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Dynorphin-(1–8) inhibits the release of substance P-like immunoreactivity in the spinal cord of rats following a noxious mechanical stimulus

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Abstract

This study was conducted to determine the effect of the opioid peptide dynorphin-(1–8) on the release of substance P-like immunoreactivity in the dorsal horn during mechanical activation of peripheral nociceptors. A push-pull cannula was used to perfuse the dorsal horn of decerebrate/spinal transected rats before, during and following the application of a noxious mechanical stimulus to the ipsilateral hindpaw and lower limb. The collected perfusates were assayed for substance P-like immunoreactivity using radioimmunoassay. Dynorphin-(1–8) applied to the spinal cord at a concentration of 1 μM reduced the basal release of substance P-like immunoreactivity by 28 ± 11% and prevented the mechanically evoked release of substance P-like immunoreactivity. This effect of dynorphin-(1–8) was reversed by 2 μM of the selective κ-opioid receptor antagonist nor-binaltorphimine. Moreover, blockade of the κ-opioid receptors by nor-binaltorphimine resulted in a 33 ± 5% increase in the basal release of substance P-like immunoreactivity. These data show that activation of nor-binaltorphimine-sensitive sites by dynorphin-(1–8) results in inhibition of the release of substance P-like immunoreactivity in the dorsal horn of the rat.

Keywords: Opioid; Nor-binaltorphimine; κ-Opioid receptor; Antinociception; Dorsal horn; Tachykinin; Nociception; Pain

1. Introduction

The involvement of the undecapeptide substance P in the processing of nociceptive information has been demonstrated by various behavioral, electrophysiological and biochemical studies (Henry, 1976; Hylden and Wilcox, 1981; Nagy and Van der Kooy, 1983; Otsuka and Yoshioka, 1993). In the spinal cord, substance P has been found in the dorsal horn (laminae I and II) in the terminals of the unmyelinated primary afferent fibers and in smaller amounts in descending neurons and interneurons (Jessel et al., 1979; Hökfelt et al., 1975). Noxious cutaneous thermal or mechanical stimuli elicit the release of substance P from the primary afferent fibers which in turn excite dorsal horn neurons that respond to noxious stimulation (Henry, 1976; Duggan et al., 1987; McCarson and Goldstein, 1991; Zachariou and Goldstein, 1996a).

There is evidence to suggest that spinal opioids modulate nociceptive transmission (Yaksh, 1981). Dynorphin-(1–8), an opioid peptide that is derived from the preprodynorphin precursor molecule, has a higher affinity for the κ-opioid receptor than other opioid receptor sub-types (Corbett et al., 1982). Autoradiographic studies demonstrate that in the spinal cord κ-opioid receptors are mainly concentrated in laminae II and III of the dorsal horn (Gouaderes et al., 1986). A possible role of this peptide in the spinal modulation of nociception has been suggested by studies showing that dynorphin-(1–8) levels and preprodynorphin mRNA increase in laminae I, II, V and VI in the spinal cord following an inflammatory response in the periphery (Iadarola et al., 1988; Ruda et al., 1988).

In previous studies in our laboratory we demonstrated that dynorphin-(1–8) inhibited the release of substance P-like immunoreactivity in the dorsal horn evoked by a noxious thermal stimulus and that this was a κ-opioid receptor-mediated effect (Zachariou and Goldstein, 1996a). The present study investigated the effect of dynorphin-(1–8) on the release of substance P-like immunoreactivity in the lumbar dorsal horn before, during and following the

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application of a noxious mechanical stimulus to the hindpaw of non-anesthetized, decerebrate/spinal transected rats.

