British Journal of Pharmacology (2003) 140, 1389–1398 © 2003 Nature Publishing Group All rights reserved 0007–1188/03 $25.00
www.nature.com/bjp

# Effect of peptidases on the ability of exogenous and endogenous neurokinins to produce neurokinin 1 receptor internalization in the rat spinal cord

*,[1]Juan Carlos G. Marvizón, [1,2]Xueren Wang, [1]Li-Jun Lao & [1]Bingbing Song

[1]Center for Neurovisceral Sciences & Women's Health, CURE: Digestive Diseases Research Center, Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, U.S.A.

**1** The ability of peptidases to restrict neurokinin 1 receptor (NK1R) activation by exogenously applied or endogenously released neurokinins was investigated by measuring NK1R internalization in rat spinal cord slices.

**2** Concentration–response curves for substance P and neurokinin A were obtained in the presence and absence of 10 $\mu$M thiorphan, an inhibitor of neutral endopeptidase (EC 3.4.24.11), plus 10 $\mu$M captopril, an inhibitor of dipeptidyl carboxypeptidase (EC 3.4.15.1). These inhibitors significantly decreased the $EC_{50}$ of substance P to produce NK1R internalization from 32 to 9 nM, and the $EC_{50}$ of neurokinin A from 170 to 60 nM.

**3** Substance P was significantly more potent than neurokinin A, both with and without these peptidase inhibitors.

**4** In the presence of peptidase inhibitors, neurokinin B was 10 times less potent than neurokinin A and 64 times less potent than substance P ($EC_{50} = 573$ nM).

**5** Several aminopeptidase inhibitors (actinonin, amastatin, bacitracin, bestatin and puromycin) failed to further increase the effect of thiorphan plus captopril on the NK1R internalization produced by 10 nM substance P.

**6** Electrical stimulation of the dorsal root produced NK1R internalization by releasing endogenous neurokinins. Thiorphan plus captopril increased NK1R internalization produced by 1 Hz stimulation, but not by 30 Hz stimulation.

**7** Therefore, NEN and DCP restrict NK1R activation by endogenous neurokinins when they are gradually released by low-frequency firing of primary afferents, but become saturated or inhibited when primary afferents fire at a high frequency.
*British Journal of Pharmacology* (2003) **140,** 1389–1398. doi:10.1038/sj.bjp.0705578

**Keywords:** Aminopeptidase; C-fiber; dorsal horn; dipeptidyl carboxypeptidase; EC 3.4.15.1; EC 3.4.24.11; neutral endopeptidase; neurokinin 1 receptor; neurokinin; primary afferent; substance P; tachykinin

**Abbreviations:** AIC, Akaike's Information Criterion; ANOVA, analysis of variance; CI, confidence interval; DCP, dipeptidyl carboxypeptidase, EC 3.4.15.1; IR, immunoreactive; $K^+$-aCSF, aCSF with 5 mM KCl; NEN, neutral endopeptidase, EC 3.4.24.11; NGS, normal goat serum; NK1R, neurokinin 1 receptor; NKA, neurokinin A; NKB, neurokinin B; SP, substance P; sucrose-aCSF, aCSF with 5 mM KCl and 215 mM sucrose instead of NaCl

## Introduction

The neurokinin 1 receptor (NK1R) is one of three receptors that recognize the neurokinins (or tachykinins) substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) (Regoli *et al*., 1994; Routh & Helke, 1995). There is ample evidence that NK1Rs in dorsal horn neurons contribute to the development of hyperalgesia and central sensitization. For example, NK1R antagonists block hyperalgesia (Traub, 1996; Henry *et al*., 1999), and hyperalgesic responses are absent in transgenic mice lacking the NK1R (De Felipe *et al*., 1998; Laird *et al*., 2000; 2001) or after selective elimination of dorsal horn neurons possessing NK1Rs (Mantyh *et al*., 1997).

NK1Rs are present throughout the spinal cord, except in lamina II neurons (Bleazard *et al*., 1994; Liu *et al*., 1994). In particular, many lamina I neurons with NK1Rs send axons to brain regions involved in pain processing, while receiving synapses from SP-containing primary afferents (Todd *et al*., 2002). Therefore, synapses between SP-containing primary afferents and NK1R-containing projection neurons appear to be crucial in determining the intensity of pain signals. Owing to this, mechanisms that control the release of neurokinins and their ability to bind to NK1Rs are extremely important. Indeed, neurokinin release is modulated by a staggering number of presynaptic receptors, including $GABA_B$,

*Author for correspondence at: Veteran Administration Greater Los Angeles Health Center, Bldg. 115, 11301 Wilshire Boulevard, Los Angeles, CA 90073, U.S.A.; E-mail: marvizon@ucla.edu

[2]Current address: Department of Anesthesiology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China.

Advance online publication: 17 November 2003

$\alpha_2$-adrenergic, opioid, cannabinoid 1, vanilloid 1, NMDA and serotonin receptors (for references, see Marvizon *et al*., 2003).

The access of neurokinins to NK1Rs could also be controlled by their degradation by peptidases. In particular, neutral endopeptidase (NEN, EC 3.4.24.11) and dipeptidyl carboxypeptidase (DCP, EC 3.4.15.1) appear to degrade neurokinins in the dorsal horn, because inhibitors of these enzymes increased the amount of released SP detected using antibody microprobes (Duggan *et al*., 1992). Other studies indicated that aminopeptidases (EC 3.4.11.) are also able to degrade SP (Harbeck & Mentlein, 1991; Mauborgne *et al*., 1991). Inhibitors of NEN, DCP and aminopeptidases increased the ability of SP and NKA to produce scratch, bite and licking behavior (Sakurada *et al*., 1990) and ventral root depolarizations (Suzuki *et al*., 1994).

NEN, DCP and aminopeptidases also efficiently degrade opioid peptides (Guyon *et al*., 1979; Chou *et al*., 1984; Yaksh & Chipkin, 1989; Hiranuma *et al*., 1998; Roques, 2000). Indeed, we found that inhibitors of NEN, DEC and aminopeptidase N increased 10 and 100 times, respectively, the potencies of dynorphin A and Leu-enkephalin to produce $\mu$-opioid receptor internalization (Song & Marvizon, 2003). It has been suggested that peptidase inhibitors could be used to treat pain, as they have analgesic effects (Noble *et al*., 1992b) and do not produce tolerance and addiction (Noble *et al*., 1992a, c; Roques, 2000). However, if peptidase inhibitors also protect neurokinins against degradation, this would produce pronociceptive effects and even long-term hyperalgesia that would compromise any analgesic effects. Therefore, it is necessary to compare the relative efficacies of peptidase inhibitors in protecting the degradation of neurokinins and opioids.

The main goal of this study was to evaluate the ability of peptidases to prevent the activation of NK1 receptors by neurokinins, either exogenously applied or endogenously released. NK1R activation was measured by their internalization, a technique validated in numerous studies (Mantyh *et al*., 1995; Allen *et al*., 1997; Liu *et al*., 1997; Marvizon *et al*., 1997; 1999a; 2003; Cao *et al*., 1998; Honore *et al*., 1999; Riley *et al*., 2001). An additional goal of this study was to compare the potencies of SP, NKA and NKB to produce NK1R internalization. We used spinal cord slices, a high-quality preparation developed for electrophysiology (Yoshimura & Jessell, 1990), that contains functional synapses between dorsal horn neurons and primary afferent terminals (Randic *et al*., 1993; Yoshimura & Nishi, 1996; Marvizon *et al*., 1997; Sandkuhler *et al*., 1997).

## Methods

### *Chemicals*

Isoflurane was from Halocarbon Laboratories, River Edge, NJ, U.S.A. Normal goat serum (NGS) was from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, U.S.A. SP was from Tocris (Ellisville, MO, U.S.A.). Other chemicals were purchased from Sigma/RBI (St Louis, MO, U.S.A.).

