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Comparing analgesia and μ-opioid receptor internalization produced by intrathecal enkephalin: Requirement for peptidase inhibition

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Abstract

Opioid receptors in the spinal cord produce strong analgesia, but the mechanisms controlling their activation by endogenous opioids remain unclear. We have previously shown in spinal cord slices that peptidases preclude μ-opioid receptor (MOR) internalization by opioids. Our present goals were to investigate whether enkephalin-induced analgesia is also precluded by peptidases, and whether it is mediated by MORs or δ-opioid receptors (DORs). Tail-flick analgesia and MOR internalization were measured in rats injected intrathecally with Leu-enkephalin and peptidase inhibitors. Without peptidase inhibitors, Leu-enkephalin produced neither analgesia nor MOR internalization at doses up to 100 nmol, whereas with peptidase inhibitors it produced analgesia at 0.3 nmol and MOR internalization at 1 nmol. Leu-enkephalin was 10 times more potent to produce analgesia than to produce MOR internalization, suggesting that DORs were involved. Selective MOR or DOR antagonists completely blocked the analgesia elicited by 0.3 nmol Leu-enkephalin (a dose that produced little MOR internalization), indicating that it involved these two receptors, possibly by an additive or synergistic interaction. The selective MOR agonist endomorphin-2 produced analgesia even in the presence of a DOR antagonist, but at doses substantially higher than Leu-enkephalin. Unlike Leu-enkephalin, endomorphin-2 had the same potencies to induce analgesia and MOR internalization. We concluded that low doses of enkephalins produce analgesia by activating both MORs and DORs. Analgesia can also be produced exclusively by MORs at higher agonist doses. Since peptidases prevent the activation of spinal opioid receptors by enkephalins, the coincident release of opioids and endogenous peptidase inhibitors may be required for analgesia.

Keywords: Aminopeptidase; Dipeptidyl carboxypeptidase; EC.3.4.11.7; EC.3.4.15.1; EC.3.4.24.11; Endomorphin; Enkephalin; Delta opioid receptor; Dorsal horn; Intrathecal; Mu opioid receptor; Neutral endopeptidase; Peptidase inhibitors; Tail-flick test

1. Introduction

Opioid receptors in the spinal cord produce strong analgesia (Budai and Fields, 1998; Jensen and Yaksh, 1984; Morgan et al., 1991; Zorman et al., 1982). Although the endogenous opioid peptides (henceforth "opioids") have been known for some time, little is known about the neuronal circuitry and pharmacological mechanisms that control their release. Yet, these issues are important to clarify the role of opioids in conditions that produce analgesia, such as stress (Yamada and Nabeshima, 1995), acupuncture (Han, 2003) or pain (Gear et al., 1999). Moreover, there is evidence that opioids produce less tolerance than morphine (Graf et al., 1979; Noble et al., 1992; Whistler et al., 1999). Hence, if it were possible to produce analgesia by stimulating opioid release, this would provide a valuable alternative to the use of opiate drugs.

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There are several unresolved issues regarding the activation of opioid receptors in the spinal cord by endogenous opioids.

First, opioids are degraded very effectively three peptidases: aminopeptidase, dipeptidyl carboxypeptidase and neutral endopeptidase (Guyon et al., 1979; Hiranuma et al., 1997, 1998b). In a previous study in spinal cord slices (Song and Marvizon, 2003a), we showed that inhibitors of these three peptidases increase the potencies of dynorphin A and Leu-enkephalin to produce MOR internalization by one and two orders of magnitude, respectively. Moreover, it only has been possible to produce MOR internalization in dorsal horn neurons by releasing opioids in the presence of peptidase inhibitors (Song and Marvizon, 2003a,b, 2005; Trafton et al., 2000). This presents us with a paradox: releasing opioids would be futile if they are degraded before they activate their receptors.

Second, there are discrepancies between measures of opioid release in the spinal cord by immunoassay and MOR internalization. MOR internalization has the advantages of indicating the areas of opioid release and showing that the released peptides have activated the receptors. It has been used to measure opioid release in the spinal cord (Song and Marvizon, 2003a,b, 2005; Trafton et al., 2000), the hypothalamus and amygdala (Eckersell et al., 1998; Mills et al., 2004; Sinchak and Micevych, 2001) and the intestine (Patierno et al., 2005). Evidence that MOR internalization is a valid measure of MOR activation by released opioids includes: (1) all naturally occurring opioids induce MOR internalization (Song and Marvizon, 2003a); (2) the dose–responses of DAMGO (a selective MOR agonist) to elicit MOR internalization (Marvizon et al., 1999), adenylyl cyclase inhibition (Keith et al., 1998, 1996) and [γ-^{35}S]GTP binding (Yabaluri and Medzihradsky, 1997) are virtually identical; and (3) DAMGO-induced analgesia correlated with MOR internalization (Trafton et al., 2000). Trafton et al. (2000) found that MOR internalization in dorsal horn neurons could not be induced by any modality of noxious stimulation. However, in previous work in which opioid release was measured by immunoassay of spinal cord superfusates, it was found that it could be elicited by thermal, chemical and mechanical noxious stimuli (Bourgoin et al., 1990; Cesselin et al., 1989; Le Bars et al., 1987a,b), or by stimulation of the sciatic nerve (Yaksh et al., 1983). This discrepancy could be explained by the fact that Trafton et al. (2000) assessed MOR internalization in the absence of peptidase inhibitors. But, if peptidases prevent the opioids released in the spinal cord from producing MOR internalization, do they also prevent opioids from producing analgesia? This question, and the paradox mentioned above, prompted us to study the ability of Leu-enkephalin to produce analgesia in the presence and absence of peptidase inhibitors, and to compare it with its ability to induce MOR internalization in the same animals.

Finally, a related question is whether the analgesia produced by enkephalins in the spinal cord is mediated by μ or δ opioid receptors: according to Takemori and Portoghese (1993), it is mediated by δ opioid receptors (DORs), whereas Budai and Fields (1998) reported that it is mediated by MORs. Hence, we also assessed the ability of MOR and DOR antagonists to inhibit enkephalin-induced analgesia. Part of this study was published previously as an abstract (Chen et al., 2006).

2. Materials and methods

2.1. Animals

Experiments were performed in adult male Sprague-Dawley rats (250–300 g, Harlan Sprague-Dawley, Indianapolis, IN). Rats were housed in standard cages on a 12 h light/dark cycle and allowed free access to food and water. Animal procedures were approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Greater Los Angeles Healthcare System, and conform to NIH guidelines. Efforts were made to minimize the number of animals and their suffering.