2. Materials and methods

2.1. Surgical procedures

Male Wistar rats (Harlan Sprague-Dawley) weighing 300–350 g were used in all the following experiments. The animals were housed individually in a room with a 12 h dark-light cycle and provided food and water ad libitum. All the protocols followed in the present study were approved by the institution's Committee on Animal Use for Research and Education. The rats were anesthetized with halothane and mechanically respired. The blood pressure of the animals was monitored using a carotid catheter. Animals with systolic blood pressure below 70 mmHg were excluded from the study. A laminectomy was performed to expose the lumbar enlargement. Following the laminectomy, the animal was decerebrated at the midcollicular level and the anesthetic was discontinued. The dura mater was incised and the dorsal roots reflected towards the midline. A spinal transection was performed about 1 cm above the lumbar enlargement. Following surgery, the rat was placed in a stereotaxic frame and immobilized with succinylcholine. A push-pull cannula was introduced into the dorsal horn at the entry zone of the fifth lumbar dorsal root, 1 mm from the midline and 700 μm deep (McCarson and Goldstein, 1991). The cannula was made from two 27-gauge needles as described elsewhere (Zachariou and Goldstein, 1996a). In order for the perfusion site to be in laminae I–III the depth of the tip of the cannula had to be 700 μm. The orientation of the cannula was parallel to the midline (McCarson and Goldstein, 1991). A peristaltic pump was used to perfuse artificial cerebrospinal fluid (CSF) through the cannula at a rate of 30 μl/min. The artificial cerebrospinal fluid contained 128.5 mM NaCl, 3.0 mM KCl, 21 mM $NaHCO_3$, 0.25 mM NaH_2PO_4, 3.4 mM glucose, 1.15 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 μM bestatin, 5 μM captopril, 1 μM leupeptin, 1 μM chymostatin and 1 μM thiorphan. The pH of the artificial cerebrospinal fluid was maintained at 7.4 by bubbling with 95% O_2 and 5% CO_2. The artificial cerebrospinal fluid was warmed to 37°C immediately before entering the cannula. The dorsal horn was perfused for 1 h prior to any collection of samples. Two hundred microliter samples of perfusate were collected (6.66 min) into assay tubes containing substance P antibody. At the end of the experiment, the lumbar spinal cord was removed and frozen. The location of the tip of the cannula in the dorsal horn was histologically verified. When the perfusion area was outside of laminae I–III there was no change in the release of the peptide and these animals were excluded from the study.

2.2. Substance P radioimmunoassay

The radioimmunoassay for substance P was performed as previously described (McCarson and Goldstein, 1991; Zachariou and Goldstein, 1996a). The substance P antibody cross-reacts with other substance P-related peptides as follows: substance P-(5–11) 30%, substance P-(3–11) 19%, substance P-(7–11) 14%, substance P-(1–4) less than 2.5% and substance P-(1–6), substance P-(1–7), substance P-(1–9), eledoisin, neurokinin A, neurokinin B, substance P-Gly and substance P-Gly-Lys all less than 1%.

2.3. Experimental protocol

Four groups of animals were used in this study: (1) control, (2) dynorphin-(1–8), (3) nor-binaltorphimine and (4) dynorphin-(1–8) + nor-binaltorphimine.

In the first group the basal release of substance P-like immunoreactivity was monitored for 40 min (baseline). There was a second 40 min monitoring of the release of substance P-like immunoreactivity that corresponded to the time a drug would be added to the perfusate in the other groups (basal). That was followed by 20 min of monitoring of the release of the peptide during the application of a noxious mechanical stimulus to the ipsilateral hindpaw and lower limb (pinch). The release of substance P-like immunoreactivity was monitored for another 20 min post stimulus.

In the second group, the dorsal horn of the animals was perfused with artificial CSF for 40 min (baseline). Following that, dynorphin-(1–8) was added to the perfusate and the basal release of the peptide was monitored for another 40 min (basal). The noxious mechanical stimulus was applied to the hindpaw and lower limb during the next 20 min of monitoring (pinch). This was followed by another 20 min of monitoring the release of substance P-like immunoreactivity post stimulus.

The dorsal horn of the rats in the third group was perfused for 40 min before the addition of nor-binaltorphimine (baseline) and for 40 min in the presence of nor-binaltorphimine (basal) to the perfusate. The changes in the release of substance P-like immunoreactivity during noxious mechanical stimulation in the presence of nor-binaltorphimine were monitored for 20 min followed by another 20 min of monitoring post stimulus.

