Depression of Windup of Spinal Neurons in the Neonatal Rat Spinal Cord In Vitro by an NK3 Tachykinin Receptor Antagonist

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Received 14 September 2000; accepted in final form 21 December 2000

Barbieri, Mario and Andrea Nistri. Depression of windup of spinal neurons in the neonatal rat spinal cord in vitro by an NK3 tachykinin receptor antagonist. J Neurophysiol 85: 1502–1511, 2001. The effects of the NK3 tachykinin receptor antagonist SR 142801 on synaptic transmission and spike windup induced by trains of stimuli applied to a dorsal root were investigated with intra- and extracellular recording from the neonatal rat spinal cord in vitro. SR 142801 (10 μM) reduced the depolarization (recorded from lumbar ventral roots) induced by senktide (an NK3 agonist) more strongly than the one evoked by substance P methyl ester (SPMeO; an NK1 agonist). Nevertheless, after a long (>2 h) application time, SR 142801 largely depressed the response to SPMeO as well. When NK1 or NK3 receptors were blocked by >50% in the presence of SR 142801, there was also a significant reduction in the cumulative depolarization induced by repeated stimuli to a single dorsal root. This blocking action by SR 142801 was also observed in the presence of the N-methyl-D-aspartate (NMDA) receptor antagonist D-aminophosphonovalerate (APV) and the calcium channel blocker nifedipine. Intracellular data from lumbar motoneurons showed that the spike windup was the first and most sensitive target for the SR 142801 blocking effect. Increasing stimulus strength to dorsal root fibers could partly surmount such a block. SR 142801 per se had no direct action on fast synaptic transmission, membrane potential, or input resistance. These findings indicate that SR 142801 could lead to an early, large reduction in the windup of action potential discharge by motoneurons, suggesting its ability to suppress the reflex component of central sensitization evoked by repeated dorsal root stimuli.

INTRODUCTION

A distinctive feature of nociceptive pathways in the spinal cord is the gradual sensitization of neuronal responses to repeated stimuli of equal strength (Baranauskas and Nistri 1998; Herrero et al. 2000). This phenomenon is typically expressed as “windup” because progressively larger numbers of action potentials are generated by interneurons and motoneurons by a train of noxious stimuli to trigger averse reflex responses (Mendell and Wall 1966). Windup is often used in animal studies either in vivo (Mendell and Wall 1966; Price et al. 1971) or in vitro (Baranauskas and Nistri 1996; Sivilotti et al. 1993; Thompson et al. 1990, 1994) for model investigations of pain processing mechanisms as it seems to reproduce the cellular responses underlying the acute pain evoked by strong, persistent stimulation.

The noxious pulse train elicits a slowly incrementing depolarization (cumulative depolarization) readily recorded extracellularly from ventral roots (VRs) or intracellularly from motoneurons or interneurons (for a recent review see Herrero et al. 2000). While the mere presence of cumulative depolarization is not predictive of action potential windup in a certain neuron, a high rate of rise of cumulative depolarization is usually associated with windup expression (Sivilotti et al. 1993; Thompson et al. 1990). The cellular mechanisms responsible for windup generation are complex and still incompletely understood. Ability to summate synaptic inputs may depend on activation of N-methyl-D-aspartate (NMDA) receptors (Davies and Lodge 1987; Thompson et al. 1990), slow voltage-dependent Ca2+ channels (Morisset and Nagy 1996; Russo and Houngsaard 1994; Russo et al. 1997), or NK1 tachykinin receptors (Baranauskas and Nistri 1996; Baranauskas et al. 1995), or a combination of them (Urban et al. 1994).

Our previous studies of the role of NK1 receptors in windup (Baranauskas et al. 1995) were made possible by the availability of the selective, nonpeptide antagonist SR 140333. Nevertheless, NK1 receptors are only one class of the multiple tachykinin receptors expressed by the spinal cord tissue. While we have found no functional involvement of NK2 receptors in synaptic transmission or windup (Baranauskas et al. 1995), the possible role of NK3 receptors remains unexplored and deserves investigation (Herrero et al. 2000) since NK3 receptors are present in the rat spinal cord (Mileusnic et al. 1999a,b; Ninkovic et al. 1984; Otsuka and Yoshioka 1993) and are localized to interneurons (Levine et al. 1993; Mantyh et al. 1989; Zerari et al. 1997) on which they are developmentally regulated (Beresford et al. 1992; Mileusnic et al. 1999a,b).

The compound SR 142801 (osanetant) has been shown to be a potent and selective blocker of NK3 receptors in a variety of tissues including the CNS (Beaujouan et al. 1997; Emonds-Alt et al. 1995). For this reason we used the neonatal rat spinal cord in vitro as a model to test the action of an NK3 antagonist on the cumulative depolarization and windup induced by dorsal root stimuli.

