Korean J Physiol Pharmacol
Vol 6: 241–246, October, 2002

Involvement of NMDA Receptor and L-type Calcium Channel in the Excitatory Action of Morphine

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We studied the excitatory action of morphine on the responses of dorsal horn neuron to iontophoretic application of excitatory amino acid and C-fiber stimulation by using the *in vivo* electrophysiological technique in the rat. In 137 of the 232 wide dynamic range (WDR) neurons tested, iontophoretic application of morphine enhanced the WDR neuron responses to N-methyl-D-aspartate (NMDA), kainate, and graded electrical stimulation of C-fibers. Morphine did not have any excitatory effects on the responses of low threshold cells. Morphine-induced excitatory effect at low ejection current was naloxone-reversible and reversed to an inhibitory action at high ejection current. NMDA receptor, calcium channel and intracellular Ca^{2+} antagonists strongly antagonized the morphine-induced excitatory effect. These results suggest that changes in intracellular ionic concentration, especially Ca^{2+}, play an important role in the induction of excitatory effect of morphine in the rat dorsal horn neurons.

Key Words: Dorsal horn neuron, Morphine, Excitatory action, NMDA receptor, Calcium channel

INTRODUCTION

Morphine is known to possess a strong inhibitory action on the nociceptive responses in the human and experimental animals (Yaksh & Rudy, 1977; Le Bars et al, 1979; Einspahr & Piercey, 1980; Knox & Dickenson, 1987). However, excitatory responses to opioids were observed in various types of neurons, including dorsal horn neuron (Willcockson et al, 1986; Knox & Dickenson, 1987), Renshaw cell (Davies & Dray, 1976), vestibular (Lin & Carpenter, 1994), cortical (Satoh et al, 1974), hippocampal (Zieglgänsberger et al, 1979) and locus coeruleus neurons (Rasmussen & Jacobs, 1985). The mechanism by which opioids induce an excitation is unclear.

Excitatory action of opioids has been verified in several different ways. Opioid substances increase spontaneous activities (Satoh et al, 1974; Lin & Carpenter, 1994) and neuronal responses to electrical (Knox & Dickenson, 1987) and chemical stimulations (Davies & Dray, 1976). Shen & Crain (1990) also reported that opioids at nanomolar concentrations prolonged the action potential duration (APD) of mouse sensory ganglion neurons, while shortening APD at micormolar concentrations. This morphine-induced APD prolongation was blocked by cholera toxin-A (Shen & Crain, 1990) and cAMP-dependent protein kinase inhibitor (Chen et al, 1988), but was further prolonged by forskolin (Crain et al, 1988; Shen & Crain, 1989). K^+-channel blockers reduce the incidence of APD prolongation (Crain et al, 1988; Shen & Crain, 1989), suggesting that APD prolongation is mediated through a decrease in K^+-conductance. In *in vitro* studies using neuroblastoma cell lines, opioid agonists increased intracellular calcium concentration ($[Ca^{2+}]_i$) which resulted from an increased Ca^{2+} influx through voltage-gated Ca^{2+} channels and/or increased Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)-sensitive calcium stores (Jin et al, 1992). The increased Ca^{2+} influx and IP_3 formation were reduced by the removal of extracellular Ca^{2+}, Ni^{2+}, nifedipine, verapamil and pertussis toxin, suggesting that both extracellular Ca^{2+} and Ca^{2+} released from IP_3-sensitive stores play an important role in the increase in $[Ca^{2+}]_i$. However, in undifferentiated NG 108-15 cells, a δ-opioid agonist dose-dependently increased $[Ca^{2+}]_i$ only through the mechanism of IP_3 formation which was blocked by thapsigargin and pertussis toxin, but not changed by the removal of extracellular Ca^{2+} and nifedipine (Jin et al, 1994; Smart & Lambert, 1996).

In the present *in vivo* study, we investigated the role of N-methyl-D-aspartate (NMDA) receptors, and intra- and extracellular Ca^{2+} in the excitatory action of morphine on the responses of dorsal horn cells to NMDA and graded electrical stimulation of afferent nerves.