Finally, in the fourth group the baseline release of substance P-like immunoreactivity was monitored for 40 min. There was another 40 min of monitoring in order to determine whether nor-binaltorphimine would alter the effect of dynorphin-(1–8) on the basal release of the peptide. The release of substance P-like immunoreactivity was monitored for 20 min during the stimulus application as well as for 20 min post stimulus. The concentration of nor-binaltorphimine used was the lowest concentration that blocked the effect of 1 μM dynorphin-(1–8) on the basal release of substance P-like immunoreactivity in our stud-

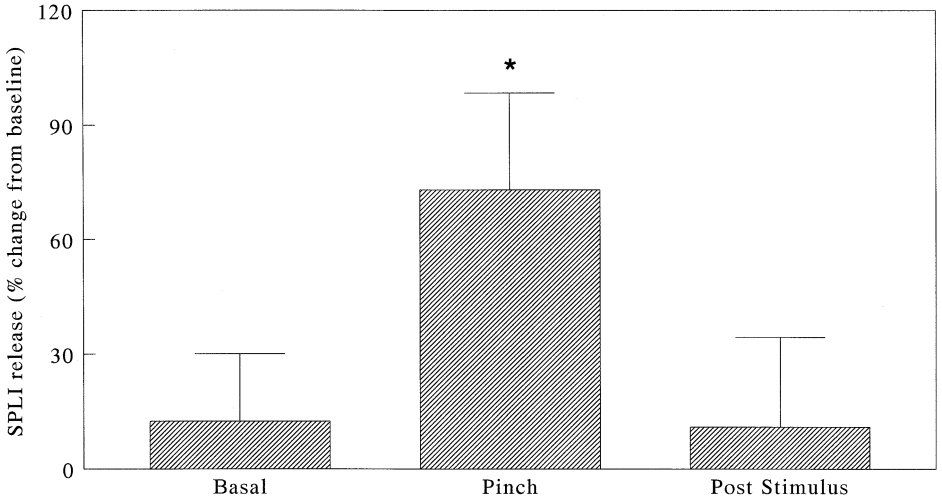

Fig. 1. Substance P-like immunoreactivity release as percent change from baseline before (basal), during (pinch) and following (post stimulus) the application of a mechanical stimulus to the ipsilateral hindpaw and lower limb of decerebrate spinal transected rats. Means ± S.E.M. ($n = 7$), * $P < 0.05$ as compared to baseline, basal and post stimulus.

ies. Nor-binaltorphimine was added to the perfusate and allowed to perfuse the dorsal horn for 60 min before monitoring the changes in the basal and evoked release of substance P-like immunoreactivity in groups 3 and 4.

The mechanical stimulus consisted of a pinch applied to various locations all over the ipsilateral hindpaw and lower limb every 20 s for 20 min with a bulldog clamp. Each area was stimulated a maximum of 2 times. The jaw opening of the clamp exerted 250 g of force. That amount of force has been characterized by McCarson and Goldstein (1991) as a medium intensity noxious stimulus.

2.4. Drugs

Nor-binaltorphimine was purchased from Research Biochemicals International. All other drugs and chemicals used were purchased from Sigma. The concentration of nor-binaltorphimine was determined in preliminary studies. Concentrations of 1.0 and 1.5 μM nor-binaltorphimine added to the perfusate 1 h prior to the addition of dynorphin-(1–8) failed to alter the effects of dynorphin-(1–8) on the release of substance P. However, a concentration 2 μM nor-binaltorphimine blocked the effects of dynorphin-(1–8) on the basal release of substance P. We, therefore, used 2 μM in these studies.

2.5. Data analysis

Substance P-like immunoreactivity release was calculated as pg of substance P-like immunoreactivity/min and presented graphically as percent of baseline release. Analysis of variance was used to compare the raw data (pg/min) for statistical significance. The least-significant difference test was used for post-hoc comparisons. A paired or unpaired Student's *t*-test was used to compare the treatments in the experiments performed to determine the lowest dynorphin-(1–8) concentration and treatments between different groups. Significance was set at $P \leq 0.05$ and all values are reported as the mean ± S.E.M.