### *Media for slices*

Artificial cerebrospinal fluid (aCSF) contained (in mM) 124 NaCl, 1.9 KCl, 26 $NaHCO_3$, 1.2 $KH_2PO_4$, 1.3 $MgSO_4$, 2.4 $CaCl_2$ and 10 glucose. $K^+$-aCSF contained 5 mM KCl. Sucrose-aCSF was aCSF in which NaCl was iso-osmotically replaced by sucrose (215 mM) and containing 5 mM KCl. These media were constantly bubbled with 95% $O_2$/5% $CO_2$.

### *Preparation of spinal cord slices*

All animal procedures were approved by the Chancellor's Animal Research Committee at UCLA, and conform to NIH guidelines. Efforts were made to minimize the number of animals used and their suffering. Slices were prepared as previously described (Randic *et al*., 1993; Marvizon *et al*., 1997; 1999a, b; Sandkuhler *et al*., 1997). Briefly, Sprague–Dawley rats 3–4-weeks old (Harlan, Indianapolis, IND) were anesthetized with isoflurane. A lumbar segment of the spinal cord was rapidly extracted, placed in ice-cold sucrose-aCSF, and cleaned of dura mater and ventral roots. The spinal cord segment was glued vertically to a block of agar in the stage of a Vibratome (Technical Products International, St Louis, MO, U.S.A.), and coronal spinal cord slices (400 $\mu$m) were cut in ice-cold sucrose-aCSF, using minimum forward speed and maximum vibration amplitude. Up to six slices were obtained from each animal in the L1–L4 region. To prepare slices with dorsal roots, the selected roots were lightly pulled to separate them from the spinal cord. The blade of the Vibratome was aimed at the entrance of the root with the help of a stereo microscope. Fiber continuity between the root and the dorsal funiculus was assessed, and only slices with more than 80% of the dorsal funiculus continuous with the root were used. After cutting, slices were kept for 1 h in $K^+$-aCSF at 35°C, and then transferred to aCSF.

### *Slice treatment*

For drug application, slices were placed in a nylon net suspended halfway inside a small beaker covered with aluminum foil, and incubated for 10 min at 35°C in aCSF (5–20 ml) constantly bubbled with 95% $O_2$/5% $CO_2$. In some experiments, 10 $\mu$M thiorphan and 10 $\mu$M captopril were added to the aCSF. The incubation was ended by placing the slices in cold fixative. Slices were used and fixed within 3 h of preparing them.

### *Electrical stimulation of the dorsal root*

One dorsal root contiguous with the slice was stimulated using previously established procedures (Malcangio *et al*., 1997; Marvizon *et al*., 1997; 1999a; Lever & Malcangio, 2002) with some modifications. Slices were placed in a custom-made slice chamber (a gift from Dr Marzia Malcangio, King's College of London, London, England) and superfused with aCSF at 35°C bubbled with 95% $O_2$/5% $CO_2$ at a rate of 4 ml $min^{-1}$, starting 5 min before the stimulation. In some experiments, aCSF contained 10 $\mu$M thiorphan and 10 $\mu$M captopril. The electrode consisted of two platinum wires (diameter 0.5 mm, separation 1 mm) located in a side compartment separated from the superfusion chamber by a movable partition. The dorsal root was drawn into the electrode compartment through a hole in the partition (sealed with vacuum grease) and placed on top of the platinum wires. The electrode compartment was then emptied of aCSF and filled with mineral oil. Contact between the root and the electrode wires was monitored with a stereo

microscope. Electrical stimulation was generated by a Master-8 stimulator and an Iso-Flex stimulus isolating unit (A.M.P. Instruments, Jerusalem, Israel), and consisted of square pulses of 20 V and 0.4 ms. Slices were kept in the chamber for 10 min after stimulation and then fixed. The side of the slice ipsilateral to the stimulated root was marked by punching a round hole in the ventral horn.

### *Immunohistochemistry in spinal cord slices*

Histological sections from spinal cord slices were prepared and labeled as described (Marvizon *et al*., 1997; 1999a; 2003). Briefly, the slices were fixed, cryoprotected, frozen on dry ice, and sectioned with a cryostat at 25 $\mu$m in the coronal plane. Free-floating sections were incubated overnight at room temperature with a 1 : 3000 dilution of a rabbit antiserum recognizing the NK1 receptor (#94168, a gift from Dr Nigel Bunnett, UCSF), previously characterized (Grady *et al*., 1996). The secondary antibody was Alexa-488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, U.S.A.), diluted 1 : 2000. Incubation with the secondary antibody was 2 h at room temperature.

### *Confocal microscopy and image processing*

Confocal images were acquired at UCLA's Carol Moss Spivak Cell Imaging Facility, using a Leica TCS-SP scanning confocal microscope and an argon (488 nm) laser. The pinhole was 1.0 Airy units, and the objective $\times 100$, with a numerical aperture of 1.4, resulting in an optical section thickness (full-width at half-maximum) of 0.62 $\mu$m. For each image, stacks of optical sections were obtained at intervals of 0.57 $\mu$m, averaging each optical section up to six times to reduce the noise. One image was obtained with a $\times 40$ objective (0.75 numerical aperture) with an optical section thickness of 2.16 $\mu$m and optical section separation of 1.79 $\mu$m. Images were processed in Adobe Photoshop 5.5., using the 'curves' feature of the program to adjust the contrast. Images were acquired at a digital size of $1024 \times 1024$ pixels, and were later cropped to the relevant part of the field without altering the original image resolution.

### *Quantification of NK1R internalization*

The amount of NK1R internalization was quantified using a standard method (Mantyh *et al*., 1995; Abbadie *et al*., 1997; Honore *et al*., 1999; Schwei *et al*., 1999; Trafton *et al*., 1999; 2001) with minor modifications (Marvizon *et al*., 1997; 1999a). Briefly, we determined the percentage of NK1R immunoreactive neurons in lamina I that show internalization in relation to the total number of NK1R neurons sampled. The person counting the neurons was unaware of the treatment. A Zeiss Axiovert 135 (Carl Zeiss, Inc., Thornwood, NY, U.S.A.) fluorescence microscope fitted with a $\times 100$ objective was used to count neurons. Neuronal somas with 10 or more endosomes were considered to have internalized receptors. At least three sections per slice were used, and all NK1R cells in lamina I in each section were counted.

### *Data analysis*

Treatments were randomized between slices, and no more than two slices from the same animal received the same treatment. Data were analyzed using Prism 4.0 for Windows (GraphPad Software, San Diego, CA, U.S.A., www.graphpad.com). In concentration–response experiments, a sigmoidal dose–response function,

$$Y = \text{bottom} + (\text{top-bottom})/(1 + 10\hat{}(\log \text{EC}_{50} - \log[X]))$$

was fitted to the data points by nonlinear regression. The 'top' and 'bottom' parameters were constrained to values $<100\%$ and $>0\%$, respectively. The statistical error associated with the calculated $\text{EC}_{50}$ (effective concentration for 50% of the maximum response) was expressed as 95% confidence interval (95% CI). To determine whether there were significant differences between $\text{EC}_{50}$ values, the data of two concentration–responses (for example, presence vs absence of peptidase inhibitors, or SP vs NKA) were fitted simultaneously to this function ('global fitting'). Akaike's Information Criterion (AIC) (Motulsky & Christopoulos, 2003) was used to calculate the probability ('Akaike's weight') that the $\log \text{EC}_{50}$ was the same or different for the two concentration–responses. The 'evidence ratio' is defined as the ratio between the probabilities of the two models (i.e., '$\text{EC}_{50}$ is the same' and '$\text{EC}_{50}$ is different'), and roughly indicates how many times one model is more likely to be correct than the other. For other statistical analyses, we used one-way or two-way ANOVA with Bonferroni's post-test; statistical significance was set at 0.05.

## Results

In untreated spinal cord slices, the NK1R was located at the cell surface in the majority of dorsal horn neurons (Marvizon *et al*., 1997; 1999a; 2003). For example, Figure 1a shows several lamina I neurons with NK1Rs in their surface. The localization of NK1R at the cell surface can be appreciated in more detail in Figure 1b, showing a high-magnification confocal image of a large lamina I neuron. In contrast, incubating spinal cord slices with SP produced the internalization of NK1Rs in neurons throughout the dorsal horn (Marvizon *et al*., 1997). For example, Figure 1d shows a lamina I neuron with NK1R internalization in a slice incubated in 100 nM SP. The number of lamina I neurons with NK1R internalization increased with the concentration of SP, following a sigmoidal concentration–response curve (Figure 2a).