METHODS

Tissue preparation and solution

Experiments were performed on spinal cord preparations (comprising a region from mid-thoracic level to conus medullaris) isolated...
from 5- to 10-day-old Wistar rats under urethan terminal anesthesia (0.2 ml ip of a 10% wt/vol solution) as previously described (Baranauskas et al. 1995). This procedure is in accordance with the regulations of the Italian Animal Welfare Act and is approved by the local authority veterinary service.

The spinal cord was fixed to the bottom of the recording chamber and superfused (12 ml/min) with Krebs solution of the following composition (in mM): 113 NaCl, 4.5 KCl, 1 MgCl₂,7H₂O, 2 CaCl₂, 1 NaH₂PO₄, 25 NaHCO₃, and 11 glucose, gassed with 95% O₂-5% CO₂; pH 7.38 at room temperature (22°C).

**Electrical stimulation and electrophysiological recording**

DC ventral root recordings (usually from L₅ VRs) were obtained with tight-fitting glass suction microelectrodes containing an Ag-AgCl pellet and filled with Krebs solution (Otsuka and Konishi 1974). Intracellular recordings were obtained with sharp (25–60 MΩ) microelectrodes filled with 3 M KCl under current-clamp conditions (Baranauskas et al. 1995). L₅ motoneurons were identified functionally by antidromic stimulation (Fulton and Walton 1986). During intracellular experiments the activity of one (or more) VR was recorded simultaneously. DC-coupled VR and motoneuron recordings were amplified, displayed on-line on a chart recorder, and digitally stored on videotape or on computer hard disk.

Dorsal root (DR) electrical stimuli were delivered via miniature bipolar suction electrodes. Stimulus intensity (1 to 20 V range; 1 ms duration) was adjusted to be at least twice above threshold defined as the minimum intensity to elicit a detectable polysynaptic response from the homolateral VR. The standard DR train protocol (Baranauskas et al. 1995; Sivilotti et al. 1993) consisted of 20 pulses (1 Hz) that generated an incrementing depolarization (cumulative depolarization) with a large increase in the number of action potentials (windup; measured as the total number of spike during the train). A typical protocol to generate cumulative depolarization and windup is shown in Fig. 1 in which intracellular traces (A) and extracellular records (B) were obtained simultaneously from the same preparation. The following parameters were measured during intracellular recording: a, amplitude of hyperpolarizing electrotonic potential (etp) evoked by hyperpolarizing current steps (0.1–0.2 nA) and used to estimate input resistance changes; b, threshold for spike generation; c, amplitude of first orthodromic spike; d, amplitude of slow synaptic potential (poly-EPSP); e, area of slow synaptic potential evoked by first pulse in the train; f, amplitude of tail of slow synaptic potential just before second pulse; g, amplitude of cumulative depolarization; h, area of depolarization decay from 1 s after end of pulse train to baseline. Parameters were measured during intracellular recording: a, amplitude of hyperpolarizing electrotonic potential (etp) evoked by hyperpolarizing current steps (0.1–0.2 nA) and used to estimate input resistance changes; b, threshold for spike generation; c, amplitude of first orthodromic spike; d, amplitude of slow synaptic potential (poly-EPSP); e, area of slow synaptic potential evoked by first pulse in the train; f, amplitude of tail of slow synaptic potential just before second pulse; g, amplitude of cumulative depolarization; h, area of depolarization decay from 1 s after end of pulse train to baseline.
ization decay from 1 s after end of pulse train to baseline. The rate of rise of cumulative depolarization (usually expressed as % of control) was calculated as the increment in baseline depolarization per second, starting after the second response to a train of 20 pulses. From VR records we measured only cumulative depolarization (and its rate of rise), and tail of the slow synaptic potential. DR stimulus trains were usually applied at intervals of 5 min.

Data were quantified as means ± SE (n is number of spinal cord preparations). Statistical significance was assessed with the Student’s t-test or ANOVA test followed by Tukey test for parametric or nonparametric data, respectively. In either case the accepted level of significance was P = 0.05.

Drugs

All agents were bath-applied via the superfusing solution at the concentrations mentioned in the text. We used senktide as a selective agonist on NK3 receptors (Maggi et al. 1993; Regoli et al. 1994) and substance P methylster (SPMeO) as selective agonist on NK1 receptors (Maggi et al. 1993; Regoli et al. 1994). To avoid response tachyphylaxis, senktide or SPMeO were applied at intervals ≥30 min; by using this application protocol, responses to either agonist could be reliably reproduced for several hours.