METHODS

Animal preparation

Sprague-Dawley male rats (300~450 g) were ane-

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ABBREVIATIONS: cAMP, adenosine cyclic monophosphate; AP, 2-amino-5-phosphonovaleric acid; APD, action potential duration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DRG, dorsal root ganglion; ECF, extracellular fluid; GABA, gamma aminobutyric acid; IP3, inositol 1,4,5-trisphosphate; L3, 3rd lumbar vertebra; MOR, morphine; Nal, naloxone; NMDA, N-methyl-D-aspartate; T13, 13th thoracic vertebra; VER, verapamil; WDR, wide dynamic range.

sthetized with urethane (1.2 g/kg, i.p.) and pentobarbital sodium was intravenously infused if needed. Their external jugular veins were cannulated with polyethylene tube (PE-60) through which pancuronium bromide (0.3 mg/kg/hr) was continuously infused to paralyze the musculature. Tracheotomy was performed and the rats were artificially ventilated by a small animal ventilator (Model 683, Harvard Apparatus, USA), and end-tidal CO_2 level was maintained between 3.5 and 4.5% by adjusting respiratory rate and volume (End-tidal CO_2 analyzar, Capstar-100, IITC Inc.). Rectal temperature was maintained near 37°C with an electrical heating blanket (Harvard Apparatus, USA). Laminectomies were carried out to expose lumbar enlargement of spinal cord between T13 and L3. The common peroneal and tibial nerves were dissected from the surrounding tissues at popliteal fossa. A liquid paraffin pool was made around the exposed spinal cord and peripheral nerves to prevent drying. After finishing all these surgical procedures, animals were placed in a stereotaxic apparatus.

Identification of dorsal horn neuron

A seven-barrel microelectrode was used for iontophoretic application of drugs and recording single unit activity in the spinal cord. Single extracellular activity of dorsal horn cells activated by electrical stimulation of afferent nerves was monitored through the central barrel which contained a low impedance carbon filament. Once single unit activity of a dorsal horn neuron was recorded, the type of dorsal horn neuron was classified according to the response pattern to mechanical stimuli applied to the receptive field. Wide dynamic range (WDR) cells which responded to both innocuous and noxious mechanical stimuli and low threshold cells which responded only to innocuous stimuli were used in this experiment. All evoked activities were amplified (WPI, DAM80) and fed into a window discriminator (Frederic Haer & Co.) whose outputs were used for compilation of poststimulus time histogram.

Iontophoretic application of drugs

One outer barrel of the 7-barreled microelectrodes was filled with 0.15 M NaCl and used for current balancing which makes the net current of all channels zero. The other outer barrels were used for iontophoretic application of drugs. Retaining current sufficient to prevent drug leakage (3~10 nA) was used between all drug applications. The following drugs were iontophoretically applied near the recorded dorsal horn cells (Neurophore BH-2 System Corp, USA): N-methyl-D-aspartate (NMDA, 0.05 M), kainate (0.02 M), morphine sulfate (0.05 M), naloxone hydrochloride (0.05 M), Mg^{2+} (0.2 M), 2-amino-5-phosphonovaleric acid (AP, 0.05 M), verapamil HCl (0.03 M), Mn^{2+} (0.2 M), and TMB-8 hydrochloride (0.05 M). All solutions were at pH 7.5~8.0. All drugs were purchased from Sigma Chemical Co. (USA). In each figure, the number next to or below the name of each drug iontophoretically applied shows the amount of ejection current (nA). The horizontal bars above each figures indicate duration of iontophoretic applications and bin-width was 1,000 msec.

Effects of drugs on the morphine-induced excitatory action

After recording the control responses of WDR cells to iontophoretically applied NMDA and kainate, and to graded electrical stimulation of afferent nerves, we investigated WDR cell responses to morphine. In this experiment, WDR cells which did not show excitatory responses to morphine were excluded. NMDA and kainate were periodically ejected for 5 sec every 15 or 20 sec. The A- or C-fibers were differentially activated by a single pulse (0.1 msec) or train of three pulses (0.5 msec, 50 Hz) every 20 seconds, respectively. The intensity of stimuli was adjusted to activate only A-fibers (10 T) or all A- and C-fibers (200~300 T). T is the threshold for activation of Aβ fibers. The number of action potentials induced by an electrical stimulation of A- or C-fibers was differentially sampled according to the differences in conduction velocity of A- and C-fibers (Chung et al, 1984). Once the responses of WDR cells to NMDA, kainate or graded electrical stimulation of afferent nerves were increased by morphine, the effects of different drugs on the morphine-induced increases in WDR cell responses were investigated. The responses induced either by NMDA and C-fiber stimulation are presented, because there was no difference in the effects of drugs on morphine-induced excitations of WDR cell responses to NMDA or to C-fiber stimulation.