2.2. Intrathecal injections

Rats were implanted with chronic intrathecal catheters for drugs injection following established procedures (LoPachin et al., 1981; Yaksh and Rudy, 1976). Rats were anesthetized with isoflurane using an induction box, and anesthesia was maintained throughout the procedure with a vaporizer delivering isoflurane (2–4%) in oxygen through a nose cone. The back of the head and neck was shaved and the rat was placed on an aluminum platform maintained at 37 °C. The head was clamped flexed forward using the ear bars of a stereotaxic holder. A midline incision was made in the skin at the back of the neck, and the muscle was cut at its juncture with the edge of the cranium. The atlanto-occipital cisternal membrane was exposed and punctured with a needle to insert an 8.0 cm polyethylene (PE-5 or PE-10) catheter, which was passed into the intrathecal space to the L3–L4 spinal segment. The volume of the catheter was 5 μl when using PE-5 tubing, and 10 μl with PE-10. The other end of the catheter was tunneled through the subcutaneous space over the cranium and externalized. Muscle and skin were sutured. The catheter was flushed with 10 μl saline and shut with an electrical cauterizer. Rats that showed motor weakness or signs of paresis were euthanized. The rats were housed separately and given an antibiotic (enrofloxacin) and an analgesic (carprofen) for 5 days. They recover for 5–7 days prior to the experiment. The day of the experiment the catheter was cut open and used for the intrathecal injections. In most experiments the volume of the injectate was 20 μl plus 10 μl of saline to flush the catheter, but it was reduced to 1 μl plus 5 μl saline flush in some experiments. Solutions were preloaded, in reverse order of administration, into a tube, and delivered with 10 μl or 100 μl Hamilton syringes within 1 min. In all the rats, the position of the catheter was examined when the spinal cord was extracted after fixation. If the catheter terminated inside the spinal cord or its tip was otherwise occluded, the animal was excluded from the study.

2.3. Tail-flick responses

Tail-flick reflex responses were measured using a Tail-Flick Analgesia Meter (Columbus Instruments, Columbus, OH). Cut-off time was set at 10 s to prevent the rat's tail from being burned. Rats were put in a Plexiglas restraint chamber and allowed to acclimate to the instrument for 30 min. Baseline latencies were measured at 5 min intervals, three times or until consistent measures were obtained. The criterion for consistency was that the difference between two consecutive latencies was less than 0.5 s. Within 2 min of establishing the baseline, drugs were injected intrathecally. Five minutes after the injection, tail-flick latencies were measured again at 5 min intervals, five times. Results were calculated as percentage of the maximum possible response [%MPE = 100 × (latency − baseline)/(cut-off − baseline) (Paronis and Holtzman, 1991), where cut-off = 10 s]. Thirty minutes after the injection the rat was fixed by aortal perfusion, and its spinal cord extracted for MOR immunohistochemistry.

2.4. Immunohistochemistry

MOR immunohistochemistry was performed as described (Marvizon et al., 1999; Song and Marvizon, 2003a,b, 2005). In brief, rats were euthanized with an overdose of isoflurane and fixed by aortal perfusion of 100 ml PBS (pH 7.4) at room temperature, followed by 400 ml of ice-cold fixative (4% paraformaldehyde, 0.18% picric acid in 0.1 M sodium phosphate buffer). Immediately after fixation, a laminectomy was performed to extract the lumbar spinal cord (L1–L6), which was cleaned of dura matter, cut into one-segment blocks, post-fixed and cryoprotected in 20% sucrose. The spinal cord segments were embedded in a drop of Tissue-Tek (Sakura Finetek USA, Inc., Torrance, CA), and frozen on dry ice. Free-floating sections (25 μm thick) were prepared with a cryostat. Sections were washed twice with PBS and twice with PBS containing 0.5% Triton X-100, 0.01% thimerosal (PBS/Triton) and 5% normal goat serum (NGS, Jackson ImmunoResearch, West Grove, PA). Sections were incubated with a rabbit antiserum (1:7000 dilution) raised against amino acids 384–398 of the cloned rat MOR-1 (DiaSorin, Stillwater, MN, catalog no. 24216). This antiserum has been characterized (Arvidsson et al., 1995) and shown to label dorsal horn neurons (Spike et al., 2002). Pre-absorption of the MOR antibody with its immunizing peptide (10 μg/ml) abolished the staining. After three washes with PBS, sections were incubated for 2 h at room temperature with the second antibody: Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) at 1:2000 dilution. After four more washes, sections were mounted on glass slides and coverslipped in Prolong Gold (Molecular Probes).

2.5. Quantification of MOR internalization

MOR internalization was quantified as previously described (Marvizon et al., 1999; Song and Marvizon, 2003a,b, 2005). Briefly, MOR neurons were counted visually, classifying them as with or without MOR internalization. This was done with a Zeiss Axio-Imager A1 (Carl Zeiss, Inc., Thornwood, NY) microscope using a fluorescence filter cube for Alexa Fluor 488 (excitation 460–500 nm, beam-splitter 505 nm, emission 510–560 nm; Chroma Technology Corporation, Rockingham, VT) and objectives of 63× (numerical aperture [NA] 1.40) and 100× (NA 1.40). Counting was done blind to the treatment. Neuronal somata with five or more endosomes were considered as having internalization. All MOR neurons of one dorsal horn were counted for each histological section, and four sections per rat (chosen randomly) were used. This amounted to 50–100 MOR neurons counted for each rat.

2.6. Confocal microscopy

Confocal images were acquired at UCLA's Carol Moss Spivak Cell Imaging Facility with a Leica TCS-SP confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Excitation was provided by an argon (488 nm) laser, and the emission window was 500–550 nm. The pinhole was 1.0 Airy unit. Low magnification images were obtained with a 20× objective (NA 0.7) and consist of two optical sections 2.53 μm thick (full width half maximum) separated 2.40 μm. High magnification images were obtained with a 100× objective (NA 1.40) and a digital zoom of 2, and consist of 1–3 optical sections 0.62 μm thick, separated 0.49 μm. Optical sections were averaged 3–6 times to reduce noise. Images were processed using Adobe Photoshop 5.5. The "curves" feature of the program was used to adjust the contrast. Images were acquired at a digital size of 1024 × 1024 pixels and cropped to the relevant part of the field.

2.7. Data analysis

Data were analyzed using Prism 4.3 (GraphPad Software, San Diego, CA). Statistical analyses consisted of two-way ANOVA and Bonferroni's post-test, with significance set at 0.05. The first variable of the two-way ANOVA, to which the Bonferroni's post-test was applied, was "drugs" (drugs injected intrathecally). The second variable was spinal cord segment for MOR internalization data, and time after injection for tail-flick data. The absence of asterisks in the figure indicates that the difference with control is not significant. Dose–response data were fitted by the logistic dose–response function: $Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10\text{^}(\text{Log EC}_{50} - \text{Log X}))$, where "top" and "bottom" are the maximum and minimum values, respectively, of the response (Y), and EC_{50} is the dose (X) that produces half of the maximum effect. Parameter constraints were top <100% and bottom >0%. The statistical error of the EC_{50} was expressed as 95% confidence interval (CI). An F-test (Motulsky and Christopoulos, 2003) was used to determine whether the Log EC_{50} of two sets of data was significantly different.

3. Results

3.1. Intrathecal enkephalin requires peptidase inhibitors to produce MOR internalization

In a previous study (Song and Marvizon, 2003a), we found that enkephalins were unable to induce MOR internalization in dorsal horn neurons unless they were delivered at high concentrations or in the presence of peptidase inhibitors. That study was done in spinal cord slices, so it remained unclear whether those results reflected the physiological situation. We have followed up that study with experiments in vivo. We injected opioid peptides and peptidase inhibitors onto the lumbar spinal cord of adult rats using chronic intrathecal catheters. To measure MOR internalization, the rat was fixed by aortal perfusion 30 min after the injection, and MOR immunohistochemistry was performed in coronal sections from lumbar spinal segments L2–L6. During the 30 min between the injection and the fixation, pain responses were measured using the tail-flick test (see next section). MOR internalization can be measured 30 min after it is induced because once these receptors are internalized it takes them about 60 min to recycle to the cell surface (Trafton et al., 2000).

