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Morphine-induced Modulation of Nociceptive Spinal Dorsal Horn Neuronal Activities after Formalin-induced Inflammatory Pain

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In this study, we examined the morphine-induced modulation of the nociceptive spinal dorsal horn neuronal activities before and after formalin-induced inflammatory pain. Intradermal injection of formalin induced time-dependent changes in the spontaneous activity of nociceptive dorsal horn neurons. In naïve cats before the injection of formalin, iontophoretically applied morphine attenuated the naturally and electrically evoked neuronal responses of dorsal horn neurons. However, neuronal responses after the formalin-induced inflammation were significantly increased by morphine. Bicuculline, $GABA_A$ antagonist, increased the naturally and electrically evoked neuronal responses of dorsal horn neurons. This increase in neuronal responses due to bicuculline after the formalin-induced inflammation was larger than that in the naïve state, suggesting that basal $GABA_A$ tone increased after the formalin injection. Muscimol, $GABA_A$ agonist, reduced the neuronal responses before the treatment with formalin, but not after formalin treatment, again indicating an increase in the GABAergic basal tone after the formalin injection which saturated the neuronal responses to GABA agonist. Morphine-induced increase in the spinal nociceptive responses after formalin treatment was inhibited by co-application of muscimol. These data suggest that formalin-induced inflammation increases $GABA_A$ basal tone and the inhibition of this augmented $GABA_A$ basal tone by morphine results in a paradoxical morphine-induced increase in the spinal nociceptive neuronal responses after the formalin-induced inflammation.

Key Words: Nociceptive dorsal horn neurons, Morphine, spinal cord, $GABA_A$ basal tone, Subacute inflammatory state

INTRODUCTION

Painful syndromes following peripheral inflammation are characterized by an increase in the spontaneous activity and expansion of the receptive field area of the dorsal horn neurons, combined with hypersensitivity to mechanical or thermal stimulation. Hypersensitivity is believed to be either an increased sensation of pain in response to a noxious stimulus (hyperalgesia) or the sensation of pain in response to a non-noxious stimulus (allodynia).

Hyperalgesia generally outlasts the initial afferent discharge produced by an injury and mild noxious stimuli are normally perceived to be more painful. It has also been reported that hyperalgesia can be caused, at least in part, by sensitization of the spinal cord neurons (Campbell et al, 1979; LaMotte et al, 1983). In current clinical practice, several drugs, such as systemic or local anaesthetics, non-steroidal anti-inflammatory drugs, and opioids (morphine, fentanyl, sufentanyl, methadone, etc.), have been used to attenuate the hyperalgesia or allodynia associated with inflammation. Opioid analgesics, particularly morphine, are currently used as specific pharmacological tools for the treatment of acute pain, as well as for the long-term treatment of severe pain. The analgesic effect of morphine is believed to result from the action of μ-opioid receptors at several sites within the central nervous system (CNS), including the various brain stem nuclei, the spinal cord, and the terminals of the primary afferent fibres (Besson and Chaouch, 1987; Dickenson and Sullivan, 1987; Inturrisi, 2002). When morphine is applied either topically or intrathecally to the spinal cord, its direct analgesic effect is assumed to be mediated by a postsynaptic action which causes hyperpolarization of the spinal neurons and by a presynaptic action which causes a decrease in the release of neurotransmitters from the primary afferent terminals. Excitation due to morphine has also been shown in the CNS (Zieglgansberger et al, 1979; Neumaier et al, 1988; Kalyuzhny and Wessendorf, 1998; Akaishi et al, 2000), where a disinhibition mechanism related to the GABAergic system has been suggested. The efficacy of morphine has been demonstrated in the inflammatory pain response, inducing the primary and secondary phases of formalin-induced inflammation, by using behavioural methods (Neil et al, 1986; Kayser and Guilbaud 1990; Kayser et al, 1991; Tjolsen et al, 1992, Hammond et al, 1998) or extracellular single-unit activity recordings (Dickenson and Sullivan, 1987). However,

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ABBREVIATIONS: CNS, central nervous system; WDR, wide dynamic range; HT, high threshold; STT, spinothalamic.

the action of morphine on pain control in the more prolonged tonic phases after the secondary phase is unclear. Peripheral inflammation causes changes in the GABAergic system in the spinal cord dorsal horn (Castro-Lopes et al, 1994, 1995), but these changes result from chronic inflammatory conditions, but not subacute inflammation. Because it is unclear whether the correlation between the opioid and the GABAergic system is affected in the subacute inflammatory state at the spinal cord level, this study was undertaken to examine the relationship between the $GABA_A$ basal tone and morphine.

METHODS

Preparation of animals

All the experimental procedures were approved by our Animal Care and Use Committee and were performed in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals.*

Extracellular single-unit recordings were made from the dorsal horn neurons. Adult cats of either sex were initially anaesthetized with atropine sulfate (0.2 mg/kg, s.c.) and ketamine hydrochloride (30 mg/kg, i.m., Ketalar; Yu-Han, Korea). Following the cannulation of the trachea and the femoral vein and artery, α-chloralose was i.v. administered (60~80 mg/kg), and pancuronium bromide (initial dose 0.4 mg, maintenance dose 0.4 mg/h; Mioblock, Organon, Netherlands) was administered to relax the systemic musculature. The depth of anaesthesia was assessed by continuously monitoring the arterial blood pressure and the pupillary diameter. The animal was ventilated artificially with an end-expiratory carbon dioxide concentration maintained in the range of 3.5~4.5% (Normocap CO_2 & O_2 Monitor, Datex, Finland). Rectal temperature was monitored and the animal was maintained at $37.5 \pm 1°C$ with an electric blanket (Homeothermic Blanket Control Unit, Harvard Apparatus, USA). The lumbosacral enlargement was exposed by laminectomy performed on the L2~S3 vertebrae. After identifying the entry of the L7 and S1 dorsal roots, the dura and arachnoid were removed under a microscopic, and several small pia holes were made so that the recording electrode could be inserted. The left sciatic nerve was dissected and exposed after a skin incision was made in the left leg. After surgery, the animal was transferred to a stereotaxic animal fixation apparatus. A pool of warmed mineral oil was used to prevent drying of the exposed nervous tissues.