SR140333 and SR142801 were generously donated by Dr. Edmonds-Alt, Sanofi Recherche, Montpellier, France, and were dissolved in DMSO (final concentration of DMSO was <0.01% and had no detectable action on spinal neurons). SPMeO was purchased from Sigma, while senktide was obtained from Research Biochemicals International. d-Aminophosphonovalerate (APV) was supplied by Tocris Neuramin. Nifedipine and all other chemicals were purchased from Sigma.

RESULTS

Effects of SR 142801 on responses induced by tachykinin agonists

Previous studies have indicated SR 142801 to be a potent and selective antagonist on NK3 receptors in a variety of tissues including the rat CNS (Beaujouan et al. 1997; Emonds-Alt et al. 1995). Our experiments were carried out to validate the applicability of this antagonism to the neonatal rat spinal cord under the present experimental conditions. Figure 2A shows control responses to bath application of receptor saturating concentrations of senktide (0.2 μM) or SPMeO (0.2 μM). These two agonists elicited different VR depolarization patterns because the response to senktide was characterized by slower onset/offset and stronger bursting. In control solution the mean depolarization area and peak amplitude for senktide responses were 227 ± 28 (SE) mV·s and 3.3 ± 0.5 mV,
respectively, while corresponding values for SPMeo were 247 ± 37 mV·s and 2.9 ± 0.5 mV (n = 12).

As shown by the histograms of Fig. 2B, SR 142801 (10 μM) induced a gradually intensifying depression of responses (expressed as depolarization area) to senktide. A significant antagonism (P < 0.05; ANOVA test) already appeared after 30 min exposure to SR 142801. At 60 min the senktide response was halved (P < 0.05%), while there was no significant decrease in the SPMeO effect (see Fig. 2B). However, when SR 142801 was applied for 120–240 min, it also significantly (P < 0.05) reduced the effect of SPMeO. Although in absolute terms the reduction in SPMeO effect was smaller than the one concerning senktide, there was no significant difference between the decreased responses to either agonist (P > 0.05; ANOVA and Tukey test).

Bursting activity was more sensitive than depolarization area to the blocking action of SR 142801 (Fig. 2C). In fact, bursts induced by senktide were almost completely suppressed (P < 0.05; Student’s t-test) after 60 min, while those due to SPMeO were significantly (P < 0.05) depressed. The varying degree of bursting block between senktide and SPMeO treatment was significantly different (P < 0.05). At later times (120–240 min) bursting could not be evoked by senktide and only minimally by SPMeO (Fig. 2C).

As shown in Fig. 2D (left pair of histograms) a 10-fold lower concentration (1 μM) of SR 142801 (>120-min application) induced a smaller but significant (P < 0.05; ANOVA test) depression of senktide responses. The effect of SPMeO was also apparently diminished even though this difference was not statistically significant. Conversely, the NK1 antagonist SR 140333 (1 μM; >120-min application) induced much stronger antagonism of SPMeO responses versus those due to senktide (Fig. 2D, right), although the latter were also significantly depressed (P < 0.05 for both agonists). The differing degree of depression between senktide and SPMeO was also statistically significant (P < 0.05). None of these antagonists per se changed the baseline polarization level of VRs.

Although the receptor selectivity of these tachykinin agonists is well established (Wormser et al. 1986), pilot experiments were carried out to confirm it under the present experimental conditions. Hence we applied a maximally effective concentration (0.2 μM) of senktide (EC50 = 12 nM) (Fox et al. 1996) or SPMeO (EC50 = 7.2 nM) (Fox et al. 1996) first separately and then together to find out whether the combined response was the simple summation of individual effects (in each experiment the response to combined agonist application was taken as 100%). The VR depolarizations induced by separate application of senktide or SPMeO were 38.7 ± 1.5 or 59.9 ± 1.5% of the combined response (n = 3 preparations); added together, these values yield 98.6%, that is almost the same as the effect produced by senktide plus SPMeO. These data indicate that the response to combined application of each agonist (at receptor saturating concentration) was equivalent to the sum of the individual responses. These observations therefore confirm that the neonatal rat spinal cord possesses distinct receptors for senktide and SPMeO (Otsuka and Yoshioka 1993).

In summary, the present results thus suggest that SR 142801 was clearly a preferential antagonist toward NK3 receptors, although it could later display broader spectrum tachykinin receptor antagonism.

