Modulation of excitatory amino acid responses by tachykinins and selective tachykinin receptor agonists in the rat spinal cord

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1 The effects of tachykinins and agonists selective for the three subtypes of neurokinin (NK) receptor have been tested on spinal neuronal responses both to the excitatory amino acids (EAAs) NMDA, AMPA and kainate, and to noxious heat stimuli. The agonists were applied by microiontophoresis in *in vivo* experiments in α -chloralose-anaesthetized, spinalized rats.

2 The NK₁-selective agonist, GR 73632, enhanced responses to all three EAAs similarly, whilst the NK₂-selective agonist, GR64349, reduced responses to AMPA and kainate without affecting those to NMDA, and the NK₃ selective agonist, senktide, enhanced responses to AMPA and kainate.

3 The endogenous ligands substance P (SP) and neurokinin A (NKA) both enhanced responses to NMDA with little effect on responses to kainate, whereas neurokinin B (NKB) selectively enhanced responses to kainate without affecting those to NMDA.

4 The effects of GR73632 on EAA responses showed some differences between the dorsal and ventral horn, with more selectivity towards enhancement of NMDA responses in the ventral horn, but a smaller maximum effect.

5 Background activity was significantly enhanced by GR73632, GR64349, SP and NKA but not by senktide or NKB. GR73632 had the greatest effect on background firing, but this action was variable between cells and was related both to the location within the spinal cord and to the degree of spontaneous activity prior to GR73632 administration.

6 Responses to noxious heat were enhanced consistently only by NKA.

7 These data show that selective agonists for the tachykinin receptors are capable of modulating EAA responses differentially. SP, NKA and NKB appear to act via more than one receptor type when modulating EAA responses *in vivo*. This indicates that NK-EAA interactions can be more specific than suggested hitherto, with the combined actions at NK₁ and NK₂ receptors biasing EAA responsiveness towards NMDA receptor mediated functions, whereas NK₃ receptor activation would have the opposite effect. The physiological role of such interactions is likely to be complex.

Keywords: Tachykinins; NK₁ receptors; NK₂ receptors; NK₃ receptors; substance P; neurokinin A; neurokinin B; NMDA; AMPA; kainate; excitatory amino acids; nociceptive; spinal cord

Introduction

The neurokinins (NKs; or tachykinins) represent a family of neuropeptides that is thought to be important in the transmission of nociceptive information and the induction/mediation of hyperalgesic changes in the spinal dorsal horn. In previous studies, intrathecal substance P (SP) induced behavioural responses indicative of pain (Hylden & Wilcox, 1981) and reduced tail flick latencies to noxious stimuli (Cridland & Henry, 1986); more recently, similar results have been obtained with agonists selective for the NK₁ and NK₂ receptor subtypes (Picard *et al.*, 1993). These results are consistent with electrophysiological data, which show that SP and related NKs can excite spinal neurones both *in vitro* (Konishi & Otsuka, 1974; Fisher *et al.*, 1994) and *in vivo* (Henry *et al.*, 1975; Fleetwood-Walker *et al.*, 1990; 1993). Substance P and neurokinin A (NKA) have been found in the nerve terminals of primary afferent fibres (Dalsgaard *et al.*, 1985), and in many of these they are co-localized with excitatory amino acids (EAA) (De Biasi & Rustioni, 1988; Merighi *et al.*, 1991). Both NKs and EAAs can be released into the spinal cord following noxious peripheral stimulation (Duggan *et al.*, 1988; 1990; Skilling *et al.*, 1988).

