Endogenous modulation of excitatory amino acid responsiveness by tachykinin NK₁ and NK₂ receptors in the rat spinal cord

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1 The effects of selective tachykinin (neurokinin, NK) NK₁ and NK₂ receptor antagonists have been examined on spinal neurones in α -chloralose anaesthetized, spinalized rats. They were tested for effects on responses both to excitatory amino acids (EAA) and to noxious heat stimuli. They were also tested for their ability to reverse the actions of selective NK agonists.

2 The NK₁-selective antagonists GR82334 (peptide) and CP-99,994 (non-peptide), when applied by microiontophoresis, both reduced responses to kainate > AMPA > NMDA. Intravenous CP-99,994 (3 mg kg⁻¹) also reduced responses to kainate but had inconsistent effects on nociceptive responses.

3 GR82334, applied microiontophoretically, reduced the enhancement by the selective NK₁ agonist, GR73632 of both responses to EAAs and background activity. Systemic CP-99,994 (≤ 10 mg kg⁻¹) failed to reverse the effects of GR73632.

4 The selective peptide NK₂ antagonist, GR103537, had no consistent effects on responses to EAAs when applied by iontophoresis. In contrast, the non-peptide NK₂ antagonist, GR159897, administered systemically (0.5–2 mg kg⁻¹, i.v.) enhanced responses to kainate (but not NMDA); responses to noxious heat were enhanced only weakly.

5 Iontophoretically-administered GR103537 attenuated the effects of the NK₂ agonist GR64349, which selectively reduced responses to kainate compared to those to NMDA. Systemically administered GR159897 (≤ 2 mg kg⁻¹, i.v.) caused little antagonism of the effects of GR64349.

6 The data indicate that under these conditions the non-peptide antagonists are not reliable at reversing the actions of selective NK agonists.

7 These results suggest that there is a tonic release of endogenous tachykinins that can modulate glutamatergic neurotransmission in the spinal cord. They provide further support for the hypothesis that release of endogenous NKs acting on NK₁ and NK₂ receptors can promote NMDA receptor mediated glutamatergic transmission.

Keywords: Excitatory amino acids; peptide tachykinin receptor antagonist; non-peptide tachykinin receptor antagonist; NK₁ receptors; NK₂ receptors; spinal cord; dorsal horn

Introduction

Substance P (SP) and neurokinin A (NKA) are tachykinins (or neurokinins, NKs) that are found in the nerve terminals of primary afferent fibres in the dorsal horn (Dalsgaard *et al.*, 1985), many of which also contain the excitatory amino acids (EAAs) glutamate or aspartate (De Biasi & Rustioni, 1988; Merighi *et al.*, 1991). Both these NKs as well as EAAs can be released following noxious peripheral stimulation (Duggan *et al.*, 1988; 1990; Skilling *et al.*, 1988) and there is considerable evidence for an interaction between the two neurotransmitter systems. SP and NKA selectively enhanced responses to N-methyl-D-aspartate (NMDA) in isolated dorsal horn neurones (Rusin *et al.*, 1992). However, SP has also been shown to enhance responses to α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), quisqualate and kainate to varying degrees when tested *in vivo* (Dougherty & Willis, 1990; 1991; Mjelle-Joly *et al.*, 1991). More recently we have shown that agonists that are selective for the NK receptor sub-types produce distinct and consistent effects on EAA responses; the naturally occurring tachykinins SP, NKA and NKB, which are less selective, had mixed NK receptor-mediated effects (see preceding paper, Cumberbatch *et al.*, 1995). Since these experiments involved exogenous application of agonists, it is difficult to interpret the physiological role of such NK-EAA

interactions. It is necessary to examine the effects of NK antagonists on EAA and synaptic responses to assess the level of endogenous modulation.

Combined application of sub-threshold doses of NK and EAA antagonists have been reported to reduce wind-up and reflex facilitation in rats (Xu *et al.*, 1992) and responses in the formalin test in mice (Seguin & Millan, 1994). Furthermore, behavioural studies have shown that selective NK₁ antagonists blocked the pro-nociceptive effects of intrathecal EAAs (Mjelle-Joly *et al.*, 1991). However, since intrathecal application of EAAs is likely to excite a high proportion of neurones in the dorsal horn, many of the effects observed may have been via a secondary release of other endogenous neurotransmitters.

