn neurones slow excitatory postsynaptic potentials which lead to the generation of the contralateral slow v.r.p.

Introduction

Substance P (SP) is concentrated in the axon terminals of certain small-diameter primary afferent fibres in the superficial layers of the spinal dorsal horn (Hökfelt *et al.*, 1975; Takahashi & Otsuka, 1975; Nagy *et al.*, 1981). SP is released upon electrical or chemical stimulation from primary afferents in a Ca^{2+}-dependent manner (Otsuka & Konishi, 1976a; Akagi *et al.*, 1980; Yaksh *et al.*, 1980). Furthermore, SP exerts a powerful excitatory action on some spinal neurones (Konishi & Otsuka, 1974; Henry *et al.*, 1975; Otsuka & Konishi, 1976b; Randić & Miletić, 1977). These lines of evidence suggest that SP acts as a neurotransmitter released from certain primary afferent fibres. However, so far the direct demonstration of SP-

[1] Present address and correspondence: Laboratory of Neuropharmacology, Mitsubishi-Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194, Japan.

mediated excitatory postsynaptic potentials (e.p.s.ps) has not been possible in the spinal cord mainly due to the complexity of the neural pathways in the superficial layers of the dorsal horn. In the prevertebral ganglia of the guinea-pig, on the other hand, there is strong evidence that SP released from axon collaterals of visceral primary afferent fibres produces in the ganglion cells non-cholinergic slow e.p.s.ps that last several tens of seconds (Dun & Karczmar, 1979; Konishi *et al.*, 1979; 1980; 1983a; Tsunoo *et al.*, 1982). Therefore, it seems reasonable to suppose that similar slow e.p.s.ps are produced in dorsal horn cells by SP released from central terminals of certain small-diameter primary afferent fibres.

In the present study we attempted to reveal in the isolated spinal cord of the neonatal rat a reflex or reflexes in which SP is involved as a neurotransmitter. We anticipated that such a reflex would be elicited by stimulation of small-diameter primary afferent fibres with a high threshold and would be depressed by two pharmacological agents that are known to inhibit SP-mediated synaptic transmission: i.e., capsaicin which causes the degeneration of the small-diameter primary afferent fibres (Nagy, 1982), and an SP antagonist, [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-SP, which has recently been developed by Rosell and his colleagues (Rosell *et al.*, 1983). Preliminary accounts of this study have been presented previously (Yanagisawa *et al.*, 1982; Akagi *et al.*, 1983; Konishi *et al.*, 1983a).

Methods

Preparation and electrophysiological experiments

Experiments were done on the isolated spinal cords of 1–4 day-old Wistar rats (Otsuka & Konishi, 1974; Yanagisawa *et al.*, 1982). The animals were anaesthetized with ether and the spinal cord below the thoracic part was isolated without hemisection together with the lumbar spinal nerves (Figure 1). In some experiments (Figures 7–9) hemisected spinal cords were also used. The cord was placed in a 0.2–0.5 ml bath which was perfused with artificial cerebrospinal fluid (CSF), at 27°C, at a rate of 3.5–6.0 ml min^{-1}. The composition of the artificial CSF was as follows (mM): NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.15, $NaHCO_3$ 20.9 NaH_2PO_4 0.58, glucose 10.0. The medium was bubbled with a mixture of 95% O_2 and 5% CO_2. In a few experiments, Ca^{2+} concentration was increased to 2.5 mM and/or Mg^{2+} concentration to 2–2.5 mM. A sciatic nerve or a dorsal root (L3–L5) was stimulated with a suction electrode, and the potential changes generated in the moto-

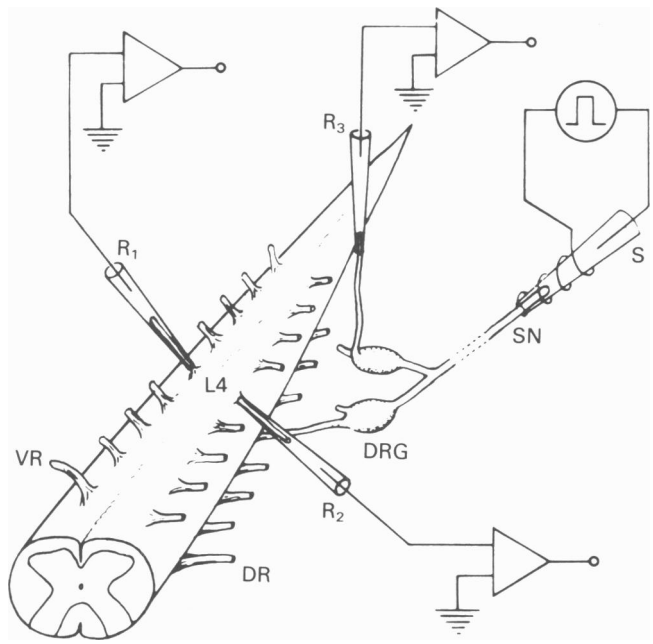

Figure 1 Diagram illustrating the experimental setup. The spinal cord of a 1–4 day-old rat was isolated together with the spinal nerves. The lumbar dorsal roots (L3–L5) retained an intact connection with a sciatic nerve (SN) which was placed in a stimulating suction electrode (S). R_1, R_2 and R_3 are the recording suction electrodes for L4 ventral roots (R_1 and R_2) and L5 dorsal root (R_3). DR, dorsal root; DRG, dorsal root ganglion; VR, ventral root.

