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Sufentanil, Morphine, Met-enkephalin, and κ -Agonist (U-50,488H) Inhibit Substance P Release from Primary Sensory-Neurons: A Model for Presynaptic Spinal Opioid Actions

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Abstract

An *in vitro* model system for analysis of presynaptic inhibitory actions of spinal opioids has been applied. Embryonic sensory neurons derived from chick dorsal root ganglia were grown in primary cell culture, and the release of substance P was evoked by electrical field stimulation during exposure to drugs with well-demonstrated affinity for opioid receptors. This allowed a pharmacologic characterization of the inhibitory actions of specific opioid agonists on the release of substance P as measured by radioimmunoassay (RIA). Sufentanil (0.5 μM), a high affinity μ receptor agonist, U-50,488H (25 μM), a selective κ receptor agonist, and morphine (10 μM), an agonist with high affinity for μ and δ receptors, inhibited the evoked release of substance P by approximately 60%, 40%, and 50%, respectively. For sufentanil the response was demonstrated to be dose-dependent. As is the case for its analgesic action *in vivo*, morphine was approximately 50-fold less potent than sufentanil on a molar basis in this assay. The actions of sufentanil, U-50-488H and morphine were mimicked by the endogenous opioid peptide met-enkephalin, and its stable synthetic analog D-ala²-met⁵-enkephalinamide (DAME). Naloxone (25 μM), an opioid receptor antagonist, blocked the inhibitory action of sufentanil (0.5 μM), morphine (5 μM), and DAME (5 μM), but not U-50,488H (10 μM). The action of U-50,488H was partially blocked by the antagonist naltrexone (25 μM). Stereo-selectivity of agonist action was confirmed by the failure of dextrorphan (50 μM), an inactive opioid isomer, to inhibit the release of substance P. Actions mediated by specific opioid receptors were thus demonstrated by high affinity responses to agonists, blockade of agonist responses by opioid antagonists, and stereoselectivity. These findings suggest that in the spinal cord presynaptic inhibition of evoked substance P release is mediated by μ , κ and δ opioid receptors located on primary sensory nerve terminals. Activation of these receptors may explain, at least in part, the spinal analgesic actions of specific opioid agonists.

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Keywords

Anesthetics, opioids; D-ala²-met⁵-enkephalinamide; dextrorphan; κ opioid agonist; U-50,488H; morphine; sufentanil. Antagonists, opioids: naloxone; naltrexone. Spinal cord: dorsal root ganglion cells; presynaptic inhibition. Pain: substance P

Although epidural and subarachnoid administration of opioid analgesics is widely used in the treatment of postoperative pain and cancer pain, the cellular and molecular basis for the antinociceptive action of these substances remains only partially understood. Afferent transmission of nociceptive impulses involves primary sensory neurons, the cell bodies of which are located in the dorsal root ganglia and the terminals of which synapse in the dorsal horn of the spinal cord. The undecapeptide, substance P, is found primarily in the terminals of small diameter unmyelinated (C) and thinly myelinated (A- δ) sensory fibers, many of which subserve nociception. Several lines of evidence indicate that substance P is a neuromodulator at this first synapse in the spinal dorsal horn¹⁻⁸ and that presynaptic inhibition of the release of substance P from sensory nerve terminals is a major mechanism by which opioids exert their spinal analgesic action.^{4,5,9-13}

Several studies have shown that opioids inhibit the release of substance P *in vivo* and *in vitro*. Yaksh *et al.* reported that intrathecal administration of morphine inhibited the electrically evoked release of substance P into the cerebrospinal fluid (CSF) only when the intensity of sciatic nerve stimulation was sufficient to activate A- δ and C fibers.⁹ Mudge *et al.* reported that dorsal root ganglia (primary sensory) neurons grown in culture released substance P on exposure to medium with a high concentration of potassium and this release of substance P was inhibited by the opioid agonist D-ala²-enkephalinamide (DAME).¹² Furthermore, Jessel and Iversen demonstrated that potassium-induced substance P release from rat trigeminal nucleus was inhibited by morphine, levorphanol, nor-morphine, DAME, and β -endorphin.¹⁰ Systematic examination of dose dependence, stereoselectivity, and reversability by antagonists was not reported in many of these earlier *in vitro* studies.