Statistical analysis

Because the size of evoked responses varied from one unit to another, data are expressed as percentage of discharges in the control state before iontophoretic application of drugs. The data are expressed as mean±SE. P values less than 0.05 were considered significant. When the experiment was completed, rats were euthanized by an overdose of pentobarbital sodium.

RESULTS

Morphine induces the excitation of WDR cell responses to excitatory amino acids and C-fiber stimulation

The responses of 232 WDR cells and 18 low threshold cells were extracellulary recorded in 53 experiments. Recordings were made at a depth between 500 μm and 1,000 μm below the dorsal surface of lumbar enlargement of rat spinal cord. Many cells were tested for more than one drug. In 137 of 232 WDR cells tested, morphine augmented WDR cell responses to iontophoretically applied NMDA (Fig. 1A) and kainate (Fig. 1B) and C-fiber stimulation (Fig. 1C). The excitatory effect induced by morphine was variable, and the maximum excitatory effect reached a few hundred percent of the control in a considerable number of WDR cells. Both types of WDR cells with or without C-fiber input were excited by iontophoretic application of morphine, but no significant relationship was found between the excitatory action of morphine and C-fiber input of WDR cells. Because there were no differences in the characteristics of morphine-induced excitatory action on the WDR cell responses to NMDA (N=50), kainate (N=24), and C-fiber stimulation (N=87), the responses either to NMDA or to C-fiber stimulation were presented in each figure.

On the other hand, iontophoretic application of morphine did not have any excitatory effects on the responses of low threshold cells to electrical stimulation (Fig. 1D).

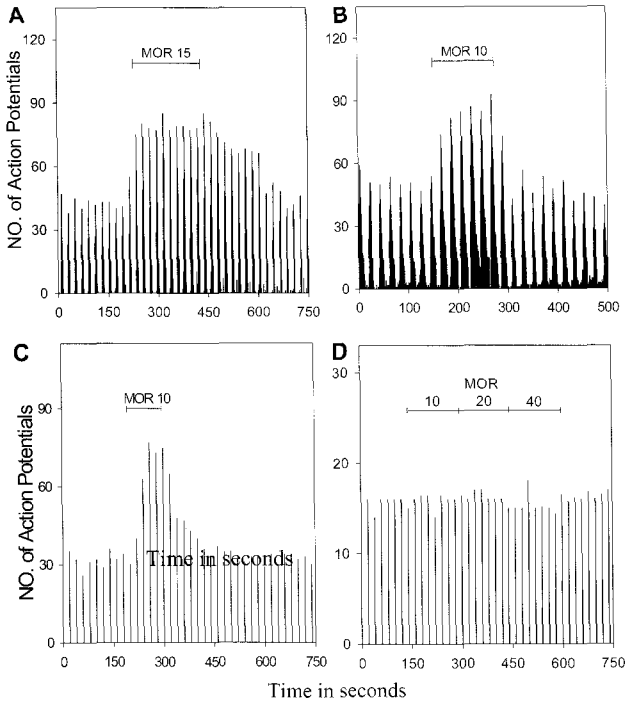

Fig. 1. Excitatory action of morphine on the responses of a rat dorsal horn cell. NO. of action potential is action potentials induced by iontophoretic application of NMDA and kainate, and electrical stimulation of C- and A-fibers. The number next to or below the name of each chemical shows the amount of ejection current (nA). The horizonal bars above each figure indicate the duration of iontophoretic application and the bin-width was 1,000 msec. The responses of a WDR cell to iontophoretically applied NMDA (A), kainate (B) or C-fiber stimulation (C), but not the responses of a low threshold cell to electrical stimulation of afferent nerve (D), were increased by the iontophoretic application of morphine (MOR).