It is possible, however, that the ability of SP and NKA to bind to the NK1R to produce its internalization is restricted by their degradation by peptidases present in the spinal cord. To investigate to what extent this occurs, we incubated spinal cord slices for 10 min with several concentrations of SP or NKA in the presence and absence of peptidase inhibitors, and measured the amount of NK1R internalization in lamina I neurons (Figure 2). The peptidase inhibitors used were thiorphan, an NEN inhibitor, and captopril, a DCP inhibitor. Inhibitors of NEN and DCP have been found to increase the release of SP (Duggan *et al*., 1992), and are routinely used to protect released SP against degradation (Malcangio & Bowery, 1993; 1994; Malcangio *et al*., 1997; Lever & Malcangio, 2002). Thiorphan and captopril were both used at 10 $\mu$M, a concentration at which they completely protect opioid peptides against degradation (Suzuki *et al*., 1997; Hiranuma *et al*., 1998; Song & Marvizon, 2003) and increase responses to SP and NKA in the spinal cord (Suzuki *et al*., 1994). Although some

of the SP or the NKA may have reached the peptidases before they were blocked by the inhibitors, this would not have affected our results, given the presence of a large supply of neurokinins outside the slices and more than enough time (10 min) for NK1R internalization to occur: it is completed in about 2 min (Wang & Marvizon, 2002). The peptidase inhibitors by themselves did not produce NK1R internalization, as can be observed in the cluster of neurons in lamina I shown in Figure 1c, and in measurements in the absence of SP ('0 M') in Figure 2a.

The peptidase inhibitors produced a left shift of the concentration–response curves of both SP and NKA (Figure 2). Thus, the $EC_{50}$ for SP decreased from a value of 32 nM (95% CI 19–55 nM) in the absence of peptidase

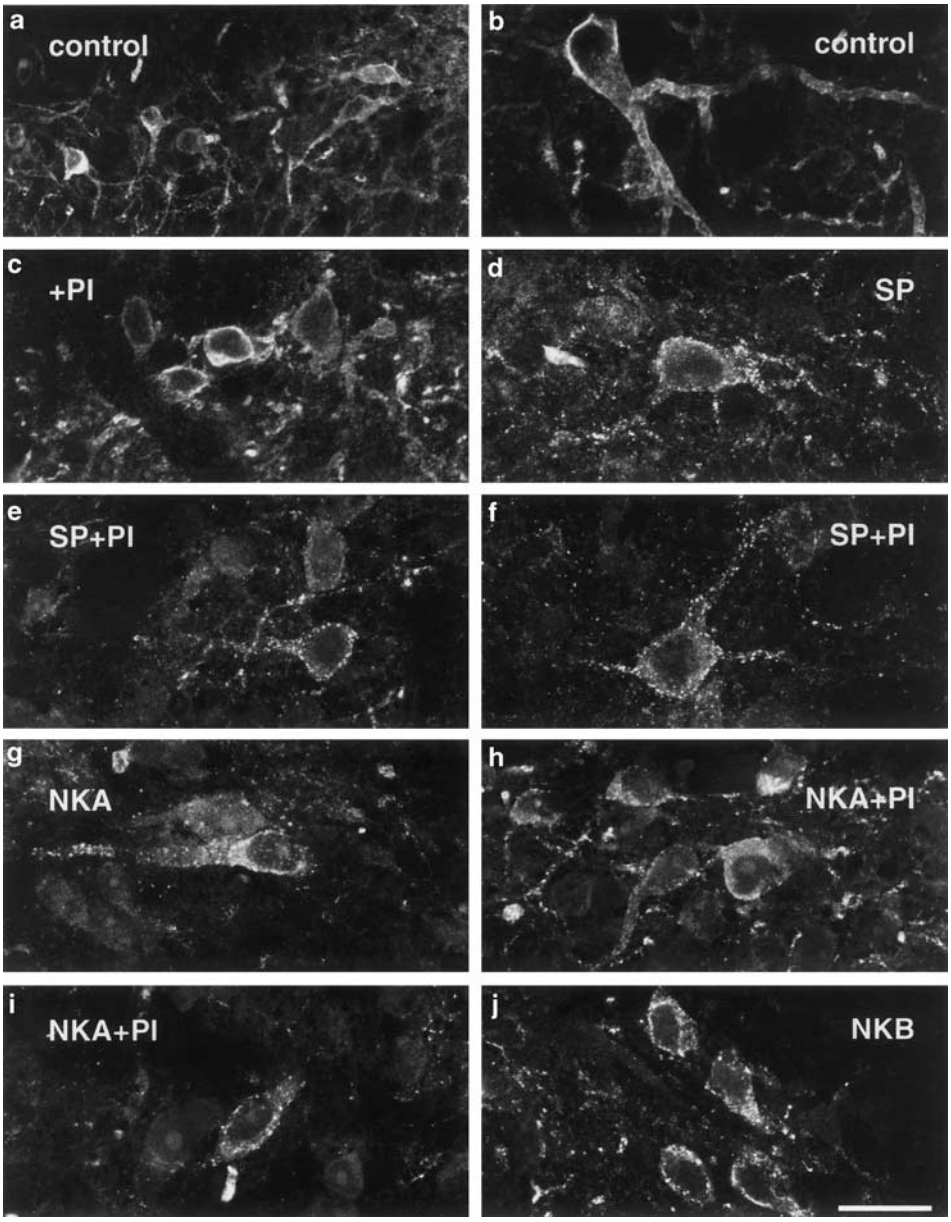


**Figure 1** Confocal images of NK1R neurons after incubation with neurokinins. The peptidase inhibitors (PI) were 10 $\mu$M thiorphan and captopril. The dorsal side is up for all panels. (a) Slice incubated in aCSF (control); this low-magnification image ($\times 40$, scale bar is 50 $\mu$m, two optical sections separated by 1.79 $\mu$m) shows six NK1R neurons in central lamina I without NK1R internalization. Images in the rest of the panels were taken at $\times 100$ (scale bar is 20 $\mu$m), and consist of two optical sections (three for b, c, h, four for g) separated 0.57 $\mu$m. (b) Slice incubated in aCSF (control); large lamina I neuron showing no NK1R internalization. (c) Slice incubated with peptidase inhibitors; these four neurons in lamina I show no NK1R internalization. (d) Slice incubated with 100 nM SP and no peptidase inhibitors; lamina I neuron showing NK1R internalization. (e) Slice incubated with 100 nM SP and peptidase inhibitors; two lamina I neurons show NK1R internalization. (f) Slice incubated with 100 nM SP and peptidase inhibitors; lamina IV neuron showing NK1R internalization. (g) Slice incubated with 100 nM NKA and no peptidase inhibitors; lamina I neuron showing NK1R internalization. (h) Slice incubated with 100 nM NKA and peptidase inhibitors; of these five neurons in lamina I, four show NK1R internalization. (i) Slice incubated with 100 nM NKA and peptidase inhibitors; lamina IV neuron showing NK1R internalization. (j) Slice incubated with 1 $\mu$M NKB and peptidase inhibitors; four lamina I neurons show NK1R internalization.

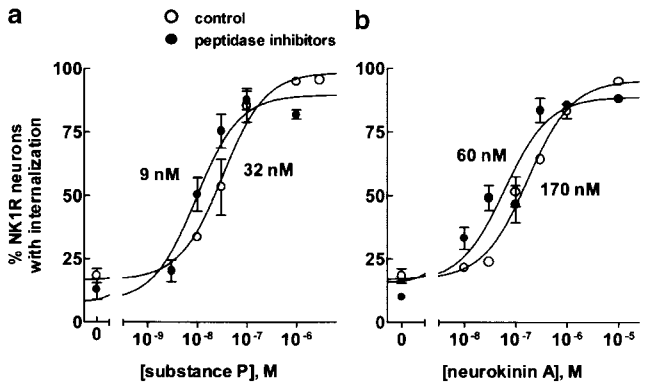


**Figure 2** Effect of peptidase inhibitors on the potencies of SP and NKA to produce NK1R internalization. Slices were incubated for 10 min at 35°C with different concentrations of SP (a) or NKA (b) in the absence (control) or presence of the peptidase inhibitors thiorphan and captopril (10 $\mu$M). Points are the mean ± s.e.m. of 3–4 slices. Curves were obtained by fitting a sigmoidal dose–response function to the data, and the calculated $EC_{50}$ values are given next to the corresponding curve.