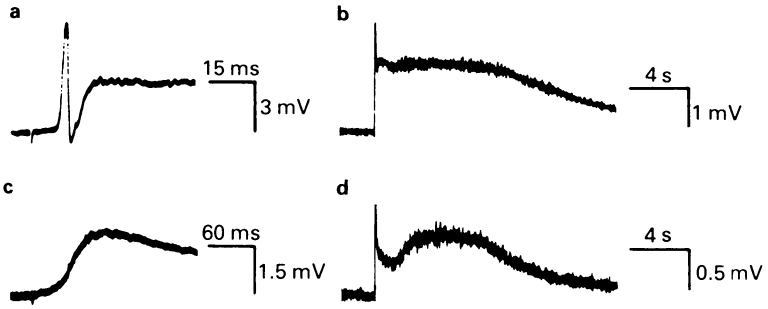

Figure 2 Extracellular recordings of spinal reflexes elicited by stimulation of a dorsal root. A single shock stimulus (10 V 0.5 ms) was given to left L4 dorsal root, and reflex responses were recorded simultaneously from the ipsilateral (a and b) and contralateral (c and d) ventral roots of the same segment with suction electrodes. (a and c) Fast components of reflexes recorded on an oscilloscope; (b and d) the slow depolarizing responses displayed on a pen recorder. Depolarization is upwards. In (b and d) fast component of each response (upward deflection) is out of scale. All records were derived from a single preparation.

neurones were recorded extracellularly from ipsilateral and contralateral ventral roots of the same segment with suction electrodes (Figure 1). The tip of the recording suction electrode was a glass capillary tube, the inner diameter of which fitted tightly about the recorded ventral roots (120–180 μm). The root was sucked into the recording electrode until the tip of the recording electrode lightly pressed against the surface of the spinal cord. For monitoring afferent volleys, a dorsal root (L4 or L5) was cut at the entry into the cord, and the propagated action potentials were recorded with another suction electrode (Figure 1).

Drug administration

Drugs were dissolved in artificial CSF and applied by perfusion for periods of 5–30 min or injected into the perfusion system using brief pulses of 0.1–2 s duration, by the use of solenoid valves driven by pulse generators (for details see Otsuka & Yanagisawa, 1980). For the *in vivo* treatment with capsaicin, the drug (50 mg kg^{-1}) was injected subcutaneously into 2 day-old rats. Control littermates received an equal volume of vehicle (10% Tween 80, 10% ethanol in 0.9% w/v NaCl solution, v/v). Two days after the injection, the spinal cords with nerve roots attached were isolated from the animals for the electrophysiological experiments and/or the analysis of the SP content. The SP antagonist, [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP, was kindly donated by Prof. S. Rosell (Karolinska Institute) and Dr M. Fujino (Takeda Chemical Industries, Ltd.).

Determination of the concentration of SP in the spinal cords and dorsal roots

The spinal cords with the nerve roots attached were isolated from the rats treated with capsaicin or vehicle,

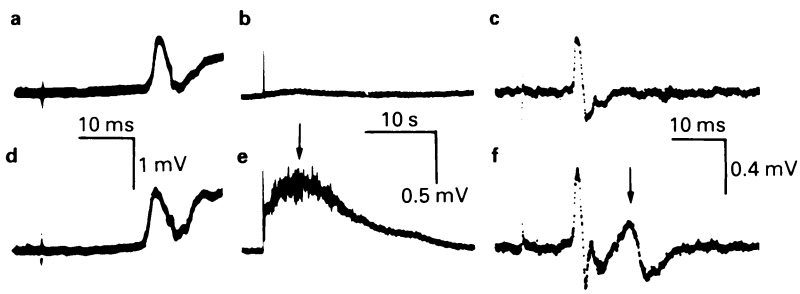

Figure 3 Responses evoked by weak and intense stimuli applied to the right sciatic nerve in a spinal cord preparation from a 3 day-old rat. Duration of stimulation pulses was 0.2 ms, and the intensity was 4.5 V for the upper records (a–c) and 9 V for the lower records (d–f). (a and d) The monosynaptic reflex recorded extracellularly from the right L4 ventral root. (b and e) The contralateral fast and slow ventral root potentials (v.r.ps) recorded from the left L4 ventral root; (c and f) afferent spike potentials recorded from the right L5 dorsal root. The distance between the site of stimulation and the site of recording was about 8 mm in (c and f). Note the concomitant appearance of the slow-conducting afferent spikes (arrow in f) and the contralateral slow v.r.p. (arrow in e).

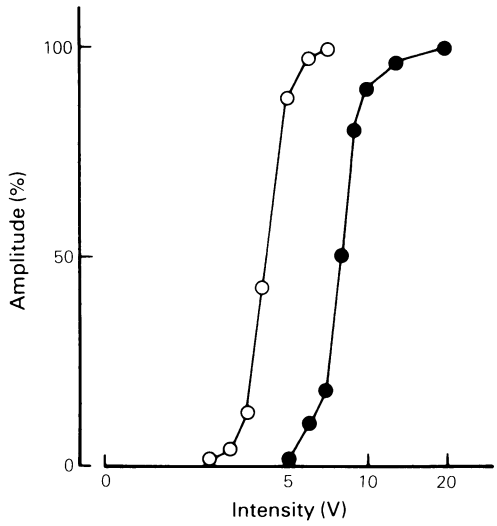

Figure 4 Relationships between stimulus intensity and the amplitudes of spinal reflex responses. A single shock stimulus of 0.1 ms duration and varied intensity was given to the L4 dorsal root every 80 s in a spinal cord preparation from a 4 day-old rat. Each point represents the relative amplitude of the evoked reflex response expressed as a percentage of the maximal response and is plotted against stimulus intensity (logarithmic scale). (○) Amplitude of the monosynaptic reflex recorded from the ipsilateral L4 ventral root; (●) amplitude of the contralateral slow ventral root potential recorded from the contralateral L4 ventral root.

placed on dry ice, and dissected to obtain the tissue samples of dorsal roots and the dorsal and ventral halves of the spinal cords of the L3–L5 segments. Each sample was homogenized in 100 μl of 2 M acetic acid with a dental drill, and the homogenate was heated in boiling water for 5 min to inactivate peptidases. After centrifugation (2000 *g* for 10 min), an aliquot of each supernatant was used for radioimmunoassay (RIA) of SP (Jessell *et al.*, 1979). The procedures and the specificity of RIA for SP have already been described (Yanaihara *et al.*, 1976).