As reported previously (Marvizon et al., 1999; Song and Marvizon, 2003a,b; Trafton et al., 2000), MOR immunoreactivity was found almost exclusively in laminae I and II of the dorsal horn (Fig. 1). This area included numerous MOR-positive neuronal somata, mostly rounded or oval in shape. Only occasionally were dendrites observed coming out of the cell body in these coronal sections, because these neurons have dendrites that run rostrocaudally (Marvizon et al., 1999; Song and Marvizon, 2003a,b). In control rats (injected with saline) MOR immunoreactivity was present at the cell surface (Fig. 1A), i.e., the receptor was not internalized. However, after activation by an agonist, MOR immunoreactivity appears inside the cytoplasm in endosomes (Fig. 1C), showing that the receptors have been internalized. We quantified MOR internalization by counting MOR-positive somata throughout the entire dorsal horn in several sections, classifying them as with or without MOR internalization. Results were then expressed as percentage of MOR neurons showing internalization (Fig. 2, right panel).

In control rats, only a small percentage (<10%) of MOR neurons showed internalization (Fig. 2, right panel). Intrathecal Leu-enkephalin at the relatively high dose of 100 nmol failed to induce MOR internalization. Even at 300 nmol (data not shown), Leu-enkephalin produced just a small amount of MOR internalization, which was significantly

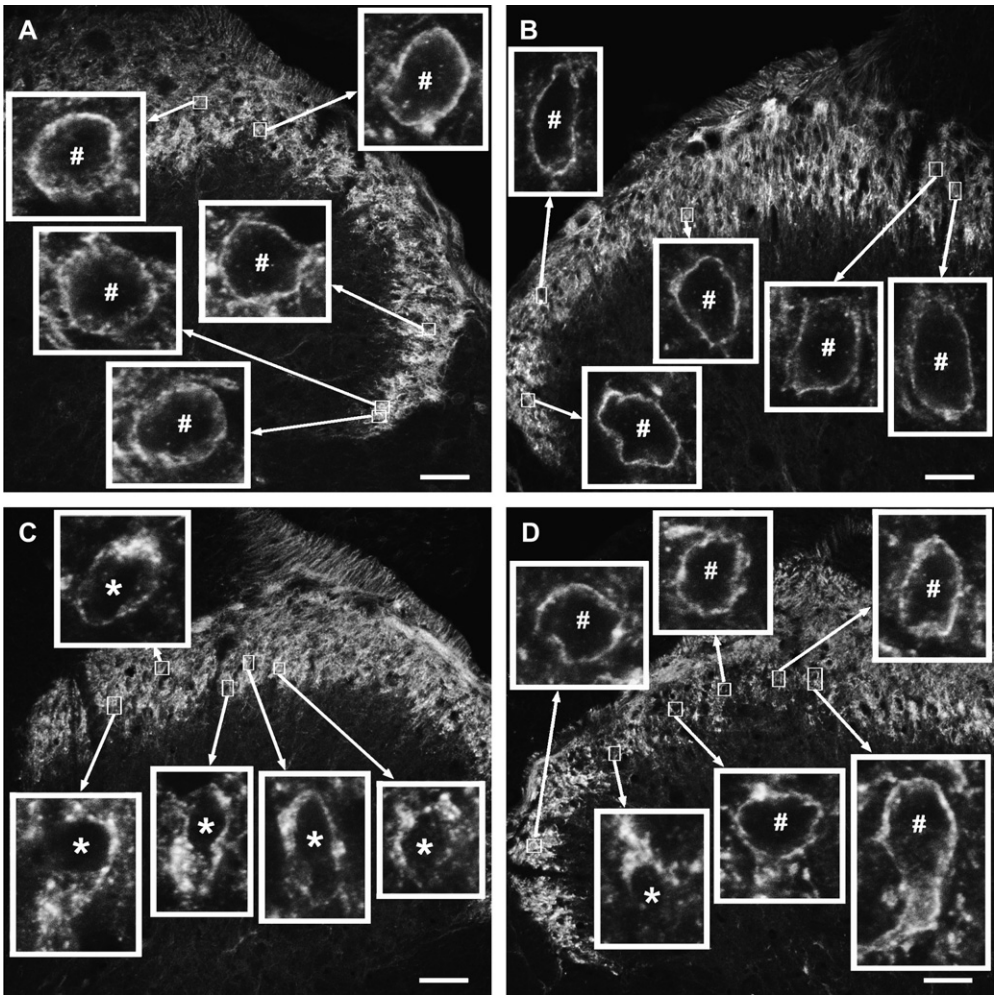

Fig. 1. Confocal images of MOR neurons in the dorsal horn after intrathecal injection. Rats received an intrathecal injection of saline (A), 100 nmol Leu-enkephalin (B), 10 nmol Leu-enkephalin plus 10 nmol peptidase inhibitors (C), or 0.3 nmol Leu-enkephalin plus 10 nmol peptidase inhibitors (D). Peptidase inhibitors were amastatin, captopril and phosphoramidon. MOR neurons with internalization are indicated by "*", and those without internalization by "#" over the cell's nucleus. Images in the main panels were taken with a 20× objective (scale bars are 50 μm), and those in the insets with a 100× objective and a digital zoom of 2 (scale bars are 5 μm).

higher than control only in segment L3. We surmised that, as we have previously observed in spinal cord slices (Song and Marvizon, 2003a), Leu-enkephalin was unable to bind to MOR because it was being rapidly degraded by peptidases.

Our previous work (Song and Marvizon, 2003a) as well as that of other groups (Guyon et al., 1979; Hiranuma et al., 1997, 1998b) showed that in order to protect enkephalins against degradation, three peptidases have to be inhibited: aminopeptidase N (EC.3.4.11.7), dipeptidyl carboxypeptidase (EC.3.4.15.1) and neutral endopeptidase (EC.3.4.24.11). Hence, to determine whether Leu-enkephalin was unable to produce MOR internalization because it was being degraded by peptidases, we co-injected Leu-enkephalin with a mixture of inhibitors of these three peptidases: amastatin, captopril and phosphoramidon. We first assessed the effect of this peptidase inhibitor mixture (at doses of 10 nmol), and found that it did not produce MOR internalization by itself (Figs. 1B and 2, right panel). In contrast, when this mixture of peptidase inhibitors was co-injected with 10 or 100 nmol Leu-enkephalin, there was 100% MOR internalization in spinal segments L1–L6. Examples of neurons with MOR internalization produced by 10 nmol Leu-enkephalin plus peptidase inhibitors are shown in Fig. 1C. At a dose of 1 nmol, Leu-enkephalin still produced an amount of MOR significantly higher than control, although it was no longer maximal. At doses of 0.3 and 0.1 nmol, Leu-enkephalin did not produce significant MOR internalization. Fig. 1D shows MOR neurons after an injection of 0.3 Leu-enkephalin and peptidase inhibitors: in five out of six neurons most MOR immunoreactivity is at the cell surface and there are few endosomes, whereas in the sixth neuron it is present in endosomes. A two-way ANOVA of the MOR internalization data (Fig. 2, right panel) revealed a significant effect of the doses of Leu-enkephalin ($p < 0.0001$), but no significant differences between spinal segments. Therefore, Leu-enkephalin is quite effective in producing MOR internalization in dorsal horn neurons, provided that its degradation by peptidases is blocked.