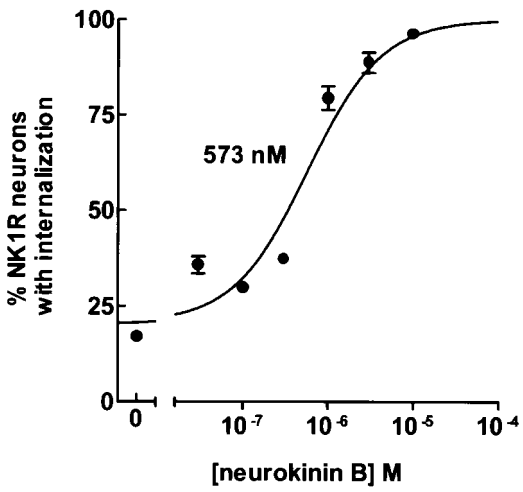


**Figure 3** Concentration–response of NKB to produce NK1R internalization. Slices were incubated for 10 min at 35°C with different concentrations of NKB in the presence of thiorphan and captopril (10 $\mu$M). Points are the mean ± s.e.m. of three slices. The curve was obtained by fitting a dose–response function to the data, and the calculated $EC_{50}$ value is given next to the curve.

inhibitors to 9 nM (95% CI 5–18 nM) in their presence. Similarly, the $EC_{50}$ for NKA decreased from 170 nM (95% CI 118–246 nM) in the absence of peptidase inhibitors to 60 nM (95% CI 29–125 nM) in their presence. To determine whether the differences between these $EC_{50}$ values were statistically significant, we performed a 'global fitting' of the data in each panel to a dose–response function (see 'Data analysis') and used the AIC to calculate the probability that the $\log EC_{50}$ were different in the absence and presence of peptidase inhibitors. The probabilities (Akaike's weights) that the $\log EC_{50}$ were different were 99% in the case of SP (evidence ratio of 97.3), and 80% in the case of NKA (evidence ratio of 4.1), showing that the peptidase inhibitors did increase the potencies of SP and NKA to produce NK1R internalization. This, in turn, indicates that a certain amount of the SP and NKA entering the slices was degraded by peptidases when peptidase inhibitors were not present.

Since a previous study (Trafton *et al*., 2001) reported that the potencies of SP and NKA to produce NK1R internalization were the same, we also used the AIC to compare the $\log EC_{50}$ of SP and NKA. The probabilities that their $\log EC_{50}$ were different were >99.99% in the absence of peptidase inhibitors, and 99.4% (evidence ratio of 154) in their presence. Therefore, SP was clearly more potent (five to seven times) than NKA to produce NK1R internalization in our experiments.

Images of NK1R neurons in the slices used for this study are shown in Figure 1. These images were taken from slices treated with 100 nM concentrations of SP or NKA, which produced submaximal amounts NK1R internalization (Figure 2). They show representative lamina I neurons with NK1R internalization after incubating the slices with SP alone (Figure 1d), SP and peptidase inhibitors (Figure 1e), NKA alone (Figure 1g) and NKA and peptidase inhibitors (Figure 1h). Although the quantitative analysis in Figure 2 was limited to lamina I neurons, SP and NKA also produced NK1R internalization in neurons in laminae III and IV. For example, lamina IV neurons with internalization after incubation with SP and peptidase inhibitors, or NKA and peptidase inhibitors, are shown in Figure 1f and i, respectively.

To determine whether neurokinin B (NKB) was able to produce NK1R internalization in dorsal horn neurons, and to compare its potency to those of SP and NKA, we incubated spinal cord slices with different concentrations of NKB (Figure 3). The experiment was done in the presence of 10 $\mu$M thiorphan and captopril to avoid any possible degradation of NKB by peptidases. Relatively high concentrations (10 $\mu$M) of NKB produced NK1R internalization in practically all NK1R neurons in lamina I. Thus, the 'top' parameter of the concentration–response curve for NKB was 100 ± 5% of NK1R cells. The calculated $EC_{50}$ for NKB was 573 nM (95% CI 329–997 nM), indicating that its potency to produce NK1R internalization was 64 times and 10 times lower than the potencies of SP and NKA, respectively. The probability (Akaike's weights) that the $\log EC_{50}$ of NKB was different from that of SP and NKA was >99.99%. Representative neurons showing NK1R internalization in slices incubated with 1 $\mu$M NKB are shown in Figure 1j. Since, in the absence of peptidase inhibitors, the potency of NKB may be still lower, NKB does not appear to contribute substantially to the activation of NK1R in lamina I neurons.

In addition to the DCP and NEN, aminopeptidases could also degrade neurokinins, restricting their ability to activate NK1R. There are several aminopeptidases in the nervous tissue that can potentially degrade neurokinins, including aminopeptidases A (EC 3.4.11.2.), N (EC 3.4.11.7.) and W (EC 3.4.11.16.). Therefore, we screened several aminopeptidase inhibitors for their ability to increase NK1R internalization produced by 10 nM SP (Figure 4). Aminopeptidase inhibitors were tested in the presence of 10 $\mu$M thiorphan and captopril in order to assess any possible effect of aminopeptidases in the absence of NEN and DCP activities, which may compete with aminopeptidases for the peptides. If the aminopeptidase inhibitors produce a decrease in the $EC_{50}$ for SP, this would be reflected in an increase in NK1R internalization elicited by 10 nM SP. Nevertheless, none of the aminopeptidase inhibitors tested increased NK1R internalization produced by SP (Figure 3). Actinonin inhibits aminopeptidase N; bestatin

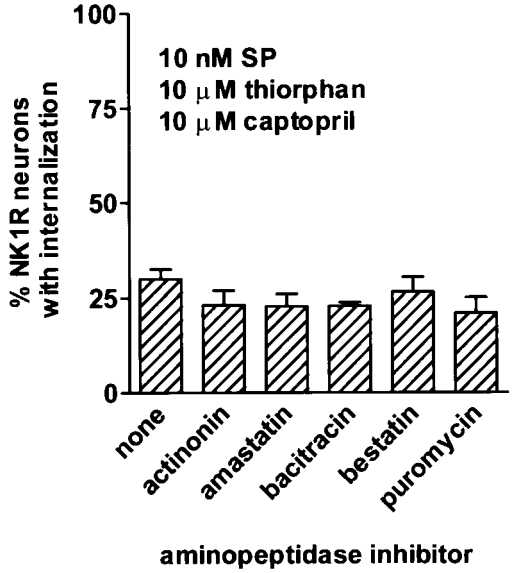


**Figure 4** Lack of the effect of aminopeptidase inhibitors on SP-induced NK1R internalization. Slices were incubated for 10 min at 35°C with 10 nM SP, 10 $\mu$M thiorphan, 10 $\mu$M captopril and the indicated aminopeptidase inhibitors (10 $\mu$M). Bars are the mean $\pm$ s.e.m. of three slices. ANOVA yielded no statistically significant differences ($P>0.05$).