In previous *in vitro* studies the release of substance P was induced by a nonphysiologic stimulus, *i.e.*, depolarization of neurons by solutions containing a high concentration of potassium. More recently, Holz *et al.*¹⁴ reported the use of graded electrical field stimulation to evoke substance P release from cultured dorsal root ganglia neurons. Electrical stimulation in this fashion generated action potentials of normal configuration and resulted in the calcium-dependent release of substance P. In this manner it has been possible to characterize the inhibitory actions of α -adrenergic and GABA receptor agonists on substance P release.¹⁵

In the current study, we have assessed the effects of opioid agonists and antagonists on the electrically evoked release of substance P from cultured dorsal root ganglia neurons. We demonstrate that the evoked release of substance P is inhibited by μ , δ , and κ opioid receptor agonists in a dose-dependent and stereoselective fashion, and that concomitant administration of opioid antagonists block this inhibition. Our findings suggest that in primary sensory neurons, presynaptic inhibition of evoked substance P release can occur via μ , κ , and δ opioid agonists.

Materials and Methods

PREPARATION OF CELL CULTURES

Chick embryos, gestational age 10–12 day, were quickly and painlessly decapitated as approved by our institutional animal care committee. Thoracic and lumbar dorsal root ganglia were dissected, incubated at 37° C for 45 min in Puck's solution (NaCl 152 mM, KCl 6.0 mM,

Na₂HPO₄ 1.2 mM, KH₂PO₄ 1.2 mM, glucose 11.2 mM, pH 7.4), and mechanically dissociated into single cell suspensions by repeated passing through tapered glass pipette tips.^{12,16,17} The cell suspension was exposed to gamma irradiation from a cesium chloride source at 550 cGy/min for 10–15 min to kill dividing (nonneuronal) cells. The dorsal root ganglia cells were then plated on 60-mm diameter, collagen-coated cell culture dishes at a density of 150,000 to 200,000 neurons per dish.¹⁴ The cells were maintained in culture for 11–12 days before experiments at which time each culture contains 5–10 ng of substance P. The culture medium contained Dulbecco's modified Eagle's minimum essential medium (GIBCO) with glutamine (1 mM), penicillin (50 U/ml), streptomycin (50 µg/ml), pro-tease-inactivated horse serum (GIBCO, 10% vol/vol), chick embryo extract (GIBCO, 3% vol/vol), and 3 µl/ml of partially purified extract of mouse salivary glands enriched in nerve growth factor.^{12,14}

For substance P release experiments, the buffer bathing the cells (release buffer, pH 7.4) contained (in mM): NaCl 132, KCl 2.5, CaCl₂ 3, MgCl₂ 0.8, N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid 25, and bovine serum albumin 6 µM. Prior to each experiment, drugs were prepared as concentrated stock solutions in H₂O. The stock solutions were diluted in release buffer to give the final desired concentrations. DAME, met-enkephalin, naloxone, and naltrexone were obtained from Sigma. Compound U-50,488H was obtained from Upjohn. Dextrorphan was obtained from Dupont. Sufentanil was donated by Janssen. Preservative-free morphine was obtained from Elkin Sinn.

STIMULATION PROTOCOL FOR SUBSTANCE P RELEASE EXPERIMENTS

Under sterile conditions, the dorsal root ganglia cell cultures were stimulated at room temperature using bipolar platinum steel electrodes. Stimulation consisted of square-wave DC pulses, 3 ms duration, 110 V for 3 min at a frequency of 1 Hz. Three successive phases of stimulation (s1–s3, fig. 1) were delivered at 2-h intervals. In between each phase of stimulation the cultures were returned to the incubator for reequilibration in culture medium. As previously reported,^{14,15} under these conditions reproducible amounts of substance P are released during each phase of stimulation. Previous experiments have shown that peptide release is linear with stimulation time over the range of 1–5 min. Furthermore, the release of substance P is repeatable provided that successive phases of stimulation are spaced at intervals of 90–120 min.^{14,15} In between each phase of stimulation, the cultures were bathed in culture medium and returned to the incubator for reequilibration.

