Peptidases Prevent μ-Opioid Receptor Internalization in Dorsal Horn Neurons by Endogenously Released Opioids

Bingbing Song and Juan Carlos G. Marvizon

Center for Neurovisceral Sciences and Women’s Health, Division of Digestive Diseases, Department of Medicine, Geffen School of Medicine at University of California Los Angeles, Los Angeles, California 90095

To evaluate the effect of peptidases on μ-opioid receptor (MOR) activation by endogenous opioids, we measured MOR-1 internalization in rat spinal cord slices. A mixture of inhibitors of aminopeptidases (amastatin), dipeptidyl carboxypeptidase (captopril), and neutral endopeptidase (phosphoramidon) dramatically increased the potencies of Leu-enkephalin and dynorphin A to produce MOR-1 internalization, and also enhanced the effects of Met-enkephalin and α-neoendorphin, but not endomorphins or β-endorphin. The omission of any one inhibitor abolished Leu-enkephalin-induced internalization, indicating that all three peptidases degraded enkephalins. Amastatin preserved dynorphin A-induced internalization, and phosphoramidon, but not captopril, increased this effect, indicating that the effect of dynorphin A was prevented by aminopeptidases and neutral endopeptidase. Veratridine (30 μM) or 50 mM KCl produced MOR-1 internalization in the presence of peptidase inhibitors, but little or no internalization in their absence. These effects were attributed to opioid release, because they were abolished by the selective MOR antagonist CTAP (D-Phe–Cys–Tyr–D-Trp–Arg–Thr–Pen–Thr–NH₂) and were Ca²⁺ dependent. The effect of veratridine was protected by phosphoramidon plus amastatin or captopril, but not by amastatin plus captopril or by phosphoramidon alone, indicating that released opioids are primarily cleaved by neutral endopeptidase, with a lesser involvement of aminopeptidases and dipeptidyl carboxypeptidase. Therefore, because the potencies of endomorphin-1 and endomorphin-2 to elicit internalization were unaffected by peptidase inhibitors, the opioids released by veratridine were not endomorphins. Confocal microscopy revealed that MOR-1-expressing neurons were in close proximity to terminals containing opioids with enkephalin-like sequences. These findings indicate that peptidases prevent the activation of extrasynaptic MOR-1 in dorsal horn neurons.

Key words: amastatin; aminopeptidase; captopril; dipeptidyl carboxypeptidase; dynorphin; endocytosis; endomorphin; endorphin; enkephalin; internalization; μ-opioid receptor; neutral endopeptidase; opioid; peptidase; phosphoramidon; rat; release; spinal cord

Introduction

Pain neurophysiology was greatly advanced by the discovery of opioid receptors and endogenous opioids (for review, see Mansour et al., 1988; Law et al., 2000; Przewlocki and Przewlocka, 2001). These findings made it possible, in principle, to trace causal links between stimuli, opioid release, opioid receptor activation, and analgesic responses. However, measuring opioid release cannot predict opioid receptor activation, because it may be produced by peptides different from the one being detected, and it is impossible to know the peptide concentration at the receptor. These problems may be avoided by using opioid receptor internalization to assess their activation by a given stimulus, independently of the particular opioid being released. This approach was successfully used to study the activation of neurokinin 1 receptors by neurokinins released by noxious stimuli (Mantyh et al., 1995; Abbadie et al., 1997; Allen et al., 1997; Liu et al., 1997; Honore et al., 1999; Trafton et al., 1999, 2001) or after primary afferent stimulation (Marvizon et al., 1997, 1999a; Allen et al., 1999). Conceivably, the internalization of μ-opioid receptors (MORs) could be used similarly to detect their activation by endogenously released opioids.

In the dorsal horn, MOR-1 is present in dorsal horn interneurons (Kemp et al., 1996), whereas the splice variants MOR-1C and MOR-1D are found in primary afferent terminals (Abbadie et al., 2001), where they control substance P release (Yaksh et al., 1980; Aimone and Yaksh, 1989). MOR-1 in dorsal horn neurons readily internalized when exposed to etorphine or [D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin (DAMGO), and the potency of DAMGO was similar to its potency to inhibit adenylyl cyclase (Marvizon et al., 1999b). Furthermore, MOR-1 internalization produced by intrathecal DAMGO correlated with its ability to elicit analgesia (Trafton et al., 2000). However, it remains to be established whether endogenous opioids, unlike morphine (Keith et al., 1998), produce MOR-1 internalization. If this were true, then MOR-1 internalization could be used as a marker of its activation by endogenous opioids. MOR-1 internalization attributable to opioid release was demonstrated in hypothalamic neurons after estrogen treatment (Eckersell et al., 1998) but has proved elusive in dorsal horn neurons. It was not elicited by primary afferent stimulation (Trafton et al., 1997) or, surprisingly, by noxious stimuli (Trafton et al., 2000) able to evoke the spinal release of Met-enkephalin (Le Bars et al., 1987a; Cesselin et al., 1989; Bourgoin et al., 1990). Furthermore, the ability of Met-enkephalin to elicit MOR-1 internalization (Trafton et al., 2000)
was much lower than its affinity for MOR-1 (Raynor et al., 1993; Zadina et al., 1997).

We hypothesized that MOR-1 internalization by endogenous opioids is prevented by their rapid degradation by peptidases. Indeed, enkephalins (Guyon et al., 1979; Chou et al., 1984; Yaksh and Chipkin, 1989; Hiranuma et al., 1997; Hiranuma et al., 1998a) and dynorphins (Hiranuma et al., 1998b) are quickly degraded in the intestine, brain, and spinal cord by three peptidases: neutral endopeptidase, dipeptidyl carboxypeptidase I, and aminopeptidases (Fig. 1). We tested our hypothesis by investigating the ability of peptidase inhibitors to protect MOR-1 internalization produced by exogenously added and endogenously released opioids. These results have been published previously in abstract form (Song and Marvizon, 2002).

Materials and Methods

**Chemicals.** Ala-pyrrolidine-nitrile (Li et al., 1995) was a gift from Dr. Sherwin Wilk (Mount Sinai School of Medicine, New York, NY). α-Neoendorphin and phosphoramidon were purchased from Bachem/Peninsula Laboratories (San Carlos, CA). Other chemicals were obtained from Sigma (St. Louis, MO).

**Media for slices.** Artificial CSF (ACSF) contained (in mM): 124 NaCl, 1.9 KCl, 26 NaHCO3, 1.2 KH2PO4, 1.3 MgSO4, 2.4 CaCl2, and 10 glucose, and was 305 mmOsm. K+−ACSF contained a higher concentration (5 mM) of KCl. Sucrose−ACSF was identical to ACSF, except that NaCl was isosmotically replaced by sucrose (215 mM) and the concentration of KCl was 5 mM. To depolarize the slices, we used ACSF (50 mM KCl) in which the concentration of K+ was increased to 50 mM and the concentration of NaCl was decreased to 74 mM. All of these media were constantly bubbled with 95% O2 and 5% CO2 to a pH of 7.4.