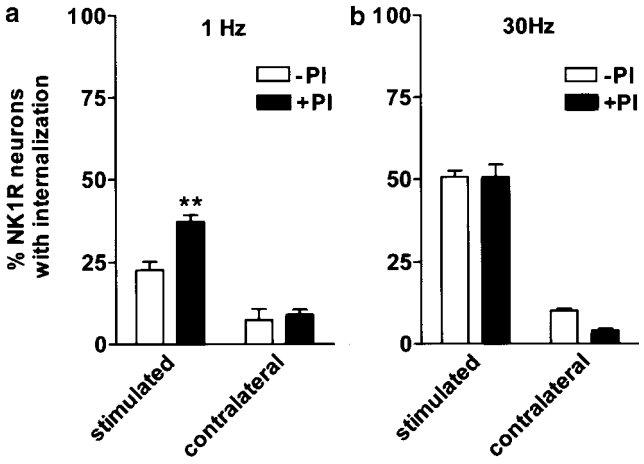


**Figure 5** Effect of peptidase inhibitors on NK1R internalization produced by endogenously released peptides. Slices with one dorsal root attached were superfused with aCSF alone (−PI) or aCSF containing 10 $\mu$M thiorphan and captopril (+PI), starting 5 min before electrical stimulation and until they were fixed 10 min after stimulation. The dorsal root was stimulated with pulses of 20 V, 0.4 ms, delivered in a single train of 1000 pulses at 1 Hz (a) or 300 pulses at 30 Hz (b). Bars are the mean $\pm$ s.e.m. of three slices. The percentage of lamina I NK1R neurons showing internalization was measured in the dorsal horn ipsilateral ('stimulated') or contralateral to the stimulated root. Statistical analyses consisted of two-way ANOVA with 'side' (contralateral *versus* stimulated) and 'peptidase inhibitors' ('−PI' *versus* '+PI') as the variables. For 1 Hz stimulation, there was a significant effect of 'side' ($P<0.0001$) and 'peptidase inhibitors' ($P=0.01$); Bonferroni's post-test revealed significant differences (**$P<0.01$) between '−PI' and '+PI'. For 30 Hz stimulation, there was a significant effect of 'side' ($P<0.0001$), but not of 'peptidase inhibitors'.

inhibits aminopeptidase W and leucine-aminopeptidase (EC 3.4.11.1) (Burley *et al*., 1991); amastatin inhibits aminopeptidases A, N and W (Tieku & Hooper, 1992; Suzuki *et al*., 1997) and puromycin and bacitracin inhibit other aminopeptidases. Our results indicate that none of these aminopeptidases were able to appreciably decrease the concentration of SP inside the slices.

To determine whether peptidases restrict the access of endogenously released neurokinins to NK1R in dorsal horn neurons, neurokinin release from primary afferent terminals was evoked by electrical stimulation of the dorsal root. Slices were superfused with 10 $\mu$M thiorphan and captopril starting 5 min before the stimulation, to allow enough time for the inhibitors to penetrate the slice and saturate the peptidases. One dorsal root entering the slice was placed on a bipolar electrode in pool of mineral oil, which served to direct the electrical current through the root. We used pulses of 20 V and 0.4 ms, an intensity and duration sufficient to recruit most C fibers in the root (Koslow *et al*., 1973; Li & Bak, 1976). Since high-frequency stimulation is more effective than low-frequency stimulation to release SP (Go & Yaksh, 1987) and to produce NK1R internalization (Marvizon *et al*., 1997), we used two stimulation patterns: (1) low frequency, consisting of 1000 pulses at 1 Hz (Figure 5a); (2) high frequency, consisting of 300 pulses at 30 Hz (Figure 5b). Both stimulation patterns produced a significant increase in NK1R internalization when comparing the side of the slice ipsilateral to the stimulated root with the contralateral side ($P<0.0001$, two-way ANOVA). However, 30 Hz stimulation produced significantly more internalization than 1 Hz stimulation, despite it being delivered with a smaller number of pulses, both in the absence ($P<0.001$, two-way ANOVA and Bonferroni's post-test) and in the presence ($P<0.05$) of peptidase inhibitors. Internalization in the contralateral side was similar to control (untreated) slices. Superfusing the slices with thiorphan and captopril significantly increased ($P<0.01$) the amount of NK1R internalization produced by 1 Hz stimulation (Figure 5a), but did not modify the internalization produced by 30 Hz stimulation.

Figure 6 shows representative NK1R neurons in lamina I ipsilateral to the stimulated root in each of these conditions. After 1 Hz stimulation, little internalization was observed in the soma of the neuron shown in Figure 6a, although some endosomes are found in its large dendrite. In the presence of peptidase inhibitors, 1 Hz stimulation produced substantial internalization in the neuron shown in Figure 6b. Stimulation at 30 Hz produced NK1R internalization both in the absence (Figure 6c) and in the presence (Figure 6d) of peptidase inhibitors.

## Discussion

Thiorphan, an inhibitor of NEN, combined with captopril, an inhibitor of DCP, moderately increased the potencies of SP and NKA to produce NK1R internalization. These findings are consistent with previous studies measuring SP release from the spinal cord. For example, Geppetti *et al*. (1989) found that thiorphan increased SP release from the guinea-pig spinal cord 2.4 times. Duggan *et al*. (1992) reported that spinal injections of the NEN inhibitor kelatorphan combined with the DCP inhibitor enalaprilat increased the amount of SP release evoked by primary afferent stimulation. However, relating SP release with NK1R activation is not straightforward: when

we compared capsaicin-induced SP release and NK1R internalization in the same slices (Marvizon *et al*., 2003), we found that NK1R internalization could be elicited by amounts of released neurokinins too low to be detected by RIA, whereas amounts of neurokinins within the range of detection of RIA saturated NK1Rs. We did not compare the relative abilities of NEN and DCP to degrade SP and NKA, because the combined effect of thiorphan and captopril was already quite small. Geppetti *et al*. (1989) found that captopril did not affect capsaicin-evoked SP release from guinea-pig dorsal horn slices, whereas thiorphan produced a 2.4-fold increase in SP release. Similarly, Duggan *et al*. (1992) found that the addition of the DCP inhibitor enalaprilat did not increase SP release from the cat spinal cord observed in the presence of the NEN inhibitor kelatorphan. Therefore, NEN appears to be the main enzyme degrading SP.

Aminopeptidases are also thought to contribute to the degradation of neurokinins. In particular, the aminopeptidase inhibitor bacitracin was reported in one study (Mauborgne *et al*., 1991) as the most potent agent protecting SP from degradation, and has been used together with inhibitors of NEN and DCP to improve the recovery of released SP (Malcangio & Bowery, 1993; 1994; Chen *et al*., 1996; Malcangio *et al*., 1997; Lever & Malcangio, 2002). However, none of the aminopeptidase inhibitors that we tested, including bacitracin, increased NK1R internalization produced by a low concentration of SP. It is difficult to reconcile our results using bacitracin with those of Mauborgne *et al*. (1991), particularly in view of the fact that they found no effect for NEN and DCP inhibitors, in contrast with our findings and those of other investigators (Geppetti *et al*. 1989; Duggan *et al*., 1992). We cannot rule out that neurokinins are degraded by aminopeptidases that are not inhibited by the compounds that we used. For example, aminopeptidase P (EC 3.4.11.9) appears to degrade SP, but is not inhibited by amastatin, bestatin, puromycin or bacitracin (Harbeck & Mentlein, 1991).

We also compared the potencies of SP, NKA and NKB to produce NK1R internalization. NKA was 5–7 times less potent than SP, whereas NKB was 10 times less potent than NKA and 64 times less potent than SP. This agrees with the established sequence of affinities of NK1R for its endogenous ligands: SP > NKA > NKB (Routh & Helke, 1995). Our $EC_{50}$ values for SP (32 nM in the absence and 9 nM in the presence of peptidase inhibitors) were similar to those reported by Trafton *et al*. (2001) in cultured dorsal horn neurons (14 nM). However, we found substantially higher $EC_{50}$ values for NKA (170 nM in the absence and 60 nM in the presence of peptidase inhibitors) than those reported by Trafton *et al*. (27 nM). This disparity may derive from differences in NK1Rs between adult neurons and the embryonic neurons used for cell cultures. Although Trafton *et al*. also reported that SP and NKA had the same potency in adult rats, they injected these peptides in the sacral spinal cord and measured NK1R internalization in the lumbar spinal cord, introducing a diffusion factor that substantially increased the $EC_{50}$ values obtained: 171 $\mu$M for SP and 210 $\mu$M for NKA. Hence, the apparently similar potencies for SP and