**Effect of SR 142801 on cumulative depolarization evoked by DR stimuli**

Repeated strong stimuli applied to a lumbar DR are known to generate cumulative depolarization recorded from VRs (Herrero et al. 2000). Tachykinin receptors are considered to play an important role in this phenomenon (Baranauskas and Nistri 1998; Herrero et al. 2000). We tested how SR 142801 affected cumulative depolarization in the rat spinal cord. Figure 3A compares the time course of the reduction in the amplitude of cumulative depolarization (see parameter g in Fig. 1; ○ in Fig. 3A) with that of depolarization areas generated by senktide or SPMeO. After applying SR 142801 the earliest time to detect a small, yet significant (P < 0.001) fall in cumulative depolarization was 20 min. Note that 50% decrease in cumulative depolarization was observed at 150 min when the area of responses to both agonists was strongly reduced. The maximum fall in cumulative depolarization leveled off at 33 ± 14% (observed at 4 h in SR 142801 solution). The relation between cumulative depolarization and responses to senktide or SPMeO is quantified in Fig. 3B in which, after applying SR 142801 (10 μM), normalized tachykinin-induced depolarizations are plotted against normalized cumulative depolarizations elicited by dorsal root pulse trains. Linear regression analysis (Fig. 3B) suggests a tight correlation between extent of reduction in cumulative depolarization and fall in tachykinin-induced responses (r = 0.99 for SPMeO responses or 0.90 for senktide responses). This association was highly significant with P < 0.001 calculated with the Pearson Product Moment Correlation (SigmaStat software, Jandel, Chicago, IL). A lower concentration of SR 142801 (1 μM; at least 2 h

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**Fig. 3.** SR 142801 slowly depressed depolarizations to senktide (SK) or substance P methylster (SPMeO) or train-evoked cumulative depolarizations (cum. depol.). A: time course of SR 142801 depression of SK (●) or SPMeO (■) response area or amplitude of cumulative depolarization (○; see g in Fig. 1; n = 29 at time 0, and n = 5 at 240 min). *, P < 0.05 vs. control; #, P < 0.01 vs. control (ANOVA tests). All cumulative depolarization time points after 60 min are significantly different (P < 0.01) from control. B: linear regression analysis of data obtained in the presence of SR 142801 (10 μM): ordinate, normalized amplitude of cumulative depolarization, abscissa, normalized response area to SK or SPMeO. r, correlation coefficient for senktide (●) or SPMeO (■) data.
application) had no significant effect on cumulative depolarization or its rate of rise \((n = 7)\).

The long-lasting recording protocol might have raised some concern about the long-term viability of the spinal cord in vitro. To explore this issue we monitored the amplitude of the fast VR reflex for at least 4 h in the presence of SR 142801 (10 \(\mu M\)) and for further 5 h after wash out of the antagonist. At 4 h the amplitude of the fast synaptic response was 99 ± 14% \((n = 5\) preparations) with respect to predrug control and was 111 ± 20% after 5 h wash out. As these observations indicated excellent long-term survival of the tissue under in vitro conditions, we examined whether there was any recovery in cumulative depolarization after wash out of SR 142801. After 1 h wash out of the antagonist, recovery of cumulative depolarization amplitude was limited to 34 ± 17% \((n = 7)\).

**Interaction of SR 142801 with NMDA receptors or slow \(Ca^{2+}\) conductances**

In in vitro spinal cord preparations, cumulative depolarization is a complex phenomenon in which several processes like, for example, activation of NMDA receptors, tachykinin receptors, or \(L\)-type \(Ca^{2+}\) conductances are thought to concur to generate sensitization to afferent impulses (Baranauskas et al. 1995; Morisset and Nagy 1999; Russo and Hounsgaard 1994, 1996). We explored whether the depressant action of SR 142801 on cumulative depolarization was indirectly mediated via decrease in one or more of these mechanisms. A typical example of cumulative depolarization induced in control solution, or in the presence of 50 \(\mu M\) APV (selective NMDA receptor antagonist) plus 20 \(\mu M\) nifedipine (selective \(L\)-type \(Ca^{2+}\) channel blocker) is presented in Fig. 4, A and B. In this representative experiment, despite the reduction in amplitude of cumulative depolarization (54%) observed with APV and nifedipine, the rate of rise of this response remained elevated (97%), indicating that summation of responses to afferent inputs was preserved (Sivilotti et al. 1993). Subsequent addition of SR 142801 (10 \(\mu M\); 120 min) further reduced (Fig. 4C) the amplitude of cumulative depolarization (37%) and largely diminished the rate of rise (56%). Hence the action of SR 142801 was present despite application of blockers for NMDA receptors and \(L\)-type \(Ca^{2+}\) channels. A quantitative summary of these data are provided in Fig. 4D in which cumulative depolarization is plotted \((\bigcirc)\) as a function of time in the presence of SR 142801 (10 \(\mu M\)). The decrease in cumulative depolarization was significant at 75 min (and subsequent times) with \(P < 0.05\) (ANOVA test).

