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Pharmacological profile of a tachykinin antagonist, spantide, as examined on rat spinal motoneurones

Mitsuhiko Yanagisawa & Masanori Otsuka

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

1 The pharmacological profile of a tachykinin antagonist, [D-Arg^1, D-Trp^{7,9}, Leu^{11}] substance P (spantide), was studied on motoneurones of the isolated spinal cord of the newborn rat. For this purpose, potentials were recorded from a lumbar ventral root extracellularly and drugs were bath-applied in the presence of tetrodotoxin (TTX).

2 Neurokinin A (NKA), a NK_2-receptor selective agonist, induced concentration-dependent depolarizations, which were antagonized by spantide. Analyses of concentration-response curves suggested a competitive type antagonism with a pA_2 of 6.5.

3 Depolarizations induced by acetyl-Arg^6-septide, a NK_1-receptor selective agonist, were also antagonized by spantide with a pA_2 of 6.5.

4 Spantide (0.5–16 μM) had no depolarizing action on the ventral root in the presence of TTX.

5 Spantide antagonized the depolarizing action of substance P (SP) when SP was applied at low concentrations (0.1–0.3 μM) or by short duration pulses in artificial cerebrospinal fluid containing TTX, but much higher concentrations of spantide (4–10 μM) were needed to exert an antagonistic action against SP than against acetyl-Arg^6-septide or NKA.

6 Thyrotrophin-releasing hormone, L-glutamate, GABA, and noradrenaline, also induced depolarizations of the ventral root in the presence of TTX but the responses to these agonists were not depressed by spantide (16 μM).

7 These results suggest that there is a subtype of tachykinin receptors on neonatal rat spinal motoneurones to which NKA, acetyl-Arg^6-septide and spantide bind competitively with high affinity. The present results also suggest the existence on rat motoneurones of another class or other classes of tachykinin receptors that are less sensitive to the antagonistic action of spantide.

Introduction

Spantide, [D-Arg^1, D-Trp^{7,9}, Leu^{11}] substance P, is one of the most potent tachykinin antagonists (Rosell *et al.*, 1983; Folkers *et al.*, 1984), and has often been used to examine possible neurotransmitter roles of tachykinins in peripheral and central nervous systems (Folkers *et al.*, 1984; Fujino *et al.*, 1987; Togashi *et al.*, 1987; Otsuka & Yanagisawa, 1988; Barthó *et al.*, 1989). Several studies suggested that spantide is an antagonist with preference for NK_1-tachykinin receptors (Chahl, 1985; Buck & Shatzer, 1988; Barthó *et al.*, 1989). Most of the studies on the pharmacological profile of tachykinin antagonists, however, have been carried out in peripheral organs rather than in the central nervous system (Håkanson & Sundler, 1985). The isolated spinal cord of the newborn rat offers an excellent means to examine pharmacological profiles of tachykinin antagonists (Akagi *et al.*, 1985; Otsuka & Yanagisawa, 1988). In particular, in the presence of tetrodotoxin (TTX), we can observe direct actions of various tachykinin agonists and antagonists on motoneurones by recording extracellular potentials from the ventral root.

During our attempts to characterize the pharmacological profile of spantide on spinal motoneurones of the newborn rat, we found rather unexpectedly that spantide antagonized the action of neurokinin A (NKA), which is believed to be an agonist with preferential action on NK_2-receptors. In this paper we present data showing the antagonism between spantide and NKA as well as other tachykinin receptor agonists as examined on spinal motoneurones of the newborn rat. We have also made some attempts to examine characteristics of tachykinin receptors on motoneurones.

Methods

We used the isolated spinal cord preparation of newborn rats. The methods are the same as described previously (Akagi *et al.*, 1985; Otsuka & Yanagisawa, 1988). The spinal cord below the thoracic region was isolated from 1–5 day-old Wistar rats, and after hemisection, was placed in a bath of 0.2 ml volume and perfused with artificial cerebrospinal fluid (CSF) at a rate of 4 ml min^{-1}. The temperature was kept at 27°C. The composition of the artificial CSF used in this study was (mM): NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 2.0, $NaHCO_3$ 21, NaH_2PO_4 0.58 and glucose 10. This composition was the same as used in previous studies (Akagi *et al.*, 1985; Otsuka & Yanagisawa, 1988) except for Mg^{2+}, the concentration of which was increased from 1.16 mM to 2 mM in order to depress spontaneous activity. The medium was saturated with a gas mixture of 95% O_2 and 5% CO_2 before perfusion.