Results

Electrophysiological properties of reflexes of slow time courses evoked by stimulation of primary afferents

When a dorsal root (L3–L5) was stimulated with a single shock, the reflex responses of various time courses were recorded from the ipsilateral and contralateral ventral roots of the corresponding segment (Figure 2). Preliminary experiments revealed that the potential changes recorded extracellularly from a ventral root reflect closely the intracellular potentials recorded from the motoneurone cell bodies of the same side and of the same segment (unpublished observation). Single shock stimulation of a dorsal root or a sciatic nerve elicited in the ipsilateral ventral root of the same segment the monosynaptic and polysynap-

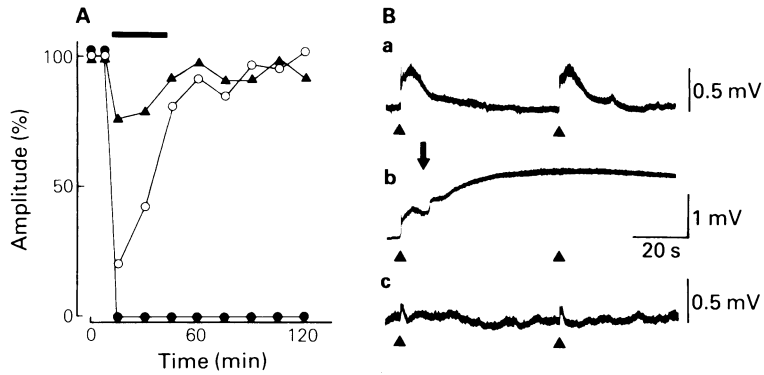

Figure 5 Effects of *in vitro* treatment with capsaicin on the reflex responses evoked by dorsal root stimulation and recorded from the ventral root. (A) Amplitudes of the reflexes plotted against time. The spinal cord of a 3 day-old rat was perfused with artificial CSF, and capsaicin (1 μM) was added to the perfusion medium during the period indicated by a black horizontal bar. A supramaximal square pulse stimulus (10 V, 0.5 ms) was given to the L4 dorsal root every 60 s to evoke the reflex responses which were recorded from the ventral roots of the both sides of the same segment. (▲) The amplitudes of the monosynaptic reflex; (○) the amplitude of the contralateral fast ventral root potential (v.r.p.); (●) the amplitude of the contralateral slow v.r.p. Each point represents the relative amplitude of a response expressed as a percentage of the control obtained before the administration of capsaicin. (B) Extracellular recordings from the L4 ventral root. The contralateral L4 dorsal root was stimulated at triangles with single shocks (10 V, 0.5 ms), (a) in normal medium; (b) at the arrow the perfusion with capsaicin (1 μM) was started and continued for 30 min; (c) 60 min after washing out capsaicin. The same experiment as shown in (A).

tic reflexes (Figure 2a), which were followed by a slow depolarizing response lasting 10–30 s (Figure 2b). The latter slow response will be referred to as ipsilateral slow ventral root potential (v.r.p.). The responses evoked by the same stimulus and recorded from the contralateral ventral root of the same segment consisted of two components, fast and slow (Figure 2c and d). The fast component, the contralateral fast v.r.p., reached its peak about 70 ms after the stimulus. The slow component, the contralateral slow v.r.p., had a time-to-peak of 2–5 s and lasted 10–30 s.

When the intensity of the stimulus was gradually increased, the spinal reflexes of fast time courses were elicited in the ipsilateral and contralateral ventral roots at a relatively low threshold (Figure 3a and b). Further increases in the stimulus intensity resulted in the generation of the ipsilateral and contralateral slow v.r.ps (Figure 3e). As shown in Figure 4, the threshold for the contralateral slow v.r.p. was about twice as high as that for the monosynaptic reflex, and it coincided with the threshold for slow-conducted afferent spikes recorded from the adjacent dorsal root (Figure 3f). The conduction velocity of these slow-conducted afferent spikes was about 0.4 m s^{-1} (Figure 3f). When the stimulus intensity was altered, the amplitude of the contralateral slow v.r.p. and that of the slow-conducted afferent spikes changed in parallel (not illustrated).

Effects of capsaicin on spinal reflexes

Figure 5 shows the effects of *in vitro* treatment with capsaicin on various types of spinal reflexes. Bath-application of capsaicin (1 μM) produced a sustained depolarization, as recorded extracellularly from the ventral root (Figure 5Bb; Theriault *et al.*, 1979), and during the continued presence of capsaicin for 30 min the depolarization subsided gradually. The contralateral slow v.r.p. was completely abolished during the application of capsaicin (Figure 5Bb), and after the removal of the drug no recovery occurred during the subsequent period of 2 h even though the d.c. potential recorded from the ventral root returned to the original level within 30 min (Figure 5Bc). Ipsilateral slow v.r.p. was also depressed by the capsaicin treatment but the degree of suppression was slight compared with that of the contralateral slow v.r.p. The monosynaptic reflex and the contralateral fast v.r.p. were transiently depressed by capsaicin probably because of the depolarization of spinal neurones, but after washing out capsaicin they recovered to their original sizes (Figure 5A).

The *in vivo* treatment of animals with capsaicin also caused a selective suppression of the contralateral slow v.r.p. When single shock stimulation was given to a dorsal root (L4 or L5) of the spinal cord preparation derived from the 4 day-old rats that had received a subcutaneous injection of capsaicin (50 mg kg^{-1}) on the 2nd day of life, the contralateral slow v.r.p. was virtually absent (Figure 6). By contrast, the amplitudes of the monosynaptic reflex and the contralateral fast v.r.p. in the spinal cords of the capsaicin-treated rats were not appreciably different from those observed in the spinal cords of the control animals. The injection of vehicle alone did not cause any noticeable changes in the sizes of fast and slow reflexes. The selective suppression of the contralateral slow v.r.p. was observed in 2 other capsaicin-treated rats. Analyses