As cumulative depolarization is generated as long as each stimulus elicits a synaptic potential sufficiently slow to allow its summation with the next response (Baranauskas and Nistri 1998), we explored whether this notion applied also to experiments with SR 142801 (10 \(\mu M\)). In the presence of APV and nifedipine, the amplitude of the synaptic potential tail (see Fig. 4D, ■) fell in parallel with the drop in cumulative depolarization (Fig. 4D, ○). This fall in tail amplitude became significant \((P < 0.05; \text{ANOVA test})\) at 75 min and was not quantitatively different \((P > 0.05)\) from the reduction in cumulative depolarization. In practice, SR 142801 had removed a potent mechanism to summate DR inputs. This phenomenon was not accompanied by a generalized depression in synaptic transmission as the peak amplitude of rapid VR reflexes (Fig. 4D, ▲) remained unchanged.

**Intracellularly recorded windup**

The main feature of the windup process is the large increment in the number of spikes generated in response to a train of constant stimuli (Herrero et al. 2000; Mendell and Wall 1966; Woolf 1983). This phenomenon can be investigated on dorsal interneurons as well as on motoneurons (Sivilotti et al. 1993; Thompson et al. 1990). The present experiments report intracellular data from lumbar motoneurons as exemplified in the traces of Fig. 5A. Repeated (1 Hz) 4 V stimuli (indicated by vertical deflections beneath the voltage trace) applied to one L5 DR induced cumulative depolarization (18 mV from −68 mV resting potential) and windup (211 spikes) recorded from one L3 motoneuron. The early component of this response is shown on a faster time base in the inset to the right of Fig. 5A.

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**Fig. 4.** Sensitization evoked by DR train in the presence of various pharmacological blockers. A: control response induced by 1-Hz train (18 V). B: response observed after 30 min application of n-aminophosphonoovalerate (APV; 50 \(\mu M\)) and nifedipine (20 \(\mu M\)). Note reduction in amplitude of cumulative depolarization but persistence of rapid rate of rise of response. C: further application of SR 142801 (10 \(\mu M\)) strongly decreases cumulative depolarization and rate of rise (120-min application). D: plot of time course changes in VR reflex amplitude (▲), cumulative depolarization (○), or tail of slow synaptic potential (■) after adding SR 142801 (time 0) to the solution containing APV and nifedipine; \(n = 4\). Asterisk indicates the earliest time (75 min) for cumulative depolarization and synaptic tail to differ significantly \((P < 0.05)\) from control. The decrease was similar \((P > 0.05)\) for both parameters (ANOVA and Tukey tests).
Recovery to baseline potential was multiphasic and slow. Simultaneous records from both L₅ VRs (not shown) indicated that cumulative depolarization also occurred at the same segmental level. Figure 5B shows that 30 min application of 10 μM SR 142801 reduced cumulative depolarization (15.5 mV) but even more strongly decreased the number of spikes (122). This effect was not associated with significant changes in resting potential (−2 mV) or resistance (9%). The inset to Fig. 5B (faster time base) indicates that the inter-stimulus period was characterized by less synaptic activity and return of membrane potential to baseline before each successive stimulus. This observation therefore accords with the data obtained with VR recording showing that the amplitude of the slow synaptic potential tail constrained evoked response summation (see Fig. 4D). Figure 5C shows that the same stimulation protocol elicited even smaller cumulative depolarization (12 mV) and windup (75 spikes) after 60 min exposure to SR 142801. Resting membrane potential remained unchanged (−1 mV) as much as input resistance (−2%). The inset in Fig. 5C shows (on faster time base) lack of slow synaptic potential.

Figure 6 shows mean data from a sample of five lumbar motoneurons after application of SR 142801 (10 μM). Various indexes of sensitization were differentially affected by SR 142801. In particular, windup (□) fell rapidly to 50% at 26 min (P < 0.05; ANOVA test) and disappeared after 90 min. At 26 min none of the other parameters was significantly reduced. In this series of experiments, the earliest times at which a significant change (P < 0.05) emerged are indicated with asterisks in Fig. 6: 45 min for cumulative depolarization (○), 60 min for the tail of synaptic potential (□) and rate of rise (○), and 75 min for the decay area (◇). At 90 min the rate of rise (○) was significantly (P < 0.05) decreased to 44% of control value, while the amplitude of cumulative depolarization (○), excitatory postsynaptic potential (EPSP) tail amplitude (□), or the slow response decay after the end of the train (◇) were less severely, although significantly (P < 0.05), depressed (73, 60, or 70% of control, respectively). Table 1 summarizes the intracellularly measured parameters during repeated stimuli after 1 or 2 h application of 10 μM SR 142801.