Potentials were recorded extracellularly from L3-5 ventral roots with a tightly fitting suction electrode and displayed via preamplifier on an oscilloscope and a d.c. pen-recorder. The dorsal root of the corresponding segment was placed in a stimulating suction electrode and used for eliciting spinal reflexes.

Agonists, such as substance P (SP), NKA, neurokinin B (NKB), SP(6-11), acetyl-[Arg^6, Pro^9]SP(6-11) which is a water soluble derivative of septide and hereafter referred to as acetyl-Arg^6-septide (Papir-Kricheli *et al.*, 1987), senktide (succinyl-[Asp^6, Me-Phe^8]SP(6-11); Papir-Kricheli *et al.*, 1987) and bombesin were dissolved in artificial CSF and applied either by perfusion for periods of 30 s or injected into the perfusion solution with short pressure pulses as described previously (Otsuka & Yanagisawa, 1988). The latter method of drug application is advantageous in that a constant amount of agonist can be repeatedly applied so that the influence of antagonists applied by perfusion can be easily and reliably detected (Otsuka & Yanagisawa, 1988), although the concentration of agonists in the bath cannot reach steady levels. The antagonist, spantide, was applied by perfusion.

Acetyl-Arg^6-septide and senktide were gifts from Prof. Z. Selinger, Department of Biological Chemistry, the Hebrew University of Jerusalem, Israel. Spantide was synthesized and

supplied by Dr M. Fujino of the Tsukuba Research Laboratories, Takeda Chemical Industries, Ltd. Japan. NKB was a gift of Professor N. Yanaihara, University of Shizuoka, School of Pharmaceutical Sciences, Japan. SP, NKA and bombesin were purchased from Peptide Institute, Inc. Osaka, Japan. SP(6-11) was purchased from Peninsula Labs.

Results

Antagonism of NKA, NKB, acetyl-Arg^6-septide and SP(6-11) by spantide

Bath application of various tachykinin agonists, such as NKA, NKB, acetyl-Arg^6-septide and SP, to the isolated spinal cord of the newborn rat for 30 s in normal artificial CSF produced a concentration-dependent depolarization of the ventral root (Figures 1,2,6A). This depolarization reflects the depolarization of motoneurone cell bodies and results from a summation of the transsynaptic action of agonists through spinal interneurones and the direct action on motoneurones (Otsuka & Yanagisawa, 1988). Tetrodotoxin eliminates the transsynaptic action. In the isolated spinal cord of the newborn rat monosynaptic and polysynaptic reflexes induced by dorsal root stimulation and recorded from the corresponding ventral root were completely blocked by TTX at 0.06 μM. In the present study, therefore, we used TTX at 0.3 μM which seems sufficient to block completely the conduction of action potentials in the spinal cord.

As shown in Figure 1, the concentration-response curve to NKA was shifted to the right by TTX (0.3 μM). In the presence of TTX, the EC_{50} of NKA on neonatal rat motoneurones was approximately 0.4 μM (cf. Brown *et al.*, 1986). Addition of spantide at 1–2 μM caused a rightward parallel shift of the concentration-response curve to NKA (Figure 1b). The effect of spantide was reversible. Thus, about 90 min after removal of spantide, the concentration-response curve to NKA returned approximately to the original position. The slope of the Schild plot (not shown) was close to unity (1.20 $\pm$ 0.1, $n = 3$) suggesting that the antagonism between spantide and NKA is competitive. The estimated pA_2 value of spantide was 6.49 $\pm$ 0.11 ($n = 3$). Spantide (0.5–16 μM) had no depolarizing action on the ventral root in the presence of TTX (0.3 μM) (Figures 3a and 5).