Although there is no direct evidence for co-release of EAAs and NKs, there is considerable evidence for interactions between the two neurotransmitter systems. NKs have been

shown to modulate both the release of endogenous EAAs (Kangrga *et al.*, 1990) and the postsynaptic responses to EAAs in spinal neurones *in vitro* (Randic *et al.*, 1990) and *in vivo* (Dougherty & Willis, 1990); SP can selectively enhance responses to N-methyl-D-aspartate (NMDA) (Dougherty & Willis, 1991; Rusin *et al.*, 1993). These various effects are however rather variable. Because this may relate to the lack of selectivity of the agonists used, we have now investigated the interaction between NK and EAA agonists on spinal neurones *in vivo*, and have compared the actions of selective and naturally occurring tachykinins. The effects of the various NKs on responses to nociceptive stimuli were tested in parallel. Some of these data have been presented previously in abstract form (Cumberbatch *et al.*, 1994a).

Methods

Animal preparation

Experiments were performed on 75 male rats (295–365 g). Anaesthesia was induced and maintained with halothane (1–2% in O₂ for maintenance) for the duration of the surgery. This involved cannulating the trachea, carotid artery and jugular vein, followed by a laminectomy from L2 to T8 and spinalization at T9 to T10. The animal was immobilised in a recording frame and was transferred to α -chloralose anaesthesia (50 mg kg⁻¹ initially followed by an infusion at

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approximately $10 \text{ mg kg}^{-1} \text{ h}^{-1}$, i.v.). The animals usually breathed spontaneously (O_2 enriched air), but in some experiments they were ventilated after paralysis with pancuronium ($1.5 \text{ mg kg}^{-1} \text{ h}^{-1}$, i.v.). In these animals end tidal CO_2 was monitored and kept close to pre-paralysis levels; the adequacy of anaesthesia was ensured by maintaining the same anaesthetic regime and by monitoring the cardiovascular responses to noxious stimuli applied rostral to the level of spinalization. Fluid therapy (isotonic saline and/or Haemaccel, Hoechst) was given at a rate of approximately $3 \text{ ml kg}^{-1} \text{ h}^{-1}$. Blood pressure was monitored throughout and systolic values remained above 90 mmHg . Core temperature was maintained close to 37°C (range 36.0 – 37.5°C).

Electrophysiology and drug administration

A seven barrelled micropipette was inserted into the spinal cord (L5–L6 segment) and the central barrel was used to record extracellularly from single dorsal or ventral horn neurones. Five of the outer barrels were used to administer drugs by microiontophoresis (Table 1). GR73632 and GR64349 (Hagan *et al.*, 1989) were gifts from Glaxo Research and Development; other NKs were obtained from Sigma and the EAAs from Tocris Neuramin. Retaining currents were applied to the outer barrels to prevent leakage of drug solutions; a standard 0.5 V was used to compensate for variability in electrode barrel resistance. A sixth barrel (200 mM NaCl) was used for automated current balancing. Because of the limitation of 5 drug barrels, only 2 NKs could be tested on responses to all 3 EAAs, or alternatively, 3 NKs could be tested on responses to 2 EAAs; the 3×3 way comparison was not possible on individual cells.

NMDA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate were ejected iontophoretically and all three excited the majority of neurones tested (Headley & Grillner, 1990). Neurones were selected that responded to all 3 agonists but that did not respond to Na^+ or Cl^- currents of at least 100 nA . NMDA, AMPA and kainate were then ejected in regular cycles of 4–5 min (mean ejection currents: NMDA 27 nA , AMPA 12 nA , kainate 21 nA). The peak firing rates (25 – 75 spikes s^{-1}) were matched for each EAA on individual cells and were always submaximal. With some cells noxious heat was also applied to the hind paw receptive field using a contact heat device with a ramp (5 s) from 35°C to the test temperature of 45.2 – 49.9°C (held for 10–15 s). Extracellular action potentials were recorded and displayed both as spikes per second (spikes s^{-1}) and as counts over the stimulus application. Any discharge between stimulus applications was termed background activity; this background baseline was subsequently subtracted from all evoked responses.