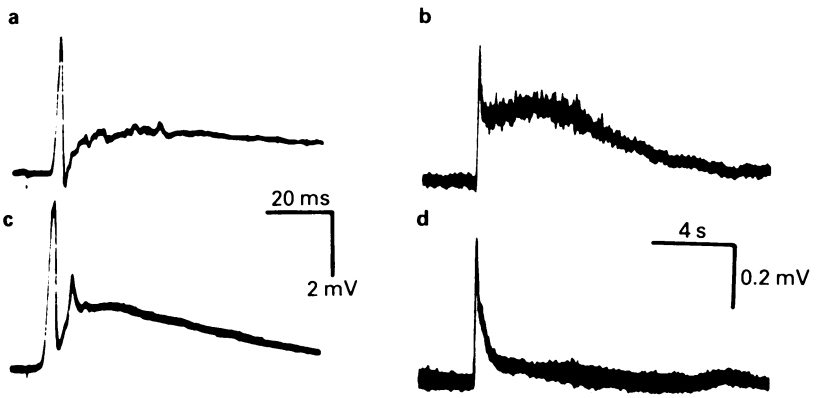

Figure 6 Effects of *in vivo* treatment with capsaicin. Rats were treated with capsaicin (50 mg kg^{-1}, s.c.) or vehicle on the 2nd day of life. Two days after the injection the spinal cords of control and capsaicin-treated animals were isolated for electrophysiological experiments. The dorsal root (L4) was stimulated with single supramaximal shocks (10 V, 0.5 ms). (a and b) Responses recorded from the spinal cord of a control animal treated with vehicle alone; (c and d) those recorded from the spinal cord of a capsaicin-treated animal. Reflex responses were simultaneously recorded from the ipsilateral L4 ventral roots (a and c) and the contralateral L4 ventral roots (b and d).

Table 1 Effects of neonatal capsaicin treatment on the amount of substance P (SP) in the spinal cords

		SP content (pg mg^{-1} protein)	*Decrease* (%)
Ventral spinal cord	Control ($n = 8$)	673 ± 38	
	Capsaicin treatment ($n = 8$)	720 ± 68	
Dorsal spinal cord	Control ($n = 5$)	1977 ± 252	
	Capsaicin treatment ($n = 5$)	760 ± 96*	61.6
Dorsal root	Control ($n = 4$)	634 ± 123	
	Capsaicin treatment ($n = 4$)	165 ± 27*	74.0

Rats were treated with capsaicin (50 mg kg^{-1}, s.c.) or vehicle on the 2nd day of life, and killed 2 days after the treatment. The isolated spinal cords with nerve roots attached were placed on dry ice and divided to obtain tissue samples of dorsal roots and the dorsal and ventral halves of the spinal cords. Tissue samples were derived from the spinal segments L3–L5. Each value represents the mean ± s.e.mean. n = number of animals. *$P < 0.01$ when compared with the control values (Student's t test).

with RIA revealed that the concentration of SP in the dorsal halves of the spinal cord and the dorsal roots was markedly reduced in these capsaicin-treated rats 2 days after the injection (Table 1; cf. Gamse *et al.*, 1981; Nagy *et al.*, 1981). Thus, it is likely that the contralateral slow v.r.p. is produced by the activation of small-diameter primary afferent fibres that are sensitive to the action of capsaicin.

Effects of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP on the responses to SP and other drugs

We have examined the specificity of the SP antagonist, [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP. In the experiment illustrated in Figure 7, SP (1 μM), applied by brief pulses (0.6 s), produced depolarizing responses of the ventral roots. The duration of the pulses was chosen so

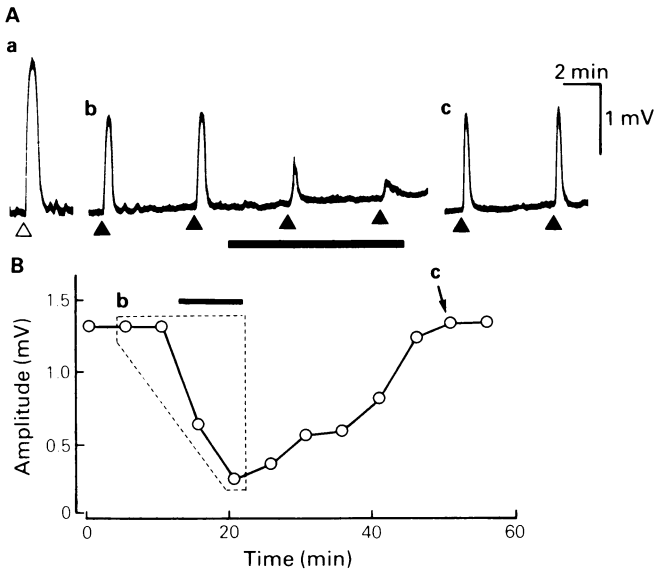

Figure 7 Effects of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P (SP) on the depolarizing responses to SP of the ventral root. Extracellular recording from L5 ventral root of a hemisected spinal cord preparation which was perfused with artificial CSF containing 1.26 mM Ca^{2+} and 2 mM Mg^{2+}. SP (1 μM) was dissolved in artificial CSF and applied by brief pulses at 5 min intervals. [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP, 5 μM, was applied by perfusion during the period indicated by black horizontal bars. (A) The d.c. records of the responses to SP of the L5 ventral root. (a) SP was applied using a 1.6 s pulse at the time indicated by (△). (b) The record taken during the periods indicated by broken lines in (B). SP was applied using 0.6 s pulses at (▲). Note that the control responses to SP shown in (b) are submaximal. (c) The record taken 30 min after the removal of the antagonist as indicated by arrow in (B). (B) The amplitudes of the responses to SP given by 0.6 s pulses were plotted against time. The same experiment as illustrated in (Ab and c).