**Spinal cord slice preparation.** All animal procedures were approved by the Chancellor’s Animal Research Committee at the University of California Los Angeles and conform to National Institutes of Health guidelines. Slices were prepared as described previously (Randic et al., 1993; Marvizon et al., 1997, 1999a,b; Sandkuhler et al., 1997). Briefly, 3- to 4-week-old Sprague Dawley rats (Harlan, Indianapolis, IN) were anesthetized with isoflurane (Halocarbon Laboratories, River Edge, NJ), and a laminectomy was performed to extract a lumbar segment of the spinal cord. The spinal cord was placed in ice-cold sucrose−ACSF in less than 1 min after the spine was pierced and cleaned of dura mater and roots. Coronal spinal cord slices (400 μm) were cut with a Vibratome (Technical Products International, St. Louis, MO) 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. After cutting, slices were kept for 1 hr in K+−ACSF at 35°C and then transferred to ACSF at 35°C. It was vital that the slices contained healthy neurons, which required the use of a Vibratome and spinal cords rapidly extracted from live rats to make the slices.

**Slice treatment.** Slices were placed in a nylon net suspended halfway inside a small beaker and incubated at 35°C with ACSF containing various compounds, constantly bubbled with 95% O2 and 5% CO2. Peptidase inhibitors (usually amastatin, phosphoramidon, and captopril) were used at 10 μM, unless otherwise indicated. Phosphoramidon was always added together with 6 μM dithiorthiol to protect it against oxidation. The incubation was ended by placing the slices in cold fixative.

**MOR-1 immunohistochemistry in spinal cord slices.** Histological sections from spinal cord slices were prepared and labeled as described previously (Marvizon et al., 1999b). Slices were fixed in 4% paraformaldehyde, 0.2% picric acid, and 0.1 M sodium phosphate, pH 7.4; cryoprotected in 20% sucrose; frozen on dry ice; and sectioned with a cryostat at 25 μm in the coronal plane. Sections were washed twice with PBS; washed twice with PBS, 0.3% Triton X-100, and 0.001% thimerosal (PBS/Triton) containing 5% normal goat serum; and then incubated at room temperature for 1 hr and at 4°C overnight (12−18 hr) with the primary antibody diluted 1:7000 in PBS/Triton. The primary antibody was a rabbit antisera raised against a synthetic peptide corresponding to amino acids 384−398 of the cloned rat MOR-1 (catalog number 24216; DiaSorin, Stillwater, MN) and was characterized previously (Arvidsson et al., 1995). Cells labeled by this antibody are neurons (Spike et al., 2002). Although the MOR-1C and MOR-1D splice variants are not recognized by this antibody, they are present in primary afferent terminals and not in dorsal horn neurons (Abbadi et al., 2001). After three washes with PBS, sections were incubated for 2 hr at room temperature with a secondary antibody (goat anti-rabbit IgG−Alexa-488; Molecular Probes, Eugene, OR) diluted 1:2000 in PBS/Triton. Sections were washed four more times with PBS and mounted in Prolong (Molecular Probes). Preabsorption of the MOR-1 antibody with its immunizing peptide (10 μg/ml) abolished the staining, and the labeling of MOR-1 in sections from slices was similar to sections from perfusion-fixed rats (Marvizon et al., 1999b).

**Double-label immunohistochemistry.** A similar procedure was used to double-label spinal cord sections for endomorphins, enkephalins, and MOR-1. Adult male rats were anesthetized with isoflurane, killed by bilateral thoracotomy, and fixed by aortal perfusion. Two spinal cord segments (L3−L2 and L4) were postfixed, cryoprotected, frozen, and bilateral thoracotomy, and fixed by aortal perfusion. Two spinal cord segments (L3−L2 and L4) were postfixed, cryoprotected, frozen, and sectioned at 25 μm in the sagittal and coronal planes, respectively. Sections were incubated simultaneously with the two primary antibodies diluted in PBS/Triton containing 1% normal serum for 1 hr at room temperature and overnight at 4°C. After three washes, sections were incubated for 2 hr at room temperature with a secondary antibody (goat anti-rabbit IgG−Alexa-488; Molecular Probes, Eugene, OR) diluted 1:2000 in PBS/Triton. Sections were washed four more times with PBS and mounted in Prolong (Molecular Probes). Preabsorption of the MOR-1 antibody with its immunizing peptide (10 μg/ml) abolished the staining, and the labeling of MOR-1 in sections from slices was similar to sections from perfusion-fixed rats (Marvizon et al., 1999b).

**Antibody characterization.** The 3-E7 mouse monoclonal antibody recognizes the C-terminal sequence Tyr−Gly−Phe−Met of β-endorphin and cross-reacts completely with Met-enkephalin and Leu-enkephalin and partially with dynorphins and α-neoendorphin, as reported previously (Wasserman et al., 1983) and confirmed by us using preabsorption controls. Staining of the spinal cord with the 3-E7 antibody was unaf-
ected by preabsorption with endomorphin-1 or endomorphin-2. The antibody directed against endomorphins recognized both endomorphin-1 (70%) and endomorphin-2 (100%) but not enkephalins or β-endorphin (<0.05%), as reported by the manufacturer. Some anti-endomorphin antibodies have been found to cross-react partially with calcitonin gene-related peptide (CGRP) (Pierce et al., 1998), probably because CGRP and endomorphins have the same C-terminal (Phe–NH₂). Staining of the dorsal horn by the endomorphin antibody was abolished by preabsorption with 1 μM endomorphin-1 or endomorphin-2 but only slightly decreased by preabsorption with 1 μM CGRP. Moreover, double-labeling of rat spinal cord sections with the endomorphin antibody and three different CGRP antibodies produced no colocalization (Marvizon and Song, 2002), indicating that this antibody does not cross-react with CGRP in our conditions. For double-labeling with the rabbit anti-endomorphin antibody, we used a goat antibody directed against MOR-1. This antibody gave a somewhat less crisp label than the rabbit anti-MOR-1 antibody, and it had to be used at a higher concentration (1:200 dilution). When the two anti-MOR-1 antibodies were used together to double-label spinal cord sections, we found good colocalization.

Confocal microscopy and image processing. Confocal images were acquired at the University of California Los Angeles Carol Moss Spivak Cell Imaging Facility with a Leica (Nussloch, Germany) TCS-SP confocal microscope with argon (476 and 488 nm) and krypton (568 nm) lasers, using a pinhole of 1.0 Airy units, and objectives of 10× (0.4 numerical aperture) or 100× (1.4 numerical aperture), giving an optical section thickness (full width half maximum) of 8.13 and 0.62 μm, respectively. A zoom factor of 2 was used with some images taken at 100× to increase the pixel resolution of the resulting digital files. For each image at 100×, stacks of 5–10 optical sections were obtained at intervals of 0.49 or 0.57 μm. Each optical section was averaged up to six times to reduce noise. Images were processed using Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA). The “curves” feature of the program was used to adjust the contrast and to balance the colors of double-label images. Images were acquired at a digital size of 1024 × 1024 pixels and were later cropped to the relevant part of the field without altering the original image resolution.