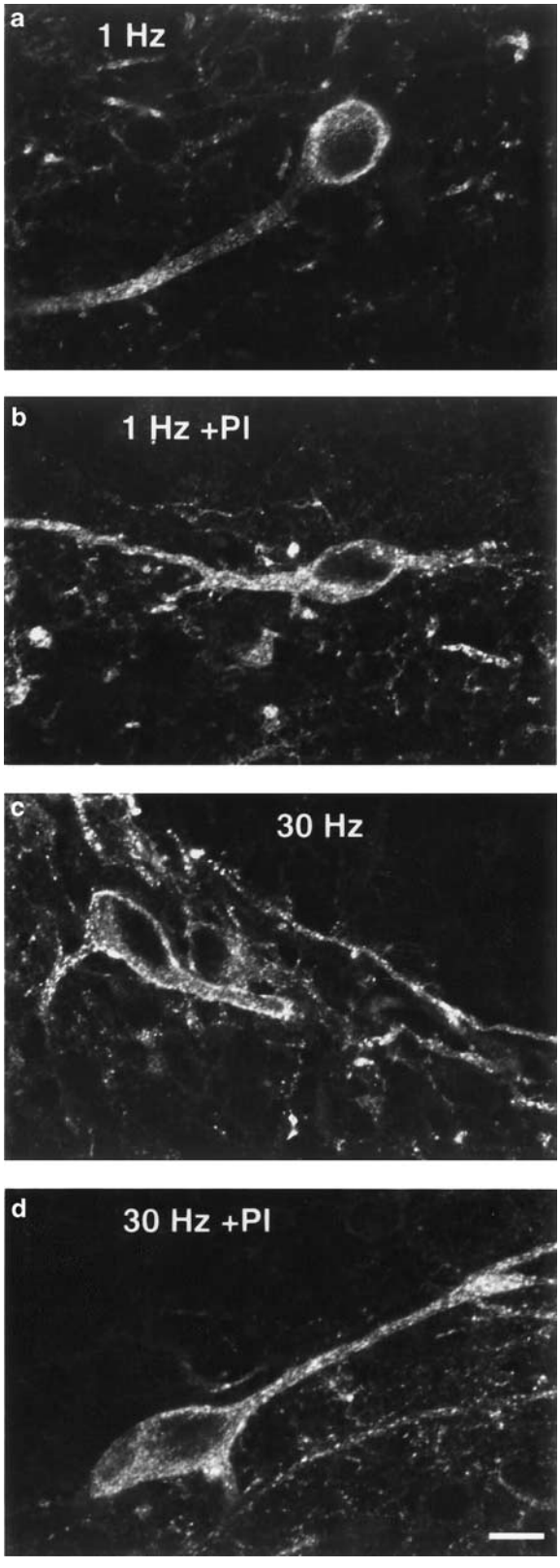


**Figure 6** Confocal images of NK1R neurons after electrical stimulation of the dorsal root. Slices with one dorsal root attached were superfused with aCSF alone (a, c) or aCSF containing 10 $\mu$M thiorphan and captopril (peptidase inhibitors, PI) (b, d) 5 min before, during, and 10 min after stimulation. The dorsal root was stimulated with pulses of 20 V, 0.4 ms, delivered in a single train of 1000 pulses at 1 Hz (a, b) or 300 pulses at 30 Hz (c, d). Images were taken at $\times$100 (scale bar is 10 $\mu$m), and consist of three optical sections (four for (d)) separated 0.57 $\mu$m. The dorsal side is up. There is little NK1R internalization in the neuron in panel (a), but substantial internalization is observed in the other panels.

NKA may have been caused by a higher susceptibility of SP to degradation by peptidases, as reported in guinea-pig airways (Martling *et al*., 1987; Devillier *et al*., 1988). Our own results support this idea: the increases in potency produced by peptidase inhibitors were greater for SP (3.5 times) than for NKA (2.8 times).

We also investigated the ability of peptidases to restrict the activation of NK1Rs by endogenous neurokinins released from primary afferent terminals. Peptidase inhibitors were able to increase NK1R internalization produced by dorsal root stimulation at 1 Hz but, surprisingly, not at 30 Hz. One possible explanation for this finding is that 1 Hz stimulation evokes a more gradual release of neurokinins, leaving enough time for peptidases to degrade them before their concentrations can substantially build up in the extracellular space. In contrast, 30 Hz stimulation produces a much rapid release of neurokinins, which may lead to the saturation of peptidase activity. Alternatively, high-frequency stimulation may release compounds that inhibit the peptidases. We have previously reported (Marvizon *et al*., 1997; 1999a) that high-frequency stimulation of the dorsal root is more efficacious than low-frequency stimulation to produce NK1R internalization, and this agrees with the findings of Go & Yaksh (1987) measuring SP release. However, another group successfully used 1 Hz root stimulation to evoke SP release (Malcangio & Bowery, 1993; 1994; Malcangio *et al*., 1997). Interestingly, Malcangio *et al*. used peptidase inhibitors, whereas Go & Yaksh did not, and neither did we in our previous studies on NK1R internalization. Thus, our finding resolves this apparent discrepancy. However, note that 1000 pulses at 1 Hz produced less NK1R internalization than 300 pulses at 30 Hz, even in the presence of peptidase inhibitors (Figure 5). Therefore, the action of peptidases does not completely account for the frequency dependence of neurokinin release.

To gain an adequate perspective on the ability of peptidases to degrade neurokinins, it is important to compare it with their ability to degrade opioid peptides. In a previous study (Song & Marvizon, 2003), we demonstrated that inhibitors of NEN, DEC and aminopeptidase N increased the potencies of dynorphin A and Leu-enkephalin 10 and 100 times, respectively, to produce $\mu$-opioid receptor internalization. In contrast, we show here that inhibitors of NEN and DEC produced a relatively smaller increase (2.8–3.5 times) in the potencies of SP and NKA to produce NK1R internalization. Moreover, whereas peptidase inhibitors are required to observe $\mu$-opioid receptor internalization in dorsal horn neurons produced by endogenously released opioids (Song & Marvizon, 2003), endogenously released neurokinins produce abundant NK1R internalization in the absence of peptidase inhibitors (Mantyh *et al*., 1995; Allen *et al*., 1997; Liu *et al*., 1997; Marvizon *et al*., 1997; 1999a; Cao *et al*., 1998; Honore *et al*., 1999; Riley *et al*., 2001). Therefore, it is possible to use peptidase inhibitors to enhance the analgesic effect of endogenous opioids (Noble *et al*., 1992b; Roques, 2000) without increasing NK1R activation by endogenous neurokinins.

In summary, our results show that peptidases limit the ability of exogenously applied or endogenously released neurokinins to activate NK1Rs. Furthermore, they confirm the idea that all three neurokinins are able to activate NK1Rs and may exert physiological actions through this receptor (Maggi & Schwartz, 1997).

This work was supported by grant R01-DA12609 to J.C.G.M. from NIDA. We thank Drs Emeran Mayer, Enrico Stefani and Ligia Toro for their support. Confocal images were obtained at the UCLA's Carol Moss Spivak Cell Imaging Facility administered by Dr Matt Schibler. The NK1R antibody was provided by Dr Nigel Bunnett, UCSF, and was prepared at the RIA/Antibody Core of CURE: DDRC, UCLA.

## References

ABBADIE, C., TRAFTON, J., LIU, H., MANTYH, P.W. & BASBAUM, A.I. (1997). Inflammation increases the distribution of dorsal horn neurons that internalize the neurokinin-1 receptor in response to noxious and non-noxious stimulation. *J. Neurosci.,* **17,** 8049–8060.

ALLEN, B.J., ROGERS, S.D., GHILARDI, J.R., MENNING, P.M., KUSKOWSKI, M.A., BASBAUM, A.I., SIMONE, D.A. & MANTYH, P.W. (1997). Noxious cutaneous thermal stimuli induce a graded release of endogenous substance P in the spinal cord: imaging peptide action *in vivo*. *J. Neurosci.,* **17,** 5921–5927.

BLEAZARD, L., HILL, R.G. & MORRIS, R. (1994). The correlation between the distribution of the NK1 receptor and the actions of tachykinins agonists in the dorsal horn of the rat indicates that substance P does not have a functional role on substantia gelatinosa (lamina II) neurons. *J. Neurosci.*, **14,** 7655–7664.

BURLEY, S.K., DAVID, P.R. & LIPSCOMB, W.N. (1991). Leucine aminopeptidase: bestatin inhibition and a model for enzyme-catalyzed peptide hydrolysis. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 6916–6920.