Table 1 depicts the design of a representative experiment. Nine cultures from a single plating were divided into three groups of three: an external control group, an agonist-treated group, and an agonist/antagonist-treated group. Prior to stimulation Phase 1, three cultures from the external control group were each bathed in 1.7 ml of release buffer for 3 min, whereupon 1.64 ml was removed as a sample for radioimmunoassay (RIA) of baseline substance P release. Next, the cultures were bathed in an additional 1.7 ml of release buffer, stimulated for 3 minutes, and again 1.64 ml was removed from each as a sample for RIA of evoked substance P release. The cultures were then bathed in culture medium and returned to the incubator for reequilibration. In stimulation Phases 1, 2, and 3, all cultures from the external control group received release buffer alone. In contrast, during phase 2 the agonist-treated group received release buffer plus an agonist for both baseline and evoked release, whereas the agonist/antagonist-treated group received release buffer plus a mixture of agonist and antagonist for both baseline and evoked release. Thus, Phases 1 and 3 provide an internal control for the drug-treated cultures. During Phase 2 the separate external control group received release buffer alone for both baseline and evoked release.

This experimental design is necessary to avoid history-dependent effects of drugs and electrical stimulation on substance P release. For example, if the intervals between successive phases of stimulation are too short, *i.e.*, <60 min, there is a decrease in the amount of substance P released

in each phase, probably related to depletion of cellular stores of substance P. However, as demonstrated by the external control group, reproducible amounts of substance P are released when successive phases of stimulation are separated by 120 min. This experimental design also allows an assessment of whether the drugs tested inhibit the release of substance P in a reversible manner, as demonstrated by the internal controls within each drug-treated group of cultures.

RADIOIMMUNOASSAY FOR SUBSTANCE P

The amount of substance P-like immunoreactivity in each sample was measured by direct radioimmunoassay of the solution bathing the cells.¹⁴ The properties of the substance P antiserum and the immunoreactive tracer were previously reported by Kream *et al.*¹⁸ The antiserum displays full recognition for substance P and its sulfoxide derivative. It also displays minimal crossreactivity to the related tachykinins, substance K and neuromedin K, and to a variety of unrelated opioid peptides. The sensitivity of the RIA was adjusted by varying the antibody dilution factor (typically 1:250,000). In most experiments the value for 50% displacement of immunoreactive tracer by non-radioactive substance P was 25 pg of substance P, and the lower limit of detection was 8 pg of substance P per assay. Standard curves were generated by assaying serial dilutions of a synthetic substance P standard (Sigma) that was diluted in the same release buffer as was used for the release experiment. The drugs tested did not interfere with binding of the tracer to the antibody. Substance P immunoreactivity generated by cultured dorsal root ganglia neurons was judged to be authentic undecapeptide after analysis by HPLC combined with RIA.¹⁴ Thus, released substance P-like immunoreactivity is henceforth considered authentic substance P.

DATA ANALYSIS

For each phase, the amount of evoked substance P release was calculated as the amount of substance P released during electrical stimulation minus the amount of substance P released in the absence of stimulation (baseline release). The release ratio for a group of cell culture plates was calculated as:

$$\text{Release ratio} = S_2 / [(S_1 + S_3) / 2]$$

where

S_1 is the amount of evoked substance P release in Phase 1,

S_2 is the amount of evoked substance P release in Phase 2, and

S_3 is the amount of evoked substance P release in Phase 3.

The % inhibition of evoked substance P release relative to internal control (Phase 1 and Phase 3) for the group of plates that received drugs during Phase 2 was defined as:

$$[1 - \text{release ratio of the drug-treated group}] \times 100\%$$

The % inhibition relative to external control (separate control group) was calculated as:

$$[1 - (\text{drug-treated group release ratio} / \text{control group release ratio})] \times 100\%$$

For each experiment, mean release ratios and % inhibition of substance P release were compared between the drug-treated group and the control group by unpaired *t* tests. Similar comparisons were performed using exact significance levels from the Wilcoxon rank sum statistic. This yielded identical conclusions in all cases concerning significant and nonsignificant differences.

Results

ELECTRICALLY EVOKED RELEASE OF SUBSTANCE P

With the protocol described above, the evoked release of substance P was reproducible for each of the repeated phases of stimulation (fig. 1). Thus, the amount of substance P released during Phases 1, 2, and 3 for the control group was not significantly different from the amount of substance P released during Phases 1 and 3 for the drug-treated groups (fig. 1).