Quantification of MOR-1 internalization. The amount of internalization was quantified essentially as described previously (Manthyl et al., 1995; Abbadie et al., 1997; Marvizon et al., 1997, 1999a,b; Trafton et al., 1999, 2001), by calculating the percentage of MOR-1 immunoreactive (IR) neurons in lamina I and II that show internalization in relation to the total number of IR neurons sampled. The person counting the neurons was unaware of the treatment given to the slice. A Zeiss Axiovert 135 (Carl Zeiss, Inc., Thornwood, NY) fluorescence microscope fitted with a 100× objective lens was used to count neurons. Neuronal somata with five or more endosomes were considered to have internalized receptors. At least three sections per slice were used, and all MOR-1 somata in these three sections were counted, representing 100–200 MOR-1 neurons per slice. An alternative method to quantify internalization consists of counting endosomes in confocal images of sample neurons for each treatment (Allen et al., 1999; Trafton et al., 2000). Although this method is more effective for examining the inside of individual neurons, it also has some problems: the selection of neurons to be imaged is a potential source of bias, and the number of endosomes depends on confounding factors such as the size of the neuronal body, the size and proximity of the nucleus to the membrane, and the number of dendrites present in the confocal plane. We found that the large number of neurons sampled with our method provides a better statistical base to discriminate subtle differences in the amount of internalization. In any event, similar results were obtained using these two methods (cf. Marvizon et al., 1999b; Trafton et al., 2000).

Statistical analysis. Treatments were randomized between slices, and no more than two slices from the same animal received the same treatment. Data were analyzed using GraphPad Prism version 3.03 for Windows (GraphPad Software, San Diego, CA). Statistical analyses consisted of one-way ANOVA followed by Tukey’s post-test or two-way ANOVA followed by Bonferroni’s post-test. Statistical significance was set at 0.05. In concentration–response experiments, a sigmoidal dose–response function, Y = bottom + (top – bottom)/(1 + 10^XY – Log(X) × nH), where nH denotes the Hill coefficient, was fitted to the data points by nonlinear regression. Data were simultaneously fitted to two models, one in which nH was fixed to 1.0, and the other in which nH was calculated by the program. The second, more complex model was chosen based on an F test if p < 0.005. The “top” parameter was fixed to 100%, because otherwise the regression often produced values >100%. The statistical error associated with the calculated EC₅₀ was expressed as a 95% confidence interval (95% CI).

Results

MOR-1 internalization produced by exogenously added opioids

It was initially observed that incubating spinal cord slices with relatively high concentrations (1 μM) of Leu-enkephalin did not produce MOR-1 internalization. A confocal image of a representative neuron in a slice exposed to Leu-enkephalin is shown in Figure 2D. MOR-1 immunoreactivity was located at the surface of the

![Figure 2](image-url)
Peptidase inhibitors increased the potencies of Leu-enkephalin and dynorphin A to produce MOR-1 internalization

The findings described above are consistent with our hypothesis that enkephalins and dynorphins are actively degraded by peptidases in the dorsal horn. The hypothesis also predicts that the inability of these opioids to produce MOR-1 internalization could be overcome at higher concentrations because they would saturate the peptidases, and that peptidase inhibitors would produce a left shift in their concentration–response curves to elicit MOR-1 internalization.

Figure 4A shows that these predictions are fulfilled for Leu-enkephalin. In the absence of peptidase inhibitors, high concentrations of Leu-enkephalin were able to produce MOR-1 internalization, yielding an EC$_{50}$ of 22 μM (95% CI, 17–30). Peptidase inhibitors (amastatin, captopril, and phosphoramidon) dramatically shifted the concentration–response curve of Leu-enkephalin to the left, increasing its potency by almost two orders of magnitude (EC$_{50}$, 0.38 μM; 95% CI, 0.31–0.47). This EC$_{50}$ value was very similar to the potency of Met-enkephalin (0.25 ± 0.6 μM) to produce outward currents in locus ceruleus neurons in the presence of the peptidase inhibitor ketalorphan (Williams et al., 1987). Figure 4B shows that the predictions of the hypothesis are also satisfied for dynorphin A, although in this case the left shift of the concentration–response curve produced by peptidase inhibitors is less dramatic: the EC$_{50}$ for dynorphin A decreased by one order of magnitude, from 5.2 μM (95% CI, 4.3–6.3) to 0.60 μM (95% CI, 0.34–1.04). Hill coefficients calculated for the concentration–response curves were significantly higher than 1 (based on an F test with p < 0.005, see Statistical analysis, above) for Leu-enkephalin with peptidase inhibitors (n$_H$, 2.8 ± 0.7) and for dynorphin A without peptidase inhibitors (n$_H$, 3.0 ± 0.6). Using a less stringent criterion in the F test (p < 0.05), n$_H$ was also >1 (n$_H$, 1.8 ± 0.5) for dynorphin A with peptidase inhibitors. These high Hill coefficients suggest the presence of positive cooperativity in processes that regulate the access of the opioids to the receptor or its internalization. The calculated EC$_{50}$ values were fairly independent of n$_H$ values. In the two cases in which n$_H$ was >1, we obtained similar EC$_{50}$ values if n$_H$ was assumed to be 1: Leu-enkephalin with peptidase inhibitors: EC$_{50}$, 0.36 μM (95%
Peptidase inhibitors did not affect the potency of endomorphins to elicit MOR-1 internalization

In contrast to the MOR-1 internalization produced by Leu-enkephalin and dynorphin A, peptidase inhibitors had no effect on the potencies of endomorphin-1 (Fig. 4C) or endomorphin-2 (Fig. 4D) to elicit MOR-1 internalization. Because dipeptidyl peptidase IV (EC 3.4.14.15) has been reported to play an important role in degrading endorphins (Shane et al., 1999), we included in the assay the dipeptidyl peptidase IV inhibitor Ala-pyrrolidine-nitrile (Li et al., 1995) (10 μM) in addition to amastatin, captopril, and phosphoramidon. However, even with this addition the potency of endomorphin-1 was the same in the presence (EC$_{50}$ 40 nM; 95% CI, 22–73) and in the absence (EC$_{50}$ 32 nM; 95% CI, 16–65) of peptidase inhibitors. Likewise, the potency of endomorphin-2 was the same with (EC$_{50}$ 10 nM; 95% CI, 6–15) and without (EC$_{50}$ 5 nM; 95% CI, 3–8) peptidase inhibitors. Diprotin A (Ile–Pro–Ile; 1 mM), another dipeptidyl peptidase IV inhibitor, did not increase the MOR-1 internalization produced by 1 nM endomorphin-2 (control, 18 ± 4%; n = 3; diprotin A, 8 ± 3%; n = 3).

Contribution of different peptidases to the degradation of Leu-enkephalin

Next, we determined the relative contribution of peptidases to preventing MOR-1 internalization by Leu-enkephalin by using different combinations of peptidase inhibitors. We took advantage of the fact that Leu-enkephalin at 1 μM elicited maximal MOR-1 internalization in the presence of peptidase inhibitors and minimal internalization in their absence (Fig. 4A). Hence, the ability of peptidase inhibitors to protect Leu-enkephalin against degradation would be reflected in the amount of MOR-1 internalization produced by 1 μM Leu-enkephalin. Figure 5A shows that in the presence of 10 μM amastatin, phosphoramidon, or captopril alone, or even combined two by two, 1 μM Leu-enkephalin remained unable to significantly stimulate MOR-1 internalization. These results indicate that each of the three peptidases targeted by these inhibitors (aminopeptidases, neutral endopeptidase, and dipeptidyl carboxypeptidase) (Fig. 1) is sufficient to degrade Leu-enkephalin substantially. Interpolating the data in Figure 5A in the left curve of Figure 4A suggests that each of these peptidases is able to reduce the concentration of Leu-enkephalin from 1 to <0.3 μM. Thoriphan, like phosphoramidon, inhibits neutral endopeptidase (Yakh and Chipkin, 1989). In the presence of 10 μM thiorphan, amastatin, and captopril, 1 μM Leu-enkephalin did produce significant MOR-1 internalization (Fig. 5A), although the effect was not as consistent as in the presence of phosphoramidon.