The present study was therefore designed to test for modulation of the responses of spinal neurones to NMDA, AMPA, kainate and noxious heat by endogenously released NKs, and to determine the relative roles of NK₁ and NK₂ receptors in this process. Some of the data have been published previously in abstract form (Chizh *et al.*, 1994).

Methods

Experiments were performed on 31 α -chloralose anaesthetized, spinalized male rats. The methods used for anaesthesia, animal preparation, electrophysiological recording and data analysis have been described fully in the preceding paper (Cumberbatch *et al.*, 1995). The solutions used for the iontophoretic

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administration of EAA and NK agonists were also given. In the present study the following NK antagonist drugs were also used: the peptide NK₁ antagonist GR82334 (Hagan *et al.*, 1991); the non-peptide NK₁ antagonist CP-99,994 (McLean *et al.*, 1993); the peptide NK₂ antagonist GR103537 (Hagan *et al.*, 1993); and the non-peptide NK₂ antagonist GR159897 (Ball *et al.*, 1994). Details of structures and of the solutions used for microiontophoresis are shown in Table 1.

Mean iontophoretic ejection currents used to elicit similar amplitude and sub-maximal EAA responses were NMDA 27 nA, AMPA 12 nA, kainate 21 nA. Iontophoretic administrations of GR159897 were abandoned because of excessive effects on spike configuration. Nociceptive responses were not tested with iontophoretic administrations of NK antagonists because of the uncertainty of drug distribution to the relevant synapses. In some experiments the NK antagonists were tested upon the responses to iontophoretically applied NK₁ or NK₂ selective agonists; in these experiments the agonist was tested once in the absence of antagonist, and, following recovery, again during concurrent ejection of the antagonist.

For the tests with the non-peptide NK antagonists administered systemically, cells were also activated by noxious heat stimuli (45.2–49.9°C) applied to the hind paw receptive field with a contact heat device. Dose-ranges used were CP-99,994 1–3 mg kg⁻¹, and GR159897 0.5–2 mg kg⁻¹, i.v. In tests of the ability of these systemic antagonists to reverse the actions of NK agonists, the agonists were tested immediately after the last cumulative dose of i.v. antagonist.

Data are shown as means ± s.e.mean. Statistical analysis was performed using either the Wilcoxon matched pairs test or the Mann-Whitney U-test. A probability level of <0.05 was assumed to be significant.

Results

Forty five cells were examined in the dorsal horn of the spinal cord (depth from pial surface 360–1360 µm).

Effects of NK₁ receptor antagonists GR82334 and CP-99,994 on EAA and nociceptive responses

The peptide NK₁ antagonist, GR82334, was tested by iontophoresis (10–40 nA, mean 15 nA) with 16 cells from the dorsal horn (11 wide dynamic range (WDR), 3 low threshold mechanoreceptive (LTMR), 1 proprioceptive and 1 with no detectable receptive field). It was ejected during ongoing cycles of matched and submaximal responses to the selective EAA agonists NMDA, AMPA and kainate. Figure 1a illustrates the effects of this NK₁ antagonist on the responses of a single dorsal horn neurone; responses to all three EAAs were reduced, with those to kainate being most affected. The pooled data (Figure 1c) show that this greater reduction of responses to kainate was consistent; the order of reduction was kainate > AMPA > NMDA. The EAA responses recovered fully and approximately in parallel; for kainate the mean time to half recovery (*t*_{1/2}) was 6 min, with a range of 3–22 min. Background activity was not affected by GR82334; the mean

spontaneous activity was 1.6 ± 1.5 spikes s⁻¹ during control and 1.8 ± 1.5 spikes s⁻¹ during ejection of GR82334.