3. Results

The average basal release of substance P-like immunoreactivity from all the experiments in this study was 0.95 ± 0.15 pg/min which is in good agreement with previous data from our laboratory (McCarson and Goldstein, 1991; Zachariou and Goldstein, 1996a). Fig. 1 shows the release pattern of substance P-like immunoreactivity in the dorsal horn when a noxious mechanical stimulus was applied to the ipsilateral hindpaw and lower limb of the rat. During the application of the mechanical stimulus the release of substance P-like immunoreactivity increased by 73 ± 25%. The mechanically evoked release of substance P-like immunoreactivity was significantly higher than the

Table 1
Effects of dynorphin-(1–8) on the basal release of substance P-like immunoreactivity in the dorsal horn

Dynorphin-(1–8) concentration (μM)	% Change from baseline	Number of observations
0	17.4 ± 12.9	5
0.001	−7.3 ± 11.3	4
0.5	18.7 ± 13	4
1	−26.0 ± 6.6 [a]	5

The release of substance P-like immunoreactivity was monitored in the absence of dynorphin-(1–8) from the perfusate and for another 40 min when dynorphin-(1–8) was added to the perfusate. Each animal received one concentration of the drug. Values are means ± S.E.M.
[a] $P < 0.05$ as compared to substance P release in the absence of the drug (baseline).

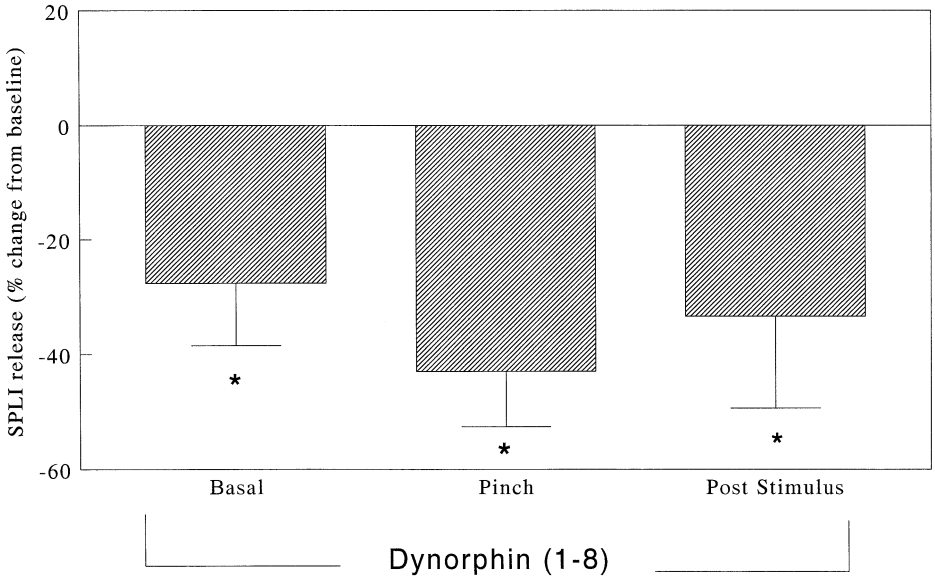

Fig. 2. Substance P-like immunoreactivity release as percent change from baseline before (basal), during (pinch) and following (post stimulus) the application of a mechanical stimulus to the ipsilateral hindpaw and lower limb when the dorsal horn is perfused with artificial CSF containing 1 μM dynorphin-(1–8). Means ± S.E.M. ($n = 4$), * $P < 0.05$ as compared to baseline.

baseline release of the peptide ($P < 0.05$). The release of substance P-like immunoreactivity returned to the baseline levels within 20 min following the stimulus application.

Table 1 shows the effect of different concentrations of dynorphin-(1–8) on the basal release of substance P-like immunoreactivity. When the dorsal horn was perfused with artificial cerebrospinal fluid containing up to 500 nM of dynorphin-(1–8) there was no significant change in the release of substance P-like immunoreactivity. The lowest concentration used that significantly reduced the basal release of substance P-like immunoreactivity was 1 μM (26 ± 6% decrease, $P < 0.05$). This was the concentration used in subsequent experiments.