CAO, Y.Q., MANTYH, P.W., CARLSON, E.J., GILLESPIE, A.M., EPSTEIN, C.J. & BASBAUM, A.I. (1998). Primary afferent tachykinins are required to experience moderate to intense pain. *Nature,* **392,** 390–394.

CHEN, J.J., BARBER, L.A., DYMSHITZ, J. & VASKO, M.R. (1996). Peptidase inhibitors improve recovery of substance P and calcitonin gene-related peptide release from rat spinal cord slices. *Peptides,* **17,** 31–37.

CHOU, J., TANG, J., DEL RIO, J., YANG, H.Y. & COSTA, E. (1984). Action of peptidase inhibitors on methionine5-enkephalin-arginine6-phenylalanine7 (YGGFMRF) and methionine5-enkephalin (YGGFM) metabolism and on electroacupuncture antinociception. *J. Pharmacol. Exp. Ther.,* **230,** 349–352.

DE FELIPE, C., HERRERO, J.F., O'BRIEN, J.A., PALMER, J.A., DOYLE, C.A., SMITH, A.J., LAIRD, J.M., BELMONTE, C., CERVERO, F. & HUNT, S.P. (1998). Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. *Nature,* **392,** 394–397.

DEVILLIER, P., ADVENIER, C., DRAPEAU, G., MARSAC, J. & REGOLI, D. (1988). Comparison of the effects of epithelium removal and of an enkephalinase inhibitor on the neurokinin-induced contractions of guinea-pig isolated trachea. *Br. J. Pharmacol.,* **94,** 675–684.

DUGGAN, A.W., SCHAIBLE, H.-G., HOPE, P.J. & LANG, C.W. (1992). Effect of peptidase inhibition on the pattern of intraspinally released immunoreactive substance P detected with antibody microprobes. *Brain Res.,* **579,** 261–269.

GEPPETTI, P., SANTICIOLI, P., RUBINI, I., SPILLANTINI, M.G., MAGGI, C.A. & SICUTERI, F. (1989). Thiorphan increases capsaicin-evoked release of substance P from slices of dorsal spinal cord of guinea pig. *Neurosci. Lett.,* **103,** 69–73.

GO, V.L.W. & YAKSH, T.L. (1987). Release of substance P from the cat spinal cord. *J. Physiol.,* **391,** 141–167.

GRADY, E.F., BALUK, P., BOHM, S., GAMP, P.D., WONG, H., PAYAN, D.G., ANSEL, J., PORTBURY, A.L., FURNESS, J.B., MCDONALD, D.M. & BUNNETT, N.W. (1996). Characterization of antisera specific to NK1, NK2, and NK3 neurokinin receptors and their utilization to localize receptors in the rat gastrointestinal tract. *J. Neurosci.,* **16,** 6975–6986.

GUYON, A., ROQUES, B.P., GUYON, F., FOUCAULT, A., PERDRISOT, R., SWERTS, J.P. & SCHWARTZ, J.C. (1979). Enkephalin degradation in mouse brain studied by a new H.P.L.C. method: further evidence for the involvement of carboxydipeptidase. *Life Sci.,* **25,** 1605–1611.

HARBECK, H.T. & MENTLEIN, R. (1991). Aminopeptidase P from rat brain. Purification and action on bioactive peptides. *Eur. J. Biochem.,* **198,** 451–458.

HENRY, J.L., YASHPAL, K., PITCHER, G.M., CHABOT, J. & CODERRE, T.J. (1999). Evidence for tonic activation of NK-1 receptors during the second phase of the formalin test in the rat. *J. Neurosci.,* **19,** 6588–6598.

HIRANUMA, T., KITAMURA, K., TANIGUCHI, T., KOBAYASHI, T., TAMAKI, R., KANAI, M., AKAHORI, K., IWAO, K. & OKA, T. (1998). Effects of three peptidase inhibitors, amastatin, captopril and phosphoramidon, on the hydrolysis of [Met5]-enkephalin-Arg6-Phe7 and other opioid peptides. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **357,** 276–282.

HONORE, P., MENNING, P.M., ROGERS, S.D., NICHOLS, M.L., BASBAUM, A.I., BESSON, J.M. & MANTYH, P.W. (1999). Spinal cord substance P receptor expression and internalization in acute, short-term, and long-term inflammatory pain states. *J. Neurosci.,* **19,** 7670–7678.

KOSLOW, M., BAK, A. & LI, C.L. (1973). C-fiber excitability in the cat. *Exp. Neurol.,* **41,** 745–753.

LAIRD, J.M., OLIVAR, T., ROZA, C., DE FELIPE, C., HUNT, S.P. & CERVERO, F. (2000). Deficits in visceral pain and hyperalgesia of mice with a disruption of the tachykinin NK1 receptor gene. *Neuroscience,* **98,** 345–352.

LAIRD, J.M., ROZA, C., DE FELIPE, C., HUNT, S.P. & CERVERO, F. (2001). Role of central and peripheral tachykinin NK1 receptors in capsaicin-induced pain and hyperalgesia in mice. *Pain,* **90,** 97–103.

LEVER, I.J. & MALCANGIO, M. (2002). CB(1) receptor antagonist SR141716A increases capsaicin-evoked release of substance P from the adult mouse spinal cord. *Br. J. Pharmacol.,* **135,** 21–24.

LI, C.L. & BAK, A. (1976). Excitability characteristics of the A- and C-fibers in a peripheral nerve. *Exp. Neurol.,* **50,** 67–79.

LIU, H., BROWN, JL., JASMIN, L., MAGGIO, J.E., VIGNA, S.R., MANTYH, P.W. & BASBAUM, A.I. (1994). Synaptic relationship between substance P and the substance P receptor: light and electron microscopic characterization of the mismatch between neuropeptides and their receptors. *Proc. NatL. Acad. Sci. U.S.A.,* **91,** 1009–1013.

LIU, H., MANTYH, P.W. & BASBAUM, A.I. (1997). NMDA-receptor regulation of substance P release from primary afferent nociceptors. *Nature,* **386,** 721–724.

MAGGI, C.A. & SCHWARTZ, T.W. (1997). The dual nature of the tachykinin $NK_1$ receptor. *Trends Pharmacol. Sci.,* **18,** 351–355.

MALCANGIO, M. & BOWERY, N.G. (1993). Gamma-aminobutyric $acid_B$, but not gamma-aminobutyric $acid_A$ receptor activation, inhibits electrically evoked substance P-like immunoreactivity release from the rat spinal cord *in vitro*. *J. Pharmacol. Exp. Ther.,* **266,** 1490–1496.

MALCANGIO, M. & BOWERY, N.G. (1994). Effect of the tachykinin NK1 receptor antagonists, RP 67580 and SR 140333, on electrically-evoked substance P release from rat spinal cord. *Br. J. Pharmacol.,* **113,** 635–641.

MALCANGIO, M., GARRETT, N.E., CRUWYS, S. & TOMLINSON, D.R. (1997). Nerve growth factor- and neurotrophin-3-induced changes in nociceptive threshold and the release of substance P from the rat isolated spinal cord. *J. Neurosci.,* **17,** 8459–8467.

MANTYH, P.W., DEMASTER, E., MALHOTRA, A., GHILARDI, J.R., ROGERS, S.D., MANTYH, C.R., LIU, H., BASBAUM, A.I., VIGNA, S.R. & MAGGIO, J.E. (1995). Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. *Science,* **268,** 1629–1632.

MANTYH, P.W., ROGERS, S.D., HONORE, P., ALLEN, B.J., GHILARDI, J.R., LI, J., DAUGHTERS, R.S., LAPPI, D.A., WILEY, R.G. & SIMONE, D.A. (1997). Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. *Science,* **278,** 275–279.

MARTLING, C.R., THEODORSSON-NORHEIM, E., NORHEIM, I. & LUNDBERG, J.M. (1987). Bronchoconstrictor and hypotensive effects in relation to pharmacokinetics of tachykinins in the guinea-pig – evidence for extraneuronal cleavage of neuropeptide K to neurokinin A. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **336,** 183–189.