We did not study the effect of each of the three peptidase inhibitors individually because this would have required the

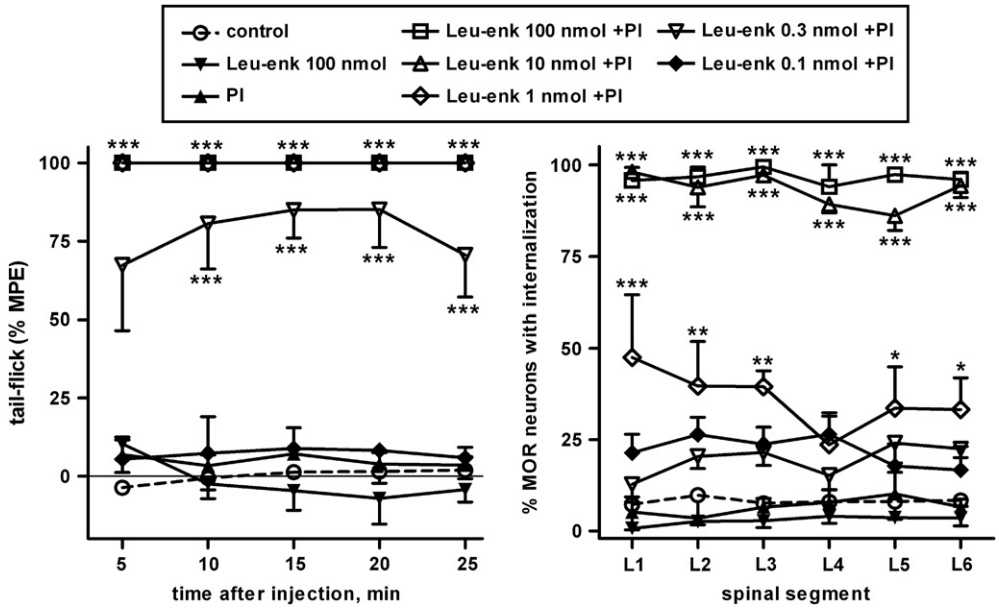

Fig. 2. Tail-flick responses and MOR internalization produced by intrathecal Leu-enkephalin and peptidase inhibitors. Left: tail-flick responses. Baseline tail-flick latencies were measured at 5 min intervals at least three times. Within 2 min, rats were injected intrathecally with Leu-enkephalin (Leu-enk, 0.1–100 nmol) and peptidase inhibitors (PI: amastatin, captopril and phosphoramidon, 10 nmol), alone or combined as indicated. Control rats received the same volume of saline. Five minutes after the injection, tail-flick latencies were measured again at 5 min intervals. The mean baseline latency of all the rats was 3.05 ± 0.01 s ($n = 56$). Right: MOR internalization in the same rats. Rats were fixed 30 min after the intrathecal injection and MOR internalization measured in spinal segments L1–L6. In both panels, data points represent the mean ± SE of 3–6 rats. A two-way ANOVA of each panel revealed significant differences between drug treatments ($p < 0.0001$) for both measures, but not between spinal segment for MOR internalization ($p = 0.99$) or between times after injection for tail-flick ($p = 0.98$). Bonferroni's post-tests: ***$p < 0.001$, **$p < 0.1$, *$p < 0.05$ compared to control.

use of a large number of additional animals. In the previous study using spinal cord slices (Song and Marvizon, 2003a), we showed that the omission of any of the three peptidase inhibitors eliminated the ability of Leu-enkephalin to induce MOR internalization. Therefore, any of the three peptidases (aminopeptidase N, dipeptidyl carboxypeptidase and neutral endopeptidase) by itself is able to effectively degrade Leu-enkephalin, as has been reported by other investigators (Hiranuma et al., 1997, 1998b).

3.2. Intrathecal enkephalin requires peptidase inhibitors to produce analgesia

It seems odd that peptidases would completely prevent the activation of opioid receptors by enkephalins, since several studies (Budai and Fields, 1998; Jensen and Yaksh, 1984; Morgan et al., 1991; Zorman et al., 1982) indicated that endogenous opioids released in the spinal cord do produce analgesia. It could be that peptidases prevent opioids from producing MOR internalization, but they do not prevent them from producing analgesia. This would mean that opioid analgesia does not require the activation of spinal MORs to a level where there is observable internalization. To explore this possibility, we investigated whether the analgesic effect of intrathecal enkephalin also requires the inhibition of peptidases. We used the tail-flick test to measure analgesia. This was done in the same rats used to measure MOR internalization in order to compare the ability of different doses of Leu-enkephalin to induce analgesia with their ability to elicit MOR internalization. After measuring baseline tail-flick responses, Leu-enkephalin was injected intrathecally with or without peptidase inhibitors. Then tail-flick responses were measured again every 5 min for 25 min. Controls included intrathecal injections of saline or peptidase inhibitors alone. The rat was sacrificed and fixed for MOR immunohistochemistry 30 min after the injection.

In controls (receiving intrathecal saline) tail-flick responses were the same as baseline (Fig. 2, left panel). The mixture of peptidase inhibitors by itself did not change the tail-flick responses. Likewise, Leu-enkephalin injected at a high dose (100 nmol) without peptidase inhibitors produced no changes in the tail-flick responses. However, the same dose of Leu-enkephalin in the presence of the mixture of peptidase inhibitors increased the latency of the tail-flick response to the cut-off time (Fig. 2, left panel). This strong analgesia was present from 5 min to at least 25 min after the injection. Then we proceeded to lower the dose of Leu-enkephalin to study its dose–response in the presence of a fixed dose of peptidase inhibitors (10 nmol). Tail-flick latencies remained at or near cut-off times with 10 and 1 nmol of Leu-enkephalin, decreased somewhat with 0.3 nmol, and returned to baseline values with 0.1 nmol. A two-way ANOVA of the tail-flick data (Fig. 2, left panel) revealed a significant effect of the doses of Leu-enkephalin ($p < 0.0001$), but no significant effect of time after injection ($p = 0.7$). Therefore, the analgesia produced by Leu-enkephalin plus peptidase inhibitors appeared in less than 5 min after the injection and persisted for more than 25 min.

3.3. Range of distribution of the intrathecal injectate

MOR internalization produced by the intrathecal injections of Leu-enkephalin and peptidase inhibitors was the same in all segments of the lumbar spinal cord (Fig. 2, right panel). This indicated a very effective rostro-caudal distribution of the injectate. We wondered what the actual extent of this distribution was, and whether the injectate could have reached the brain. In three rats injected intrathecally with 10 nmol Leu-enkephalin plus peptidase inhibitors, MOR internalization was found in $60 \pm 21\%$ ($n = 3$) of the MOR cells in the cervical spinal cord (C1–C2). Therefore, a substantial fraction of the injectate was able to reach the cervical cord and possibly the brainstem. It is unlikely that this was caused by the 30 min delay between the injection and the fixation of the rat: when rats were fixed just 5 min after the intrathecal injection of 10 nmol Leu-enkephalin and peptidase inhibitors, MOR internalization was found in $80 \pm 10\%$ ($n = 3$) of the MOR cells in the cervical spinal cord (C1–C2). The other possibility was that the diffusion of the injectate was caused by the volume of the injection (20 µl plus 10 µl to flush the catheter). Intrathecal injection volumes of 5–15 µl (plus a 10 µl flush) are routinely used by many investigators (Aimone et al., 1987; Jensen and Yaksh, 1984; Kondo et al., 2005; West et al., 1993; Zorman et al., 1982). When they developed the intrathecal catheterization method used here, Yaksh and Rudy (1976) reported that there was little rostro-caudal diffusion of injectate volumes up to 20 µl. They assessed this with intrathecal injections of bromophenol blue dye, [^{3}H]-naloxone and [^{14}C]-urea. Very low levels of these markers (<0.5%) were found in the brain, even 1 h after the injection.