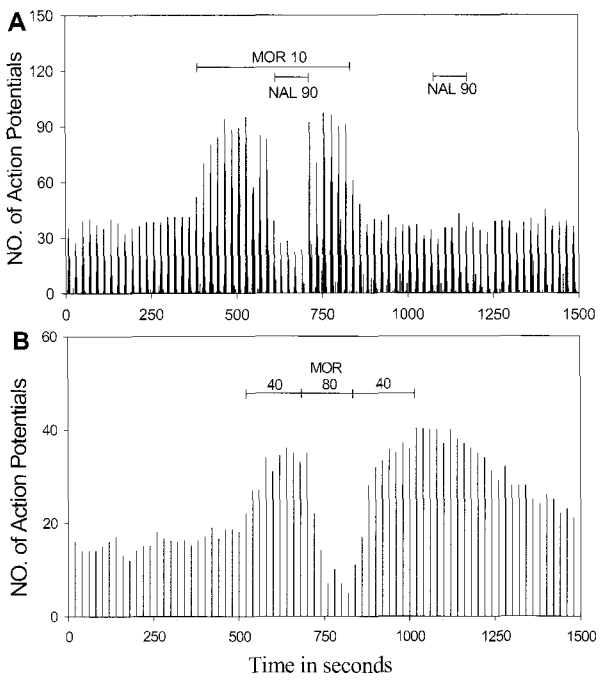

Fig. 2. Excitatory action of morphine is mediated through opioid receptors. Iontophoretic application of naloxone (NAL) antagonized the excitatory action of morphine on the responses of a WDR cell to NMDA (A). At a low ejection current, morphine induced an excitatory action on the C-fiber responses while morphine produced an inhibitory action when ejected with high current (B).

Activation of opioid receptor is necessary for the excitatory action of morphine

Excitatory actions of morphine were antagonized by naloxone (N=12, 90~120 nA), suggesting that excitatory action was mediated through activation of opioid receptors (Fig. 2A). In general, greater excitatory action of morphine was produced in the WDR cell responses to NMDA than to C-fiber stimulation (Fig. 2A, 2B). Maximum excitatory actions of morphine on the WDR cell responses to NMDA and C-fiber stimulation were induced when morphine was ejected with approximately 5~10 nA and 20~30 nA, respectively. Even at an ejection current of 5nA (N=18), WDR cell responses to NMDA were enhanced to 201.5±10.5% of the control by iontophoretic application of morphine while at an ejection current of 20 nA, the C-fiber responses were augmented to 182.8±11.4% of the control (N=18). In general, morphine-induced excitatory action at the lower range of an ejection current, less than about 60 nA, was current-dependent and converted to inhibitory action at an ejection current, which was higher than approximately 40 nA (Fig. 2B, N=12). However, the ejection currents that could induce maximal excitatory and inhibitory actions were different from one cell to another.

Implication of NMDA receptors in the morphine-induced excitation

Fig. 3 shows that the activation of NMDA receptors plays an important role in the mediation of morphine-induced excitatory action. After the iontophoretic application of Mg^{2+} (N=9, 90 nA) and 2-amino-5-phosphonovaleric acid (N=8, 10 nA), the morphine-induced excitatory effect on the WDR cell responses to NMDA (176.3±6.9% of the control, 15 nA) was strongly antagonized to 31.2±8.2% and 28.8±4.9% of the control. WDR cell responses to C-fiber stimulation were enhanced by morphine (N=23), and excitatory effect of morphine on the C-fiber responses (177.4±7.7% of the control, 15 nA) was also suppressed to 62.5±11.4% and 105.9±3.8% of the control by Mg^{2+} (90 nA) and 2-amino-5-phosphonovaleric acid (120 nA), respectively. Mg^{2+} and 2-amino-5-phosphonovaleric acid had greater inhibitory action on the NMDA response than on the response to C-fiber stimulation.

Calcium channel blockers antagonize the excitatory action of morphine

Calcium ions are also implicated in the excitatory action of morphine on the WDR cell responses to NMDA and C-fiber stimulation (Fig. 4). Calcium channel blockers, Mn^{2+} and verapamil, inhibited the excitatory effect of morphine on the WDR cell responses. The C-fiber responses augmented by morphine (186.0±9.0% of the control, 20 nA) were suppressed to 55.4±9.8% and 54.1±6.5% of the