INHIBITION OF SUBSTANCE P RELEASE BY OPIOIDS

Figure 1 illustrates the inhibition of the electrically evoked release of substance P by sufentanil (0.5 μM). Inhibition of the evoked release of substance P by sufentanil (0.5 μM) was demonstrated in three of three release experiments using nine cultures from three different platings. Morphine, DAME, met-enkephalin, and the selective κ agonist-U50,488H were also shown to inhibit the evoked release of substance P from cultured dorsal root ganglia neurons (table 2). All inhibitory compounds tested were observed to have inhibitory effects ranging from 13% to 75% of control. Inhibition of substance P release by sufentanil was dose-dependent. As shown in table 2, the inhibition of substance P release by sufentanil increased from 15% to 59% at 0.02 to 0.5 μM , respectively. Consistent with clinical analgesic actions of these compounds, sufentanil exhibited significantly higher inhibitory potency than morphine. Morphine showed saturable action with maximal inhibitory effect at 50%.

SPECIFIC OPIOID RECEPTOR AGONISTS

Both sufentanil, a specific μ agonist, and U-50,488H, a specific κ agonist, inhibited the evoked release of substance P (table 2). Moreover, met-enkephalin, which exhibits relative preference for δ receptors, also inhibited substance P release.

BLOCKADE BY ANTAGONISTS

Naloxone (25 μM) completely blocked the effect of sufentanil (0.5 μM), *i.e.*, the percent inhibition calculated for the sufentanil/naloxone group was not significantly different from the control group (tables 2 and 3). As summarized in table 3, naloxone (25 μM) also blocked the inhibitory actions of morphine (5 μM) and DAME (5 μM). However, the inhibitory action of κ agonist, U-50,488H (10 μM), on the evoked substance P release was not blocked by naloxone (25 μM). The inhibitory effect of U-50,488H was partially blocked by naltrexone at 25 μM .

STEREOSELECTIVITY FOR OPIATE AGONISTS

Dextrorphan (50 μM), an inactive opioid isomer, has no significant inhibition or enhancement of substance P release (table 4). Concomitant addition of dextrorphan did not alter the extent of inhibition of substance P release by sufentanil (0.5 μM), nor did it alter the antagonism by naloxone. These findings indicate that the inhibitory effects of opioid agonists on the evoked release of substance P from cultured dorsal root ganglia are due to actions on stereoselective opioid receptors and are not mediated *via* nonspecific interactions.

Discussion

A model for presynaptic actions of certain opioids at the spinal level was explored in this study. Cultured dorsal root ganglion neurons from chick embryos were electrically stimulated in the presence or absence of opioids, and the release of substance P, a putative neuromodulator in nociceptive pathways, was measured by radioimmunoassay. Using this approach, we have shown that opiate alkaloids and an endogenous opioid peptide inhibit the electrically evoked release of substance P in a high affinity and stereoselective fashion. As previously noted, several types of opioid receptors, *e.g.*, μ , κ , δ , may be involved in the spinal actions of opioids.

¹⁹ Because analgesia *in vivo* may be due to both presynaptic and postsynaptic sites of action, it is of interest to determine which opioid subclasses modulate presynaptic events. With our data on presynaptic primary sensory neurons, we speculate a role for μ , δ , and κ opioid agonists in spinal analgesic mechanisms.

Previous studies have demonstrated that opioid ligand-receptor interactions display an extraordinary degree of stereoselectivity. Goldstein *et al.* originally demonstrated the stereospecific binding of levorphanol by incubating membranes with or without its inactive enantiomer dextrorphan.²⁰ In subsequent studies examining opioid receptor-mediated responses, the action of the active enantiomer levorphanol has been compared with that of the inactive enantiomer dextrorphan. In this study we have demonstrated that the inhibitory actions of opioids on the evoked release of substance P are mediated through stereoselective opioid receptors. Dextrorphan by itself did not affect the release of substance P, nor did dextrorphan alter the effect of sufentanil or naloxone.