To determine whether the analgesia produced by intrathecal 10 nmol Leu-enkephalin plus peptidase inhibitors could be caused by them acting on the brainstem, we reduced the volume of the intrathecal injection as much as it was feasible. The use of a thin PE-5 catheter allowed us to inject the drugs in 1 µl and to flush the catheter with 5 µl saline. This injection produced maximal tail-flick analgesia (Fig. 3, left panel), but little MOR internalization in the nucleus of the trigeminal track (Fig. 3, right panel). Still, MOR internalization was found in 50% of the MOR cells in segment C1, showing that there was a sizable rostro-caudal distribution of the injectate even with this small injection volume. In any case, the low MOR internalization in the nucleus of the trigeminal track indicates that the analgesia was not caused by an effect of the injectate in the brainstem. With lower concentrations of Leu-enkephalin, MOR internalization was low even in the lumbar spinal cord (Fig. 2, right panel), so it is highly unlikely that the analgesia observed in those cases was produced at the brainstem.

3.4. Comparing analgesia and MOR internalization produced by enkephalin

To investigate the relationship between analgesia and the activation of MORs in dorsal horn neurons, we compared

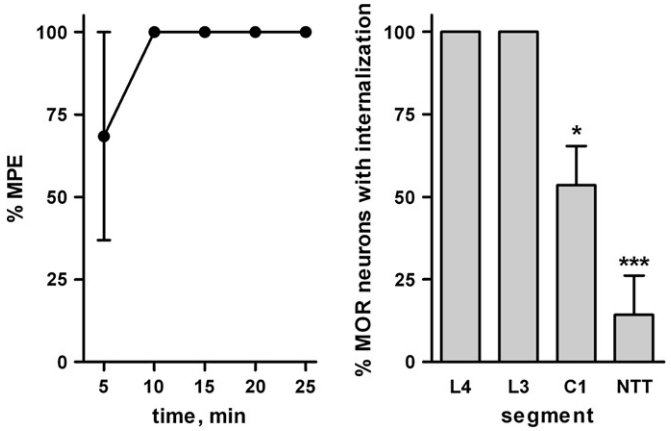

Fig. 3. Tail-flick responses and MOR internalization produced by a 1 µl intrathecal injection of Leu-enkephalin and peptidase inhibitors. Three rats received an intrathecal injection of 10 nmol Leu-enkephalin and peptidase inhibitors (10 nmol amastatin, captopril and phosphoramidon) in a volume of 1 µl, followed by 5 µl saline to flush the catheter. Data points are the mean ± SE. Left: tail-flick responses. Five minutes after the injection, tail-flick latencies were measured at 5 min intervals. Right: MOR internalization was measured in spinal segments L4, L3, C1 and the nucleus of the trigeminal track (NTT). ANOVA revealed significant differences between segments ($p = 0.0002$). Bonferroni's post-tests: ***$p < 0.001$, *$p < 0.05$, compared to L4.

the tail-flick responses (Fig. 2, left panel) and MOR internalization (Fig. 2, right panel) produced by the different doses of Leu-enkephalin with and without peptidase inhibitors. First, we studied the correlation between the tail-flick responses and MOR internalization for each rat, using tail-flick values at 10 min and MOR internalization values in segment L3. A significant correlation ($p < 0.0001$, Pearson's correlation coefficient = 0.695) was found between tail-flick analgesia and MOR internalization. However, points corresponding to mid-range doses of Leu-enkephalin (1 and 0.3 nmol) deviated from linearity, because these doses of Leu-enkephalin produced near-maximal analgesia but little MOR internalization (Fig. 2).

In view of this, we obtained dose–response curves for Leu-enkephalin from the data in Fig. 2. Our goal was to determine whether the potency of Leu-enkephalin to produce tail-flick analgesia was different from its potency to produce MOR internalization. Only data obtained in the presence of peptidase inhibitors (including the zero dose of Leu-enkephalin) were used. Fig. 4 shows that the curve corresponding to tail-flick responses appears to the left of the curve corresponding to MOR internalization, indicating that Leu-enkephalin is more potent to produce analgesia than to produce MOR internalization. Applying an F-test to the non-linear regression fitting procedure (Motulsky and Christopoulos, 2003) revealed that the Log EC_{50} values (tail-flick: -9.79 ± 0.18, MOR internalization, -8.93 ± 0.08) were significantly different between the two sets of data ($p = 0.0001$). The corresponding EC_{50} values for Leu-enkephalin were 0.16 nmol for tail-flick and 1.17 nmol for MOR internalization. Hence, Leu-enkephalin was one order of magnitude more potent to produce tail-flick analgesia than to produce MOR internalization.

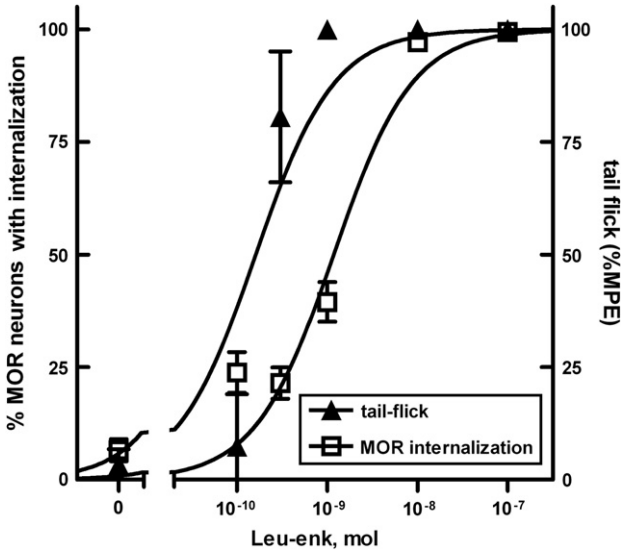

Fig. 4. Leu-enkephalin dose–responses. Tail-flick data (right Y-axis) or MOR internalization values (left *Y*-axis) were plotted against the dose of Leu-enkephalin administered by intrathecal injection. Data are a subset of those shown in Fig. 2: tail-flick values correspond to the 10 min time after injection, and MOR internalization values were from spinal segment L3. Peptidase inhibitors (10 nmol amastatin, captopril and phosphoramidon) were co-injected with Leu-enkephalin. The 0 nmol dose of Leu-enkephalin correspond to an injection of peptidase inhibitors alone. Error bars are SEM. Curves were obtained by non-linear regression fitting of the logistic equation to the data, yielding the following EC_{50} values: tail-flick, 0.16 nmol (95% C.I. 0.07–0.38 nmol); MOR internalization, 1.17 nmol (95% C.I. 0.80–1.71 nmol). The "bottom" and "top" parameters were constrained to 0% and <100%, respectively. The Log EC_{50} for the tail-flick and MOR internalization data were significantly different ($p < 0.0001$, F-test).