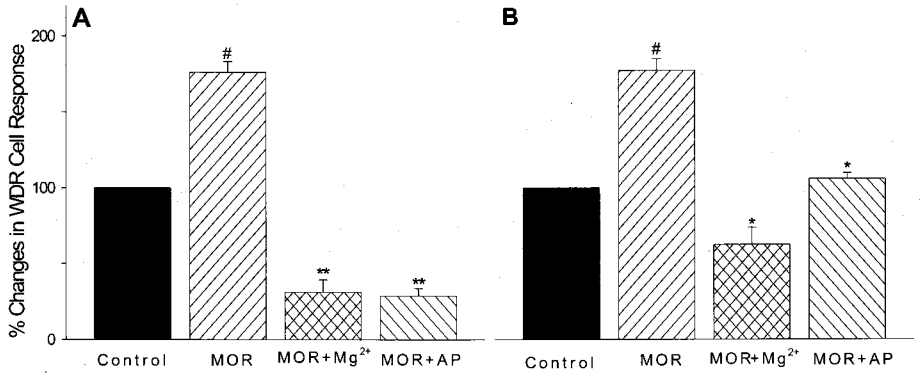

Fig. 3. Morphine (MOR)-induced excitation of the WDR cell response to NMDA (A) and C-fiber stimulation (B) was suppressed by NMDA receptor antagonists, Mg^{2+} and 2-amino-phosphono-valeric acid (AP). Data are presented as a percentage of the control response and mean±S.E. Data were analysed using ANOVA followed by the Newman-Keuls test. #indicates $P<0.005$, compared with the control and *$P<0.05$, ** $P<0.01$, comoared with MOR group.

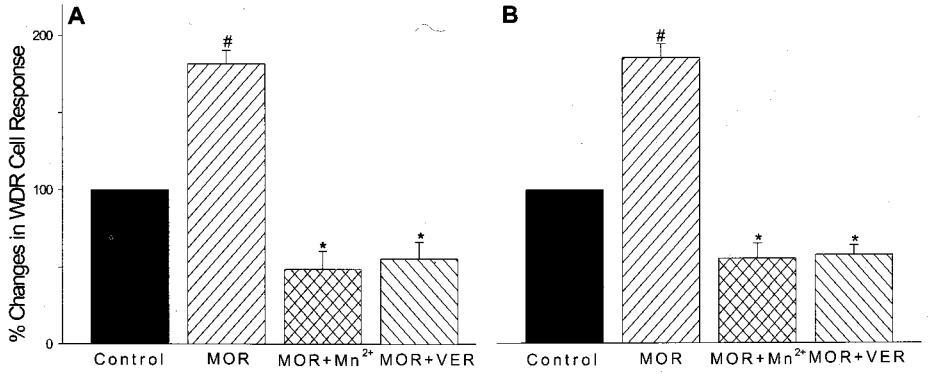

Fig. 4. Effects of calcium channel blockers on morphine (MOR)-induced excitatory action on WDR cell responses. Iontophoretic application of Mn^{2+} and verapamil (VER) antagonized excitatory effects of morphine on the responses of WDR cells to NMDA (A) and C-fiber stimulation (B). Data are presented as a percentage of the control response and mean±S.E. Data were analyzed using ANOVA followed by the Newman-Keuls test. #indicates $P<0.005$, compared with the control and *$P<0.05$, compared with MOR group.

control by Mn^{2+} (N=10, 60 nA) and verapamil (N=12, 30 nA), respectively. Ca^{2+} channel antagonists also suppressed morphine-induced excitatory effect on the NMDA responses to a similar extent to the excitatory effect on C-fiber responses. The inhibitory actions of Mn^{2+} was short-lasting and verapamil had a little longer inhibitory action than Mn^{2+}.

Effects of TMB-8 hydrochloride on the C-fiber responses and the excitatory action of morphine

Intracellular Ca^{2+} antagonist, TMB-8 hydrochloride, current-dependently inhibited WDR cell responses to C-fiber stimulation and its inhibitory action was very strong. At an ejection current of 60 nA, TMB-8 hydrochloride suppressed C-fiber responses to 33.7±5.7% of the control (Fig. 5A, N=12) and also reduced the excitatory action of morphine on the C-fiber responses of WDR cells. The augmented C-fiber responses (185.3±15.3% of the control at 20 nA) were suppressed to 63.4±7.8% of the control after iontophoretic application of TMB-8 hydrochloride with an ejection current of 60 nA (Fig. 5B, N=9).