Electrodes

Glass multibarrel pipettes (seven-barrel; World Precision Instruments, inc. USA) with carbon filaments were used to record the single-unit activity from the dorsal horn ipsilateral field to the cutaneous receptive field. The microelectrode had a tip resistance of 1~3 MΩ and was made using an electrode puller (PE-2, Narishige, Japan). Iontophoretic barrels were independently filled with the following dialysing solutions: normal saline containing morphine sulfate (MOR; 10 mM, pH 4.5~5.0), naloxone hydrochloride (NLX; 50 mM, pH 5.0~5.5), muscimol (MUS; 5 mM, pH 3.5~4.0), bicuculline (BIC; 0.5 mM), l-glutamic acid (GLU; 500 mM, pH 8.5), or normal saline which served as the independent vehicle and pH control. To preclude the toxic action or non-specific alkaloid effects of morphine, the iontophoretic barrel filled with morphine was separated by at least 50 μm from the recording barrel with a carbon filament (Jones et al, 1990). All the drugs were freshly prepared before each experiment and were dissolved either in normal saline or in distilled deionized water. Holding currents of 0.5~2.0 nA were used to minimize the passive leakage of the drugs from the iontophoretic barrels. An additional barrel was filled with 2 M NaCl for current balance. All the drugs were iontophoretically ejected from the multibarrel pipettes with a cathodic or anodic current of 10~100 nA, depending on the characteristics of the drugs.

Stimulation and recording

The responses of the dorsal horn neurons to the noxious mechanical or electrical stimuli (A- and C-intensity, respectively) applied to the peripheral receptive field and the sciatic nerve were recorded using carbon-filament microelectrodes. When a single-unit activity with sufficient amplitude was isolated extracellularly, it was characterized with regard to its primary afferent input. Graded mechanical and electrical stimuli were then applied to the cutaneous receptive field. Usually, the depth of the recording site was approximately 200~1,200 or 1,500~3,000 μm. The signals were picked up by the recording electrode, amplified by an AC amplifier (DAM 80, WPI, USA), and fed into an oscilloscope and a window discriminator interfaced with a computer and a data acquisition system (CED 1401 *plus*) for storage and analysis. The spike size and configuration were continuously monitored to confirm that the unit activity of the same cell was recorded throughout the experiment. The neurons were classified in the following, according to their responses to mechanical stimuli applied to the most sensitive part of their receptive field for 10 s (20 s between stimuli): wide dynamic range (WDR) or high threshold (HT) neuronal activity (Chung et al, 1986). Calibrated von Frey filaments were used to determine the thresholds for mechanical stimuli. Noxious mechanical stimuli were given by applying a pinch to the receptive field with serrated forceps. After triple platinum poles were established at the exposed sciatic nerve as stimulating electrodes, A-intensity (1 mA intensity, 0.1 ms width) or C-intensity (10 mA, 0.5 ms) single or triple square pulses, generated by the stimulator (Pulse Master A300, WPI, USA), were applied to the sciatic nerve. Post-stimulus time histograms were analysed by their latencies to distinguish the responses to A- and C-fibre inputs. Cellular responses that appeared in less than 100 ms were considered to be A-inputs, and those appearing after 100 ms were considered to be C-inputs. The activity of the spinothalamic tract (STT) neurons was identified by antidromic activation from the ventral posterior lateral nucleus of the thalamus, using a square-current pulse (2 Hz, 1~2 mA, 0.2 ms) search stimulus. Antidromic activation was recognized by the criteria previously discussed (Trevino et al, 1973; Lin et al, 1996). The recording and stimulation sites in both the dorsal horn and the ventroposterolateral region of the thalamus were marked by injecting a direct current at the end of each experiment. The lumbosacral spinal cord and the brain were removed and fixed in 4% formalin for a week or longer. The recording and antidromic stimulation sites were identified using conventional histological examination. In all cases, animals were treated with a single injection of either

3% or 4% formalin (50 μL). At the end of each experiment, the animals were killed with anaesthetic.

Statistical analysis of data

The results were analysed by compiling single-pass time histograms. All the values are expressed as means $\pm$ SEM. Statistical significance was ascertained by a paired t-test at a p-value of <0.05.

RESULTS

A total of 69 cells were examined in 69 experiments. The recording sites were located from 200 to 3,000 μm below the surface of the spinal cord. These micrometer readings suggest that all the cells were within the dorsal horn. After identifying the single-unit activity from the dorsal horn (L3 ~ S2), the mechanical thresholds and responses to peripheral mechanical (brush, pressure, pinch, squeeze, and von Frey filaments) and electrical nerve stimulations were observed. In all cases, the nociceptive dorsal horn neurons were excited by iontophoretic bicuculline, which is a $GABA_A$ receptor antagonist, in their spontaneous and evoked activity. This indicates a high degree of tonic GABAergic inhibition of the nociceptive dorsal horn cells that were recorded in the spinal cord (Lin et al, 1996). Bicuculline currents applied *via* iontophoresis were carefully manipulated until there was an increase in electrical-stimulus-evoked activity of greater than 30 ~ 40%, because the excitation of unit activity by bicuculline occurs in a current-dependent manner. No changes in the amplitude of the action potential were observed in the presence of morphine, muscimol, or naloxone, implying that the inhibitory effects on unit activity produced by these agents were not local anaesthetic effects (Jones et al, 1990). From these results, 27 cells were classified as HT and 42 as WDR. Neuronal activity was investigated on a numerical rating scale before and after the formalin injections.