The non-peptide NK₁ antagonist, CP-99,994, was tested by iontophoresis (10–20 nA, mean 11 nA) on 8 cells (7 WDR, 1 LTMR) and had effects very similar to those of GR82334. The example of Figure 1b is on the same cell as the GR82334 test of Figure 1a. These effects of CP-99,994 were consistent, with the order of reduction again being kainate > AMPA > NMDA (Figure 1c). As with GR82334, background activity was not affected by CP-99,994; mean values were 2.8 ± 2.7 spikes s⁻¹ during the control period and 2.7 ± 2.5 spikes s⁻¹ in the presence of CP-99,994. The *t*_{1/2} recovery was 3–8 min.

CP-99,994 was also tested systemically (1–3 mg kg⁻¹, i.v.) on 10 cells (9 WDR, 1 high threshold mechanoreceptive; HTMR). Even high doses had only small effects, reducing responses to kainate at 3 mg kg⁻¹ (to 85 ± 7% control; *P* < 0.05) without affecting responses to NMDA (93 ± 7% control). It was not tested on responses to AMPA. Background activity was reduced slightly (from 3.1 ± 1.1 to 2.0 ± 0.7 spikes s⁻¹) but this was not significant. CP-99,994 was also tested on responses to noxious heat with 7 cells, but these responses were affected very variably (108 ± 17% control; range 52–190% control). The variability could not be correlated with receptive field properties or cell location.

Tests were also performed to assess the ability of the these NK₁ antagonists to reverse the actions of the selective NK₁ agonist, GR73632, which enhanced EAA responses non-selectively (Figure 2; see also preceding paper, Cumberbatch *et al.*, 1995). GR82334 blocked or attenuated this action of GR73632, as is shown for a single cell in Figure 2. Figure 2a shows the direct excitation and enhancement of EAA responses by GR73632 and Figure 2b shows that this effect was attenuated following pretreatment with an 8 min ejection of the antagonist. Note that the GR73632 ejection was twice as long as during the control test. This effect of the antagonist was consistent on 8 cells tested, as is shown in the pooled data of Figure 2c. Similar tests were attempted with CP-99,994, but were abandoned because the higher ejection currents required caused excessive attenuation of spikes.

To control for the possibility that the effects of GR82334 on EAA were due to antagonism of GR73632 that was leaking out of the micropipette (thereby enhancing EAA responses), GR73632 was excluded from the electrodes with 7 cells. GR82334 had the same effect on these cells as on those tested when GR73632 was in the pipette: responses to kainate were reduced to 26 ± 6% control, those to AMPA to 52 ± 6%, and those to NMDA to 74 ± 6% (all *P* < 0.05 vs. control).

CP-99,994 was examined by systemic administration for its ability to antagonize the effects of GR73632. At the very high doses of 3–10 mg kg⁻¹ (*n* = 5) it did not antagonize the effects of GR73632, whether on background activity or on EAA enhancements.

Effects of the NK₂ receptor antagonists GR103537 and GR159897 on EAA and nociceptive responses

The peptide NK₂ antagonist GR103537 was tested by iontophoretic ejection (5–40 nA, mean 21 nA) upon responses to

Table 1 Structures of the neurokinin (NK) antagonists and solutions used for microiontophoresis

Drug	Receptor	(mM)	pH	Ejected as	Full name
GR82334	NK ₁	1/50	4.5–5.0	cation	Trp-Leu-SLac-D-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met.NH ₂
CP-99,994	NK ₁	20/20	4.5–5.0	cation	(+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine
GR103537	NK ₂	1/50	4.5–5.0	cation	CyCO-Gly-Ala-D-Trp-PheRNMe ₂
GR159897	NK ₂	20/20	4.5–5.0	cation	(R)-1-[2-(5-fluoro-1H-indol-3-yl)ethyl]-4-methoxy-4-[(phenylsulphonyl)methyl]-piperidine

mM refers to the concentration of the NK in either 20 mM or 50 mM saline. Equivalent data for the NK and excitatory amino acid agonists used in this study are given in Table 1 of Cumberbatch *et al.*, 1995.