The effects of dynorphin-(1–8) on the mechanically evoked release of substance P-like immunoreactivity are shown in Fig. 2. Dynorphin-(1–8) at a concentration of 1 μM reduced the basal release of substance P-like immunoreactivity by 28 ± 11% ($P < 0.05$) and prevented the in-

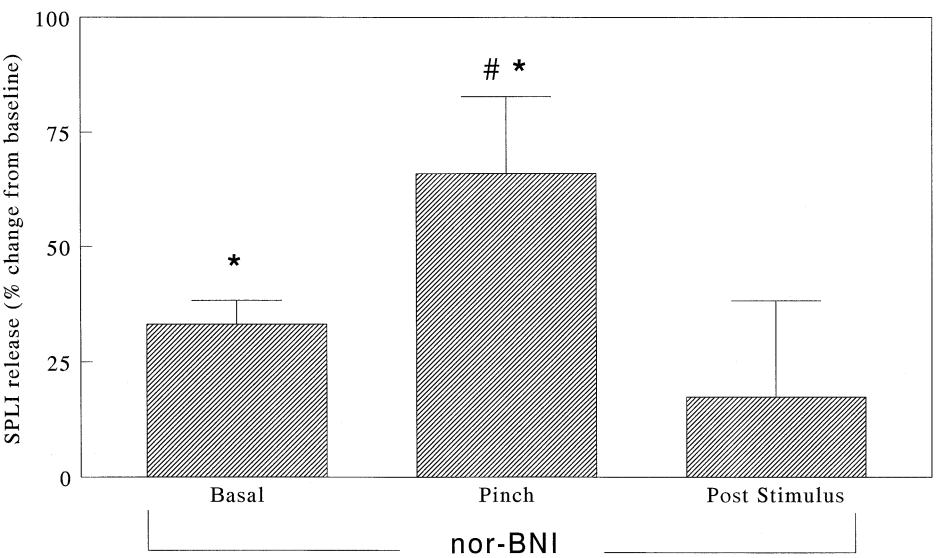

Fig. 3. Substance P-like immunoreactivity release as percent change from baseline before (basal), during (pinch) and following (post stimulus) the application of a mechanical stimulus to the ipsilateral hindpaw and lower limb when the dorsal horn is perfused with artificial CSF containing 2 μM nor-binaltorphimine. Means ± S.E.M.($n = 6$), * $P < 0.05$ as compared to baseline, # $P < 0.05$ as compared to basal.

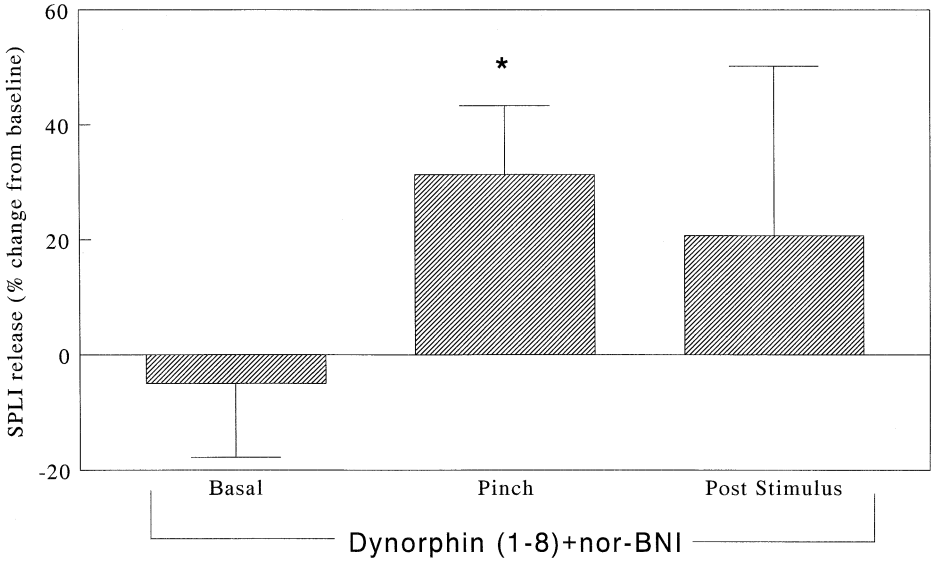

Fig. 4. Substance P-like immunoreactivity release as percent change from baseline before (basal), during (pinch) and following (post stimulus) the application of a mechanical stimulus to the ipsilateral hindpaw and lower limb when the dorsal horn is perfused with artificial CSF containing 1 μM dynorphin-(1–8) and 2 μM nor-binaltorphimine. Means ± S.E.M. ($n = 4$), * $P < 0.05$ as compared to baseline, basal, post stimulus.

crease in the release of the peptide during the application of a noxious mechanical stimulus. The release of substance P-like immunoreactivity remained decreased as compared to baseline during the post-stimulus period ($33 \pm 16\%$, $P < 0.05$). Removal of the drug from the perfusate resulted in an immediate return of the substance P-like immunoreactivity release to baseline levels (data not shown).