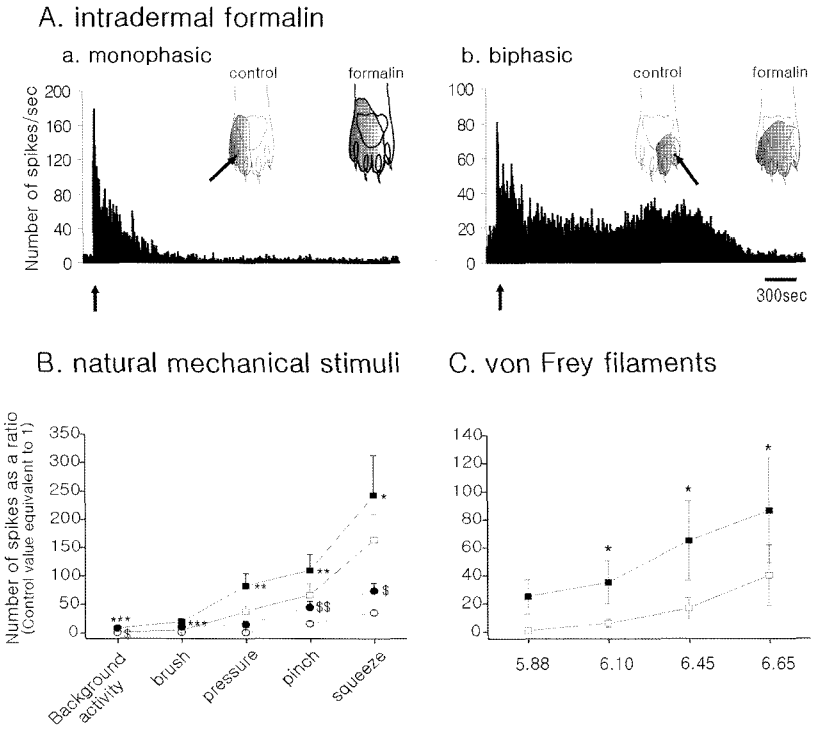

Fig. 1. Time-dependent changes in spontaneous activity after formalin treatment and the responses to natural mechanical and von Frey filaments. (A) Injection of 50 μL of formalin into the corresponding receptive field of the ipsilateral hind paw evoked distinctive phases: a monophasic (a, n=35) or biphasic (b, n=34) pattern. Arrows indicate the time and sites of intradermal injection of formalin. Injection of formalin also produced an expansion of the cutaneous receptive fields (dashed area). (B) Graphs show the normalized values (mean $\pm$ SEM) of the grouped data for WDR (square, n=42) and HT neuron (circle, n=27) responses to mechanical stimuli before (open square and circle, respectively) and 2 h after formalin treatment (filled square and circle, respectively). A comparison of the groups revealed significant differences between the control and formalin groups (*p, $^{\$}p < 0.05$, **p, $^{\$\$}p<0.01$, ***$p<0.001$). (C) Responses to the von Frey filaments before (open squares) and 2 h after formalin treatment (filled squares). The vertical axis represents the normalized mean number of spikes as a ratio in 10 s periods. The asterisk (*) indicates that the value of each group is significantly different from the control value. Four handle markings from 5.88 to 6.65 mean Log_{10} of (1000* Force in grams), which in turn represent 75.9, 125.9, 281.8, and 446.7, respectively.

Time-dependent changes in the spontaneous activity of nociceptive dorsal horn neurons after intradermal formalin injection

The administration of 3~4% formalin into the corresponding receptive field in the ipsilateral hind paw resulted in an increase in the spontaneous activity of all 69 cells (Fig. 1A), showing either distinctive monophasic (22 WDR and 13 HT) or spontaneous biphasic patterns (20 WDR and 14 HT), irrespective of the modality of the projection into the thalamus. In inflamed animals, the receptive fields were larger than those in intact animals (data not shown). Expansion of the receptive field area was observed in all experiments, irrespective of either a monophasic or biphasic pattern. Two hours after treatment with intradermal formalin to allow the full expression of both early- and late-phase responses to formalin, the responses to the peripheral mechanical stimuli were found to increase in both the 42 WDR neurons and the 27 HT neurons ($p<0.001$, Fig. 1B), and the mechanical threshold using von Frey filaments of the nociceptive neurons was reduced ($p<0.05$, Fig. 1C), showing a leftward shift in the stimulusresponse curves. When presented as the number of spikes given per unit background activity in WDR neurons (Fig. 1B), the values were 4.8 ± 1.2 (brush), 37.4 ± 10.0 (pressure), 66.2 ± 19.4 (pinch), and 163.9 ± 44.6 (squeeze) in intact animals. In the inflamed group, the values increased to 19.8 ± 4.0 ($p<0.001$), 82.3 ± 21.8 ($p<0.01$), 109.6 ± 28.2 ($p<0.01$), and 242.8 ± 69.5 ($p<0.05$), respectively. In HT neurons, the values were 16.4 ± 3.9 (pinch) and 36.2 ± 4.4 (squeeze). In the inflamed group, these values increased to 44.7 ± 10.3 ($p<0.01$) and 73.8 ± 13.4 ($p<0.05$), respectively. Although the responses of HT neurons to brush (1.1 ± 0.1) and pressure (1.1 ± 0.2) in the intact group seemed to increase to 10.7 ± 3.9 ($p=0.11$) and 14.8 ± 6.4 ($p=0.19$), respectively, the differences in the inflamed group were not significant, compared with background activity after treatment with formalin (8.8 ± 3.1). Overall, there was a significant increase (mean 8.5 ± 1.7; $p<0.001$) in background activity in the subacute phase.