Figure 1 Microiontophoretic tests of the effects of two tachykinin NK₁ receptor antagonists, GR82334 and CP-99,994, on responses to the excitatory amino acids NMDA (N), AMPA (A) and kainate (K) on spinal neurones of α -chloralose anaesthetized, spinalized rats. (a) Original record of the effect of GR82334 on a single neurone. (b) Action of CP-99,994 on the same unit. (c) Pooled data of the actions of GR82334 ($n=11$) and CP-99,994 ($n=7$) on responses to NMDA (solid columns), AMPA (open columns) and kainate (hatched columns). Data are expressed as a percentage of the mean (with s.e.mean) of 3 pre-drug control responses. A significant difference from control is indicated by * $P<0.05$, ** $P<0.01$, *** $P<0.005$. A significant difference between the effects on AMPA/kainate vs. NMDA responses is indicated by # $P<0.05$, ## $P<0.01$.

EAA responses with 11 cells (6 WDR, 2 LTMR and 3 with no detectable receptive field). The effects were variable, with either increases or decreases of responses to all three EAAs; the pooled data show little change (Figure 3b). Background activity remained unchanged; mean values were 2.7 ± 1.9 spikes s⁻¹ during the control period compared to 3.0 ± 2.2 spikes s⁻¹ in the presence of GR103537.

The non-peptide NK₂ receptor antagonist, GR159897, was tested by i.v. administration (0.5 – 2 mg kg⁻¹) on 9 neurones (8 WDR, 1 with no receptive field) from the dorsal horn. Responses to kainate were selectively enhanced in a dose-dependent fashion, whilst responses to NMDA remained unchanged. An example on a single neurone is shown in Figure 3a, whilst the pooled data of Figure 3b demonstrate that this effect was consistent and reached significance at 2 mg kg⁻¹ ($135 \pm 8\%$

control). Background activity remained unchanged, being 2.7 ± 1.1 spikes s⁻¹ during the control period and 2.8 ± 1.2 spikes s⁻¹ after 2 mg kg⁻¹ GR159897. Responses to noxious heat were tested with 6 cells. Responses were increased by the 2 mg kg⁻¹ dose with 5 cells (to a mean $138 \pm 21\%$ control, not significant; range 111–222%) but were depressed in the other cell (to 29% control) giving a mean non-significant increase for all 6 cells of $120 \pm 25\%$ control. EAA responses remained enhanced until the end of the experiment (up to 1 h after the last dose). This is presumably due to slow metabolism of this drug; the $t_{1/2}$ recovery was approximately 90 min in the guinea-pig (Ball et al., 1994).

The NK₂ antagonists were examined over the same dose-range for their ability to antagonize the actions of the NK₂ agonist, GR64349. This agonist consistently reduced responses

Figure 2 (a) The effects of the NK₁ agonist, GR73632, on the background discharge and on responses to NMDA (N), AMPA (A) and kainate (K) of a dorsal horn neurone. (b) Following recovery, a second ejection of GR73632 was made in the presence of the NK₁ antagonist, GR82334. Note that GR82334 ejection started 7 min before GR73632, and that the agonist ejection was twice as long as in (a). (c) Pooled data for the effects of the agonist on responses to NMDA (solid columns) and kainate (hatched columns) in the absence (left) and presence (right) of GR82334 ($n=8$). Control refers to the last three cycles prior to the first ejection of GR73632. A significant effect of the NK₁ agonist vs. control is shown by $*P<0.05$; a significant difference from this effect in the presence of the antagonist is indicated by $\$P<0.05$.

to AMPA and kainate but not NMDA (Figure 4a; see also Cumberbatch *et al.*, 1995). The peptide antagonist GR103537 reduced the actions of GR64349 when tested by iontophoresis on 3 neurones. In the example of Figure 4a GR64349 depressed responses to kainate but not to NMDA and this effect was blocked following 7 min pretreatment with GR103537 (Figure 4b). In contrast, the non-peptide antagonist GR159897 administered systemically ($0.5\text{--}2\text{ mg kg}^{-1}$ i.v., $n=7$) only weakly reversed the actions of GR64349 on EAA responses; kainate responses were $49\pm 3\%$ control with GR64349 alone and $64\pm 7\%$ control following the administration of GR159897 2 mg kg^{-1} (not significantly different).