When the dorsal horn was perfused with artificial cerebrospinal fluid containing the selective κ-opioid receptor antagonist nor-binaltorphimine, there was a $33 \pm 5\%$ ($P < 0.05$) increase in the basal release of substance P-like immunoreactivity (Fig. 3). The release of the peptide increased to $66 \pm 16\%$ ($P < 0.05$) of baseline when the hindpaw was mechanically stimulated. Following the stimulus application the release of substance P-like immunoreactivity returned to baseline levels.

Fig. 4 shows the effect of dynorphin-(1–8) on the release of substance P-like immunoreactivity in the dorsal horn in the presence of nor-binaltorphimine. Dynorphin-(1–8) failed to decrease the basal release of substance P-like immunoreactivity when the κ-opioid receptors were blocked by nor-binaltorphimine. The release of substance P-like immunoreactivity increased by $31 \pm 12\%$ during the application of the mechanical stimulus ($P < 0.05$). This increase in the release of the peptide was not significantly different from the increase during the application of the mechanical stimulus in the control group. Following the stimulus application the release of substance P-like immunoreactivity approached baseline levels.

4. Discussion

The present study demonstrates that activation of κ-opioid receptors by dynorphin-(1–8) results in inhibition of the basal and mechanically evoked release of substance P-like immunoreactivity in the dorsal horn of decerebrate/spinal transected rats.

Changes in the release of substance P-like immunoreactivity during mechanical activation of cutaneous nociceptors have been demonstrated by several investigators. Duggan et al. (1988), using antibody microprobes, showed that the release of substance P-like immunoreactivity in anesthetized cats increased following mechanical stimulation of the hindpaw. Similar findings were reported using the push-pull cannula method in anesthetized rabbits (Kuraishi et al., 1989) and in non-anesthetized/spinal transected rats (McCarson and Goldstein, 1991). As reported by McCarson and Goldstein (1991) application of a medium intensity noxious mechanical stimulus to the ipsilateral hindpaw and lower limb every 30 s for 20 min resulted in a 33% increase in the release of substance P-like immunoreactivity in the dorsal horn. In the present study, the same stimulus applied every 20 s for 20 min resulted in a 70% increase in the release of the peptide. The difference in the percent increase in the release of substance P-like immunoreactivity between the two studies can be explained by the different duration and frequency of the stimulus application.

A number of behavioral studies have shown that synthetic κ-opioid receptor agonists possess antinociceptive action against thermal, chemical and mechanical stimuli (Millan, 1990; Nakazawa et al., 1991). Moreover, antisense oligodeoxynucleotides to the κ-opioid receptor blocked the analgesic effect of the κ-opioid receptor agonist 3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrolodinyl)-cyclohexyl]-benzacetamide (U50-488H) (Chien et al., 1994). Although synthetic κ-opioid receptor ligands are antinociceptive, not all dynorphin peptides possess analgesic proper-

ties (Fujimoto et al., 1990). The present study demonstrated that activation of the κ-opioid receptors by dynorphin-(1–8) inhibits mechanically evoked release of substance P-like immunoreactivity in the dorsal horn. Previously we have demonstrated that dynorphin-(1–8) had the same effect against noxious thermal stimulation. Similar findings on the effect of κ-opioid receptor agonists on the release of substance P-like immunoreactivity have been shown by Chang et al. (1989) who demonstrated that the synthetic κ-opioid receptor agonist U50488H inhibits the release of substance P from cultured primary sensory neurons. The antinociceptive activity of dynorphin (1–8) against mechanical stimuli has been previously demonstrated in behavioral studies where i.c.v. administration of the peptide resulted in an increase in the nociceptive pressure threshold in the rat (Hayes et al., 1983). Since dynorphin-(1–8) failed to inhibit the release of substance P-like immunoreactivity in the presence of the selective κ-opioid receptor antagonist nor-binaltorphimine, the effect of dynorphin-(1–8) is mediated though activation of the κ-opioid receptor.