3.5. Both MOR and DOR are required for the analgesia produced by low doses of enkephalin

Enkephalins bind to both MOR and DOR with similar affinities, but are inactive at κ-opioid receptors (Raynor et al., 1993). Therefore, the tail-flick analgesia produced by low doses (1 and 0.3 nmol) of Leu-enkephalin could be mediated by DORs and not by MORs, explaining why there was little MOR internalization at these doses. To investigate this possibility, we determined whether the tail-flick analgesia produced by 0.3 nmol of Leu-enkephalin (in the presence of peptidase inhibitors) was blocked by selective antagonists of MORs and DORs. CTAP, a MOR antagonist with a 2500-fold selectivity for MORs over DORs (Kazmierski et al., 1988; Kramer et al., 1989) was used at a dose of 10 nmol, similar to the doses used in previous studies [2.7 nmol (Chen and Pan, 2006), 34 nmol (Dawson-Basoa and Gintzler, 1997)]. SDM-25N (*N*-2-methylallyl-noroxymorphindole, purchased from Tocris, Ellisville, MO) is a DOR antagonist structurally related to naltrindole but showing higher (100–1000 times) selectivity for DOR over MOR in several functional assays, including [^{35}S]GTPγS binding and smooth muscle assays (McLamore et al., 2001). We could not find any studies that used intrathecal injections of SDM-25N, so used it at the same dose as CTAP (10 nmol). The antagonists were co-injected with Leu-enkephalin and the peptidase inhibitor mixture. Fig. 5 (left panel) shows that both the MOR and the DOR antagonists completely blocked the analgesia produced by this low dose of Leu-enkephalin, which indicates that it requires the activation of both receptors. In agreement with the data in Fig. 2, this dose of Leu-enkephalin produced only a small amount of MOR internalization (Fig. 5, right panel).

3.6. Comparing analgesia and MOR internalization produced by the selective MOR agonist endomorphin-2

To investigate whether MOR activation is able to produce analgesia in the absence of concurrent of DOR activation, we used the selective MOR agonist endomorphin-2 (Zadina et al., 1997) to exclusively activate MOR. Endomorphin-2 was injected intrathecally in the absence of peptidase inhibitors, because we previously found that it is not appreciably degraded by peptidases in the spinal cord (Song and Marvizon, 2003a). Endomorphin-2 did produce tail-flick analgesia, but at higher doses than Leu-enkephalin (Fig. 6, left panel): it produced no effect at a dose of 10 nmol, some analgesia (but still not significantly different from control) at 50 nmol, and maximal analgesia at 100 nmol. A previous study (Trafton et al., 2000) used an intrathecal dose of endomorphin-1 of 41 nmol to induce MOR internalization. Another difference with the effect of Leu-enkephalin was that the analgesia produced by endomorphin-2 started to decrease 20 min after the injection (Fig. 6, left panel) whereas the effect of Leu-enkephalin remained for at least 25 min after the injection. The analgesia produced by 100 nmol endomorphin-2 was not significantly reduced by the DOR antagonist SDM-25N (10 nmol), confirming that it did not require the activation of DOR.

MOR internalization was measured in spinal segments L3–L4 of the same rats. The ability of endomorphin-2 to produce MOR internalization paralleled its ability to produce analgesia (Fig. 6, right panel): at doses of 10 and 50 nmol it produced internalization that was not significantly different from control, but at 100 nmol it produced maximal internalization. The DOR antagonist SDM-25N (10 nmol) did not decrease MOR internalization produced by 100 nmol endomorphin-2.

To investigate the relationship between the analgesia and the MOR internalization in dorsal horn neurons produced by endomorphin-2, we studied the correlation between these two measures (using tail-flick values at 10 min). The correlation was significant ($p = 0.0001$, Pearson correlation coefficient = 0.748). As we did for Leu-enkephalin, we constructed dose–response curves from the data in Fig. 6 to determine whether endomorphin-2 had the same potency to produce tail-flick analgesia and MOR internalization. As can be observed in Fig. 7, the two dose–response curves overlap. Applying an F-test to the non-linear regression fitting procedure (Motulsky and Christopoulos, 2003) revealed that the Log EC_{50} values (tail-flick: -7.45 ± 0.53, MOR internalization, -7.49 ± 0.46) were not significantly different between the two sets of data ($p = 0.83$). The corresponding EC_{50} values for endomorphin-2 were 36 nmol for tail-flick and 32 nmol for MOR internalization. Therefore, endomorphin-2 produces

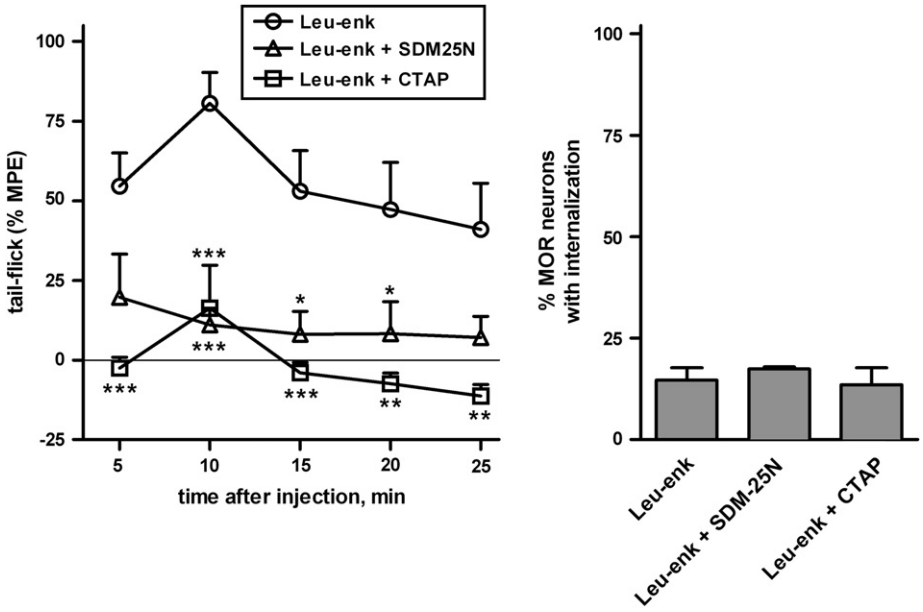

Fig. 5. Effect of opioid antagonists on the tail-flick responses produced by intrathecal Leu-enkephalin with peptidase inhibitors. Left: baseline tail-flick latencies were measured at 5 min intervals at least three times. Within 2 min, rats were injected intrathecally with 0.3 nmol Leu-enkephalin (Leu-enk) together with 10 nmol peptidase inhibitors (amastatin, captopril and phosphoramidon), alone or combined as indicated with the δ-opioid receptor antagonist SDM-25N (10 nmol) or the MOR antagonist CTAP (10 nmol). Five minutes after the injection, tail-flick latencies were measured at 5 min intervals. Data points are the mean ± SE of 6–7 rats. Two-way ANOVA revealed significant effects of the antagonists ($p < 0.0001$) and time after injection ($p = 0.008$). Bonferroni's post-tests: ***$p < 0.001$, **$p < 0.1$, *$p < 0.05$ compared to Leu-enkephalin alone. Right: MOR internalization in the same rats, there were no statistically significant differences (ANOVA).