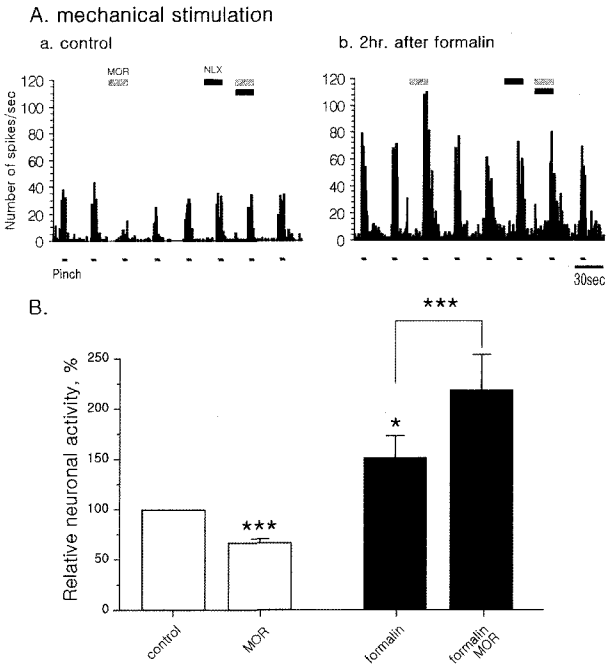

Fig. 2. The effects of morphine (MOR) on the noxious-mechanical-stimulus-evoked responses of dorsal horn neurons. (Aa) In a spinothalamic tract cell identified by antidromic stimulation, morphine (45 nA) reduced the response (rate of decrease of 80.2% compared with the prior response) to pinch in a naloxone (NLX, 56 nA)-reversible manner (b). Morphine (45 nA) enhanced the response (rate of increase, 37.2%) to pinch 2 h after formalin treatment. The results are expressed as a percentage of the initial control response. The responses to the pinch-evoked responses are shown in (B), sequentially from the left to the right of the graph (mean±SEM): $66.9\pm3.8\%$, $151\pm21.3\%$, and $219\pm34.9\%$. The asterisk (*) indicates that the value of each group is significantly different from the control value before and after formalin treatment (*$p<0.05$, ***$p<0.001$).

Morphine enhanced the responses of nociceptive transmission cells to noxious mechanical stimuli, following subacute inflammation

Following formalin injection, a stronger response to noxious stimuli developed at the site of the injection. Fig. 2A shows the effect of the iontophoretic application of morphine on the responses of nociceptive neurons (STT) to the pinch stimulus. Morphine reduced neuronal excitability in response to a noxious stimulus in a naloxone-reversible manner. However, the response to pinch increased, when morphine was applied 2 h after formalin treatment and the response to the non-noxious mechanical stimulus had little effect on this measure 2 h after the treatment.

In summary, an injection of iontophoretically applied morphine significantly increased the neuronal response to pinch ($140\pm7.5\%$, $p<0.001$) in 60 of the 69 cells after treatment with formalin, compared with before formalin ($66.9\pm3.8\%$, $p<0.001$, Fig. 2B). When the changes in evoked firing were examined with regard to cell type, 35 WDR cells showed increased firing, five WDR cells showed decreased firing, and two were unaffected by morphine. Twenty five HT cells showed increased firing and two showed decreased firing, and 13 STT cells (12 WDR, one HT) showed increased firing, two STT cells (one WDR, one HT) showed decreased firing, and one STT cell (WDR) was unchanged by morphine. The above result indicates that the excitatory effect of morphine described in this study may be due to a centralized action, because peripheral sensitization of the nociceptors is unlikely after spinal administration.

Morphine enhanced the unmyelinated C-fibre activity evoked by electrical stimuli in formalin-induced subacute phases, but not myelinated A-fibre activity

In 56 of the 69 cells that showed neuronal excitability in response to mechanical stimuli, further studies were performed to examine the excitatory effects of morphine on mechanical stimuli in terms of the relationship between afferent fibre activity and nociceptive transmission cells at the spinal cord level.

Fig. 3A shows a representative example of the effects of iontophoretic morphine on the electrical-stimuli-evoked myelinated A- and unmyelinated C-fibre activities before and after treatment with formalin. Vigorous responses to the electrical stimuli were observed for all neurons in the study, whereas, the evoked A- and C-fibre activities were reduced by the application of morphine in the control. The rate of

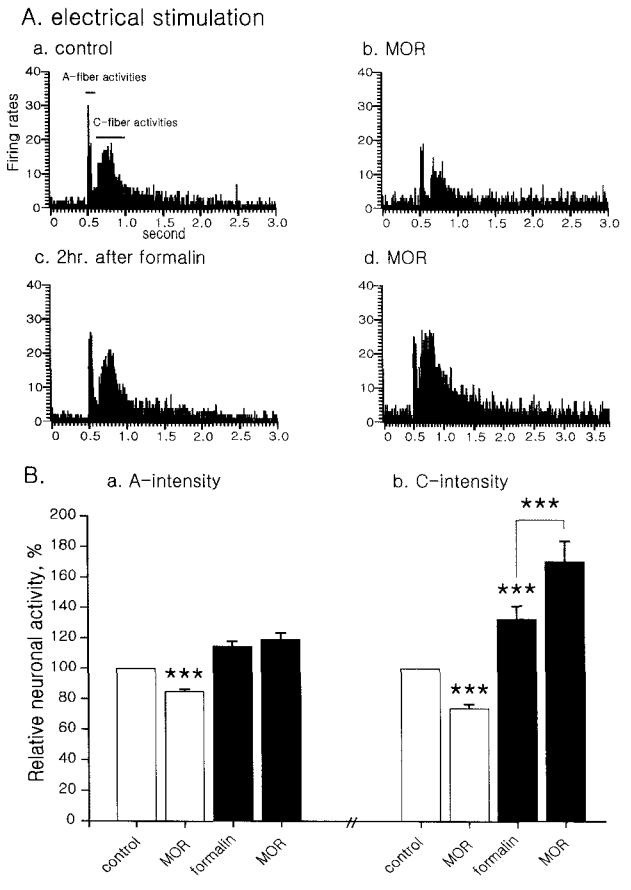

Fig. 3. Post-stimulus time histograms illustrating the effects of morphine on A- and C-fibre activity. The results show the cumulative response to a train of 10 stimuli. Changes in the evoked responses to A- and C-intensity electrical stimulation, following the application of morphine, are shown in (A). (A) When the results are expressed as a percentage of the initial control response, the changes in the evoked response to A-intensity electrical stimulation are shown from (b) to (d) on the graph: 68.5%, 107.9%, and 131.5% of the control value, respectively. Changes in the evoked responses to C-intensity electrical stimulation, following the application of the morphine (65 nA) 2 h after treatment with formalin, are shown from (b) to (d) on the graph: 58.2%, 115.4%, and 206.9% of the control value. (B) The histograms summarize the grouped dorsal horn neuronal responses to electrical stimuli. The results are expressed as a percentage of the initial control response. Responses to A-evoked responses are shown in (a), sequentially from left to right of the graph (mean±SEM): 84.9±1.5%, 114.3±3.6%, and 118.8±4.7%. Responses to the C-evoked responses are shown in (b), sequentially from left to right of the graph: 73.8±2.8%, 132.4±8.8%, and 170.3±13.3%. Comparison of the groups reveals significant differences (***$p<0.001$).