To further characterize the aminopeptidase(s) responsible for the degradation of enkephalins in the spinal cord, we substituted different aminopeptidase inhibitors for amastatin in our inhibitor mixture (Fig. 5B). Antipain, arphamenine, bacitracin, proctolin, and puromycin were ineffective. Other studies found that bacitracin (Tseng et al., 1986; Ozaki et al., 1994) and puromycin (Aoki et al., 1984) did not protect enkephalins against degradation. Only antipain (Fig. 1F) and amastatin (Fig. 1E) protect the effect of Leu-enkephalin at 10 μM. The effectiveness of amastatin was reduced at 1 μM but remained significant. Amastatin inhibits aminopeptidases N (EC 3.4.11.7), A (EC 3.4.11.2), and W (EC 3.4.11.16), with IC$_{50}$ values of 1–20 μM, whereas actinonin inhibits aminopeptidase N (IC$_{50}$ 2 μM) but not aminopeptidases A and W (Tieku and Hooper, 1992). Bestatin was ineffective at 10 μM but protected the effect of Leu-enkephalin at 100 μM (Fig. 1G). Bestatin potently inhibits aminopeptidase W (IC$_{50}$ 8 μM), inhibits aminopeptidase N at higher concentrations (IC$_{50}$ 89 μM), and does not inhibit aminopeptidase A (Tieku and Hooper, 1992; Suzuki et al., 1997). Together, these results show that the pharmacological profile of the aminopeptidase that degrades Leu-enkephalin matches that of aminopeptidase N.

Contribution of different peptidases to the degradation of dynorphin A

The effect of combinations of peptidase inhibitors on dynorphin A (Fig. 5C) was different from their effect on Leu-enkephalin (Fig. 5A). Amastatin plus phosphoramidon was as effective as all three peptidase inhibitors in protecting the effect of dynorphin A, indicating that dynorphin A is degraded by aminopeptidases and neutral endopeptidase. Moreover, significant (p < 0.05) protection could be achieved with amastatin alone. Actinonin could substitute for amastatin, which is consistent with the idea that...
aminopeptidase N is involved in the degradation of dynorphins as well as enkephalins. No protection of dynorphin A was obtained with phosphoramidon plus captopril, and the addition of captopril did not increase the effect of amastatin, which shows that the MOR-1 internalization produced by dynorphin A is not appreciably decreased by dipeptidyl carboxypeptidase. This is probably attributable to the fact that dipeptidyl carboxypeptidase starts cleaving dynorphin A at its extended C terminal (Fig. 1), producing peptides containing the Leu-enkephalin sequence that are still able to activate MOR-1.

Relative localization of opioid peptides and MOR-1 in the dorsal horn

The localization of opioid-containing terminals relative to MOR-1 IR neurons was explored using double-label immunofluorescence. To detect opioid peptides, we used a monoclonal antibody (3-E7) that recognized β-endorphin, enkephalins, dynorphins, and α-neoendorphin (Gramsch et al., 1983) but not endorphins (data not shown).

Costaining with the 3-E7 and the MOR-1 antibodies (Fig. 6A) showed that 3-E7 IR is very dense in laminas I and II, where MOR-1 IR cells were located, and also slightly deeper in the dorsal horn and in the dorsolateral funiculus, where MOR-1 staining was absent. High-magnification confocal images in sagittal sections (Fig. 6B) showed that 3-E7 IR terminals are dispersed around MOR-1 IR neurons and dendrites. Some 3-E7 IR terminals were in close apposition with MOR-1 IR neurons or colocalized with it (Fig. 6B, arrows), indicating the presence of synapses and presynaptic MOR-1.

Using an antibody that recognizes endorphin-1 and endorphin-2, we found that endorphin-like immunoreactivity did not colocalize with 3-E7 immunoreactivity (data not shown), suggesting that endorphins are contained in terminals different from other opioids. Endorphin-like immunoreactivity was found primarily in lamina I and the dorsolateral funiculus, whereas most MOR-1 IR neurons were found in lamina II (Fig. 6), separated from endorphin IR fibers and varicosities (data not shown). However, Spike et al. (2002) recently reported that endorphin-2 was present in substance-P-containing axons contacting MOR-1 neurons in the dorsal horn.

MOR-1 internalization produced by endogenously released opioids

To elicit opioid release, spinal cord slices were incubated with 30 μM veratridine or 50 mM KCl in the presence or absence of peptidase inhibitors (amastatin, captopril, and phosphoramidon). Veratridine increases Na⁺ fluxes through voltage-dependent Na⁺ channels (Satoh and Nakazato, 1991). These stimuli have been shown previously to elicit Met-enkephalin (Uzumaki et al., 1984; Yaksh and Chipkin, 1989) and dynorphin (Przewlocka et al., 1990) release from the spinal cord.

A short (2 min) incubation with 30 μM veratridine in the presence of peptidase inhibitors produced MOR-1 internalization in approximately two-thirds of MOR-1 IR cells (Fig. 7A). However, veratridine did not produce MOR-1 internalization in the absence of peptidase inhibitors. These data were analyzed with a two-way ANOVA with veratridine and peptidase inhibitors as the two variables, which showed significant (p < 0.0001) effects of the variables and their interaction. Bonferroni’s post-test revealed a significant effect (p < 0.001) of veratridine combined with peptidase inhibitors but no significant effects of peptidase inhibitors alone or veratridine without peptidase inhibitors.

Depolarization with high K⁺ in the presence of peptidase inhibitors elicited MOR-1 internalization in one-third of the MOR-1 IR neurons (Fig. 8A). In this experiment, slices were incubated for 20 min in ACSF containing 50 mM KCl, decreasing the concentration of NaCl by the same amount to avoid increasing the osmotic pressure. In the absence of peptidase inhibitors, 50 mM KCl produced MOR-1 internalization in a small percentage of MOR-1 IR neurons (Fig. 8A). Two-way ANOVA revealed significant effects of the variables high K⁺ and peptidase inhibitors (p < 0.0001) and their interaction (p < 0.01). Bonferroni’s post-test yielded a significant effect for 50 mM KCl without (p < 0.05) and with (p < 0.001) peptidase inhibitors.

Confocal images of neurons representative of these observations are shown in Figure 9. Neurons exposed to peptidase inhibitors (A) or veratridine (B) alone showed crisp surface staining and no endosomes. Figure 9F shows a neuron exposed to 50 mM KCl in the absence of peptidase inhibitors; it presents a somewhat discontinuous labeling of the membrane, possibly indicating some MOR-1 clustering. In the presence of peptidase inhibitors, veratridine produced clear internalization in most neurons, as shown by the group of three neurons in Figure 9C. The neuron in the bottom left corner does not have the nucleus in the confocal plane, and part of a fourth neuron is visible in the bottom right corner. Note the presence of intensely labeled endosomes and the decrease in surface staining. Figure 9G shows a neuron presenting internalization after treatment with high K⁺ and peptidase in-
hibitors. This neuron has a nucleus occupying most of the soma, which is common in MOR-1 IR neurons in the dorsal horn (see also Figs. 2, 6B). However, there is almost no surface staining, and several endosomes are visible in the cytoplasm and along one dendrite.