Acetyl-Arg^6-septide is known as a selective agonist for the NK_1-tachykinin receptor (Papir-Kricheli *et al.*, 1987). The effects of acetyl-Arg^6-septide on potentials of ventral roots in normal artificial CSF before and after adding TTX were similar to those of NKA as shown in Figures 2a and 6a. TTX caused a rightward shift of the concentration-response curve to acetyl-Arg^6-septide and at the same time a slight decrease of the amplitude of the maximum response (Figures 2a and 6a). In the presence of TTX, spantide again caused a rightward parallel shift of the concentration-response curve to acetyl-Arg^6-septide. The slope of the Schild plot was again close to unity (1.23 $\pm$ 0.14, $n = 3$) and the pA_2 estimate for spantide was 6.51 $\pm$ 0.04 ($n = 3$).

Figure 3 shows the time course of the antagonistic action of spantide against acetyl-Arg^6-septide, NKA, NKB and SP (6-11). In these experiments, the agonists were applied by brief pressure pulses at 10 or 20 min intervals. Perfusion with spantide at 2 μM markedly depressed the agonist-induced depolarizations, and after removal of spantide the recovery occurred slowly and often incompletely within 1–2 h. The responses to

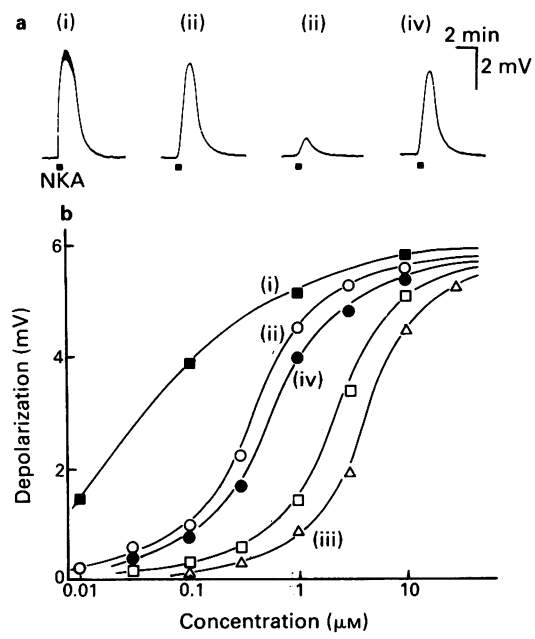

Figure 1 Effects of tetrodotoxin (TTX) and spantide on the responses of motoneurones to neurokinin A (NKA). Potentials were recorded extracellularly from the L5 ventral root of an isolated hemisected spinal cord of a 1 day-old rat. NKA was applied by perfusion for 30 s. (a) Sample records of the NKA-induced depolarizations, which correspond to the points indicated in (b). Horizontal black bars under records indicate the periods of application of NKA. (i) In normal artificial CSF; (ii) after adding 0.3 μM tetrodotoxin (TTX); (iii) after adding 2 μM spantide in the presence of TTX; (iv) 90 min after removal of spantide in continued presence of TTX. (b) Concentration-response curves to NKA. Ordinate scale, peak amplitude of depolarization induced by NKA. Abscissa scale, logarithmic concentration of NKA. (■) In normal artificial CSF; (○) in the presence of TTX (0.3 μM); (□) and (△) after addition of spantide at 1 and 2 μM respectively in the presence of TTX; (●) 60–90 min after removal of spantide in continued presence of TTX.