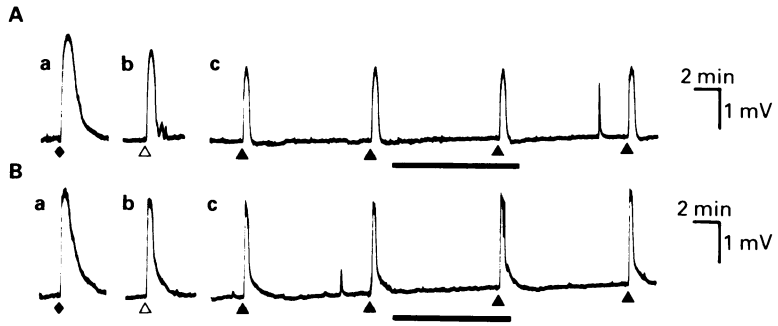

Figure 8 Effects of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P on the depolarizing responses of the ventral root (L5) to neurotensin and thyrotrophin releasing hormone (TRH). The hemisected spinal cord preparation was perfused with artificial CSF containing 2 mM Mg^{2+}. The peptides were applied by brief pulses. (A) Neurotensin (2 μM) was applied using a 2 s pulse at (◆), a 0.7 s pulse at (△) and 0.5 s pulses at (▲). (B) TRH (1 μM) was applied using a 1.6 s pulse at (◆), a 0.7 s pulse at (△) and 0.4 s pulses at (▲). The substance P (SP) antagonist, 5 μM, was applied during the periods marked with black horizontal bars. All records were taken from a single preparation. Note that the responses to the peptides given at (▲) are submaximal.

that the response to SP was submaximal (cf. Figure 7Aa). The response to SP was markedly depressed during perfusion with [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP, 5 μM, and after the removal of the SP antagonist the size of the response recovered with a relatively slow time course. The submaximal responses to neurotensin and thyrotrophin releasing hormone (TRH), by contrast, were not affected by the SP antagonist at 5 μM (Figure 8). Noradrenaline and 5-hydroxytryptamine applied using brief pulses also produced depolarizing responses recorded from the ventral root, but these responses were not affected by the SP antagonist (Figure 9A and B). The response to acetylcholine was slightly reduced by the SP antagonist at 10 μM (Figure 9C).The SP antagonist by itself exerted no noticeable depolarizing action on the

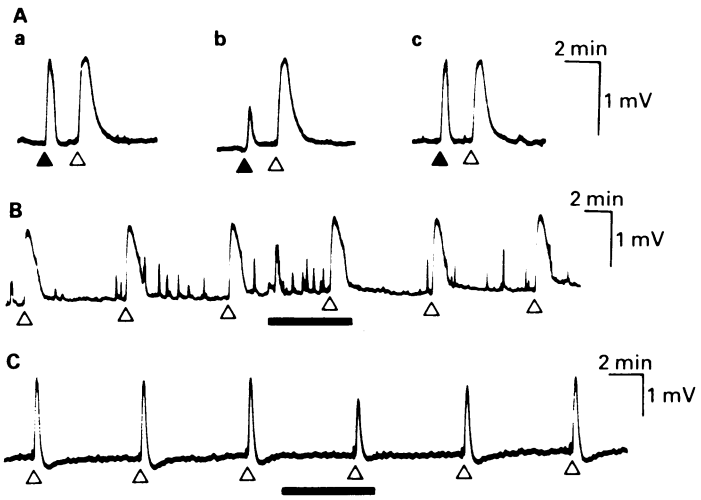

Figure 9 Effects of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P on the depolarizing responses to substance P (SP), noradrenaline, 5-hydroxytryptamine (5-HT) and acetylcholine. The d.c. potentials were recorded extracellularly from L4 or L5 ventral root of hemisected spinal cord preparations. (A) SP (1 μM) was applied using brief pulses of 0.7 s duration with 10 min intervals at (▲), and noradrenaline (0.1 mM) was alternately applied using 0.75 s pulses at (△). (a) Control record in normal artificial CSF; (b) in the presence of the SP antagonist (10 μM); (c) 10 min after the removal of the antagonist. (B) 5-HT, 0.2 mM, was applied using 0.6 s pulses with 7 min intervals at (△). (C) Acetylcholine, 0.5 mM, was applied using 1.8 s pulses with 6 min intervals at (△). Artificial CSF with 1.26 mM Ca^{2+} and 2.5 mM Mg^{2+} was used in (A), with 1.26 mM Ca^{2+} and 2 mM Mg^{2+} in (B), and with 0.1 mM Ca^{2+} and 2 mM Mg^{2+} in (C). The SP antagonist (10 μM) was applied by perfusion during the periods marked with black horizontal bars in (B and C).

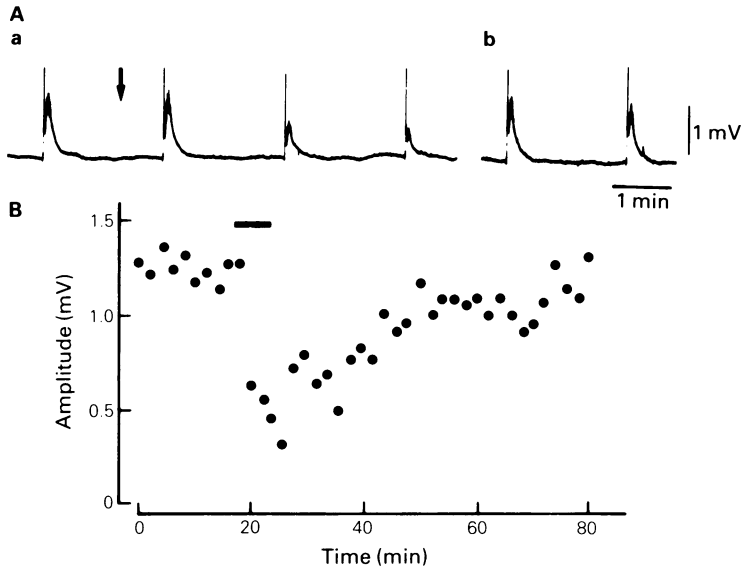

Figure 10 Effects of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P (SP) on the contralateral slow ventral root potential (v.r.p.). (A) Sample records of reflex responses. Single shock stimulus (10 V, 1 ms) was given to L4 dorsal root every 120 s and the reflex responses were recorded extracellularly from L4 ventral root of the contralateral side. (a) At the arrow the perfusion with artificial CSF containing [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP (5 μM) was started and continued for 6 min. (b) About 50 min after the removal of the SP antagonist. (B) The peak amplitudes of the contralateral slow v.r.p. were plotted from the same experiment as illustrated in (A). The SP antagonist was applied during the period marked with a black horizontal bar.

ventral root in concentrations of 5–10 μM. To summarize, the SP antagonist at relatively low concentrations (5–10 μM) exhibited a considerable, though not absolute, specificity towards SP.