The selective MOR-1 antagonist CTAP (1 μM) abolished MOR-1 internalization produced by veratridine (Fig. 7B) or 50 mM KCl (Fig. 8A) in the presence of peptidase inhibitors. Representative neurons are shown in Figure 9D (veratridine) and Figure 9F (high K⁺). This observation indicates that the internalization was attributable to the interaction of agonists with
MOR-1. Moreover, if the internalization produced by veratridine and high K\(^+\) was attributable to the release of endogenous opioids, we predicted that it would be inhibited in the absence Ca\(^{2+}\) as well. However, it is possible that the mechanism of MOR-1 internalization itself requires Ca\(^{2+}\). Indeed, in the nominal absence of Ca\(^{2+}\) (no CaCl\(_2\) was added), the MOR-1 internalization produced by 1 \(\mu\)M endomorphin-2 was decreased from 100% of MOR-1 neurons with internalization (Fig. 4D) to 55 ± 18% (n = 5). However, a 10-fold reduction in Ca\(^{2+}\) concentration (from 2.4 to 0.2 mM) did not affect the MOR-1 internalization produced by 0.1 \(\mu\)M endomorphin-2, which was present in 97 ± 2% of the MOR-1 IR neurons (n = 3). Therefore, the MOR-1 internalization process can take place if a small amount of Ca\(^{2+}\) is present in the extracellular medium. Yet when the Ca\(^{2+}\) concentration was decreased to 0.2 mM, the MOR-1 internalization produced by 50 mM KCl (Figs. 7A, 8I) or veratridine (Figs. 6B, 8E) was abolished or greatly reduced, respectively. These results confirm that veratridine and high K\(^+\) produced MOR-1 internalization by eliciting the Ca\(^{2+}\)-dependent release of endogenous opioids.

**Contribution of different peptidases to the degradation of endogenously released opioids**

To determine which peptidases contributed to the degradation of the opioids released by veratridine or high K\(^+\), we studied the effect of different combinations of peptidase inhibitors. In the case of high K\(^+\) (Fig. 8B), only the mixture of the three inhibitors significantly increased (p < 0.05) MOR-1 internalization above that produced by 50 mM KCl alone. However, phosphoramidon plus captopril produced an effect that was not significantly different from the combination of the three inhibitors, and showed a trend toward increased internalization. In the case of veratridine (Fig. 7C), phosphoramidon plus captopril or phosphoramidon plus amastatin produced MOR-1 internalization significantly above control levels (p < 0.05). However, phosphoramidon by itself or amastatin plus captopril did not significantly increase internalization above the effect of veratridine alone. Mixtures of three inhibitors, including either amastatin or actinonin as aminopeptidase inhibitors, produced robust MOR-1 internalization (p < 0.01). These results indicate that neutral endopeptidase is the main enzyme degrading opioids released in the spinal cord, with a substantial contribution of aminopeptidases and dipeptidyl carboxypeptidase.

**Discussion**

In summary, in the dorsal horn: (1) peptidases restrict MOR-1 internalization by exogenously applied enkephalins and dynorphins, (2) endomorphins are not substantially degraded by peptidases, and (3) peptidases prevent MOR-1 internalization by endogenously released opioids.
enkephalin–degrading aminopeptidases are sensitive to puromycin (Shimamura et al., 1983; Dyer et al., 1990; Hui et al., 1998). However, we cannot rule out the involvement of aminopeptidases with a pharmacological profile similar to aminopeptidase N, like one associated with opioid receptors (Hui et al., 1985).

The ability of dynorphin A to produce MOR-1 internalization was decreased by aminopeptidases and neutral endopeptidase, but not appreciably by dipeptidyl carboxypeptidase, probably because their cleavage of dynorphin A produces active peptides containing the Leu-enkephalin sequence. Likewise, although dynorphin A does have a fairly high affinity for MOR-1 (Raynor et al., 1993; Zadina et al., 1997), we cannot rule out that MOR-1 internalization produced by dynorphin A was mediated through its conversion to Leu-enkephalin by peptidase EC 3.4.24.15 (Bell and Traynor, 1998). Importantly, in the absence of peptidase inhibitors, dynorphin A was more potent than Leu-enkephalin in producing MOR-1 internalization.

Endomorphin-1 and endomorphin-2 are not degraded by peptidases
Shane et al. (1999) showed that ventricular administration of an inhibitor of dipeptidyl peptidase IV enhanced the analgesic effect of endomorphin-2. In contrast, we found that inhibitors of this and other peptidases did not affect the abilities of endomorphins to produce MOR-1 internalization. It is possible that dipeptidyl peptidase IV activity in the dorsal horn is lower than in the brain. However, Tomboly et al. (2002) showed that endomorphins are degraded at slow rates in the brain. Unlike endomorphins, the opioids released by veratridine or high K⁺ were degraded by peptidases. It is possible that the marginal internalization elicited in the absence of peptidase inhibitors by high K⁺ (but not veratridine) was attributable to endomorphin release, but it could also be mediated by β-endorphin. Therefore, endomorphins, despite their high affinity, seem to contribute little to the activation of extrasynaptic MOR-1 in dorsal horn neurons.

Endogenously released opioids
The MOR-1 internalization produced by veratridine or high K⁺ was abolished by the MOR-1 antagonist CTAP and was Ca²⁺ dependent, indicating that it was attributable to the release of MOR-1 agonists. Veratridine-evoked MOR-1 internalization required peptidase inhibitors, and internalization produced by high K⁺ was greatly reduced in their absence, showing that most of the released opioids are degraded by peptidases. This is consistent with studies showing that peptidase inhibitors decreased ventral root potentials (Suzuki et al., 1997) and responses of dorsal horn neurons to C fiber activity (Dickenson et al., 1987).

Neutral endopeptidase appears to be the main enzyme degrading released opioids, because phosphoramidon plus amastatin or captopril, but not amastatin plus captopril, protected the effect of veratridine. However, phosphoramidon alone did not produce significant protection, indicating that the contribution of aminopeptidases and dipeptidyl carboxypeptidase is also important. In contrast, the internalization produced by Leu-enkephalin was similarly decreased by all three peptidases, whereas the effect of dynorphin A was not decreased by dipeptidyl carboxypeptidase. This discrepancy suggests that the opioids released are not the enkephalin pentapeptides or dynorphin A alone, but a mixture of opioid peptides of various lengths (Lucas and Yaksh, 1990), resulting in a mixed susceptibility to peptidases.

Our results show that MOR-1 internalization can be used to investigate mechanisms of opioid release. Opioids are not appreciably released from primary afferents, because prolonged dorsal root stimulation while superfusing the slices with peptidase inhibitors did not produce MOR-1 internalization (Marvizon and Song, 2002). Therefore, the opioid release detected here is probably from interneurons (Todd and Spike, 1993) and/or bulbospinal fibers (Babbaun and Fields, 1984; Le Bars et al., 1987b; Fields et al., 1991; Budai and Fields, 1998).

Physiological relevance
Our results likely reflect the situation in vivo. Amastatin, captopril, and phosphoramidon increased the analgesic effects of intrathecal or intracerebral opioids (Kishioka et al., 1994; Kitamura et al., 2000), but deletion of any of the inhibitors substantially decreased this effect. The peptidase inhibitor RB-101 [N-[(R,S)-2-benzyl-3-[S]-2-amino-4-methylthio]butyl dithio]-1-oxopropyl]-L-phenylalanine benzyl ester] injected systemically also produced analgesia (Noble et al., 1992a).