DISCUSSION

Predominant effects of opioids have been known to be inhibitory on the neuronal firing rate (Yaksh & Rudy, 1977; Le Bars et al, 1979; Einspahr & Piercey, 1980; Knox & Dickenson, 1987) and transmitter release (Yaksh et al, 1980; Lembeck & Donnerer, 1985). These inhibitory effects have been reported to be mediated by an increase in K^{+} conductance (Williams et al, 1982; North et al, 1987) and a decrease in Ca^{2+} conductance (Werz & Macdonald, 1983; Attali et al, 1989), resulting in the hyperpolarization of nerve cells and in shortening of the Ca^{2+} action potentials. However, there is growing evidence that opioids produce an excitatory action in a various types of neuronal cells. These opioid-induced excitatory actions include the increase in neuronal activities (Satoh et al, 1974; Davies & Dray, 1976; Willcockson et al, 1986) and transmitter release (Suarez-Roca & Maixner, 1993; Neal et al, 1994), para-

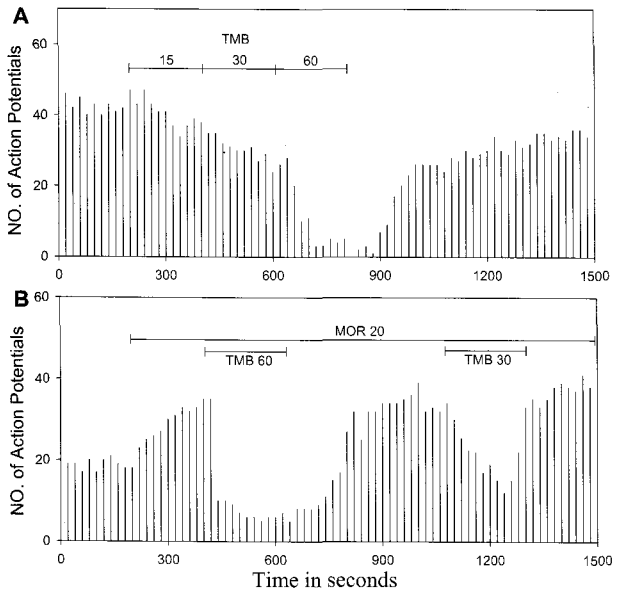

Fig. 5. Effect of an intracellular Ca^{2+} antagonist on the excitatory action of morphine. TMB-8 hydrochloride (TMB) by itself inhibited C-fiber responses (A) and also strongly suppressed the morphine (MOR)-induced excitatory action on the C-fiber responses of WDR cell (B).

doxical hyperalgesia (Kayser et al, 1987), and prolongation of action potential duration (APD) of dorsal root ganglion (DRG) neurons in culture (Shen & Crain, 1989, 1990). The mechanism by which opioids induce excitatory actions is still a matter of debate.

To the best of our knowledge, the findings of this study are the first *in vivo* electrophysiological evidence demonstrating that NMDA receptor and Ca^{2+} channel are implicated in the excitatory action of morphine on dorsal horn cell responses to NMDA and C-fiber inputs. Morphine-induced excitatory effect was naloxone-reversible, suggesting that the excitation was mediated through the activation of opioid receptors (Shen & Crain, 1989; Jin et al, 1994). There are a few proposed mechanisms by which opiates induce an excitatory action. Zieglgänsberger et al (1979) suggested the disinhibition mechanism on the basis of experimental findings that iontophoretic applications of opioid peptides excite hippocampal pyramidal neurons which is antagonized by γ-aminobutyric acid (GABA) antagonist, bicuculline. This indicates that opiates induce excitatory action indirectly by the inhibition of neighboring inhibitory interneurons.

However, Lin & Carpenter (1994) reported that [D-Ala^2] leucine enkephalin and morphine induce the increase in spontaneous activities of rat medial vestibular neurons. This enhanced spontaneous firing is sustained even after GABA receptors are blocked by bicuculline and synaptic transmission is blocked by low Ca^{2+} and high Mg^{2+}. These results indicate that the excitation is not due to disinhibition, but is mediated through direct postsynaptic action. The direct excitatory effect has also been reported to be produced in DRG neurons which are devoid of synaptic inputs (Crain et al, 1988; Shen & Crain, 1990).

Many non-nociceptive dorsal horn neurons were reported to be excited by iontophoretic application of morphine whereas nociceptive neurons were inhibited (Belcher & Ryall, 1978). This is in sharp contrast with our experimental finding that low threshold cells which responded only to innocuous stimuli did not show any excitatory responses to morphine. This difference may be due to the presence or absence of μ-opioid receptors in WDR and low threshold cells, respectively.