MARVIZON, J.C., GRADY, E.F., STEFANI, E., BUNNETT, N.W. & MAYER, E.A. (1999a). Substance P release in the dorsal horn assessed by receptor internalization: NMDA receptors counteract a tonic inhibition by GABA(B) receptors. *Eur. J. Neurosci.,* **11,** 417–426.

MARVIZON, J.C., MARTINEZ, V., GRADY, E.F., BUNNETT, N.W. & MAYER, E.A. (1997). Neurokinin 1 receptor internalization in spinal cord slices induced by dorsal root stimulation is mediated by NMDA receptors. *J. Neurosci.,* **17,** 8129–8136.

MARVIZON, J.C., WANG, X., MATSUKA, Y., NEUBERT, J.K. & SPIGELMAN, I. (2003). Relationship between capsaicin-evoked substance P release and NK1 receptor internalization in the rat dorsal horn. *Neuroscience,* **118,** 535–545.

MARVIZON, J.C.G., GRADY, E.F., WAZSAK-MCGEE, J. & MAYER, E.A. (1999b). Internalization of $\mu$-opioid receptors in rat spinal cord slices. *NeuroReport,* **10,** 2329–2334.

MAUBORGNE, A., BOURGOIN, S., BENOLIEL, J.J., HAMON, M. & CESSELIN, F. (1991). Is substance-P released from slices of the rat spinal-cord inactivated by peptidase(s) distinct from both enkephalinase and angiotensin-converting enzyme. *Neurosci. Lett.,* **123,** 221–225.

MOTULSKY, H. & CHRISTOPOULOS, A. (2003). *Fitting Models to Biological Data Using Linear and Nonlinear Regression*. San Diego, CA: GraphPad Software, Inc..

NOBLE, F., CORIC, P., FOURNIE-ZALUSKI, M.C. & ROQUES, B.P. (1992a). Lack of physical dependence in mice after repeated systemic administration of the mixed inhibitor prodrug of enkephalin-degrading enzymes, RB101. *Eur. J. Pharmacol.,* **223,** 91–96.

NOBLE, F., SOLEILHAC, J.M., SOROCA-LUCAS, E., TURCAUD, S., FOURNIE-ZALUSKI, M.C. & ROQUES, B.P. (1992b). Inhibition of the enkephalin-metabolizing enzymes by the first systemically active mixed inhibitor prodrug RB 101 induces potent analgesic responses in mice and rats. *J. Pharmacol. Exp. Ther.,* **261,** 181–190.

NOBLE, F., TURCAUD, S., FOURNIE-ZALUSKI, M.C. & ROQUES, B.P. (1992c). Repeated systemic administration of the mixed inhibitor of enkephalin-degrading enzymes, RB101, does not induce either antinociceptive tolerance or cross-tolerance with morphine. *Eur. J. Pharmacol.,* **223,** 83–89.

RANDIC, M., JIANG, M.C. & CERNE, R. (1993). Long-term potentiation and long-term depression of primary afferent neurotransmission in the rat spinal cord. *J. Neurosci.,* **13,** 5228–5241.

REGOLI, D., DRAPEAU, G., DION, S. & COUTURE, R. (1994). Receptors and antagonists for substance P and related peptides. *Pharmacol. Rev.,* **46,** 551–599.

RILEY, R.C., TRAFTON, J.A., CHI, S.I. & BASBAUM, A.I. (2001). Presynaptic regulation of spinal cord tachykinin signaling *via* GABA(B) but not GABA(A) receptor activation. *Neuroscience,* **103,** 725–737.

ROQUES, B.P. (2000). Novel approaches to targeting neuropeptide systems. *Trends Pharmacol. Sci.,* **21,** 475–483.

ROUTH, V.H. & HELKE, C.J. (1995). Tachykinin receptors in the spinal cord. *Prog. Brain Res.,* **104,** 93–108.

SAKURADA, T., TAN-NO, K., YAMADA, T., SAKURADA, S. & KISARA, K. (1990). Phosphoramidon potentiates mammalian tachykinin-induced biting, licking and scratching behaviour in mice. *Pharmacol. Biochem. Behav.,* **37,** 779–783.

SANDKUHLER, J., CHEN, J.G., CHENG, G. & RANDIC, M. (1997). Low-frequency stimulation of afferent A$\delta$-fibers induces long-term depression at primary afferent synapses with substantia gelatinosa neurons in the rat. *J. Neurosci.,* **17,** 6483–6491.

SCHWEI, M.J., HONORE, P., ROGERS, S.D., SALAK-JOHNSON, J.L., FINKE, M.P., RAMNARAINE, M.L., CLOHISY, D.R. & MANTYH, P.W. (1999). Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. *J. Neurosci.,* **19,** 10886–10897.

SONG, B. & MARVIZON, J.C. (2003). Peptidases prevent $\mu$-opioid receptor internalization in dorsal horn neurons by endogenously released opioids. *J. Neurosci.,* **23,** 1847–1858.

SUZUKI, H., YANAGISAWA, M., YOSHIOKA, K., HOSOKI, R. & OTSUKA, M. (1997). Enzymatic inactivation of enkephalin neurotransmitters in the spinal cord of the neonatal rat. *Neurosci. Res.,* **28,** 261–267.

SUZUKI, H., YOSHIOKA, K., YANAGISAWA, M., URAYAMA, O., KURIHARA, T., HOSOKI, R., SAITO, K. & OTSUKA, M. (1994). Involvement of enzymatic degradation in the inactivation of tachykinin neurotransmitters in neonatal rat spinal cord. *Br. J. Pharmacol.,* **113,** 310–316.

TIEKU, S. & HOOPER, N.M. (1992). Inhibition of aminopeptidases N, A and W. A re-evaluation of the actions of bestatin and inhibitors of angiotensin converting enzyme. *Biochem. Pharmacol.,* **44,** 1725–1730.

TODD, A.J., PUSKAR, Z., SPIKE, R.C., HUGHES, C., WATT, C. & FORREST, L. (2002). Projection neurons in lamina I of rat spinal cord with the neurokinin 1 receptor are selectively innervated by substance p-containing afferents and respond to noxious stimulation. *J. Neurosci.,* **22,** 4103–4113.

TRAFTON, J.A., ABBADIE, C. & BASBAUM, A.I. (2001). Differential contribution of substance P and neurokinin A to spinal cord neurokinin-1 receptor signaling in the rat. *J. Neurosci.,* **21,** 3656–3664.

TRAFTON, J.A., ABBADIE, C., MARCHAND, S., MANTYH, P.W. & BASBAUM, A.I. (1999). Spinal opioid analgesia: how critical is the regulation of substance P signaling? *J. Neurosci.,* **19,** 9642–9653.

TRAFTON, J.A., ABBADIE, C., MAREK, K. & BASBAUM, A.I. (2000). Postsynaptic signaling *via* the mu-opioid receptor: responses of dorsal horn neurons to exogenous opioids and noxious stimulation. *J. Neurosci.,* **20,** 8578–8584.

TRAUB, R.J. (1996). The spinal contribution of substance P to the generation and maintenance of inflammatory hyperalgesia in the rat. *Pain,* **67,** 151–161.

WANG, X. & MARVIZON, J.C. (2002). Time-course of the internalization and recycling of neurokinin 1 receptors in rat dorsal horn neurons. *Brain Res.,* **944,** 239–247.

YAKSH, T.L. & CHIPKIN, R.E. (1989). Studies on the effect of SCH-34826 and thiorphan on [Met5]enkephalin levels and release in rat spinal cord. *Eur. J. Pharmacol.,* **167,** 367–373.

YOSHIMURA, M. & JESSELL, T. (1990). Amino acid-mediated EPSPs at primary afferent synapses with substantia gelatinosa neurones in the rat spinal cord. *J. Physiol.,* **430,** 315–335.

YOSHIMURA, M. & NISHI, S. (1996). Blind patch-clamp recordings from substantia gelatinosa neurons in adult rat spinal cord slices: pharmacological properties of synaptic currents. *Neuroscience,* **53,** 519–526.

*(Received June 26, 2003*
*Revised September 22, 2003*
*Accepted October 17, 2003)*