The relationship between MOR activation and its internalization is complex. Although internalization requires activation, the converse is not always true, because morphine activates MOR-1 without producing its internalization (Keith et al., 1998). Nevertheless, it has been suggested that endogenous opioids are able to produce MOR-1 internalization (Whistler et al., 1999). Our results support this idea, because all of the opioids tested produced MOR-1 internalization. Moreover, in the presence of peptidase inhibitors, the potency of Leu-enkephalin to produce MOR-1 internalization was remarkably similar to the potency of Met-enkephalin to evoke outward currents in locus ceruleus neurons (Williams et al., 1987). Therefore, MOR-1 internalization provides a good indicator of its activation by endogenous opioids.

In view of this, it is puzzling that noxious stimuli that elicited spinal enkephalin release (Yaksh and Elde, 1981; Cesselin et al., 1985; Le Bars et al., 1987a,b; Cesselin et al., 1989; Bourgoin et al., 1990) did not produce MOR-1 internalization in dorsal horn neurons (Trafton et al., 2000). We show here that this was probably because of opioid degradation, but the question remains as to why peptidases did not degrade opioids detected in release studies that did not use peptidase inhibitors. However, in other studies (Yaksh and Chipkin, 1989) peptidase inhibitors did produce a 10-fold increase in Met-enkephalin release. Peptidases are bound to the extracellular surface of neurons (Aoki et al., 1984; Roques, 2000) and may be associated with MOR (Hui et al., 1985), degrading opioids in their microenvironment. Thus, opioids diffusing away from the tissue would not be degraded as readily as those near the receptors. Conversely, exogenous opioids will be degraded as they approach MOR-1.

Our findings indicate that opioids, unlike neurokinins (Mantyh et al., 1995; Abbadie et al., 1997; Allen et al., 1997; Marvizon et al., 1997, 1999a; Trafton et al., 2001), do not normally operate by “volume transmission,” (i.e., by activating extrasynaptic receptors over a large region) (Fuxe and Agnati, 1991). Although released opioids inhibited dorsal horn neurons by activating MOR (Budai and Fields, 1998), this may be because of the activation of synaptic MOR. Indeed, we found opioid-containing terminals in close proximity to MOR-expressing dorsal horn neurons. Whether synaptic and extrasynaptic MORs are differently associated with peptidases needs to be investigated.

Yet, it is unlikely that the abundant extrasynaptic MOR-1 in dorsal horn neurons serves no function; hence, there may be some conditions in which opioids act by volume transmission. First, during inflammation and hyperalgesia, dynorphin and enkephalins are upregulated (Draisci et al., 1991; MacArthur et al., 1999; Wang et al., 2000). However, although the spinal release of
dynorphin was increased during inflammation, Met-enkephalin release was reduced (Pohl et al., 1997), and noxious stimulation during inflammation failed to produce MOR-1 internalization (Trafton et al., 2000). Second, peptidase activity could be downregulated in some conditions. For example, the activity of a substance P endopeptidase in the spinal cord was altered during morphine tolerance and withdrawal (Zhou et al., 2001). Moreover, an enkephalin-degrading aminopeptidase can be switched between cytosolic and membrane-bound forms (Dyer et al., 1990). Third, these peptidases also degrade other neuropeptides, notably neurokinins (Duggan et al., 1992), and could become saturated when neuropeptides are released in large amounts or inhibited otherwise. For example, enkephalin-degrading aminopeptidases are inhibited by substance P (Hersh, 1985; Shimamura et al., 1991).

**Therapeutic implications**

It has been suggested that peptidase inhibitors could be used to treat pain, because they have analgesic effects (Noble et al., 1992a) and do not produce tolerance and addiction (Noble et al., 1992b,c; Whistler et al., 1999; Roques, 2000). This may be because pain causes the release of opioids in the dorsal horn but not in brain areas involved in addiction. Although these peptidases also degrade neurokinins (Duggan et al., 1992), peptidase inhibitors had a comparatively small effect on their ability to produce neurokinin 1 receptor internalization in dorsal horn neurons (X. Wang and J. C. G. Marvizon, unpublished observations). Our findings underscore the importance of inhibiting dipeptidyl carboxypeptidase, in addition to aminopeptidase N and neutral endopeptidase (Noble et al., 1992a,b; Roques, 2000), to fully protect from the effect of endogenously released opioids.

**References**

Abbadie C, Trafton J, Liu H, Mantyh PW, Basbaum AI (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.

Abbadie C, Pasternak GW, Aicher SA (2001) Presynaptic localization of the carboxy-terminal epitopes of the μ opioid receptor splice variants MOR-1C and MOR-1D in the superficial laminae of the rat spinal cord. Neuroscience 106:853–842.

Aimone LD, Yuksh TL (1989) Opioid modulation of capsaicin-evoked release of substance P from rat spinal cord in vivo. Peptides 10:1127–1131.

Allen BJ, Rogers SD, Ghilardi J, Menning PM, Simone DA (1998) 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.

Allen BJ, Li J, Menning PM, Rogers SD, Ghilardi J, Mantyh PW, Simone DA (1999) Primary afferent fibers that contribute to increased substance P receptor internalization in the spinal cord after injury. J Neurophysiol 81:1379–1390.

Aoki K, Kajiwara M, Oka T (1984) The role of bestatin-sensitive aminopeptidase, angiotensin converting enzyme and thiopran-sensitive “enkephalainase” in the potency of enkephalins in the guinea–pig ileum. Jpn J Pharmacol 36:39–65.

Arvidsson U, Riedl M, Chakrabarti S, Lee JH, Nakano AH, Dado RJ, Loh HH, Law PY, Wessendorf MW, Elde R (1995) Distribution and targeting of a μ-opioid receptor MOR1 in brain and spinal cord. J Neurosci 15:3328–3341.

Basbaum AI, Fields HL (1984) Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annu Rev Neurosci 7:309–338.

Bell KM, Traynor JR (1998) Dynorphin A 1–8: stability and implications for in vitro opioid activity. Can J Physiol Pharmacol 76:325–333.

Bewley TA, Li CH (1985) Tertiary structure in deletion analogues of human β-endorphin: resistance to leucine aminopeptidase action. Biochemistry 24:6568–6571.

Bourgon S, Le Bars D, Clot AM, Hamon M, Cesselin F (1990) Subcutaneous formalin induces a segmental release of Met-enkephalin-like material from the rat spinal cord. Pain 41:323–329.

Budai D, Fields HL (1998) Endogenous opioid peptides acting at μ-opioid receptors in the dorsal horn contribute to midbrain modulation of spinal nociceptive neurons. J Neurophysiol 79:677–687.

Burghard JP, De Kloet ER (1982) Proteolysis of β-endorphin in brain tissue. Peptides 3:451–453.

Cesselin F, Le Bars D, Bourgoin S, Artaud F, Gozlan H, Clot AM, Besson JM, Hamon M (1985) Spontaneous and evoked release of methionine-enkephalin-like material from the rat spinal cord in vivo. Brain Res 339:305–313.

Cesselin F, Bourgoin S, Clot AM, Hamon M, Le Bars D (1989) Segmental release of met-enkephalin-like material from the spinal-cord of rats, elicited by noxious thermal stimuli. Brain Res 484:71–77.