Once a series of stable response cycles was obtained a NK agonist was ejected in a cumulative current-doubling regime (5 – 80 nA) until either a clear effect was seen or, if no effect, until the maximum current of 80 nA was reached. Each

current was ejected over at least 2 stimulus cycles (i.e. at least 6–8 min) and drug effects were measured for all parameters within the same cycle. Drug effects were expressed either as percentages of control (where control was the mean of the last three pre-test responses, based on spike counts), or as the change in the number of EAA-evoked spikes. The time to half recovery (t_1) following the termination of the drug application was also calculated; since each stimulus was necessarily at a different point in the 3–4 min cycle, the resolution of these t_1 values is limited. Statistical analysis was performed on untransformed data using either the Wilcoxon matched pairs test or the Mann-Whitney U-test. Comparisons were made either between test and the three pre-test control counts (* in figures) or, between the effects of a NK on different EAA responses (#). A probability level of <0.05 was deemed significant.

Results

Effects of selective tachykinin receptor agonists on responses to excitatory amino acids

The NK₁ agonist, GR73632, was tested upon 61 neurones from the dorsal and ventral horns (5 – 80 nA , mean maximum current 14 nA). Figure 1a illustrates that with cells on which NMDA, AMPA and kainate were cycled together, GR 73632 enhanced responses to all three EAAs to a similar degree. This effect was consistent and, on the 11 cells that were tested with more than one ejection current, the effect was current-dependent. The population data (Figure 2a) show that responses to all three EAAs were enhanced significantly, although there was a significantly greater enhancement of responses to NMDA than of those to AMPA. There was no difference between the effects of GR73632 on responses to each EAA on dorsal vs. ventral horn neurones. However, in the ventral but not dorsal horn, there was a significantly greater enhancement of responses to NMDA relative to those to both AMPA or kainate (Figure 3a). The effects of GR73632 on EAA responses did not correlate with the change in the background activity. Responses remained enhanced for some time after cessation of ejection (mean time of half recovery (t_1) was 10 min, range 3–32 min).

The NK₂ agonist, GR64349, was tested on 51 neurones from the dorsal and ventral horns (5 – 80 nA , mean maximum 30 nA) and had effects markedly different from those of the NK₁ agonist. Figure 1b shows for one neurone that GR64349 significantly depressed responses to kainate more than AMPA, with little effect on responses to NMDA. This was consistent for all cells ($n=18$; Figure 2a), and was current-dependent on 16 of the 17 units tested. Responses recovered rapidly (t_1 mean 5 min, range 3–16 min). When analysed separately on dorsal and ventral horn neurones, GR64349 did not affect responses to NMDA on dorsal horn neurones, whereas these responses were slightly enhanced with ventral horn neurones (Figure 3b).

Table 1 The neurokinin and excitatory amino acid compounds used in this study

Drug	Receptor	(mM)	pH	Ejected as	Full name
GR73632	NK ₁	1/50	4.5–5.0	cation	δ -Ava-Phe-Phe-Pro-Leu(Me)-Met.NH ₂
Substance P	NK ₁	1/50	4.5–5.0	cation	Arg-Pro-Lys-Pro-Gln-Glu-Phe-Phe-Gly-Leu-Met.NH ₂
GR64349	NK ₂	1/50	4.5–5.0	cation	Lys-Asp-Ser-Phe-Val-Rlactam-Met.NH ₂
Neurokinin A	NK ₂	1/50	4.5–5.0	cation	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met.NH ₂
Senktide	NK ₃	1/50	8.0–8.5	anion	Succinyl-Asp-Phe-MePhe-Gly-Leu-Met.NH ₂
Neurokinin B	NK ₃	1/50	8.0–8.5	anion	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met.NH ₂
NMDA	NMDA	100/100	7.5–8.0	anion	<i>N</i> -methyl-D-aspartic acid
AMPA	AMPA	5/200	7.5–8.0	anion	(RS)- α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
Kainic acid	Kainate	5/200	7.5–8.0	anion	Kainic acid

mM refers to the concentration of the drug in a solution of 50, 100 or 200 mM NaCl. The pH of solutions was adjusted with HCl or NaOH.