Effects of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP on spinal reflexes

Figure 10 shows the effects of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP on the contralateral fast and slow v.r.ps. The size and the duration of the contralateral slow v.r.p. were markedly reduced by the SP antagonist in a concentration of 5 μM. The effects of the SP antagonist on the contralateral slow v.r.p. outlasted the period of application but gradually disappeared 20–40 min after the removal of the drug (Figure 10, see also Yanagisawa *et al.*, 1982). At a higher concentration (16 μM) of the SP antagonist, the effect on the contralateral slow v.r.p. was more pronounced (Yanagisawa *et al.*, 1982), but at this concentration, the SP antagonist often slightly depressed the responses to neurotensin and TRH. The ipsilateral slow v.r.p. was slightly but consistently depressed by the SP antagonist (5–16 μM). The monosynaptic reflex and the polysynaptic reflexes of fast time course, the dorsal root potential and contralateral fast v.r.p. were not affected by the SP antagonist even at a concentration of 16 μM.

Discussion

The present study showed that stimulation of high-threshold and slow-conducting (therefore probably small-diameter) primary afferent fibres induces in the contralateral ventral root of the rat isolated spinal cord preparation a reflex of slow time course which we have called contralateral slow v.r.p., and the reflex is depressed by two pharmacological tools, capsaicin and the SP antagonist, [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP. These results taken together with other lines of evidence (see Introduction), suggest that SP is involved, as a neurotransmitter, in the contralateral slow v.r.p.

The present results obtained in the isolated spinal cord of newborn rats form a striking parallel with those obtained in the prevertebral ganglia of the guinea-pig where SP acts as a neurotransmitter of a non-cholinergic slow e.p.s.p. that is recorded from principal ganglion cells (Konishi *et al.*, 1979; 1980; 1983b; Tsunoo *et al.*, 1982). In both places, the slow synaptic responses lasting tens of seconds elicited by

stimulation of primary afferent fibres were depressed by capsaicin and the SP antagonist, [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP. Therefore, it seems reasonable to suppose that a slow e.p.s.p. similar to that observed in the guinea-pig prevertebral ganglia is produced by SP released from certain small-diameter primary afferent fibres in the dorsal horn neurones of the spinal cord of the newborn rat.

However, it should be noted that the two pharmacological tools used in the present study are by no means absolutely specific. Recent immunohistochemical studies (Jancsó *et al.*, 1981) suggested that capsaicin depletes not only SP but also other peptides such as somatostatin, vasoactive intestinal polypeptide (VIP) and gastrin/CCK in the nerve terminals of primary afferents in the dorsal horn. Therefore, it is conceivable that some of these peptides are also involved in generation of the contralateral and ipsilateral slow v.r.ps. However, in the rat isolated spinal cord the action of somatostatin appears to be inhibitory (Suzue *et al.*, 1981; Murase *et al.*, 1982). In fact, the contralateral slow v.r.p. was markedly depressed by a low concentration of somatostatin (1–10 μM), which produced no depolarizing effect on motoneurones (unpublished observation). The action of VIP is excitatory but relatively weak on the spinal neurones of the newborn rat (unpublished observation) and furthermore the action of VIP is probably not antagonized by the SP antagonist, [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP (see below; Jensen *et al.*, 1984). The recent experiments of Schultzberg *et al.* (1982) and Marley *et al.* (1982) showed that CCK-octapeptide does not exist in the axon terminals of primary sensory neurones. Taken together, it is likely that SP, but not somatostatin, VIP and CCK-octapeptide, plays an important role as an excitatory transmitter initiating the contralateral slow v.r.p. in the isolated spinal cord of the newborn rat.

After the subcutaneous injection of capsaicin, the contralateral slow v.r.p. was abolished when the SP content of the dorsal spinal cord of the neonatal rat has been reduced by 62%. By contrast the SP content of the ventral spinal cord was not altered by the capsaicin injection (Figure 6 and Table 1, see also Nagy *et al.*, 1981). These results suggest that the SP-releasing terminals which are involved in the contralateral slow v.r.p. are probably located in the dorsal horn. Capsaicin is known to cause a selective degeneration of small-diameter primary afferent fibres (Nagy, 1982). Gilbert *et al.* (1982) have shown that about 25% of the SP contents in the dorsal horn and about 90% of the SP contents in the ventral horn are of supraspinal origin, and this may explain at least partly the remaining SP contents of the ventral and dorsal spinal cord after the capsaicin injection.

The new SP antagonist, [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP, exhibited a considerable degree of specificity in the ileum and the sympathetic ganglia of the guinea-pig (Rosell *et al.*, 1983; Konishi *et al.*, 1983b). Recently, Jensen *et al.* (1984) have shown that this SP antagonist inhibits the stimulation of amylase release from dispersed pancreatic acini by SP and bombesin but not CCK-octapeptide and VIP. The specificity of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP was extensively examined in the rat spinal cord in the present study. The SP antagonist at low concentrations (5–10 μM) depressed the response to SP, but not the responses to neurotensin, TRH, noradrenaline, 5-hydroxytryptamine (Figures 7, 8 and 9) and γ-aminobutyric acid (GABA) (Yanagisawa *et al.*, 1982). However, the SP antagonist at 10 μM slightly reduced the action of acetylcholine (Figure 9C). The anticholinergic action of the SP antagonist may reflect a similar action of SP on Renshaw cells (Ryall, 1982) and adrenal chromaffin cells (Clapham & Neher, 1984). These cholinergic mechanisms are probably not involved in the contralateral slow v.r.p., because the slow reflex was not affected by the cholinergic antagonists, atropine (0.1 μM) and dihydro-β-erythroidine (4 μM) (unpublished observation).