Once the blocking effect by SR 142801 (10 μM) had reached apparent steady-state conditions (2 h), 1-h application of the NK₁ antagonist SR 140333 (1 μM) further decreased cumulative depolarization (by 16 ± 9%; P < 0.05; ANOVA and Tukey test) and EPSP tail (by 45 ± 9%; P < 0.05; ANOVA and Tukey test; n = 3).

Finally, we explored whether, after reaching apparent steady-state block of windup by SR 142801, increasing the stimulus strength could reverse this condition. Figure 7 shows one such experiment in which the top record is from an L₅ VR and the bottom one is an intracellular trace from an L₅ motoneuron. After 120 min from the start of SR 142801, there was large reduction in windup (2 spikes) and cumulative depolarization (1.5 mV; same cell shown in Fig. 5). Increasing the stimulus strength to 4.5, 6, or 7 V partly overcame the depression. For instance, the windup now comprised 85 spikes with the 7 V stimulus train (compare it with 211 spikes in control solution with 4 V stimulation), and the associated cumulative depolarization was 17 mV (almost the same as in control solution; 18 mV). Similar data were obtained from three cells.
effect of 1-Hz trains at 4, 4.5, 6, or 7 V intensity.

apparent loss of selectivity appeared in parallel with intensification generated by tachykinin-sensitive dorsal horn interneurons and interneurons (Fisher et al. 1994).

interneuronal network while NK1 receptors are almost functionally absent from motoneurons but present in the spinal cord, even partial antagonism of these receptors was sufficient to depress the spike windup of motoneurons.

Functional pharmacology of tachykinin receptors

When applied to the rat spinal cord at receptor-saturating concentrations, the selective NK3 agonist senktide, or the selective NK1 agonist SPMeO induced distinct responses. The action of senktide had slower onset and was often associated with more intense bursting. Bursting is a network-based activity generated by tachykinin-sensitive dorsal horn interneurons (Sandkuhler and Eblen-Zajjur 1994). Our previous study has shown that in the neonatal rat spinal cord, NK3 receptors are shown to depress the spike windup of motoneurons.

**DISCUSSION**

The principal finding of the present study is the demonstration that in the immature rat spinal cord the NK3 receptor antagonist SR 142801 induced early, strong block of the spike windup and late depression of the cumulative depolarization. The selectivity of this compound toward NK3 receptors gradually changed during sustained application because antagonism of NK1 receptor-mediated responses emerged late. This phenomenon coincided with depression of cumulative depolarization and other components of sensitization. Our observations cast new light onto the role of NK3 receptors in spinal cord sensitization as, at least in the case of the neonatal rat spinal cord, partial antagonism of these receptors was sufficient to depress the spike windup of motoneurons.

**TABLE 1.** Values of intracellularly recorded windup parameters recorded from five motoneurons

<table>
<thead>
<tr>
<th></th>
<th>Resting Potential, mV</th>
<th>Input Resistance, MΩ</th>
<th>Spike Threshold, mV</th>
<th>Spike Amplitude, mV</th>
<th>polyEPSP Amplitude, mV</th>
<th>polyEPSP Area, %</th>
<th>polyEPSP Tail, %</th>
<th>Cumulative Depolarization, %</th>
<th>Decay Area, %</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>−72 ± 2</td>
<td>53 ± 6</td>
<td>−48 ± 2</td>
<td>87 ± 1</td>
<td>28 ± 5</td>
<td></td>
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<td></td>
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<tr>
<td>SR 142801 (60 min)</td>
<td>−71 ± 2</td>
<td>56 ± 1</td>
<td>−47 ± 2</td>
<td>89 ± 2</td>
<td>26 ± 4</td>
<td>98 ± 2</td>
<td>76 ± 7*</td>
<td>76 ± 6*</td>
<td>76 ± 5*</td>
</tr>
<tr>
<td>SR 142801 (120 min)</td>
<td>−69 ± 5</td>
<td>50 ± 1</td>
<td>−46 ± 2</td>
<td>92 ± 3</td>
<td>30 ± 5</td>
<td>72 ± 7*</td>
<td>68 ± 3*</td>
<td>65 ± 8*</td>
<td>62 ± 18*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 with ANOVA test followed by Tukey test.

It is not unusual for tachykinin antagonists to take quite a long time to block their receptors (Guo et al. 1998; Nagy et al. 1994); thus, in this sense, the slowly incrementing antagonism of NK3 receptors by SR 142801 was not particularly unexpected. Nevertheless, a previous electrophysiological study of the rat spinal cord did not find a blocking action by SR 142801 of NK1 receptor–mediated responses (Guo et al. 1998). This discrepancy may be due to the different age of rats used for experiments and to the length of application of SR 142801.