Responses to AMPA were affected variably on ventral horn neurones, whereas responses to kainate were reduced similarly with both cell groups.

The NK₃ agonist, senktide, was tested on 19 neurones from the dorsal horn (5–40 nA, mean maximum 16 nA) and had effects essentially opposite to those of the NK₂ agonist. Figure 1 illustrates the effect of senktide on the responses of one neurone. Responses to AMPA and kainate were selectively enhanced compared to those to NMDA. This effect was con-

sistent as shown by the pooled data in Figure 2a. AMPA and kainate responses recovered rapidly (t_1 mean 1 min, maximum 4 min). Senktide was not tested on ventral horn neurones.

Effects of naturally occurring tachykinins on responses to NMDA and kainate

The NK₁ preferring tachykinin, substance P (SP), was tested upon 17 neurones from the dorsal and ventral horns (5–

Figure 1 Single cell records of the effects of neurokinins on the responses of spinal wide dynamic range neurones to the excitatory amino acid agonists NMDA (N), AMPA (A) and kainate (K). The amino acids were ejected iontophoretically in regular cycles and ejection currents were adjusted to elicit similar amplitude, submaximal, responses. Thereafter a selective neurokinin (NK) agonist was ejected over 2 cycles at each current tested: (a) the NK₁ agonist, GR73632, (b) the NK₂ agonist, GR64349 and (c) the NK₃ agonist, senktide. Each NK agonist had a distinctive effect upon the excitatory amino acid responses. Data from 3 neurones in the ventral (a) or dorsal horns (b,c) of 3 α -chloralose-anaesthetized, spinalized rats.

40 nA, mean maximum 17 nA). With 11 of 17 cells, responses to NMDA were enhanced (to $146 \pm 7\%$ control, mean $\pm$ s.e.mean; $P < 0.001$ vs. control) and with the remaining 6 cells were reduced (to $63 \pm 11\%$ control; $P < 0.05$); this variability did not correlate with either location in the spinal cord or background activity. The pooled data show no significant change in the NMDA response (Figure 2b). Responses returned to control values with a mean $t_{1/2}$ of 5 min (range 3–12 min). Responses to kainate were not affected significantly.

The NK₂ preferring tachykinin, neurokinin A (NKA, 5–20 nA, mean maximum 8 nA) had effects similar to SP, but was more consistent. When tested upon 10 neurones from the dorsal horn, responses to NMDA were enhanced significantly (Figure 2b), whilst responses to kainate were relatively unchanged; the difference between NMDA and kainate was significant. Responses to NMDA recovered rapidly ($t_{1/2}$ mean 1 min, maximum 4 min).

Figure 2 Population data of the effects of selective NK agonists (a) and endogenous neurokinins (b) upon the responses of spinal neurones to the excitatory amino acids NMDA (solid columns), AMPA (open columns) and kainate (hatched columns). Data are expressed as the percentages of the mean of three pre-test control responses, after correcting responses by subtracting background activity. (a) Effects of the NK₁ agonist, GR73632 (5–40, mean 9 nA, $n=28$), the NK₂ agonist, GR64349 (5–40, mean 14 nA, $n=18$) and the NK₃ agonist, senktide (5–20, mean 11 nA, $n=8$). (b) Effects of the three endogenous neurokinins, substance P (SP, 5–40, mean 15 nA, $n=17$), neurokinin A (NKA, 5–20, mean 8 nA, $n=9$) and neurokinin B (NKB, 5–40, mean 16 nA, $n=8$). A significant difference from control is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, whilst a significant difference between the effects of a neurokinin on responses to AMPA or kainate vs. NMDA is indicated by # $P < 0.05$, ## $P < 0.01$, ### $P < 0.005$.