decrease was 26.4% and 40.8% in the evoked A- and C-fibre activities, respectively. On the other hand, both the A- and C-fibre activities were enhanced by morphine 2 h after formalin treatment. The rate of increase was 10.2% and 39.2% for the evoked A- and C-fibre activities, respectively.

In summary, typical post-stimulus time histograms show significant inhibition of the myelinated A-(n=56, 84.9±1.5% of the control value, $p<0.001$) and unmyelinated C-fibre responses (n=52, 73.8±2.8% of the control value, $p<0.001$) by morphine in the naïve cats (Fig. 3B). However, morphine enhanced the evoked C-fibre response (131.6±8.9%, $p<0.001$) after the application of formalin, but not the A-fibre activity. The change in the C-fibre response due to morphine was completely reversed by naloxone (data not shown). Examination of the change in the evoked C-fibre activity with regard to cell type during morphine application after formalin injection showed that firing increased in 21 WDR cells (162.5±20.3%, $p<0.004$), 12 WDR cells were unaffected by morphine (<10%), 13 HT cells showed increased firing (121.2±2.8%), 6 HT cells were unchanged, 6 STT cells (five WDR, one HT) showed increased firing (147.2±3.1%, $p<0.001$), and 2 STT cells (two WDR) were not affected by morphine. The 52 cells that showed increased or unchanged C-fibre activity as a result of morphine treatment also showed enhanced firing rates in response to mechanical stimuli in the presence of morphine (131.6±8.9%, $p<0.001$). Three cells that showed decreased fibre activity also showed a decreased firing rate. In contrast, the addition of morphine was effective in the control, but was unable to induce the desired effects on formalin-induced inflammation. No correlation was noted between the morphine-induced excitatory effects and either the change in the spontaneous activity caused by formalin or the cell classification.

Effects of $GABA_A$ antagonist and agonist on the response of the nociceptive dorsal horn neurons before and after formalin injection

To study the effects of morphine on $GABA_A$ basal tone at the spinal cord level, further experiments were performed in 11 of the 56 cells that showed increased firing in response to pinch and electrical stimulation. Fig. 4 shows a typical experiment, in which morphine was added with bicuculline, a $GABA_A$ receptor antagonist, or muscimol, a $GABA_A$ receptor agonist. Bicuculline enhanced the A-fibre response in the naïve and subacute phases (Fig. 4Ac, Bc), and the rates of increase were 19.8% and 19.7%, respectively. Bicuculline also clearly enhanced the C-fibre activity in the naïve and subacute phases (Fig. 4Ac, Bc), and the rates of increase were 60.1% and 88.6%, respectively. Morphine reduced the bicuculline-mediated increase in the A-response before and after formalin treatment (Fig. 4Ad, Bd), and the rates of decrease were 11.9%, and 10.6%, respectively. Morphine also significantly reduced the bicuculline-mediated increase in the C-response before and after treatment with formalin (Fig. 4Ad, Bd) and the rates of decrease were 19.2% and 28.4%, respectively. As shown in Fig. 4Ae, muscimol inhibited A- and C-activity to 80.9% and 79.7% of the control value, respectively. However, no inhibitory effects of muscimol on the A- and C-response were observed in the subacute phase (100.5% and 99.6%, respectively; Fig. 4Be). Morphine co-applied with muscimol also reduced the C-response to 52.6% of the control value (Fig. 4Af). The A-response of the same neuron was altered to 77.8% of the control value by morphine and muscimol before formalin treatment, but was altered to 90.1% of the control value after treatment with formalin (Fig. 4Bf). Fig. 4Bb shows the excitatory effect of morphine on the C-fibre response, which increased to 151.7% of the control value after formalin treatment. However, the enhanced C-response induced by morphine was reduced by the co-application of muscimol to 83.5% of the control value after formalin treatment (Fig. 4Bf).

The results are summarized in Fig. 5. The A- and C-fibre responses were reduced by morphine and/or muscimol exo-