The reason for the late, gradual block of NK1 receptors remains unclear. Even though VR depolarizations induced by senktide or SPMeO included indirect effects due to release of endogenous transmitters (Maehara et al. 1995), these should not account for the time-dependent loss of receptor selectivity by SR 142801 because our experiments confirmed the well-established notion (Maggi et al. 1993; Regoli et al. 1994) that the action of SPMeO and that of senktide were mediated by distinct receptors. In the spinal cord, tachykinin receptors are known to undergo relatively fast modulatory changes, for example, when exposed to substance P (Honore et al. 1999). It is possible that sustained block of NK3 receptors by SR 142801 triggered a gradual reduction in NK1 receptor activity perhaps because it suppressed NK3 receptor–mediated generation of intracellular second messengers, like cyclic AMP that stabilizes NK1 receptor function in rat spinal neurons (Abrahams et al. 1999). To the best of our knowledge, the issue of time-dependent changes in receptor selectivity of tachykinin antagonists has not been systematically examined before. This matter should be further considered in future studies especially as even the NK1 antagonist 140333 slowly lost its selectivity, thus suggesting time-related adaptive changes in tachykinin receptor subclasses following sustained block of a certain receptor group.

Guo et al. (1998) have not detected any direct agonist action by SR 142801 on tachykinin receptors or any antagonism toward receptors for transmitters like glutamate, glycine, or GABA. The fact that SR 142801 did not change resting potential, input resistance, spike amplitude, or fast synaptic transmission of motoneurons indicates that this agent did not possess nonspecific actions on active or passive membrane properties or other transmitter receptors. SR 142801 can depress the late component of the electrically evoked VR potentials (Guo et al. 1998); while this observation implies NK3 receptor activity contributing to slow synaptic potentials, it is...
also possible that slow buildup of antagonism of NK1 receptors played a role.

SR 142801 depressed cumulative depolarization

By monitoring in parallel changes in cumulative depolarization and sensitivity to senktide or SPMeO following SR 142801, it became apparent that strong reduction in cumulative depolarization (down to \( \frac{1}{3} \) of control) was manifested only when responses to senktide and SPMeO were both largely reduced. A close correlation was found between pharmacological antagonism of tachykinin receptors and size of cumulative depolarization. These results suggest that a relatively robust cumulative depolarization could still be observed as long as one tachykinin receptor class was not largely blocked. It might therefore be inferred that NK1 as well as NK3 receptors could contribute to the mechanism responsible for cumulative depolarization.

The depressant action by SR 142801 on cumulative depolarization was not secondary to interference with other contributors to this phenomenon, namely NMDA receptors and L-type Ca\(^{2+} \) channels. In fact, SR 142801 retained its inhibitory effect even in the presence of the NMDA antagonist APV and the dihydropyridine blocker nifedipine. At the concentrations used in the present study, nifedipine is known to be a full, selective blocker of L-type Ca\(^{2+} \) channels in the spinal cord in vitro (Morisset and Nagy 1999; Russo and Houngaard 1996), while APV is a well-established, selective antagonist of NMDA receptors on rat spinal neurons (Beato et al. 1997; D’Hooge et al. 1999; Jiang et al. 1990; Pinco and Lev-Tov 1993). The presence of a strongly incrementing cumulative depolarization in the presence of an NMDA receptor blocker and an L-type Ca\(^{2+} \) channel blocker is not surprising as similar observations have repeatedly been obtained before (reviewed by Baranaukas and Nistri 1998; Herrero et al. 2000). These data concur to suggest sensitization of spinal neurons to afferent stimuli as due to a multifarious mechanism of action inclusive of tachykinin receptors, NMDA receptors, and high-threshold Ca\(^{2+} \) channels.

SR 142801 induced depression of windup

Intracellular data concerning the windup responses were obtained from motoneurons even if interneurons are considered to be the first elements in the process of synaptic integration that leads to the windup expression. The choice of motoneurons was due to their unambiguous identification with electrophysiological recording, and to the close similarity in cellular characteristics of the windup (onset, duration, amplitude) between motoneurons and interneurons (Sivilotti et al. 1993; Thompson et al. 1993). Furthermore, as windup is a network-based mechanism with incompletely understood circuit connections, the likelihood of detecting windup may depend on the position of a certain neuron within this network. Downstream cells like motoneurons are more likely to exhibit strong windup and are therefore most advantageous to monitor this process (Sivilotti et al. 1993) even though motoneurons simply remain a model for investigating this complex network response.