Chou J, Tang J, Del Rio J, Yang HY, Costa E (1984) Action of peptidase inhibitors on methionine-enkephalin-arginine-phenylalanine (YGGFM) and methionine-enkephalin (YGGFM) metabolism and on electroacupuncture antinociception. J Pharmacol Exp Ther 230:349–352.

Dickerson AH, Sullivan AF, Fournie-Zaluski MC, Roques BP (1987) Prevention of degradation of endogenous enkephalins produces inhibition of nociceptive neurones in rat spinal cord. Brain Res 408:185–191.

Draisci G, Kajander KC, Dubner R, Bennett GJ, Iadara M (1991) Up-regulation of opioid gene expression in spinal cord evoked by experimental nerve injuries and inflammation. Brain Res 560:186–192.

Duggan AW, Saible H-G, Hope PJ, Lang CW (1992) Effect of peptidase inhibition on the pattern of intraspinally released immunoreactive substance P detected with antibody microprobes. Brain Res 579:261–269.

Dyer SH, Slaughter CA, Orth K, Moonaw CR, Hersh LB (1990) Comparison of the soluble and membrane-bound forms of the purumycin-sensitive enkephalin-degrading aminopeptidases from rat. J Neurochem 54:547–554.

Eckersell CB, Popper P, Micevych PE (1998) Estrogen-induced alteration of µ-opioid receptor immunoreactivity in the medial preoptic nucleus and medial amygdala. J Neurosci 18:3967–3976.

Fields HL, Heinricher MM, Mason P (1991) Neurotransmitters in nociceptive modulatory circuits. Annu Rev Neurosci 14:219–245.

Fuxe K, Agnati LF (1991) Two principal modes of electrochemical communication in the brain: volume versus wiring transmission. In: Volume transmission in the brain (Fuxe K, Agnati LF, eds), pp 1–9. New York: Raven.

Graf L, Paldi A, Patthy A (1985) Action of neutral metalloendopeptidase (“enkephalinase”) on θ-endorphin. Neuropeptides 6:13–19.

Graumich C, Meo T, Riethmuller G, Herz A (1983) Binding characteristics of a monoclonal θ-endorphin antibody recognizing the N-terminus of opioid peptides. J Neurochem 40:1220–1226.

Guyon A, Roques BP, Guyon F, Foucault A, Perdrisot R, Swerts JP, Schwartz JC (1979) Enkephalin degradation in mouse brain studied by a new HPLC method: further evidence for the involvement of carboxypeptidase. Life Sci 25:1605–1611.

Hersh LB (1985) Characterization of membrane-bound aminopeptidases from rat brain: identification of the enkephalin-degrading aminopeptidase. J Neurochem 44:1427–1435.

Hersh LB, Aboukhair N, Watson S (1987) Immunohistochemical localization of aminopeptidase M in rat brain and periphery: relationship of enzyme localization and enkephalin metabolism. Peptides 8:523–532.

Hiranuma T, Oka T (1986) Effects of peptidase inhibitors on the [Met5]- enkephalin hydrolysis in ileal and striatal preparations of guinea-pig: almost complete protection of degradation by the combination of amastatin, captopril and thiorphan. J Pharmacol Exp Ther 41:437–446.

Hiranuma T, Iwao K, Kitamura K, Matsumiya T, Oka T (1997) Almost complete protection of degradation of endogenous enkephalins produces inhibition of nociceptive neurones in rat spinal cord. J Neurosci 17:5921–5927.

Kajiwara M, Oka T (1986) Stability and implications for enkephalinase on Met-endorphin in brain tissue. J Pharmacol Exp Ther 281:769–774.

Hiranuma T, Kitamura K, Taniguchi T, Kobayashi T, Tamaki R, Kanai M, Akahori K, Iwao K, Oka T (1998a) Effects of three peptidase inhibitors, amastatin, captopril and phosphoramidon, on the hydrolysis of [Met5]-enkephalin-Arg6-Phe7 and other opioid peptides. Naunyn Schmiedebergs Arch Pharmacol 357:276–282.

Hiranuma T, Kitamura K, Taniguchi T, Kanai M, Arai Y, Iwao K, Oka T (1998b) Protection against dynorphin-(1–8) hydrolysis in membrane
preparations by the combination of amastatin, captopril and phosphoramidon. J Pharmacol Exp Ther 286:863–869.

Honore P, Menning PM, Rogers SD, Nichols ML, Basbaum AI, Besson JM, Mantyh PW (1999) Spinal cord substance P receptor expression and internalization in acute, short-term, and long-term inflammatory pain states. J Neurosci 19:7670–7678.

Hui KS, Gioannini T, Hui M, Simon EJ, Lajtha A (1985) An opiate receptor-associated aminopeptidase that degrades enkephalin. Neurochem Res 10:1047–1058.

Hui KS, Saito M, Hui M (1998) A novel neuron-specific aminopeptidase in rat brain synaptosomes. Its identification, purification, and characterization. J Biol Chem 273:31053–31060.

Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet-Agius G, Stewart PL, Evans CJ, von Zastrow M (1998) ß-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. Mol Pharmacol 53:377–384.

Kemp T, Spike RC, Watt C, Todd AJ (1996) The ß- opioid receptor (MOR1) is mainly restricted to neurons that do not contain GABA or glycine in the superficial dorsal horn of the rat spinal cord. Neuroscience 75:1231–1238.

Kishioka S, Miyamoto Y, Fukunaga Y, Nishida S, Yamamoto H (1994) Effects of a mixture of peptide inhibitors (aminastatin, captopril and phosphoramidon) on Met-enkephalin-, ß-endorphin-, dynorphin-(1–13), and electroacupuncture-induced antinociception in rats. Jpn J Pharmacol 66:337–345.

Kitamura K, Akahori K, Yano H, Iwao K, Oka T (2000) Effects of peptide inhibitors on anti-nociceptive action of dynorphin-(1–8) in rats. Naunyn Schmiedebergs Arch Pharmacol 361:273–278.

Law PY, Wong YH, Loh HH (2000) Molecular mechanisms and regulation of opioid receptor signaling. Annu Rev Pharmacol Toxicol 40:389–430.

Le Bars D, Bourgoin S, Clot AM, Hamon M, Cesselin F (1987a) Noxious mechanical stimuli increase the release of Met-enkephalin-like material heterosynaptically in the rat spinal cord. Brain Res 402:188–192.

Le Bars D, Bourgoin S, Villanueva L, Clot AM, Hamon M, Cesselin F (1987b) Involvement of the dorsolateral funiculi in the spinal release of Met-enkephalin-like material triggered by heterosynaptic noxious mechanical stimuli. Brain Res 412:190–195.

Li J, Wilk E, Wilk S (1995) Aminoclycprolyliden-2-nitrites: potent and stable inhibitors of dipetidyl-peptidase IV (CD 26). Arch Biochem Biophys 323:148–154.

Liu H, Mantyh PW, Basbaum AI (1997) NMDA-receptor regulation of substance P release from primary afferent nociceptors. Nature 386:721–724.

Lucas D, Yaksh TL (1990) Release in vivo of Met-enkephalin and encrypted forms of Met-enkephalin from brain and spinal cord of the anesthetized cat. Peptides 11:1119–1125.

MacArthur L, Ren K, Pfaffenroth E, Franklin E, Ruda MA (1999) Descending modulation of opioid-containing nociceptive neurons in rats with peripheral inflammation and hyperalgesia. Neuroscience 88:499–506.