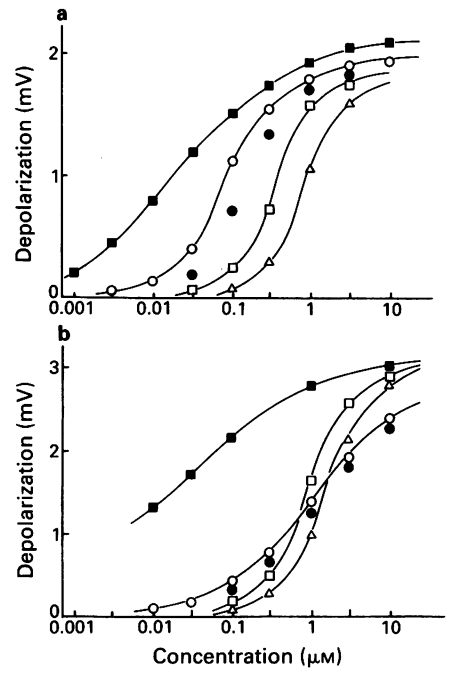

Figure 2 Effects of spantide on concentration-response curves to acetyl-Arg^6-septide (a) and substance P (SP) (b). These tachykinin receptor agonists were applied by perfusion for 30 s. Extracellular recordings from L5(a) and L4(b) ventral roots of hemisected spinal cords of 1 day-old rats. The peak amplitude of depolarizations of the ventral root was plotted against logarithmic concentrations of the agonists shown on the abscissa scale. (a) Concentration-response curves to acetyl-Arg^6-septide: (■) in normal artificial CSF; (○) in the presence of tetrodotoxin (TTX, 0.3 μM); (□) and (△) after addition of spantide at 1 and 2 μM respectively in the presence of TTX; (●) 90–130 min after removal of spantide in continued presence of TTX. (b) Concentration-response curves to SP: (■) in normal artificial CSF; (○) in the presence of TTX (0.3 μM); (□) and (△) after addition of spantide at 4 and 8 μM respectively in the presence of TTX; (●) 120–160 min after removal of spantide in continued presence of TTX.