The windup itself was an early target for SR 142801 depression as 50% reduction was already noted after approximately 30 min administration, when NK3 receptors (assessed in terms of their sensitivity to senktide) were blocked by approximately 30%. Other parameters were more slowly (and less intensely) affected; thus the rate of rise of cumulative depolarization fell by 50% after about 90 min, while decrease in the tail of the polysynaptic EPSP and in the cumulative depolarization (and its decay) lagged behind. While windup was severely curtailed, cumulative depolarization was less diminished (including the slow EPSP tail), indicating that summation of slow synaptic potentials during the stimulus train could still occur. Our data thus indicate that, for studying changes in the process of central sensitization elicited by afferent inputs, action potential windup is a more sensitive index than cumulative depolarization.

The relation between cumulative depolarization and windup was highly nonlinear. For example, a nearly 50% decrease in windup (after 30-min SR 142801 application) was associated with an approximately 10% reduction in cumulative depolarization and its rate of rise. It is interesting that the only other electrophysiological response rather sensitive to SR 142801 block was the bursting activity (unlike neuronal depolarization) induced by tachykinin agonists.

Mode of action of NK3 receptors in the windup process

Studies of the laminar distribution of NK3 receptors in the rat spinal cord have indicated that, while such receptors are concentrated in laminae II–III, they are also more widely distributed than any other tachykinin receptor in various laminae, including motor nuclei (Beresford et al. 1992; Linden et al. 2000; Mileusnic et al. 1999a; Seybold et al. 1997). This distribution makes it difficult to identify a certain class of NK3-sensitive neurons as the key element for induction and generation of windup. Nevertheless, such interneurons seem to be in a strategic position to influence the windup mechanism not only at the level of the superficial dorsal horn but also in the proximity of the output elements of the windup response, namely the motoneurons.

High-threshold afferent fibers release glutamate acting on neonatal rat interneurons via \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and NMDA receptors (King and Lopez-Garcia 1993). Excitation produced by glutamate is expected to recruit peptidergic cells to release tachykinins, like substance P or neurokinin B, which are the endogenous agonists for the NK3 receptors present on a variety of interneurons. Although the precise mode of action of NK3 receptors in the spinal cord is currently unclear, it may involve activation of a nonspecific cationic current (Hardwick et al. 1997) and/or block of various conductances (Fisher and Nistri 1993; Inoue et al. 1995; Lepre et al. 1996; Murase et al. 1989) that directly or indirectly lead to complex modulation of glutamate receptors of network interneurons. In particular, it has been noted how NK3 receptor activity can bias glutamatergic transmission toward AMPA/kainate receptor activity and down-regulate an NMDA receptor–mediated one (Cumberbatch et al. 1995). As electron microscopy investigations have shown that in the rat spinal cord NK3 receptors are mainly located inside glomeruli surrounding dendritic spines (Zerari et al. 1997), these receptors indeed appear to possess a strategic location to modulate glutamatergic transmission that takes place chiefly within these structures. NK3 receptors would therefore up-regulate AMPA/kainate receptors in a fashion
similar to the action of other endogenous substances, the main representative of which is the neurotrophin NT-3 (Arvanov et al. 2000). Enhanced glutamatergic activity might then enable nonlinear summation of synaptic inputs during windup. This condition may be subjected to further amplification because NK3 receptors facilitate release of substance P from spinal afferent terminals (Schmid et al. 1998), and endogenous substance P too can apparently facilitate central sensitization (Liu et al. 1997; Urban and Nagy 1997) mediated by glutamatergic transmission (Randic et al. 1993; Svendsen et al. 1998).

Within this scheme, one might account for the reason why, in the present experiments, the windup phenomenon developed despite block of NMDA receptors and was exquisitely sensitive to NK3 receptor block. It has been noted that at least two broad classes of spinal interneuron, separated on the basis of their tonic or phasic firing pattern, are probably implicated in windup (reviewed by Herrero et al. 2000). Perhaps diverse groups of interneurons rely, to a different degree, on mechanisms ranging from tachykinin receptors to NMDA receptors or high-threshold Ca$^{2+}$ channels. This hypothesis is compatible with the present observation that even a severe reduction in windup by the NK3 antagonist could be reversed by stronger stimuli applied to the same segmental DR. This finding may imply that recruitment of additional interneurons (or cellular processes operated by the same class of cells) could compensate for the deficit in NK3-mediated activity by using diverse mechanisms like NMDA receptor activity or high-threshold Ca$^{2+}$ currents to restore windup.

This work was supported by grants from Ministero dell’Università and della Ricerca Scientifica e Tecnologica (co-finanziamento) and from Istituto Nazionale di Fisica della Materia.

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