Mansour A, Khachaturian M, Ishii K, Kuno Y, Hiranuma T, Matsumiya T (1990) Spinal cord substance P receptor expression and electroacupuncture-induced antinociception in rats. J Neurosci 10:1047–1058.

Noble F, Banisadr G, Jardinad F, Popoviciti T, Lai-Kuen R, Chen H, Bischoff L, Parsadantiz SM, Fournie-Zaluski MC, Roques BP (2001) First discrete autoradiographic distribution of aminopeptidase N in various structures of rat brain and spinal cord using the selective iodinated inhibitor [35S]BB 129. Neuroscience 105:479–488.

Numata H, Hiranuma T, Oka T (1988) Inactivation of dynorphin-(1–8) in isolated preparations by three peptides. Jpn J Pharmacol 47:417–423.

Oka T, Aoki K, Kajiwara M, Ishii K, Kuno Y, Hiranuma T, Matsumiya T (1986) Inactivation of Leu5-enkephalin in three isolated preparations: relative importance of aminopeptidase, endopeptidase-24.11 and peptidyl dipeptidase A. NIDA Res Monogr 75:259–262.

Ozaki M, Miyamoto T, Kishioka S, Masuda Y, Yamamoto H (1994) Effect of some peptide inhibitors on exogenous and endogenous opioid actions in guinea-pig ileum. Biol Pharm Bull 17:62–69.

Pierce TL, Grahek MD, Wessendorf MW (1998) Immunoreactivity for endomorphin-2 occurs in primary afferents in rats and monkey. NeuroReport 9:385–389.

Pohl M, Ballet S, Collin E, Mauborgne A, Bourgoin S, Benoliel JJ, Hamon M, Cesselin F (1997) Enkephalinergic and dynorphinergic neurons in the spinal cord and dorsal root ganglia of the polyrhythmic rat: in vivo release and cDNA hybridization studies. Brain Res 749:18–28.

Przewlocka B, Lason W, Dziedzicka M (1990) Modulation of prodynorphin peptides release from the rat spinal cord in vitro. Neuropeptides 16:201–206.

Przewlocki R, Przewlocka B (2001) Opioids in chronic pain. Eur J Pharmacol 429:79–91.

Randic M, Jiang MC, Cerne R (1993) Long-term potentiation and long-term depression of primary afferent neurotransmission in the rat spinal cord. J Neurosci 13:5228–5241.

Raynor K, Kong H, Chen Y, Yasuda K, Yu L, Bell GI, Reisine T (1993) Pharmacological characterization of the cloned ë-, Í- and ß- opioid receptors. Mol Pharmacol 45:330–334.

Roques BP (2000) Novel approaches to targeting neuropeptide systems. Trends Pharmacol Sci 21:475–483.

Sandkuhler J, Chen JG, Cheng G, Randic M (1997) Low-frequency stimulation of afferent Aδ-fibers induces long-term depression at primary afferent synapses with substantia gelatinosa neurons in the rat. J Neurosci 17:6483–6491.

Sato E, Nakazato Y (1991) Effects of monensin and veratridine on acetylcholine release and cytosolic free Ca2+ levels in cerebrocortical synaptosomes of rats. J Neurochem 57:1270–1278.

Shane R, Wilk S, Bodnar RJ (1999) Modulation of endomorphin-2-induced analgesia by dipetidyl peptidase IV. Brain Res 815:278–286.

Shimamura M, Hazato T, Katayama T (1983) A membrane-bound aminopeptidase isolated from monkey brain and its action on enkephalin. Biochim Biophys Acta 756:223–229.

Shimamura M, Hazato T, Iwaguchi T (1991) Enkephalin-degrading aminopeptidase in the longitudinal muscle layer of guinea pig small intestine: its properties and action on neuropeptides. J Biochem (Tokyo) 109:492–497.

Song B, Marzviton JCG (2002) Peptidases restrict ß- opioid receptor activation by enkephalins but not endorphins in the rat dorsal horn. Eur J Pharmacol 453:300–334.

Song and Marzviton JCG • Peptidases Prevent MOR Internalization by Opioids J. Neurosci, March 1, 2003 • 23(5):1847–1858 • 1857
re-evaluation of the actions of bestatin and inhibitors of angiotensin converting enzyme. Biochem Pharmacol 44:1725–1730.

Todd AJ, Spike RC (1993) The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. Prog Neurobiol 41:609–645.

Tomboly C, Peter A, Toth G (2002) In vitro quantitative study of the degradation of endomorphins. Peptides 23:1573.

Trafton J, Abbadie C, Liu H, Basbaum AI (1997) Internalization of the μ opioid receptor (MOR) in spinal cord neurons of the rat in vivo. Soc Neurosci Abstr 23:175.16.

Trafton JA, Abbadie C, Marchand S, Mantyh PW, Basbaum AI (1999) Spinal opioid analgesia: how critical is the regulation of substance P signaling? J Neurosci 19:9642–9653.

Trafton JA, Abbadie C, Marek K, Basbaum AI (2000) Postsynaptic signaling via the μ-opioid receptor: responses of dorsal horn neurons to exogenous opioids and noxious stimulation. J Neurosci 20:8578–8584.

Trafton JA, Abbadie C, Basbaum AI (2001) Differential contribution of substance P and neurokinin A to spinal cord neurokinin-1 receptor signaling in the rat. J Neurosci 21:3656–3664.

Tseng LF, King RC, Fujimoto JM (1986) Lack of inhibitory effect of aprotinin and bacitracin on the spinal release of Met-enkephalin induced by intraventricular β-endorphin. Peptides 7:369–371.

Uzumaki U, Govoni S, Facchin E, Pasinetti G, Missale C, Trabucchi M (1984) Neuropeptidergic inhibitory regulation of Met-enkephalin immunoreactive material release from rat spinal cord in vitro. Peptides 5:849–852.

Wang H, Wang R, Nie H, Zhang R, Qiao JT (2000) Neurokinin A, calcitonin gene-related peptide, and dynorphin A (1–8) in spinal dorsal horn contribute to descending inhibition evoked by nociceptive afferent pathways: an immunocytochemical study. Regul Pept 89:7–12.

Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M (1999) Functional dissociation of μ opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron 23:737–746.

Williams JT, Christie MJ, North RA, Roques BP (1987) Potentiation of enkephalin action by peptidase inhibitors in rat locus ceruleus in vitro. J Pharmacol Exp Ther 243:397–401.

Yaksh TL, Chipkin RE (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.

Yaksh TL, Elde RP (1981) Factors governing release of methionine enkephalin-like immunoreactivity from mesencephalon and spinal cord of the cat in vivo. J Neurophysiol 46:1056–1073.

Yaksh TL, Jessell TM, Gamse R, Mudge AW, Leeman SE (1980) Intrathecal morphine inhibits substance P release from mammalian spinal cord in vivo. Nature 286:155–157.

Zadina JE, Hackler L, Ge L-J, Kastin AJ (1997) A potent and selective endogenous agonist for the μ-opiate receptor. Nature 386:499–502.

Zhou Q, Karlsson K, Liu Z, Johansson P, Le Greves M, Kiuru A, Nyberg F (2001) Substance P endopeptidase-like activity is altered in various regions of the rat central nervous system during morphine tolerance and withdrawal. Neuropharmacology 41:246–253.