Discussion

Effectiveness of antagonists at reversing agonist actions

In the preceding paper (Cumberbatch *et al.*, 1995) we showed that NK agonists had selective actions on responses to EAA. NK₁ agonists enhanced EAA responses non-selectively, NK₂

agonists reduced kainate and AMPA but not NMDA responses and NK₃ agonists increased responses to AMPA and kainate. In the present study we have tested the ability of peptide and non-peptide NK₁ and NK₂ antagonists to reverse these actions. NK₃ antagonists were not tested as there was none suitable at the time these experiments were performed. The peptide antagonists GR82334 (NK₁) and GR103537 (NK₂) were effective when administered iontophoretically. The non-peptide antagonists, CP-99,994 and GR159897, could not be tested successfully by iontophoresis because the high ejection currents required altered spike configuration. When given intravenously, they had only weak antagonist actions, even at high doses.

The results indicate that under these conditions the non-peptide antagonists used are not entirely suitable, at the doses tested, for assessing actions of endogenously-released neurokinin agonists. CP-99,994 has been shown to have variable effects *in vivo* (McLean *et al.*, 1993; Jackson *et al.*, 1994) and the non-peptide NK₁ antagonists in general tend to have non-specific effects on neurotransmission (Wang *et al.*, 1994). Concerning GR159897, other reports (Ball *et al.*, 1994;

Figure 3 (a) Effects of cumulative intravenous doses of GR159897, a selective NK₂ receptor antagonist, on responses of a dorsal horn neurone to the excitatory amino acids NMDA (N) and kainate (K) and to noxious heat stimuli (H) applied to the receptive field on the paw. (b) Pooled data for the effects of the NK₂ antagonists, GR159897 and GR103537 on responses to NMDA (solid columns) and kainate (hatched columns). GR159897 was given intravenously (0.5 mg kg⁻¹, *n* = 6; 1 mg kg⁻¹, *n* = 9; 2 mg kg⁻¹, *n* = 7). GR103537 was administered iontophoretically (5–40, mean 20 nA; *n* = 11). A significant change from control is indicated by **P* < 0.05.

Figure 4 (a) Reduction by the NK₂ agonist, GR64349 of responses to kainate (K) but not NMDA (N) on a single spinal neurone. (b) Blockade of these effects, in a subsequent test on the same neurone, by iontophoretic co-ejection of the peptide NK₂ antagonist, GR103537.

Stratton *et al.*, 1994) do indicate that the systemic doses used in the present study should have been adequate for NK₂ antagonism. A likely explanation for the relative weakness of the systemically administered antagonists at reversing the effects of iontophoretically-administered NK agonists is that the focal concentrations of agonists are high relative to the (presumably lower and more uniform) concentration of systemically administered antagonists.

Actions of NK antagonists on responses to EAA

The NK₁ receptor antagonists reduced the responses to kainate > AMPA > NMDA when tested iontophoretically, and CP-99,994 reduced responses to kainate but not NMDA when tested i.v. The simplest explanation is that there was a tonic release of endogenous tachykinins that was facilitating the responses to EAA via an action at NK₁ receptors. Release of tachykinins in the dorsal horn under similar conditions has already been established (Duggan *et al.*, 1988; 1990). The effect was not an artefact due to leakage of GR73632 from the electrodes causing an artificial enhancement of the EAA responses, because when the agonist was omitted from the electrodes the two NK₁ antagonists were still effective. Since GR82334 and CP-99,994 are structurally quite different, and similar (albeit weak) effects were seen with systemic CP-99,994, the effects produced are unlikely to have been artefacts caused by non-NK receptor-mediated mechanisms (see also Cumberbatch *et al.*, 1995).

The NK₂ antagonist, GR159897, caused a small enhancement of kainate responses, an effect opposite to that of the NK₁ agonist, GR64349. This implies that there is also tonic release of endogenous tachykinin(s) acting at NK₂ receptors. The lack of effect of the peptide antagonist GR103537 on EAA responses is hard to explain.