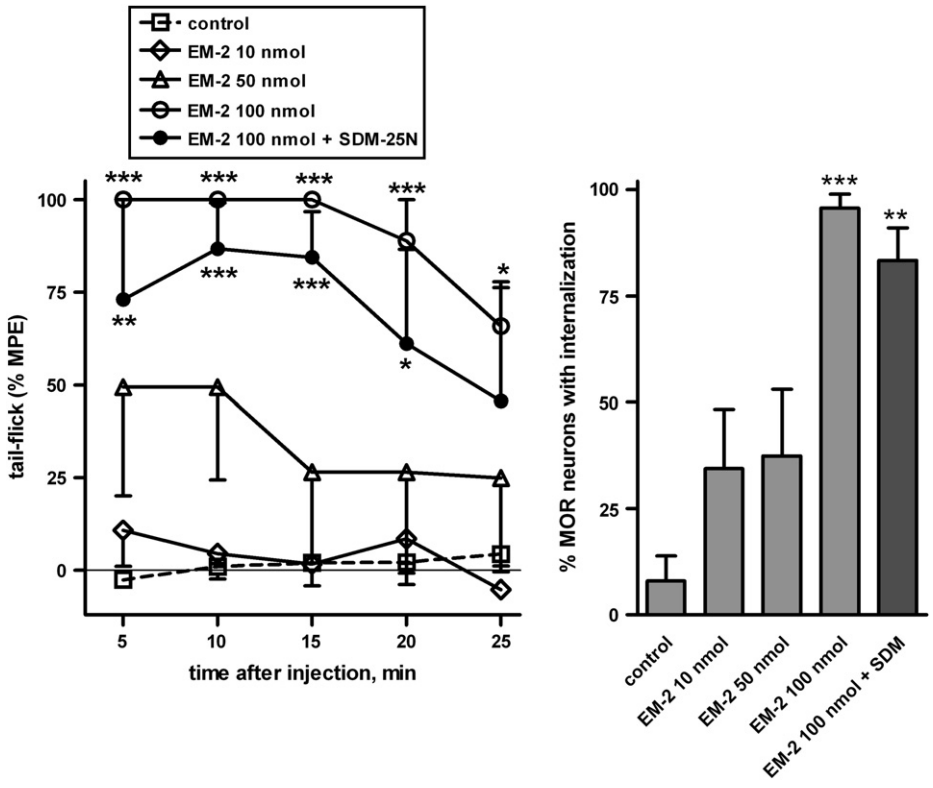

Fig. 6. Tail-flick analgesia and MOR internalization produced by endomorphin-2. Left: tail-flick responses. After measuring baseline latencies, rats were injected intrathecally with saline (control) or 10, 50 or 100 nmol endomorphin-2 (EM-2), without or with the DOR antagonist SDM-25N (10 nmol). No peptidase inhibitors were used. Five minutes after the injection, tail-flick latencies were measured at 5 min intervals. Data points are the mean ± SE of four rats. Two-way ANOVA revealed significant effects of drugs ($p = 0.0001$) and time after injection ($p = 0.025$). Right: MOR internalization in the same rats (spinal segments L3–L4). One-way ANOVA revealed significant differences ($p = 0.0001$). Bonferroni's post-tests: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control.

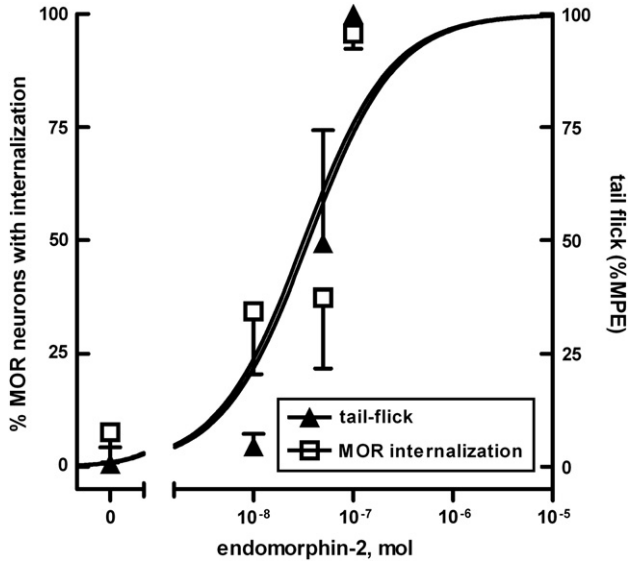

Fig. 7. Endomorphin-2 dose–responses. Tail-flick data (right *Y*-axis) or MOR internalization values (left *Y*-axis) were plotted against the dose of endomorphin-2 administered by intrathecal injection. Data are a subset of those shown in Fig. 6: tail-flick values correspond to the 10 min time after injection, and MOR internalization values were from spinal segments L3–L4. The 0 nmol dose corresponds to an intrathecal injection of saline. Error bars are SEM. Curves were obtained by non-linear regression fitting of the logistic equation to the data, yielding the following EC_{50} values: tail-flick, 36 nmol (95% C.I. 2.6–487 nmol); MOR internalization, 32 nmol (95% C.I. 3.3–312 nmol). The "bottom" and "top" parameters were constrained to 0% and <100%, respectively. The Log EC_{50} for the tail-flick and MOR internalization data were not significantly different ($p = 0.88$, F-test).

tail-flick analgesia and MOR internalization with the same potency. It is worth noting that the potency of Leu-enkephalin (with peptidase inhibitors) to produce analgesia is more than one order of magnitude higher than the potency of endomorphin-2. In contrast, Leu-enkephalin and endomorphin-2 produced MOR internalization with similar potencies.

4. Discussion

4.1. Physiological significance of opioid degradation by peptidases

The cleavage of neuropeptides by peptidases is generally regarded as a mechanism to terminate their action. However, this study presents evidence that peptidases degrade enkephalins so effectively that they completely prevent them from activating opioid receptors in the spinal cord. We found that whereas Leu-enkephalin did not produce analgesia when injected alone, even at high doses, it produced strong analgesia at low doses when co-injected with peptidase inhibitors. These findings are consistent with a previous study (Kishioka et al., 1994) in which peptidase inhibitors increased the analgesia induced by Met-enkephalin, dynorphin and electroacupuncture. Our findings also show that the inability of opioids, either exogenously applied or released, to induce MOR internalization unless peptidases are inhibited (Song and Marvizon, 2003a,b, 2005; Trafton et al., 2000) truly reflects their inability to activate opioid receptors in the spinal cord.

The peptidases that degrade enkephalins also degrade substance P. However, peptidase inhibitors increased the potency of Leu-enkephalin to induce MOR internalization by two orders of magnitude (Song and Marvizon, 2003a), and that of substance P to induce neurokinin 1 receptor internalization only by threefold (Marvizon et al., 2003). These findings suggest that peptidases inactivate enkephalins much more effectively than substance P.

Besides enkephalins, peptidases prevent the activation of MORs by other opioids present in the spinal cord, notably dynorphin and α-neoendorphin (Hiranuma et al., 1997, 1998a,b; Song and Marvizon, 2003a). Peptidase inhibitors also increased the antinociception produced by intrathecal (Kishioka et al., 1994) and intra-ventricular dynorphin (Kitamura et al., 2000). β-Endorphin and endomorphins are far less susceptible to peptidase degradation (Bewley and Li, 1985; Song and Marvizon, 2003a). However, the dorsal horn does not contain endorphins (Tsou et al., 1986), and there are conflicting data concerning the presence of endomorphins in the dorsal horn. Thus, although endomorphin immunoreactivity was found in substance P-containing primary afferent terminals (Martin-Schild et al., 1998; Nydahl et al., 2004; Pierce et al., 1998; Spike et al., 2002), endomorphins were not co-released with substance P during dorsal root stimulation (Song and Marvizon, 2003b). This discrepancy may be due to the cross reactivity of anti-endomorphin antibodies with other neuropeptides with an amidated phenylalanine at the C-terminus (Marvizon and Song, 2002; Pierce et al., 1998).