Brief, pulsed electrical stimulation was used in this study to generate action potentials and to evoke substance P release. We believe this procedure is more physiologic than depolarization with medium containing elevated concentrations of potassium. Electrical stimulation produces action potentials, and the transmitter release that results is triggered by calcium influx through voltage-gated calcium channels.¹⁴ In contrast, potassium-induced transmitter release results from a prolonged depolarization of the membrane, which triggers a brief phase of calcium influx through voltage-gated ion channels, followed by a rise in intracellular calcium due to uptake processes unrelated to action potential propagation. The experimental design is notable in that it employs a three-phase stimulation protocol using both internal and external controls. Using this experimental design, it was recently demonstrated that evoked substance P release is also inhibited by α -adrenergic agonists,¹⁵ ligands with well-demonstrated spinal analgesic actions.^{19,21}

In the present study, all of the opioids tested inhibited the evoked release of substance P by at most 75%. One possible explanation for this finding is that dorsal root ganglia neurons grown in primary cell culture represent a heterogeneous population of cells, as previously noted in studies examining the electrophysiologic actions of opioids on these neurons.^{22,23} Specifically, it was reported that some dorsal root ganglia neurons do not respond to opioid agonists, and those that do respond exhibit differential sensitivity to the specific, μ , δ , or κ agonists tested.^{22,23} Therefore, the incomplete inhibition of substance P release by the opioid agonists we have tested might be expected if a subpopulation of dorsal root ganglia neurons release substance P but do not express functional opioid receptors.

The results presented here clearly demonstrate an inhibition of the evoked release of substance P by specific μ receptor agonists and by the specific κ receptor agonist U-50,488H. Met-enkephalin and DAME, which exhibit an incomplete preference for δ opioid receptors, also exhibited potent inhibitory activities in this model system. Although this observation is as expected if δ receptors mediate presynaptic inhibition of evoked neurosecretion, unequivocal demonstration of such receptors will require the testing of more specific δ receptor ligands. Technical factors ascribed to ligand chemical reactivity in the *in vitro* system prohibited interpretation of experiments employing the more selective δ agonist, D-pen²-D-pen⁵-enkephalin (data not shown).

Even before the discovery of opioid receptors, researchers have been interested in identifying opioids with desirable pharmacologic actions such as analgesia and anesthesia, but without undesirable actions such as respiratory depression and physical dependence. The popularity of the epidural and spinal narcotics also call for agents that provide analgesia without respiratory depression. Among the opioid receptor subtypes, the κ receptor is of particular interest. The

initial description of the actions of κ opioid receptors by Martin had suggested that κ agonists may have a respiratory-sparing effect.²⁴ Recently, a study examining rats with chronically implanted lumbar or cerebral ventricular catheters demonstrated less respiratory depression with κ agonist U-50,488H compared to that with morphine.²⁵ In the dorsal root ganglia neuron system, we observed significant inhibition of the evoked release of substance P with the administration of κ agonist U-50,488H at 5–25 μM . In several experiments, this inhibition was not blocked by naloxone (25 μM), but, naltrexone (25 μM), another opioid antagonist, was partially effective. Naloxone has been shown to have higher affinity for the μ opioid receptor than δ or κ opioid receptors (higher pA_2 values against μ agonists).^{26,27,**} The inability of naloxone in blocking κ receptors has also been previously reported.²⁸ Our data suggest the presence of functional κ receptors on these cells. Recently, Ruda *et al.* demonstrated that Dynorphin A, an endogenous opioid peptide prototypic for κ receptors, is present in neurons of the superficial dorsal horn in the same area as presynaptic substance P containing nerve terminals.²⁹ Our findings suggest a potential role for κ receptors in the transmission of nociceptive information via synaptic contact in the superficial dorsal horn.

In summary, we have examined an *in vitro* model of a presynaptic modulation of spinal opioids on the electrically evoked release of substance P from cultures by chick embryo dorsal root ganglia. It appears that μ , δ , and κ opioid receptors mediate this inhibition. This system will allow an analysis of the presynaptic actions of other analgesic agents considered for intrathecal or epidural administration.

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FIG. 1.

Substance P release during three phases of electrical field stimulation: Diagrams showing the result of a typical experiment. Nine cultures from a single plating were divided into three groups of three as shown in table 1. Each culture was stimulated for 3 min at 1 Hz. Baseline (shaded bars) and evoked (blank bars) levels of substance P immunoreactivity were determined by direct RIA of the release buffer for three repeated phases of stimulation. S1, 2, and 3 stand for the amount of substance P released in Phases 1, 2, and 3, respectively. The upper diagram shows results for the external control group. In the absence of any drug, the amount of evoked substance P release was not significantly different for the three repeated phases. The middle diagram shows the result of the agonist-treated (sufentanil 0.5 μM) group. The presence of sufentanil during Phase 2 inhibited the evoked substance P release by 54% ($P < 0.05$) as compared with Phase 1 and 3 of the same group. The lower diagram shows the result of the agonist/antagonist-treated (sufentanil 0.5 μM and naloxone 25 μM) group. Naloxone completely blocked the inhibitory effect of sufentanil. Error bars indicate the mean $\pm$ SD ($n = 3$) for released substance P as determined for three cultures of a single group.