The NK₃ preferring tachykinin, neurokinin B (NKB) was tested upon 8 neurones from the dorsal horn (5–40 nA, mean maximum 16 nA) and had effects opposite to those of both SP and NKA; as with senktide, responses to kainate were enhanced significantly (Figure 2b), whilst those to NMDA were less affected. Responses recovered to control values with a mean $t_{1/2}$ of 5 min (range 3–20 min).

The effects of the tachykinins on background activity

The NK₁ and NK₂ agonists, GR73632, GR64349, SP and NKA all enhanced background activity significantly; conversely, the NK₃ agonists, senktide and NKB had little effect (Figure 4a). The selective NK₁ agonist, GR73632, had by far the greatest mean effect, although this hides considerable variability; some cells showed a large increase in the background activity (e.g. Figure 2a in Chizh *et al.*, 1995) whereas others remained quiescent even when the responses to the EAAs were much enhanced. There was some correlation ($r=0.34$; $P < 0.05$) between GR73632-evoked activity and the depth of the neurone relative to the pial surface (Figure 4b); GR73632 evoked an increase of more than 5 spikes s^{-1} with 67% of 18 neurones in the ventral horn compared to only 40% of 43 neurones in the dorsal horn. There was a population of neurones in the deep dorsal horn (21 of the 43 cells, Figure 4b) with which there was little change in the background activity (< 1 spike s^{-1}).

Figure 3 Comparison of the effects of the NK₁ agonist GR73632 (a) and the NK₂ agonist GR64349 (b) upon responses to NMDA (solid columns), AMPA (open columns) and kainate (hatched columns) on dorsal horn neurones (DHN; $n=15$ for GR73632, $n=14-44$ for GR64349) and on ventral horn neurones (VHN; $n=13$ for GR73632, $n=4-7$ for GR64349). A significant difference between control and test counts is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$. A difference between the effects of the NK agonist on AMPA or kainate vs NMDA responses is shown by # $P < 0.05$, ## $P < 0.01$, ### $P < 0.005$.

Figure 4 Effects of neurokinins on background activity of spinal neurones. (a) Spontaneous activity prior to drug administration (stippled columns) and neurokinin evoked activity (hatched columns). Data are presented as mean firing rate determined over a period of 60 s. GR73632 $n = 61$, substance P (SP) $n = 17$, GR64349 $n = 50$, NKA $n = 10$, senktide (Senk) $n = 19$ and NKB $n = 8$. A significant difference between spontaneous and test counts is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. (b) The effect of GR73632 on background activity was correlated with the depth of the electrode relative to the cord surface. To give a clear indication of the cells on which GR73632 did not alter spontaneous activity, data are expressed as a change in the mean firing rate above spontaneous levels (*ie.* evoked firing rate less control firing rate). The slope represents the linear regression ($r = 0.34$; $P < 0.05$). (c) GR73632 evoked activity related to the initial spontaneous activity. The slope indicates the linear regression ($r = 0.55$; $P < 0.001$).

Figure 5 The effects of selective and endogenous neurokinin agonists on responses to noxious heat (stippled columns; 45.5–49.9°C; 15–20 s) on dorsal and ventral horn neurones. These responses were compared to those to NMDA (solid columns) and kainate (hatched columns) on the same neurones. Data are shown for the NK₁ agonist, GR73632 (5–10 nA, mean 6 nA, $n = 9$); the endogenous NK₁ preferring agonist, substance P (SP; 5–20 nA, mean 9 nA, $n = 9$); the NK₂ agonist, GR64349 (5–80 nA, mean 28 nA, $n = 16$); the NK₂-preferring agonist, neurokinin A (NKA; 5–20 nA, mean 9 nA, $n = 7$); the NK₃ agonist senktide (5–20 nA, mean 11 nA, $n = 6$) and the NK₃-preferring agonist, neurokinin B (NKB; 5–40 nA, mean 14 nA, $n = 6$). A significant effect of a neurokinin on nociceptive responses, compared to pre-drug control, is indicated by * $P < 0.05$. See Figure 2 for the significance levels of the effects of the NK agonists on responses to amino acids.