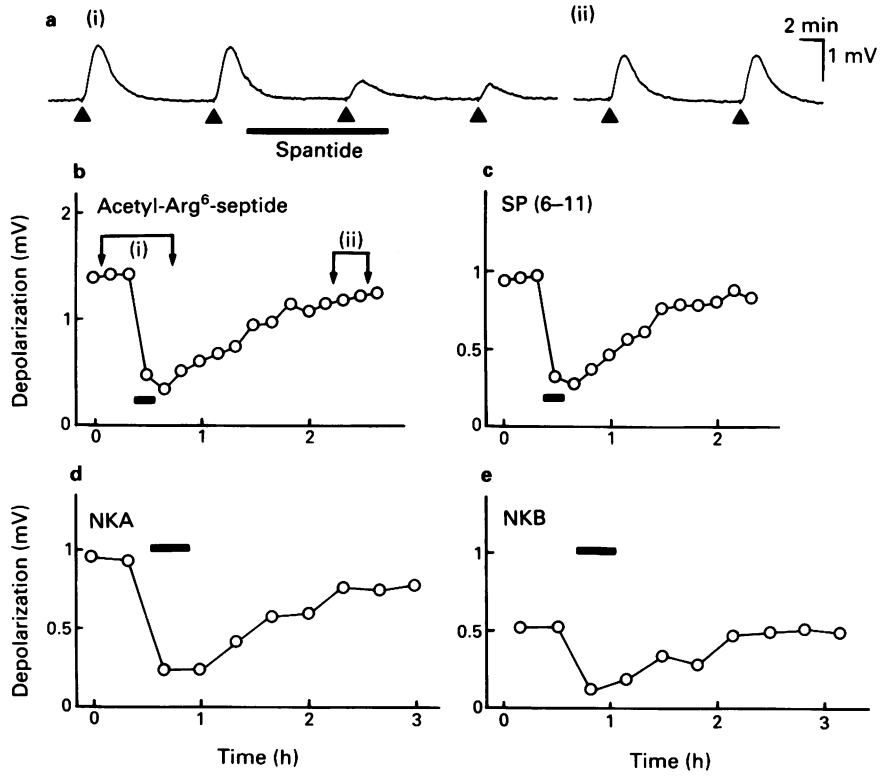

Figure 3 Effects of spantide on the responses of motoneurones to acetyl-Arg^6-septide, SP(6-11), neurokinin A (NKA) and neurokinin B (NKB) in artificial CSF containing 0.3 μM tetrodotoxin. Extracellular recordings from L4 or L5 ventral root of isolated hemisected spinal cords of 1 and 2-day old rats. (a) Sample records showing the effect of spantide on the responses to acetyl-Arg^6-septide. Acetyl-Arg^6-septide (5 μM) was applied by brief pressure pulse of 0.35 s at (▲): (i) during the period shown by the black horizontal bar, spantide (2 μM) was bath-applied; (ii) 100 min after the removal of spantide, the same experiment as shown in (b). Records (i) and (ii) were taken during the periods indicated by arrows in (b). (b–e) Open circles show the amplitudes of depolarizing responses induced by brief pulse application of acetyl-Arg^6-septide (5 μM, 0.35 s), SP(6–11) (5 μM, 0.6 s), NKA (5 μM, 0.5 s) and NKB (5 μM, 0.5 s). The peptide solutions were pressure injected into the perfusion solution at intervals of 10 or 20 min. Spantide (2 μM) was applied by perfusion during the periods indicated by black horizontal bars.

senktide and bombesin were almost completely abolished by TTX. Therefore, we did not examine further the effect of spantide on the responses to these agonists. Des-Met^{11}-SP(1-11) was inactive at 10 μM both in the absence (Otsuka & Konishi, 1977) and presence of TTX (cf. Figure 2b), suggesting that the C-terminal is important for the action of tachykinins on spinal neurones.

Antagonism between spantide and substance P

SP is a tachykinin agonist with preference for NK_1-receptors (Iversen *et al.*, 1987; Buck & Shatzer, 1988). Our previous study showed that spantide depressed the SP-induced depolarization of the ventral root of newborn rat spinal cord in normal artificial CSF (Otsuka & Yanagisawa, 1988). In the presence of TTX, however, Wienrich & Harting (1985) and Brown *et al.* (1986) observed that the SP-induced depolarization of the ventral root was potentiated by spantide. We confirmed the latter observation, as shown in Figure 4. In this experiment the response to acetyl-Arg^6-septide was depressed by spantide whereas that to SP was potentiated by the antagonist (see also Figure 2b). The amplitudes of responses to SP applied for 30 s at 3–10 μM were increased by spantide (16 μM) to 122.0 $\pm$ 3.9% (mean $\pm$ s.e.mean, $n = 4$) of the controls. This increase was statistically significant ($P < 0.02$).

Figure 2b shows the effects of TTX and spantide on the concentration-response curve to SP. SP was bath-applied by perfusion for periods of 30 s. The SP-induced depolarizations in normal artificial CSF were markedly depressed by TTX (0.3 μM), which agrees with the notion that SP exerts both transsynaptic and direct actions to induce the depolarization of motoneurones (Otsuka & Yanagisawa, 1988). In the presence of TTX, the SP-induced depolarization was depressed

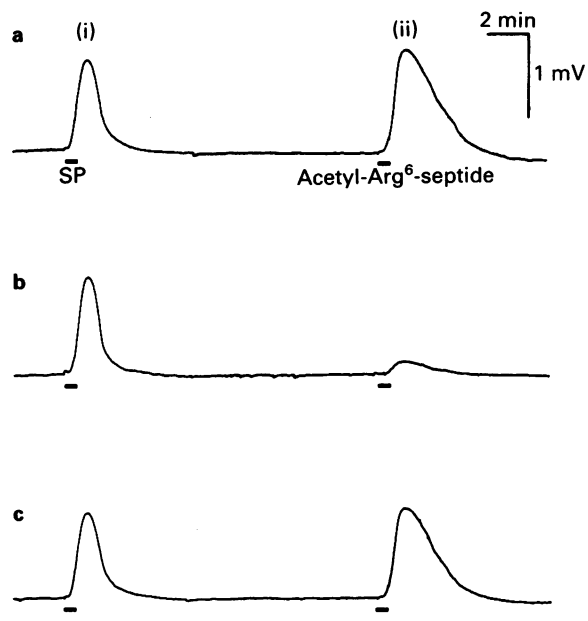

Figure 4 Effects of spantide on the depolarizing responses of motoneurones to substance P (SP) (i) and acetyl-Arg^6-septide (ii) in artificial CSF containing 0.3 μM tetrodotoxin (TTX). Potentials were recorded from L4 ventral root of an isolated hemisected spinal cord of 1 day-old rat. SP (4 μM) and acetyl-Arg^6-septide (3 μM) were bath-applied for 30 s during the periods marked by horizontal bars under the records at 15 min intervals. (a) Responses to SP and acetyl-Arg^6-septide in artificial CSF containing TTX; (b) 7–26 min after addition of spantide (10 μM); (c) responses about 150 min after the removal of spantide.