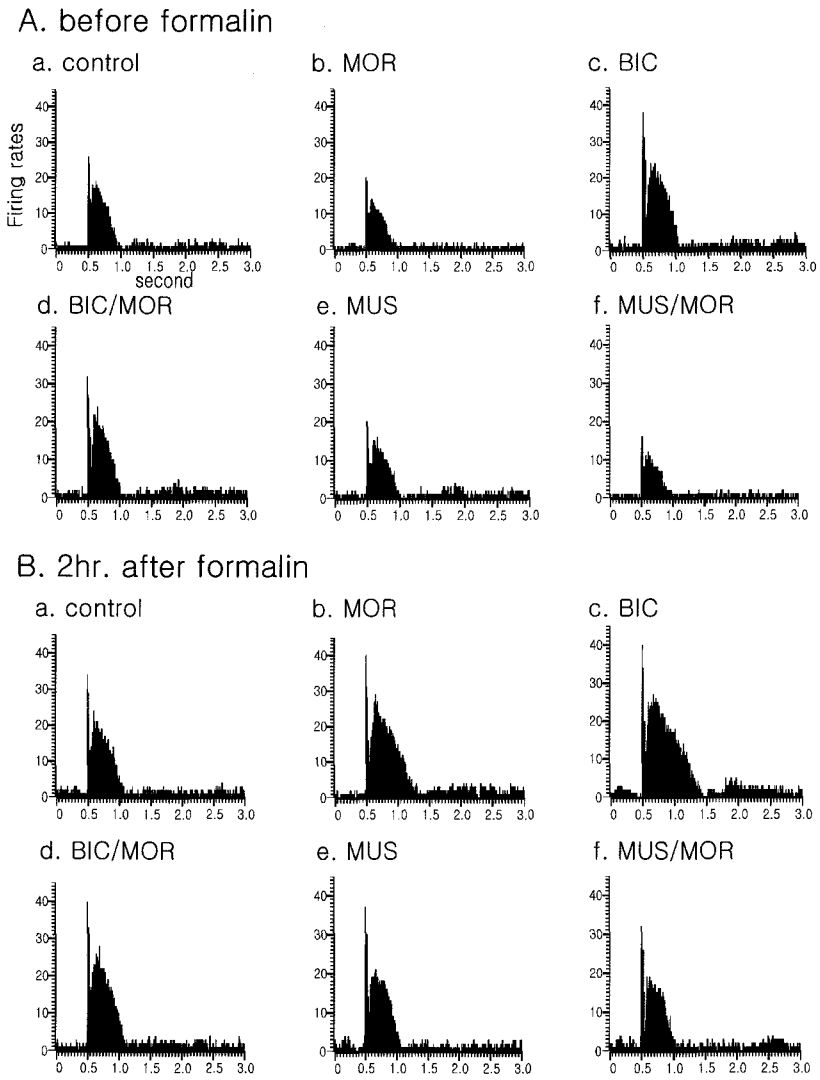

Fig. 4. Examples of post-stimulus time histograms, showing the effects of morphine co-applied with either bicuculline (BIC) or muscimol (MUS). (A) When the results are expressed as a percentage of the initial control response, the changes in A- (C-) fibre activity are sequentially shown from (b) to (f) of the graph: 93.2% (65.6%), 119.8% (160.1%), 125.3% (129.3%), 80.7% (79.7%), and 77.8% (52.6%) of the control value. (B) When the results are expressed as a percentage of the control response 2 h post-formalin, the changes in A- (C-) fibre activity are, respectively, from (b) to (f) of the graph: 106.6% (151.7%), 119.7% (188.6%), 122.1% (135.1%), 100.5% (99.6%), and 90.1% (83.5%) of the control value.

genously applied prior to the formalin injection, but showed no significant inhibition after the formalin injection. Iontophoretic bicuculline increased the neuronal response (A- and C-fibre) in naïve and subacute inflammation. The post-bicuculline responses in the A- and C-fibres increased to $121.7 \pm 5.4\%$ and $141.2 \pm 2.6\%$ of the control values before the formalin injection, respectively, and to $117.5 \pm 5.9\%$ and $163.1 \pm 5.4\%$ after the formalin injection, respectively. In particular, the increase in the C-fibre response caused by bicuculline 2 h after treatment with formalin was greater than that of the naïve phase ($p < 0.005$). Bicuculline-stimulated A-fibre excitability in the naïve and subacute phases was attenuated by the presence of morphine (Fig. 5A, $p < 0.05$). C-fibre excitability in the naïve and subacute phases also showed a significant decrease (Fig. 5B, $p < 0.001$). The increase in the C-fibre response ($122.3 \pm 6.9\%$, $p < 0.01$) observed after a single application of morphine disappeared, when muscimol and morphine were co-applied. Moreover, the co-application of muscimol and morphine reduced the C-fibre response ($85.5 \pm 3.2\%$, $p < 0.005$), compared with the response to a single application of exogenous muscimol (Fig. 5B). Fig. 5B also shows the change in $GABA_A$ basal tone in the C- fibre response after treatment with formalin. A decrease in GABAergic tone due to spinal bicuculline administration led to an increase in neuronal activities. There was a significant difference in the response of C-activity to bicuculline in the naïve and inflamed groups, normalized to the responses of each C-

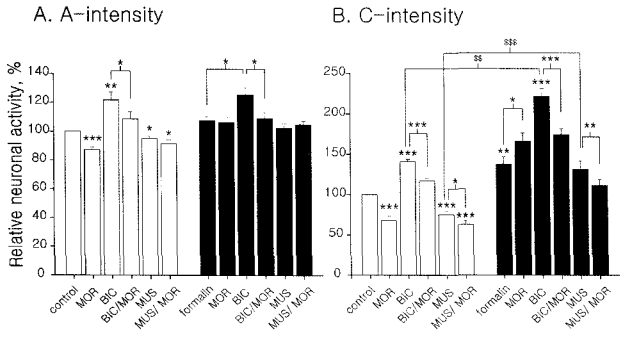

Fig. 5. Histograms showing the effects of morphine co-applied with bicuculline or muscimol and the changes in GABAergic tone on A- and C-fibre activity. The results are expressed as a percentage of the initial control response. Responses to the A-evoked responses are shown in (A), from the left to the right of the graph (mean±SEM): 87.3±1.9%, 121.7±5.4%, 108.6±4.9%, 94.9±1.8%, 91.4±2.7%, 107.5±2.6%, 106.3±3.2%, 125.4±5.1%, 109.1±4.2%, 102.5±3.0%, and 104.8±2.6%. The responses to the C-evoked responses are shown in (B), from the left to the right of the graph: 67.3±6.4%, 141.2±2.6%, 117.2±3.4%, 75.0±3.9%, 62.8±4.5%, 137.7±9.4%, 165.9±10.3%, 221.3±10.0%, 174.3±7.3%, 131.5±10.5%, and 111.2±8.0%. A comparison of the groups revealed significant differences (*$p<0.05$, **$p<0.005$, ***$p<0.001$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$).

activity before and after formalin (141.2±2.6% vs 163.1±5.4%, $p<0.01$). However, there was no difference in the bicuculline-induced excitability of the A-response before and after treatment with formalin (121.7±5.4% vs 117.5±5.9%, respectively). The response of the C-activity to muscimol also showed a significant difference between the groups (75.0±3.9% before formalin vs 95.2±2.2% after formalin, $p<0.001$). A muscimol-associated decrease was observed in the control, but not in the inflamed group. There was no difference in the muscimol-induced inhibition of the A-response between the control and inflamed groups (94.9±1.8% vs 95.6±2.9%, respectively). The change in the C-response after formalin in the presence of muscimol or bicuculline indicates an altered sensitivity to the $GABA_A$ basal tone in the C-fibre activity.