Recently, Matsuto *et al.* (1984) showed that [D-Arg[1], D-Pro[2], D-Trp[7], Leu[11]]-SP depressed the responses of the ventral root to neurokinin A and neurokinin B (for nomenclature of the peptides, see Iversen, 1985). Neurokinin A (also known as substance K or neuromedin L) and neurokinin B (neuromedin K) are particularly interesting because they have been shown to occur in mammalian spinal cord (Kangawa *et al.*, 1983; Kimura *et al.*, 1983; Maggio *et al.*, 1983; Nawa *et al.*, 1983). In addition, Minamino *et al.* (1983; 1984) demonstrated the occurrence of two bombesin-like peptides in porcine spinal cord (neuromedin B and neuromedin C). Therefore, some of the endogenous neuropeptides other than SP may also play a physiological role similar to that of SP. However, Akagi *et al.* (1980) showed, by the use of high-performance liquid chromatography combined with RIA for SP, that the immunoreactive SP released from the spinal cord of the newborn rat consists mainly of the undecapeptide SP. Therefore, the undecapeptide SP is no doubt released from certain primary afferent fibres, although other neuropeptides such as neurokinin A might also be released from some primary afferents (cf. Nawa *et al.*, 1983). To examine whether other neuropeptides are also involved in the generation of the contralateral slow v.r.p., further improvement of SP antagonists and other neuropeptide antagonists with respect to specificity is desirable.

We wish to thank Prof. S. Rosell of the Karolinska Institute and Dr M. Fujino of Takeda Chemical Industries, Ltd for the generous gifts of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP. Part of this work was supported by research grants from the Ministry of Education, Science and Culture of Japan.

References

AKAGI, H., KONISHI, S., YANAGISAWA, M. & OTSUKA, M. (1983). Effects of capsaicin and a substance P antagonist on a slow reflex in the isolated rat spinal cord. *Neurochem. Res.*, **8,** 795–796.

AKAGI, H., OTSUKA, M. & YANAGISAWA, M. (1980). Identification by high-performance liquid chromatography of immunoreactive substance P released from isolated rat spinal cord. *Neurosci. Lett.*, **20,** 259–263.

CLAPHAM, D.E. & NEHER, E. (1984). Substance P reduces acetylcholine-induced currents in isolated bovine chromaffin cells. *J. Physiol.*, **347,** 255–277.

DUN, N.J. & KARCZMAR, A.G. (1979). Actions of substance P on sympathetic neurons. *Neuropharmacology*, **18,** 215–218.

GAMSE, R., LACKNER, D., GAMSE, G. & LEEMAN, S.E. (1981). Effect of capsaicin pretreatment on capsaicin-evoked release of immunoreactive somatostatin and substance P from primary sensory neurons. *Naunyn-Schmiedebergs Arch. Pharmac.*, **316,** 38–41.

GILBERT, R.F.T., EMSON, P.C., HUNT, S.P., BENNETT, G.W., MARSDEN, C.A., SANDBERG, B.E.B., STEINBUSCH, H.W.M. & VERHOFSTAD, A.A.J. (1982). The effects of monamine neurotoxins on peptides in the rat spinal cord. *Neuroscience*, **7,** 69–87.

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

HÖKFELT, T., KELLERTH, J.-O., NILSSON, G. & PERNOW, B. (1975). Experimental immunohistochemical studies on the localization and distribution of substance P in cat primary sensory neurons. *Brain Res.*, **100,** 235–252.

IVERSEN, L.L. (1985). *Nature*, (in press).

JANCSÓ, G., HÖKFELT, T., LUNDBERG, J.M., KIRALY, E., HALÁSZ, N., NILSSON, G., TERENIUS, L., REHFELD, J., STEINBUSCH, H., VERHOFSTAD., A., ELDE, R., SAID., S. & BROWN, M. (1981). Immunohistochemical studies on the effect of capsaicin on spinal and medullary peptide and monamine neurones using antisera to substance P, gastrin/CCK, somatostatin, VIP, enkephalin, neurotensin and 5-hydroxytryptatmine. *J. Neurocytol.*, **10,** 963–980.

JENSEN, R.T., JONES, S.W., FOLKERS, K. & GARDNER, J.D. (1984). A synthetic peptide that is a bombesin receptor antagonist. *Nature*, **309,** 61–63.

JESSELL, T., TSUNOO, A., KANAZAWA, I. & OTSUKA, M. (1979). Substance P: Depletion in the dorsal horn of rat spinal cord after section of the peripheral processes of primary sensory neurons. *Brain Res.*, **168,** 247–259.

KANGAWA, K., MINAMINO, N., FUKUDA, A. & MATSUO, H. (1983). Neuromedin K and B: Novel neuropeptides with smooth muscle stimulant activity identified in pig spinal cord. In *Peptide Chemistry 1983, Proceedings of the 21st Symposium on Peptide Chemistry*. ed. Munekata, E., pp. 309–314. Osaka: Protein Research Foundation.

KIMURA, S., OKADA, M., SUGITA, Y., KANAZAWA, I. & MUNEKATA, E. (1983). Novel neuropeptides, neurokinin α and β, isolated from porcine spinal cord. *Proc. Jap. Acad.*, **59,** Ser. B, 101–104.

KONISHI, S., AKAGI, H., YANAGISAWA, M. & OTSUKA, M. (1983a). Enkephalinergic inhibition of slow transmission in the rat spinal cord. *Neurosci. Lett.*, **Suppl. 13,** S107.

KONISHI, S. & OTSUKA, M. (1974). Excitatory action of hypothalamic substance P on spinal motoneurones of newborn rats. *Nature*, **252,** 734–735.

KONISHI, S., OTSUKA, M., FOLKERS, K. & ROSELL, S. (1983b). A substance P antagonist blocks non-cholinergic slow excitatory postsynaptic potential in guinea-pig sympathetic ganglia. *Acta physiol. scand.*, **117,** 157–160.