It is puzzling that peptidases completely prevent opioids from activating MORs, because then it would seem that opioid release in the spinal cord serves no purpose whatsoever. Yet, there is compelling evidence that analgesia can be produced by the activation of spinal MORs by endogenously released compounds. Thus, stimulation of the RVM (Zorman et al., 1982) or the PAG (Jensen and Yaksh, 1984; Morgan et al., 1991) produces analgesia that is blocked by spinal application of MOR antagonists (Budai and Fields, 1998). Therefore, there must be some conditions that allow enkephalins to bind to MORs without being degraded. These may include: (1) release of opioids in quantities large enough to overcome peptidase degradation; (2) changes in peptidase activity, or (3) inhibition of the peptidases by endogenous compounds. Concerning the first possibility, our results indicate that the concentrations of released enkephalins would need to be increased by orders of magnitude to overcome peptidase degradation, whereas increases in enkephalins during inflammation are quite modest (Draisci et al., 1991; MacArthur et al., 1999). In fact, noxious stimuli failed to produce MOR internalization in dorsal horn neurons even after inflammation (Trafton et al., 2000). Concerning the second possibility, peptidase activity is known to change in conditions like morphine tolerance (Zhou et al., 2001) and by switching between cytosolic and membrane-bound forms (Dyer et al., 1990). However, preventing the degradation of enkephalin would require switching off the activity of three different peptidases.

The third possibility is the most likely. Three endogenous inhibitors of aminopeptidases and neutral endopeptidase (two of the enzymes that degrade enkephalin) have been identified so far: spinorphin (LVVYPWT), isolated from bovine spinal cord (Nishimura and Hazato, 1993); sialorphin (QHNPR), identified in the rat submandibular gland and prostate (Rougeot et al., 2003), and opiorphin (QRFSR), isolated from human saliva (Wisner et al., 2006). Spinorphin increases the antinociceptive effects of Leu-enkephalin (Honda et al., 2001), and sialorphin and opiorphin have analgesic properties. Sialorphin secretion is stimulated by stress (Rougeot et al., 2003), a condition known to produce opioid-mediated analgesia (Lewis et al., 1981). Therefore, the release of endogenous peptidase inhibitors may be as important for the induction of analgesia as the release of opioids. Thus, the opioid receptor-mediated analgesia induced by PAG and RVM stimulation (Jensen and Yaksh, 1984; Morgan et al., 1991; Zorman et al., 1982) may require the simultaneous release of opioids and endogenous peptidase inhibitors. The existence of such a coincidence mechanism for the activation of opioid receptors would be of great physiological significance.

4.2. Opioid receptors that mediate the analgesic effect of enkephalin

The fact that Leu-enkephalin was 10 times more potent to produce analgesia than to produce MOR internalization suggested an involvement of DORs in enkephalin-induced analgesia. To clarify this issue, we determined whether MOR or DOR antagonists could block the analgesic effect of low doses of Leu-enkephalin. Both antagonists did this, suggesting that the analgesia required the activation of both receptors. Previous studies used only one antagonist: Takemori and Portoghese (1993) found that the DOR antagonist naltriben decreased enkephalin-induced tail-flick analgesia, whereas Budai and Fields (1998) found that MOR antagonists reversed the decrease in neuronal responses to noxious heat produced by PAG stimulation. Our results are consistent with both of these findings. However, the fact that the analgesia produced by Leu-enkephalin lasts 25 min while most MORs are internalized within 10 min (Marvizon et al., 1999), an effect not observed with endomorphin-2, suggests that DORs are involved in the late phase of Leu-enkephalin-induced analgesia. While we cannot completely rule out that CTAP antagonizes DORs when using a low dose of Leu-enkephalin, CTAP displays a 2500-fold selectivity for MOR over DOR (Kazmierski et al., 1988; Kramer et al., 1989) and was used at a dose generally assumed to be selective for MORs (Chen and Pan, 2006; Dawson-Basoa and Gintzler, 1997).

But then, why didn't the activation of MORs by low doses of enkephalin produce MOR internalization? Possible explanations include the involvement of MOR/DOR heterodimers (Gomes et al., 2000) or MOR–DOR synergism (Malmberg and Yaksh, 1992). The first possibility is unlikely because in MOR/DOR heterodimers DOR antagonists increase signaling by MOR agonists, which is the opposite of what we found. The presence of MOR–DOR synergism is suggested by the fact that both MOR and DOR antagonists were able to completely block enkephalin-induced analgesia. Synergism between a few MORs and DORs activated by the low dose of enkephalin may produce a signal amplification resulting in the analgesic effect, whereas the number of activated MORs would be too small to produce enough MOR-containing endosomes to be registered as internalization. It is also possible that the synergism between MORs and DORs occurs in primary afferent terminals, which contain both receptors (Abbadie et al., 2001, 2002; Kondo et al., 2005).

The ability of endomorphin-2 to produce analgesia, even in the presence of a DOR antagonist, demonstrates that MOR activation is sufficient to produce analgesia without concurrent activation of DORs. However, doses of endomorphin-2 10 times higher than those of Leu-enkephalin were required, even though endomorphin-2 has higher affinity for MORs than Leu-enkephalin (Zadina et al., 1997). And, unlike Leu-enkephalin, endomorphin-2 had the same potencies to produce analgesia and MOR internalization. These findings indicate that it is possible to produce analgesia by activating MORs exclusively, but this requires higher agonist doses. It is likely that a synergistic or additive interaction between MORs and DORs detected at low concentrations of enkephalins reflects the true physiological situation, because peptidases would only allow the presence of low concentrations of opioids.

Interestingly, whereas analgesia produced by Leu-enkephalin persisted for at least 25 min, analgesia produced by endomorphin-2 started to decrease 20 min after the injection. One possible explanation for this finding is that long-term analgesia involves a sequential mechanism in which MORs are activated first and internalize in about 10 min (Marvizon et al., 1999), and then DORs are trafficked to the cell surface (Cahill et al., 2001; Morinville et al., 2003) in time to replace them. Thus, we speculate that MOR receptors mediate the short-term effect of opioids, whereas the role of DOR would be to sustain analgesia for longer times. Alternative explanations for the shorter effect of endomorphin-2 are that it is cleared from the tissue faster than Leu-enkephalin, or differences in the desensitization of opioid receptors produced by endomorphin-2 and Leu-enkephalin.

4.3. Internalization as a measure of MOR activation

Our findings show that MOR internalization reflects its activation, except when there is co-activation of DORs. When only MOR activation was involved (i.e., by endomorphin), there was a strong correlation between MOR-mediated function and MOR internalization. This agrees with the results of Trafton et al. (2000), who found a correlation between hot plate analgesia and MOR internalization produced by DAMGO, another MOR selective agonist. We found that endomorphin-2 had exactly the same dose–response curves to produce analgesia and MOR internalization. The fact that MOR internalization induced by either exogenous or released opioids required peptidase inhibitors (Song and Marvizon, 2003a) could be raised as an objection against using of MOR internalization to measure MOR activation, but we now show that the same

requirement for peptidase inhibition applies to analgesia induced by Leu-enkephalin. Still, it is intriguing that low doses of Leu-enkephalin could produce analgesia without inducing MOR internalization. This seems to require an interaction between MORs and DORs, possibly involving a small number of MORs whose internalization went undetected.

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