Morphine decreased glutamate-stimulated neuronal excitability in both control and inflamed groups

The effect of morphine on the glutamate-evoked response was studied in 3 cells that showed an excitatory effect as a result of morphine administered 2 h after formalin. Fig. 6A shows a representative example of the effects of iontophoretically applied morphine on glutamate-evoked neuronal firing before and after treatment with formalin. The mean firing rate increased from the control level of 10.5±1.9 Hz to 48.9±5.1 Hz in the presence of iontophoretic glutamate before formalin treatment (Fig. 6Aa). In the inflamed group, the mean firing rate increased from the control level of 19.5±0.9 Hz to 101.9±8.6 Hz in the presence of iontophoretic glutamate (Fig. 6Ab). The glutamate-evoked activity was inhibited by the presence of morphine before and after formalin treatment to 24.6±1.0 Hz (a rate of decrease of 50.4%) and 47.2±5.5 Hz (a rate of 46.3 %), respectively. These changes were completely reversed by naloxone.

In summary, morphine significantly inhibited the neuronal excitability induced by glutamate in both the naïve and

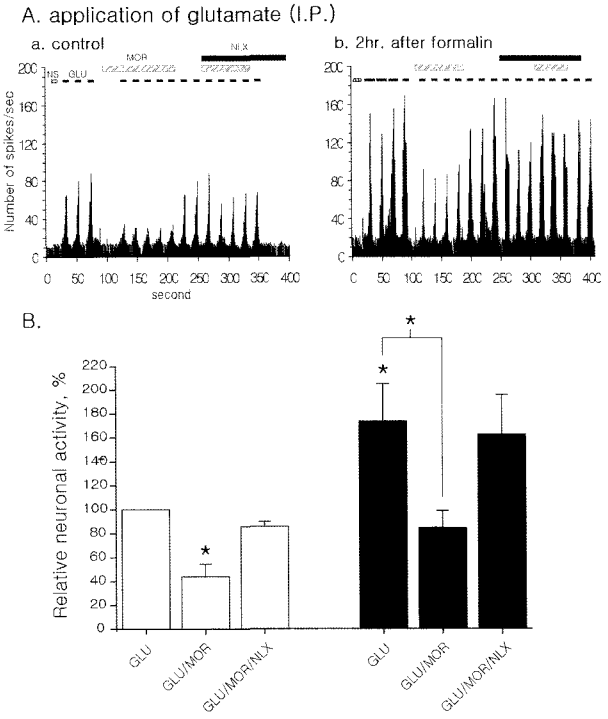

Fig. 6. Effects of morphine on glutamate (GLU)-evoked responses of dorsal horn neurons. (A) Spontaneous activity did not change under control conditions (normal saline, NS). Morphine reduced the GLU-evoked responses in both the control and inflamed groups in a naloxone-reversible manner. (B) The results are expressed as the percentage of the initial control response before (open square) and 2 h after treatment with formalin (closed square), from the left to the right of the graph (mean±SEM): 44.0±10.5%, 85.7±4.2%, 173.4±31.4%, 84.7±14.0%, and 162.3±33.4%. The asterisk (*) indicates that the value of each group is significantly different from the control value before and after treatment with formalin (*$p<0.05$).

inflamed groups to 44.0±10.5% ($p<0.05$) and 49.4±2.1% ($p<0.05$), respectively (Fig. 6B).

DISCUSSION

In the present study, the central role of iontophoretic morphine played in modulating the responses of nociceptive dorsal horn neurons in the subacute phase was investigated 2 hours after treatment with formalin. The results showed that the observed nociceptive dorsal horn neurons (WDR and HT) in the subacute phase received stronger input from GABAergic interneurons than those in the naïve phase, because neuronal excitability was further increased when the cells in the subacute phase were treated with bicuculline. This indicates that a tonic release or the presence of endogenous GABA, which affects the neurons that are inhibited by the exogenously applied GABA or muscimol, may be responsible for the observed action of bicuculline before and after treatment with formalin. Consequently, using *in vivo* extracellular recordings, this study examined the central effects of morphine on the $GABA_A$ basal tone which is susceptible to inflammation at the spinal cord level.

Iontophoretic morphine resulted in significant attenuation of the naturally, electrically, and iontophoretic-glutamate-evoked neuronal responses in naïve cats. This is in good agreement with earlier studies showing that morphine generally produces an inhibitory modulation of neuronal activities (North, 1986; Kemp et al, 1996). In contrast, however, the neuronal activity caused by mechanical stimuli and C-fibre activity in the formalin-induced inflamed group was significantly increased by spinal morphine, although A-fibre activity in the inflamed group showed no significant increase in response to morphine. In the present study, we observed no significant decrease in either A- or C-fibre activity after treatment with formalin. These results indicate that even if the transmission of painful input into the spinal cord is conducted *via* both myelinated- and unmyelinated afferent fibres, the excitatory effects of morphine on neuronal activity in response to noxious mechanical stimuli may be due to a change in C-fibre-related spinal sensory processing (Dickenson & Sullivan, 1987). Although it is reasonable to speculate that morphine selectively inhibits the nociceptive input in normal animals (Belcher & Ryall, 1978), further study in need is to clarify whether some of the effects of morphine reflect any of the changes near the nociceptive dorsal horn neurons in inflamed animals.