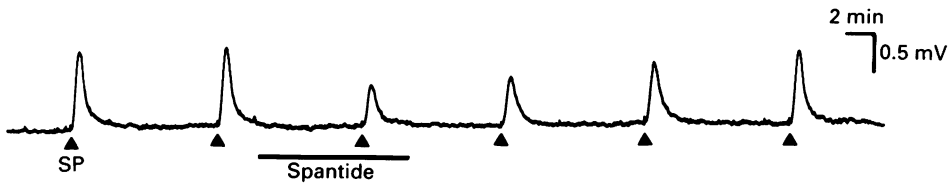

Figure 5 Effects of spantide on the responses of motoneurones to substance P (SP). Extracellular recordings from L4 ventral root of a hemisected spinal cord preparation of a 1 day-old rat which was perfused with artificial CSF containing 0.3 μM tetrodotoxin. SP (10 μM) was applied by brief pressure pulses of 0.3 s duration at (▲) with 10 min intervals. Spantide (10 μM) was applied by perfusion during the period indicated by a black horizontal bar.

by spantide (4–10 μM) at a low concentration range of SP (0.1–0.3 μM), but was potentiated at a high concentration range of SP (3–10 μM). Similar results were obtained in 4 other preparations.

When SP was applied by short pulses in the artificial CSF containing TTX (0.3 μM), the SP-induced depolarization was depressed by spantide at 10 μM (Figure 5). It was noted, however, that higher concentrations (4–10 μM) of spantide were needed to obtain the antagonistic effect against SP than against acetyl-Arg^6-septide or NKA (cf. Figures 1, 2a and 3).

Specificity of spantide

In a previous paper we examined the specificity of the action of spantide as a tachykinin antagonist in the neonatal rat spinal cord in normal artificial CSF, where spantide depressed the responses to SP, NKA and bombesin, but not those to noradrenaline, γ-aminobutyric acid (GABA), neurotensin and thyrotrophin-releasing hormone (TRH) (Otsuka & Yanagisawa, 1988). Under such conditions, we are dealing with actions of drugs on both spinal interneurones and motoneurones. In the present study, therefore, we re-examined the specificity of spantide in the presence of TTX in order to eliminate the involvement of interneurones. As shown in Figure 6, addition of TTX depressed the depolarization of the ventral root induced by TRH, L-glutamate, acetylcholine and noradrenaline, but not that induced by GABA. In the presence of TTX, spantide (16 μM) markedly depressed the depo-

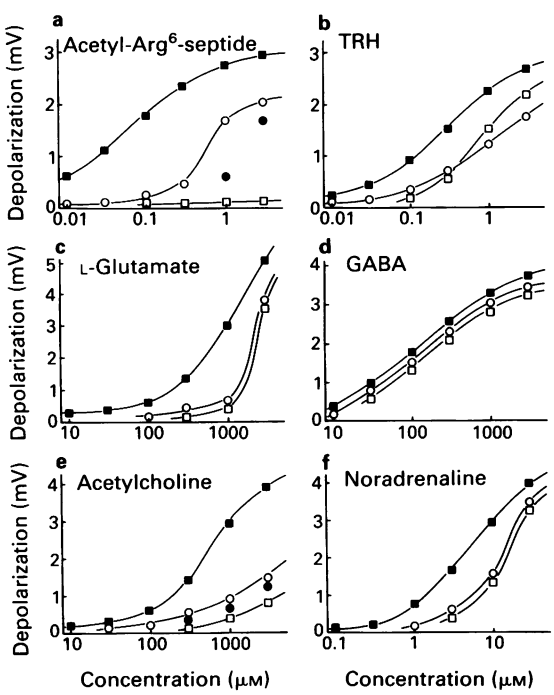

Figure 6 Effects of tetrodotoxin (TTX) and spantide on the concentration-response curves to acetyl-Arg^6-septide, thyrotrophin-releasing hormone (TRH), L-glutamate, γ-aminobutyric acid (GABA), acetylcholine and noradrenaline. The amplitudes of depolarizations of the ventral root were plotted against logarithmic concentrations of agonists shown on the abscissa scale: (■) in normal artificial CSF; (○) in the presence of 0.4 μM TTX; (□) after addition of spantide at 16 μM in the presence of TTX; (●) about 2 h after the removal of spantide in continued presence of TTX.