Perfusion of the dorsal horn with artificial cerebrospinal fluid containing nor-binaltorphimine alone resulted in an increase in the basal release of substance P-like immunoreactivity; providing evidence for a tonic regulatory role of these receptors on the substance P-containing neurons. This is consistent with previous findings in our laboratory (Zachariou and Goldstein, 1996a). Collin et al. (1992) reported that the basal release of substance P-like immunoreactivity was not affected by nor-binaltorphimine. This difference can be explained by the limited time the antagonist was added to the perfusate before the sample collection. Although studies in mice (Horan et al., 1991) indicated that 20 min was the time of peak antagonistic effect of nor-binaltorphimine, the studies in our laboratory as well as studies by Takemori et al. (1988) determined that 1 h was necessary for nor-binaltorphimine to block the antinociceptive actions of κ-opioid receptor ligands. It should also be noted that Collin et al. (1992) were using intact anesthetized animals. There is also a difference in the perfusing area, since the place of the cannula was the substantia gelatinosa and in their study it was an intrathecal cannula.

Although the basal release of substance P-like immunoreactivity increased in the presence of nor-binaltorphimine, the release of the peptide during the application of the mechanical stimulus increased as much as in the control group. These observations suggest that endogenous opioids acting at nor-binaltorphimine-sensitive sites exert a tonic inhibitory influence on the basal release of substance P-like immunoreactivity but they do not affect the mechanically evoked release of the peptide in the dorsal horn of decerebrate/spinal transected rats.

In previous studies we reported that following a noxious thermal stimulus the release of substance P-like immunoreactivity remained elevated in the presence of nor-binaltorphimine (Zachariou and Goldstein, 1996a). This was attributed to the long-lasting antagonistic actions of nor-binaltorphimine which have been observed by many investigators (Horan et al., 1992; Jones and Holtzman, 1992). Interestingly, following the mechanical stimulus, blockade of the κ-opioid receptors did not prevent the release of substance P-like immunoreactivity from returning to baseline levels suggesting that (unlike the tonic release of substance P-like immunoreactivity) the release of the peptide evoked by mechanical stimuli is not regulated by endogenous ligands for the nor-binaltorphimine binding site. In previous studies, when the thermal or mechanical stimulus was delivered in the presence of the selective δ-opioid receptor antagonist naltrindole we observed the opposite effect: blockade of the δ-opioid receptors prevented the release of substance P-like immunoreactivity to return to baseline levels following a mechanical but not a thermal stimulus (Zachariou and Goldstein, 1996b). The above observations may suggest that the thermally and mechanically evoked responses are mediated by two different populations of substance P-containing neurons that both contain κ- and δ-opioid receptors but are regulated by different endogenous opioids. Neurons activated by thermal stimuli appear to be regulated by endogenous κ-opioid receptor ligands and neurons activated by mechanical stimuli appear to be regulated by endogenous δ-opioid receptor ligands. However, previous studies where the release of met-enkephalin was monitored using intrathecal catheters in halothane-anesthetized rats do not support this hypothesis. These studies revealed that the release of met-enkephalin in the spinal zone receiving the nociceptive input increases during thermal but not during mechanical stimulation (LeBars et al., 1987; Cesselin et al., 1989). Monitoring the release of endogenous δ- or κ-opioid receptor ligands during mechanical or thermal activation of peripheral nociceptors, using the present paradigm, would provide more evidence for the above hypothesis.

In summary, this study demonstrated that the opioid peptide dynorphin-(1–8) decreased the basal release of substance P-like immunoreactivity and prevented the mechanically evoked release of substance P-like immunoreactivity in the dorsal horn of decerebrate/spinal transected rats. This effect was mediated through κ-opioid receptors which are also involved in the tonic regulation of the release of substance P-like immunoreactivity in the spinal cord.

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