Increase of basal GABAergic tone in the spinal cord

Spinal bicuculline increased the neuronal responses to electrical stimuli in spinal neurons in both naïve and formalin-induced inflammation, which may result from a loss of GABAergic tone due to spinal bicuculline. The increased neuronal responses caused by bicuculline during inflammation were greater than those in the naïve state. These data show that, at least in part, GABAergic (particularly the $GABA_A$) tonic inhibition in C-fibre-related spinal sensory processing was developed after subcutaneous injection of formalin. This might be due to a formalin-induced increase in the activity of the GABAergic system at the spinal cord level. The results presented thus far are consistent with the proposal that spinal neuronal responses are influenced by the GABAergic as well as the glycinergic inhibitory tone (Melzack & Wall, 1965; Carlton et al, 1992; Mascias et al, 2002), and that spinal bicuculline can produce enhanced A- and C-fibre responses in the deep dorsal horn cells (Reeve et al, 1998). We are unaware of any firm evidence that the change in $GABA_A$ basal tone in C-fibre-related spinal sensory processing is due to an increase in the affinity of the $GABA_A$ receptors or to the recruitment of the $GABA_A$ system newly produced by inflammation. However, it is highly likely that the effect of morphine would be altered when the $GABA_A$ basal tone is changed.

Inhibitory effects of morphine on excitatory synaptic transmission

The effect of morphine on the evoked activity in response to iontophoretically applied glutamate was examined before and after the injection of formalin. The effects of glutamate were observed immediately after its iontophoretic application. The effects of iontophoretic glutamate were not restricted to the activity of the recorded unit, and it was impossible to investigate whether glutamate acts directly on the recorded neurons. However, glutamate could act, at least in part, on cells synaptically connected to the recorded unit or cells that have a demonstrable postsynaptic action on the recorded neurons. The profound excitation of neuronal activity that occurred as a result of iontophoretic glutamate was significantly inhibited by iontophoretic morphine. Therefore, we suggest that morphine exerts inhibitory effects on excitatory neurotransmitter-mediated synaptic transmission in both the naïve and subacute phases.

Paradoxical excitatory effects of morphine

Earlier studies showed that spinal dynorphin may be associated with nociceptive behaviour or inflammation-induced hyperalgesia (Knox & Dickenson, 1987; Hylden et al, 1991; Tan-No et al, 2002). This indicates that morphine may act as an excitatory drug in the postsynaptic neuron *via* the dynorphine receptor. It has also been reported that opiates can activate N-methyl-L-aspartate (NMDA) processes, resulting in a sustained increase in glutamate synaptic effectiveness (Celerier et al, 1999; Rivat et al, 2002) and an increase of the neuronal firing in the CNS *via* direct excitatory actions, including toxic actions or non-specific alkaloid effects (Jones et al, 1990; Lin et al, 1994; Akaishi et al, 2000). Furthermore, there is species difference between cats and rats: The excitatory effects of morphine on some spinal dorsal horn neurons have often been observed in cats (Dostrovsky & Pomeranz, 1976; Piercey et al, 1980), but less often in rats. However, the excitatory effects of morphine in these experiments are considered to be due to toxic effects of high morphine concentrations in the vicinity of the neuron being recorded. In the present study, we used extended recording electrodes to avoid any non-specific excitatory effects of iontophoretic morphine (see Methods) and iontophoretically applied the same ejection currents of morphine in both the naïve and subacute phases. Therefore, the excitatory effects on unit activity produced by morphine in the subacute phase are not likely to be due to direct consequence on the cell body of its close contact to high concentrations of morphine.

In neurons with an endogenously enhanced $GABA_A$ basal tone after treatment with formalin, the application of morphine resulted in excitatory responses. It is possible that morphine precludes the inhibition of cells *via* the $GABA_A$ system by preventing the release of GABA, which is increased and triggered by inflammation, thereby increasing the excitability of cells. Muscimol reduced the neuronal response (A- and C-fibre activity) before treatment with formalin, but not after formalin treatment. This also indicates a change in the GABAergic basal tone. Clearly, the most reproducible morphine-associated increased responses in both noxious mechanical (data not shown) and C-fibre-related spinal sensory processing were inhibited by muscimol. This result is considered to reflect the full activation of the GABAergic system due to iontophoretically applied muscimol in the vicinity of the nociceptive cells. When the tonic GABA input was blocked with bicuculline and morphine was then added to determine if it would cause any changes, there was significant inhibition of the bicuculline-induced excitability, compared with the effects of bicuculline alone. Therefore, it is most likely that morphine does not directly stimulate the postsynaptic cells, but has an inhibitory effect on the GABAergic input during the increase in inflammation-induced tonic inhibition. This study confirms earlier studies (Zieglgansberger et al, 1979; Neumaier et al, 1988; Kalyuzhny & Wessendorf, 1998; Moran

et al, 2002) that showed that the inhibition of the GABAergic system may underlie some of the excitatory effects of opioids on the CNS.

A morphine-associated excitatory response was also observed, indicating that morphine increases the release of substance P from the primary afferent neurons (Suarez-Roca & Maixner, 1993, 1995) and exerts excitatory effects on some rat lamina I and II neurons (Magnuson & Dickenson, 1991). There is no evidence of morphine- mediated presynaptic excitatory effects in the deep dorsal horn neurons in the subacute phase. However, it is suggested that spinal morphine exerts consistent inhibitory effects on muscimol, bicuculline, and glutamate in both the naïve and subacute phases.

Although these results exclude $GABA_B$ and glycinergic inhibition within the spinal cord, they show that activation of the GABA system could be affected by morphine. This suggests that morphine can affect the GABAergic system, which is involved in the development of formalin-induced hypersensitivity, which in turn counteracts the tonic inhibition enhanced by inflammation in the spinal cord. This means that the paradoxical excitatory effect of morphine may be due to a loss of formalin-induced enhanced GABAergic tone as a result of the central action of morphine. This study may provide the scientific basis for improved pain management with the opiate analgesics.

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