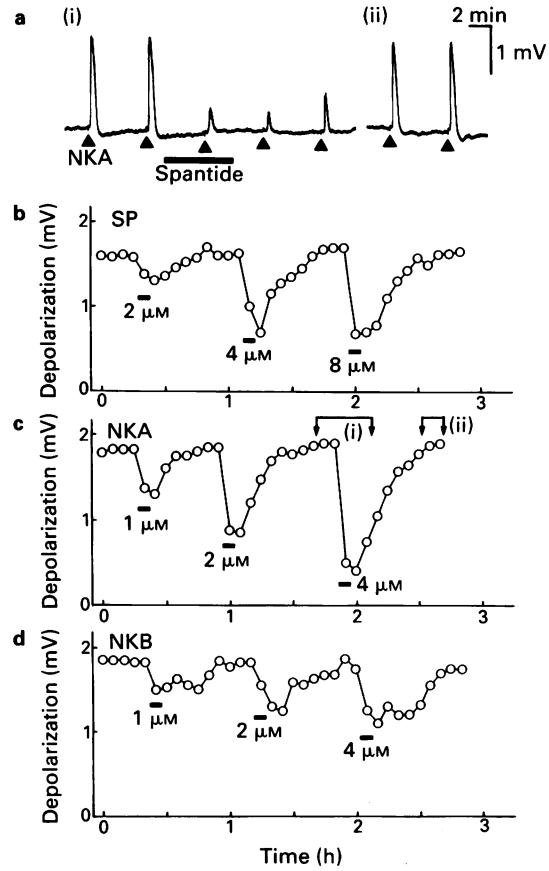

Figure 7 Effects of spantide on the responses of motoneurones to substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) in normal artificial CSF. Potentials were recorded from the L3 ventral root of a hemisected spinal cord preparation of a 1 day-old rat. SP, NKA and NKB were applied by brief pressure pulses. For each agonist, spantide was applied by perfusion in three different concentrations during the periods indicated by black horizontal bars. (a) Sample records of the responses to NKA (the same experiment as shown in (c)); (i) and (ii) are the records taken during the periods indicated by arrows in (c). NKA (2 μM) was applied by brief pressure pulses of 0.2 s duration at (▲) with 5 min intervals. (ii) During the period indicated by the black horizontal bar, spantide (4 μM) was bath-applied; (ii) 40 min after the removal of the antagonist. (b–d) Open circles show the amplitudes of depolarizing responses induced by brief pulse application of SP (2 μM, 0.2 s), NKA (2 μM, 0.4 s) and NKB (2 μM, 0.23 s). The peptide solutions were pressure injected into the perfusion solution at intervals of 5 min. Spantide was applied by perfusion during the periods indicated by black horizontal bars at concentrations indicated under the bars.

larization induced by acetyl-Arg^6-septide, but did not affect the depolarizations induced by L-glutamate, GABA and noradrenaline. The depolarization induced by acetylcholine was slightly depressed by spantide, which confirms the previously reported antinicotinic action of tachykinin antagonists (Akagi *et al.*, 1985; Simasko *et al.*, 1985; Boksa & Livett, 1985; Otsuka & Yanagisawa, 1988). The depolarizing action of TRH was slightly potentiated by spantide (Figure 6b).