The effect of GR73632 on background activity was also compared with the level of pre-drug spontaneous activity, an indicator of initial cell excitability. Figure 4c indicates that there was some correlation between the two ($r = 0.55$; $P < 0.001$). GR73632 increased the background activity of 76% of the 29 cells that had a level of spontaneous firing higher than 1 spike s⁻¹, but only of 38% of the 32 cells that had very low or no spontaneous activity (< 1 spike s⁻¹).

With the NK₂ and NK₃ agonists there was no correlation between their effects on background activity and either depth or initial spontaneous activity.

The effects of the tachykinins on responses to noxious heat

Only NKA consistently affected responses to noxious heat, enhancing responses to $126 \pm 7\%$ control ($n = 7$, $P < 0.05$, Figure 5). The other endogenous tachykinins and selective agonists had effects that were variable from cell to cell. There was no correlation between the effects of each agonist on heat responses when compared to depth in the cord, pre-agonist spontaneous activity or agonist-evoked background activity. Figure 5 summarizes these data and for comparison shows the effects of the NKs on the responses to NMDA and kainate tested on the same cells.

Discussion

The selective NK receptor agonists clearly caused diverse, receptor-specific modulations of EAA responses in the rat spinal cord, implying that NK receptors are differentially involved in the fine-tuning of EAA receptor-mediated neurotransmission.

Effects of the selective NK agonists on EAA responses

The NK₁ agonist, GR73632, enhanced responses to all three EAAs consistently and to similar degrees, and background activity was also enhanced with many cells. The non-selectivity of the enhancements suggests that they may have been secondary either to a direct depolarizing effect that would sum with other inward currents, or to an increase in membrane

resistance that would increase the effectiveness of such currents. The effects are likely to have been mediated via NK₁ receptors, both because this agonist has been shown to be highly selective (Hagan *et al.*, 1989) and because the effects were blocked by the NK₁ receptor antagonist, GR82334 (Chizh *et al.*, 1994; 1995). However, since the NK₁ and NK₂ agonists were ejected from acidified solutions, the possibility of effects being mediated by proton ejection needs to be considered. Ejection of protons can cause direct excitation, but this is only seen at pH values below about 3 (Curtis & Watkins, 1960) whereas in these experiments the NK₁ and NK₂ agonists were ejected from solutions of pH 4.5–5.0. Protons can also modulate EAA responses directly, preferentially decreasing NMDA responses (Vyklícký *et al.*, 1990; Traynelis & Cull-Candy, 1991). Such effects of protons seem unlikely here, firstly because the similarly acidic NK solutions of GR73632 and GR64369 had differential effects on EAA responses, and secondly because the effects of GR73632 were prevented by ejection of an antagonist from a solution of similar pH (Chizh *et al.*, 1994; 1995).

The magnitude of the effect of GR73632 was variable between individual neurones, and one possibility is that this could relate to the distribution of NK₁ receptors in the spinal cord. Although they occur throughout the dorsal and ventral horns, they are concentrated in the superficial dorsal horn and on motoneurons (Yashpal *et al.*, 1988; Mantyh *et al.*, 1989; Brown *et al.*, 1993; Bleazard *et al.*, 1994). In the dorsal horn there is a relatively uniform distribution of NK₁ receptors over the soma and dendrites, indicating that there are extrasynaptic receptors (Liu *et al.*, 1994) but this is not necessarily the case in the ventral horn. Variable local concentrations of receptors may, therefore, have contributed to the inter-cell variability.

The hypothesis of a primary depolarizing effect of NK₁ agonists is consistent with numerous reports of excitation by substance P (SP). However, if NK₁ receptor activation causes depolarization, the question arises why GR73632 increased the spike firing rate with only about one third of the cells in the present experiments. The most likely explanation is that with many silent cells there was a depolarization that remained subthreshold for evoking spikes; this is consistent with the finding that background activity was enhanced with nearly all cells that were spontaneously active (Figure 4c). It remains possible that some NK₁ receptors did not induce depolarizing currents, which would be consistent with the report that SP can enhance responses to EAAs under voltage-clamp conditions (Randic *et al.*, 1990).