KONISHI, S., TSUNOO, A. & OTSUKA, M. (1979). Substance P and noncholinergic excitatory synaptic transmission in guinea pig sympathetic ganglia. *Proc. Jap. Acad.*, **55,** Ser. B, 525–530.

KONISHI, S., TSUNOO, A., YANAIHARA, N. & OTSUKA, M. (1980). Peptidergic excitatory and inhibitory synapses in mammalian sympathetic ganglia: Roles of substance P and enkephalin. *Biomedical Res.*, **1,** 528–536.

MAGGIO, J.E., SANDBERG, B.E.B., BRADLEY, C.V., IVERSEN, L.L. SANTIKARN, S., WILLIAMS, D.H., HUNTER, J.C. & HANLEY, M.R. (1983). A novel tachykinin in mammalian spinal cord. *Irish J. Med. Sci.*, **152,** (Suppl. 1), 45.

MARLEY, P.D., NAGY, J.I., EMSON, P.C. & REHFELD, J.F. (1982). Cholecystokinin in the rat spinal cord: distribution and lack of effect of neontal capsaicin treatment and rhizotomy. *Brain Res.*, **238,** 494–498.

MATSUTO, T., YANAGISAWA, M., OTSUKA, M., KANAZAWA, I. & MUNEKATA, E. (1984). The excitatory action of the newly-discovered mammalian tachykinins, neurokinin α and neurokinin β, on neurons of the isolated spinal cord of the newborn rat. *Neurosci. Res.*, **2,** 105–110.

MINAMINO, N., KANGAWA, K. & MATSUO, H. (1983). Neuromedin B: A novel bombesin-like peptide identified in porcine spinal cord. *Biochem. biophys. Res. Commun.*, **114,** 541–548.

MINAMINO, N., KANGAWA, K. & MATSUO, H. (1984). Neuromedin C: A bombesin-like peptide identified in porcine spinal cord. *Biochem. biophys. Res. Commun.*, **119,** 14–20.

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.

NAGY, J.I. (1982). Capsaicin's action on the nervous system. *Trends in Neuro. Sci.*, **5,** 362–365.

NAGY, J.I., HUNT, S.P., IVERSEN, L.L. & EMSON, P.C. (1981). Biochemical and anatomical observations on the degeneration of peptide-containing primary afferent neurons after neonatal capsaicin. *Neuroscience*, **6,** 1923–1934.

NAWA, H., HIROSE, T., TAKASHIMA, H., INAYAMA, S. & NAKANISHI, S. (1983). Nucleotide sequences of cloned cDNAs for two types of bovine brain substance P precursor. *Nature*, **306,** 32–36.

OTSUKA, M. & KONISHI, S. (1974). Electrophysiology of mammalian spinal cord *in vitro*. *Nature*, **252,** 733–734.

OTSUKA, M. & KONISHI, S. (1976a). Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat. *Nature*, **264,** 83–84.

OTSUKA, M. & KONISHI, S. (1976b). Substance P and excitatory transmitter of primary sensory neurons. *Cold Spring Harb. Symp. quant. Biol.*, **40,** 135–143.

OTSUKA, M. & YANAGISAWA, M. (1980). The effects of substance P and baclofen on motoneurones of isolated

spinal cord of the newborn rat. *J. exp. Biol.*, **89,** 201–214.

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

ROSELL, S., BJÖRKROTH, U., XU, J.-C. & FOLKERS, K. (1983). The pharmacological profile of a substance P (SP) antagonist. Evidence for the existence of subpopulations of SP receptors. *Acta physiol. scand.*, **117,** 445–449.

RYALL, R.W. (1982). Modulation of cholinergic transmission by substance P. In *Substance P in the Nervous System.* ed. Porter, R. & O'Connor, M., pp. 267–280. London: Pitman.

SCHULTZBERG, M., DOCKRAY, G.J. & WILLIAMS, R.G. (1982). Capsaicin depletes CCK-like immunoreactivity detected by immunohistochemistry, but not that measured by radioimmunoassay in rat dorsal spinal cord. *Brain Res.*, **235,** 198–204.

SUZUE, T., YANAIHARA, N. & OTSUKA, M. (1981). Actions of vasopressin, gastrin releasing peptide and other peptides on neurons of newborn rat spinal cord *in vitro*. *Neurosci. Lett.*, **26,** 137–142.

TAKAHASHI, T. & OTSUKA, M. (1975). Regional distribution of substance P in the spinal cord and nerve roots of the cat and the effect of dorsal root section. *Brain Res.*, **87,** 1–11.

THERIAULT, E., OTSUKA, M. & JESSELL, T. (1979). Capsaicin-evoked release of substance P from primary sensory neurons. *Brain Res.*, **170,** 209–213.

TSUNOO, A., KONISHI, S. & OTSUKA, M. (1982). Substance P as an excitatory transmitter of primary afferent neurons in guinea-pig sympathetic ganglia. *Neuroscience*, **7,** 2025–2037.

YAKSH, T.L., JESSELL, T.M., GAMSE, R., MUDGE, A.W., & LEEMAN, S.E. (1980). Intrathecal morphine inhibits substance P release from mammalian spinal cord *in vivo*. *Nature*, **286,** 155–157.

YANAGISAWA, M., OTSUKA, M., KONISHI, S., AKAGI, H., FOLKERS, K. & ROSELL, S. (1982). A substance P antagonist inhibits a slow reflex response in the spinal cord of the newborn rat. *Acta physiol. scand.*, **116,** 109–112.

YANAIHARA, C., SATO, H., HIROHASHI, M., SAKAGAMI, M., YAMAMOTO, K., HASHIMOTO, T., YANAIHARA, N., ABE, K. & KANEKO, T. (1976). Substance P radioimmunoassay using N^{α}-tyrosyl-substance P and demonstration of the presence of substance P-like immunoreactivities in human blood and porcine tissue extracts. *Endocrinol. Japon.*, **23,** 457–463.

(Received July 26, 1984.
Revised November 5, 1984.
Accepted November 22, 1984.)