Antagonism between spantide and tachykinin receptor agonists in normal artificial CSF

In order to explore the action of spantide on spinal interneurones, we studied the antagonism between spantide and tachykinin receptor agonists in the absence of TTX. Under such conditions, depolarizing responses of the ventral root to various tachykinin agonists are mainly due to transsynaptic action of agonists through interneurones (Figures 1 and 2). SP, NKA and NKB were applied by brief pressure pulses. As shown in Figure 7, spantide depressed the responses to all of these agonists but the response to NKA was most susceptible to spantide. In this context, Matsuto *et al.* (1984) obtained similar results with another tachykinin antagonist, [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]SP.

Discussion

In the presence of TTX, the responses of motoneurones to NKA and acetyl-Arg^6-septide were depressed by spantide at 1–2 μM. The pA_2 value of spantide against NKA and that against acetyl-Arg^6-septide were about the same, i.e. 6.5. This, together with the fact that the slope of the Schild plot was close to unity in both the antagonism between spantide and NKA and that between spantide and acetyl-Arg^6-septide, suggests that NKA, acetyl-Arg^6-septide and spantide bind to the same subtype of tachykinin receptors. This subtype of tachykinin receptor on motoneurones is similar to the NK_1-subtype in that it binds to acetyl-Arg^6-septide but is different from NK_1-receptors in that it binds to NKA with high affinity. Whether or not this represents a new class of tachykinin receptors remains to be clarified.

The pA_2 value of spantide was estimated at 6.65–6.9 against SP in guinea-pig ileum (Rosell *et al.*, 1983; Chahl, 1985). These values represent the pA_2 value on the NK_1-receptor, because the SP response of guinea-pig ileum is predominantly due to NK_1-receptor (Iversen *et al.*, 1987; Regoli *et al.*, 1988). There are several other studies reporting much lower pA_2 values of spantide. In our previous study we analysed the effect of spantide on the SP-induced depolarization of the ventral root of newborn rat spinal cord in normal artificial CSF, and obtained the pA_2 estimate of 5.4. Similar pA_2 values of spantide (5.2–5.4) were obtained when measured on the atropine-sensitive response to SP or tachykinin-stimulated [^{3}H]-acetylcholine release in the guinea-pig ileum (Chahl, 1985; Featherstone *et al.*, 1986). These relatively low values of pA_2 for spantide probably reflect the action of spantide on NK_3- and possibly other types of receptors. There is evidence suggesting the presence of NK_3-receptors on dorsal horn cells of the neonatal rat spinal cord (Ireland *et al.*, 1988) and myenteric neurones of guinea-pig ileum (Hanami *et al.*, 1988; Regoli *et al.*, 1988).

The effects of spantide on SP action are complicated. Spantide depressed the SP response when SP was applied at low concentrations or with short pulses (Figures 2b and 5), whereas the spantide potentiated the SP response when SP was applied at high concentrations for 30 s (Figures 2b and 4). It is likely that SP acts on more than one class of tachykinin receptors, including the one on which spantide acts with a pA_2 of 6.5.

The present results on the action of spantide against acetyl-Arg^6-septide and SP agree partly with those of Wienrich & Harting (1985) and Wienrich *et al.* (1988), who found that [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]SP depressed the depolarizing action of septide but not that of SP on the newborn rat motoneurones in the presence of TTX.

The present study suggests that there is more than one class of tachykinin receptors on motoneurones of the newborn rat spinal cord. For one class of tachykinin receptors, spantide has a high pA_2 value of 6.5. For another class or other classes of tachykinin receptors, spantide appears to be a weak antagonist or rather a potentiator. In the isolated spinal cord of the new born rat, slow responses to C-fibre stimulation were depressed by spantide, but relatively high concentrations (4–16 μM) were needed (Otsuka & Yanagisawa, 1988; Nussbaumer *et al.*, 1989). Depression of SP response by spantide in normal artificial CSF as well as in the TTX-containing solution also required high concentrations (Figures 2b, 5 and 7; Otsuka & Yanagisawa, 1988). These responses probably involve tachykinin receptors of a subtype or subtypes other than the one on which spantide acts with pA_2 of 6.5. We suppose that if there are tachykinin antagonists with potent action on the former type of tachykinin receptor which is less sensitive to the antagonistic action of spantide, they may exert potent antinociceptive action at low concentrations.

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