NK receptors have been linked to various second messenger systems. Intracellular Ca²⁺ levels can be enhanced by NKs (Womack *et al.*, 1988) via an inward Ca²⁺ current that may be linked to second messenger systems (Murase *et al.*, 1989). Moreover, SP can enhance responses to NMDA via a mechanism involving protein kinase C and cyclic AMP (Rusin *et al.*, 1992; 1993) and NK₁ receptor activation can selectively enhance inositol phosphate production in primary cultures of spinal cord neurones (Parsons *et al.*, 1993). NK₁ receptors may therefore mediate their effects on EAA responses via more than one mechanism, again contributing to the inter-cell variability.

The NK₂ receptor-selective agonist, GR64349, had effects on EAA responses that were consistently opposite to those of the NK₃ agonist, senktide. Neither agonist had a major effect on background activity, as in a previous study (Fleetwood-Walker *et al.*, 1993). This suggests that, unlike the NK₁ agonist effects, the changes in EAA responses were not secondary to changes in cell excitability, but the mechanism for the interactions cannot be predicted from these experiments. Since the effects reversed rapidly they are likely to involve a change in the EAA receptor or channel kinetics rather than receptor up/down-regulation.

Binding studies indicate that NK₂ and NK₃ receptors are preferentially localized to the superficial dorsal horn (Yashpal *et al.*, 1988; Mantyh *et al.*, 1989) yet the effects observed were consistent throughout the dorsal horn and, where tested, in the

ventral horn. Since GR64349 and senktide had opposite effects, and as they were ejected with opposite polarities from solutions that were acidic and alkaline respectively, the possibility of current or proton artefacts must again be assessed. Firstly all cells were tested for resistance to current at 100 nA (see Methods). Secondly the actions of the NK₂ agonist are not consistent with proton effects (see above) and were in addition reversed on some cells by a NK₂ receptor antagonist (Chizh *et al.*, 1994; 1995). Thirdly the NK₃ solutions were within half a pH unit of extracellular values. These considerations indicate that it is unlikely that current or proton effects were responsible for the actions observed.

Effects of the naturally occurring tachykinins on EAA responses

The effects on EAA responses of the NK₁-preferring endogenous neurokinin, SP, were variable between cells, as reported previously (Dougherty & Willis, 1990; Randic *et al.*, 1990; Rusin *et al.*, 1993). Although SP and NKA are NK₁ and NK₂ receptor-preferring respectively, the selectivities of these agonists are poor compared to those of GR73632 and GR64349 (Hagan *et al.*, 1989). One might expect that, at the high localized concentrations that occur with iontophoresis, there will be little receptor selectivity between NK₁ and NK₂ receptors by either SP or NKA. The combination of an NK₁ mediated enhancement of responses to all three EAAs and an NK₂ mediated depression of kainate and AMPA responses could have resulted in a net selective enhancement of NMDA responses. This was just what was observed in the current experiments with NKA, although the SP effect was more variable and did not reach significance.

This greater variability with SP compared to either NKA or GR73632 could be related to a more rapid peptidase inactivation (Hooper *et al.*, 1985; Theodorsson-Norheim *et al.*, 1987; see also Duggan *et al.*, 1992). Moreover the N-terminal sequence of SP has been demonstrated to modulate responses to EAAs via a non-NK receptor mechanism (Larson & Sun, 1992; Budai *et al.*, 1992). Such non-NK effects were not responsible for the EAA effects of GR73632 since these effects were reversed by a NK₁ antagonist (Chizh *et al.*, 1994; 1995).

NKB had effects similar to those of the selective NK₃ agonist, senktide. However, the lower selectivity between kainate and NMDA responses with NKB vs. senktide suggests that the endogenous ligand was causing some NK₁-mediated effects.