In the present study, morphine showed dual modulatory action on the responses of rat dorsal horn neurons. Although the ejection currents at which the excitatory action of morphine was reversed to the inhibitory were different from one neuron to another, morphine evoked an excitatory action at low ejection currents, but an inhibitory action on the dorsal horn cell responses at high ejection currents. This dual modulatory effect was also reported in DRG neurons in culture (Shen & Crain, 1989, 1990), NG 108-15 cells (Jin et al, 1992) and dorsal horn neurons (Knox & Dickenson, 1987). Nanomolar concentration of μ-, δ-, and κ-opioid agonists increased the Ca^{2+} influx through voltage-gated Ca^{2+} channels in NG 108-15 cells and prolonged APD of DRG neurons, while Ca^{2+} influx was decreased and APD was shortened at micromolar concentrations of opioids (Shen & Crain, 1990; Jin et al, 1992). Shen & Crain (1992) proposed that the dual modulation by a low or a high concentration of opioid agonists is mediated through excitatory or inhibitory opioid receptor subtypes which are activated dose-dependently.

The excitatory effect of morphine appeared to be mediated by the increased ionic influx through NMDA receptors and voltage-gated Ca^{2+} channels. The selective NMDA receptor antagonist, 2-amino-5-phosphonovaleric acid and Mg^{2+} strongly suppressed excitatory effect on the responses of dorsal horn neurons to NMDA and electrical stimulation of C-fibers. NMDA receptor is known to be permeable to cations, especially Ca^{2+} when it is activated. Morphine-induced excitation was also antagonized by Ca^{2+} channel blockers, Mn^{2+} and verapamil, and the intracellular Ca^{2+} antagonist, TMB-8 hydrochloride, indicating that the extracellular and intracellular Ca^{2+} played an important role in the excitatory action of morphine. An increased Ca^{2+} influx has been known to induce a series of intracellular events which lead to the activation of protein kinase C and phospholipase A_2, and liberation of arachidonic acid (Coderre, 1992). Consistent with these experimental findings are the observations that δ-opioid agonists increased the formation of inositol 1,4,5-trisphosphate, intracellular Ca^{2+} concentration, and the release of Ca^{2+} from intracellular Ca^{2+} stores in neuroblastoma cells (Jin et al, 1992, 1994; Smart & Lambert, 1996). From the experimental findings that APD prolongation by nanomolar concentrations of opioids was blocked by cholera toxin A and protein kinase A inhibitor, and that forskolin further prolonged APD, Shen & Crain (1989, 1990) and Chen et al (1988) suggested that opioid-induced excitation appeared to be mediated by the changes in adenylate cyclase/cAMP-dependent ionic conductance: a decrease in K^+ conductance (Williams et al, 1982; North et al, 1987) and an increase in Ca^{2+} conductance (Werz & Macdonald, 1983; Attali et al, 1989). The morphine-induced excitation of WDR cell responses was antagonized by H7, a nonselective protein kinase inhibitor and further increased by phorbol 12,13-dibutyrate (unpublished data). A study to determine which kind of protein kinase is implicated in morphine-induced excitation of rat dorsal horn cells is now in progress in our laboratory.

In morphine tolerant DRG neurons in culture, the inci-

dence of morphine-induced excitation was greater than that in the naive neurons, and the threshold concentration for induction of excitation was decreased (Crain et al, 1988; Shen & Crain, 1992). These results suggest that opioid-induced excitatory action has something to do with the development of tolerance to opioid inhibitory effect. Evidence in support of this proposal is that ultra-low dose of naloxone selectively blocks excitatory receptors, which increases analgesic effect of opioid and reduces the development of tolerance (Taiwo et al, 1989). This proposal is further supported by results that in 129/SvEv mice, which are devoid of excitatory opioid receptors, low dose of opioids induces strong analgesic effect and less tolerance is developed even after chronic administration of morphine (Crain & Shen, 2000, 2001). However, chronic treatment of neuroblastoma cell with opioids enhanced the efficacy of excitatory opioid receptor function, which increase the development of tolerance (Wu et al, 1995).

These results demonstrate that morphine induces naloxone-reversible excitation of WDR cell responses to NMDA and electrical stimulation of C-fibers which was mediated through the activation of NMDA receptor and voltage-dependent Ca^{2+} channel, and Ca^{2+} appeared to play an important role in the morphine-induced excitation.

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