TABLE 1

Experimental Design

Treatment ^a	Stimulation phase	Phase 1	Rest → 2h	Phase 2	Rest → 2h	Phase 3
Cultured neurons						
Control		Buffer				Buffer
Agonist		Buffer	Agonist			Buffer
Agonist + antagonist		Buffer	Agonist + antagonist			Buffer

TABLE 2

Agonist Pharmacology

Agonist Tested	% Inhibition by Internal Control	% Inhibition by External Control
Sufentanil 0.02 μM (n = 3)	$13 \pm 2^{*\dagger}$	$15 \pm 2^{*\dagger}$
Sufentanil 0.1 μM (n = 3)	$35 \pm 6^*$	$37 \pm 5^*$
Sufentanil 0.5 μM (n = 9)	$54 \pm 13^*$	$59 \pm 12^*$
Morphine 5 μM (n = 6)	$17 \pm 7^*$	$13 \pm 8^*$
Morphine 10 μM (n = 3)	$51 \pm 6^*$	$49 \pm 6^*$
Morphine 50 μM (n = 3)	$49 \pm 15^*$	$48 \pm 15^*$
U-50,488H 5 μM (n = 6)	$22 \pm 15^*$	$30 \pm 15^*$
U-50,488H 10 μM (n = 9)	$32 \pm 7^*$	$30 \pm 10^*$
U50,488H 25 μM (n = 3)	$43 \pm 8^*$	$40 \pm 8^*$
met-Enkephalin 20 μM (n = 3)	$72 \pm 4^*$	$75 \pm 4^*$
DAME 5 μM (n = 3)	$53 \pm 2^*$	$54 \pm 2^*$
DAME 10 μM (n = 3)	$55 \pm 6^*$	$56 \pm 6^*$

Values are mean $\pm$ SD.

* Significantly different from control ($P \leq 0.05$, by both t test and Wilcoxon rank sum statistic).

† Significantly different from sufentanil 0.1 μM ($P \leq 0.05$).

TABLE 3

Antagonist Pharmacology

Agonist/Antagonist Tested	% Inhibition by Internal Control	% Inhibition by External Control
Sufentanil 0.5 μM		
+ naloxone 25 μM (n = 6)	-13 ± 10	-2 ± 9
Morphine 5 μM		
+ naloxone 25 μM (n = 6)	7 ± 8	2 ± 9
U-50,488H 10 μM		
+ naloxone 25 μM (n = 5)	$33 \pm 8^*$	$27 \pm 9^*$
U-50,488H 10 μM		
+naloxone 25 μM (n = 5)	6 ± 16	$12 \pm 9^\dagger$
DAME 5 μM		
+naloxone 25 μM (n = 3)	-5 ± 13	-5 ± 13

Values are mean $\pm$ SD.

* Significantly different from control ($P < 0.05$).

† Significantly different from both the agonist (U-50,488H 10 μM) treated group ($P < 0.05$) and the control ($P < 0.05$).

TABLE 4

Stereoselectivity of Opioid Effect

Drug Tested	% Inhibition by Internal Control	% Inhibition by External Control
Sufentanil 0.5 μM *		
+ dextrophan 50 μM (n = 3)	$43 \pm 5^{\dagger}$	$48 \pm 5^{\dagger}$
Sufentanil 0.5 μM *		
+ naloxone 25 μM		
+ dextrophan 50 μM (n = 3)	-13 ± 4	-2 ± 4
Naloxone 25 μM (n = 5)	-7 ± 11	1 ± 14
Dextrophan 50 μM (n = 5)	-6 ± 11	-3 ± 20

Values are mean $\pm$ SD.

* The experiment was done with dextrophan 50 μM in the release buffer.

† Significantly different from control ($P \leq 0.05$).