The data therefore suggest that SP, NKA and NKB acted via more than one receptor type under these conditions. Given that the synaptic concentration of these agonists is likely to be high, endogenously released NKs may also have actions via more than one NK receptor. Moreover, NKA and SP are co-localized in some primary afferents (Dalsgaard *et al.*, 1985). Conversely, NKB is not found in primary afferent fibres but is localized instead in ascending fibres and interneurons (Ogawa *et al.*, 1985; Too & Maggio, 1991; Moussaoui *et al.*, 1992). The functional effects of these three endogenous tachykinins may therefore be anatomically related.

Effects of neurokinins on nociceptive responses

Enhancement of NMDA responses might be expected to promote nociceptive responses, since NMDA receptors have been linked with the generation and maintenance of pro-nociceptive states (review by Meller & Gebhart, 1993). On the other hand, AMPA/kainate receptors are involved in mediating both nociceptive and non-nociceptive responses (Dougherty *et al.*, 1992; Cumberbatch *et al.*, 1994b). Alteration of AMPA/kainate responsiveness would therefore be expected to alter both nociceptive and non-nociceptive transmission.

Selective NK₁ agonists administered intrathecally can indeed facilitate nociceptive reflexes in behavioural tests (Picard *et al.*, 1993), but, despite the increase of EAA responses, GR73632 did not enhance nociceptive responses in the spinal

neurones tested in this study. It is possible that intrathecal administration promotes a sufficiently widespread excitation to cause the release of other endogenous neurotransmitters that in turn modulate neurotransmission. Iontophoretic application of NK₁ agonists will have had a far more localized effect and may not even have reached the distal dendrites of the single neurone under study. Under these conditions the effects on synaptic responses may be expected to be much less than following intrathecal administration.

The selective NK₂ agonist, GR64349, depressed AMPA/kainate responses, so that one would predict that it would also have depressed synaptic responses. Previous studies have shown that higher ejection currents of GR64349 did depress responses to noxious heat, although at lower currents the responses were enhanced (Fleetwood-Walker *et al.*, 1993). We saw no evidence for such a bivalent action; GR64349 had no detectable effect on nociceptive responses. With NK₃ agonists, intrathecal administration can cause antinociception and motor flaccidity (Papir-Kricheli *et al.*, 1987). Here senktide enhanced AMPA/kainate responses, but failed to affect nociceptive responses; failure to reach synaptic sites could again underlie this lack of effect. It is therefore difficult to interpret the present findings on NK-AMPA/kainate receptor interactions with respect to physiological mechanisms.

Concerning the endogenous ligands, the preferential NK₂ agonist, NKA selectively enhanced NMDA responses, and also increased responses to noxious heat. These effects are consistent with behavioural experiments showing that intrathecal NKA was pro-nociceptive (Hylden & Wilcox, 1981;

Cridland & Henry, 1986). The lack of effect of SP on nociceptive responses may be for the same reasons as its lesser effect on NMDA responses as discussed above. The absence of effect of NKB on nociceptive responses again correlates with its lack of effect on NMDA responses.

Conclusions

Agonists selective for the NK receptor sub-types are capable of modulating responses to EAAs differentially. Endogenous NKs appear to mediate their effects via more than one type of NK receptor, and possibly via other non-neurokinin mechanisms as well. Release of endogenous tachykinins may alter the balance of NMDA vs. AMPA/kainate receptor mediated transmission. Thus SP/NKA may shift glutamatergic transmission towards NMDA receptors, and this could selectively promote nociceptive responses. Conversely, endogenously released NKB may promote neurotransmission via AMPA/kainate receptors. The physiological relevance of the endogenous modulations of EAA responses and of glutamatergic neurotransmission can only be elucidated with selective NK antagonists. Such studies have been reported previously in brief (Chizh *et al.*, 1994) and are described in more detail in the following paper (Chizh *et al.*, 1995).

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