The selective NK₁ agonist GR73632 caused a relatively non-selective enhancement of responses to all 3 EAAs (Cumberbatch *et al.*, 1995), whereas both NK₁ antagonists preferentially depressed responses to kainate. This implies that more than NK₁ receptor antagonism was occurring. Since the effects of the NK₁ antagonists were similar to those of the NK₂ agonist, GR64349 (compare Figures 1a and 4a) it is possible that the antagonism of the NK₁ mediated effects somehow altered the effectiveness of the tonic NK₂ receptor mediated component. There are two possible explanations. Firstly either the agonists or the antagonists may not be selective (but see Hagan *et al.*, 1991; McLean *et al.*, 1993). Secondly NK₁ receptors are more abundant in the dorsal horn than NK₂ receptors (Yashpal *et al.*, 1988; Mantyh *et al.*, 1989; Brown *et al.*, 1993; Bleazard *et al.*, 1994). Synaptically released SP and NKA (which are not particularly selective between NK₁ and NK₂ receptors) will therefore be more likely to act via the more abundant NK₁ receptors. Blocking this NK₁ effect could allow a weaker NK₂-mediated component to become more prominent. We consider this second possibility more likely.

These data therefore suggest a tonic release of tachykinins, and activation of NK₁ and NK₂ receptors, under these conditions. It is, however, possible that this does not reflect normal basal release. The surgery was distant from the receptive fields of the units studied, and there was no detectable inflammation within the receptive field, so that it is unlikely that NKs will have altered the synaptic input onto the neurones studied by peripheral mechanisms. The preparatory surgery is, however, likely to have enhanced general spinal excitability

(see Hartell & Headley, 1991). By analogy with the finding that inflammation-induced spinal hyperexcitability can be blocked by NK antagonists (Birch *et al.*, 1992; Kellstein & Proffitt, 1992), it is likely that spinal release of tachykinins would be enhanced under the conditions of electrophysiological experiments such as these.

Relative lack of effectiveness of NK antagonists on synaptic transmission

Since the NK antagonists modulated EAA responses, one would predict that they would also modify synaptic transmission, whether spontaneous or evoked. Moreover both NK₁ and NK₂ agonists enhanced background activity (Cumberbatch *et al.*, 1995), and endogenously released tachykinins would be expected to have similar actions. The ineffectiveness of the NK antagonists at reducing background activity cannot be explained by a lack of tonic release of endogenous NKs, because EAA responses were clearly affected by the antagonists. It is therefore more likely that the NK antagonists did not reach sufficient concentrations at the active synapses to block transmission/modulation by endogenous NKs. This is entirely likely with iontophoretic applications, which cause only very focal concentrations close to the electrode, and which may therefore not reach the relevant synapses. Systemically administered NK antagonists, on the other hand, should achieve a uniform distribution. Nonetheless, neither i.v. CP-99,994 nor GR159897 significantly reduced background activity at the relatively high doses tested.

Iontophoretically-administered antagonists were not tested on nociceptive responses because of the technical limitations of access to the relevant synaptic sites. Systemically-administered antagonists were examined, but were relatively ineffective and/or inconsistent at altering nociceptive responses evoked by peripheral heat stimuli. CP-99,994 had extremely variable effects on nociceptive responses, with the mean effect being not significant; it was not possible to relate the variations to any experimental variable. GR159897 increased nociceptive responses with 5 of 6 units, consistent with its effect on EAA responses, but this effect did not reach statistical significance. These enhancements by GR159897 differ from the results of another study using NK₂ selective compounds, which indicated that spinal thermal nociceptive responses were depressed by NK₂ antagonists (Fleetwood-Walker *et al.*, 1993).

In conclusion, these data show that there is a tonic release of endogenous tachykinins under the conditions of these electrophysiological experiments. These tachykinins act via both NK₁ and NK₂ receptors to modulate neuronal responses to EAAs acting at kainate, AMPA and NMDA receptors, and these interactions are receptor specific. The data are consistent with the hypothesis, based on NK agonist action (Cumberbatch *et al.*, 1995), that NK₁ and NK₂ receptor activation by endogenous tachykinins will push glutamatergic neurotransmission towards NMDA receptor activity, an action that could promote nociception. Further experiments will be required to establish how this EAA modulation influences synaptic responses to nociceptive and non-nociceptive inputs, both in the normal state and during pathological conditions such as peripheral inflammation, when NK and EAA release may be enhanced.

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