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Antagonists of NMDA Receptor, Calcium Channel and Protein Kinase C Potentiate Inhibitory Action of Morphine on Responses of Rat Dorsal Horn Neuron

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The present study was designed to examine whether the co-application of morphine with Ca^{2+} channel antagonist (Mn^{2+}, verapamil), N-methyl-D-aspartate (NMDA) receptor antagonist (2-amino-5-phosphonopentanoic acid[AP_5], Mg^{2+}) or protein kinase C inhibitor (H-7) causes the potentiation of morphine-induced antinociceptive action by using an in vivo electrophysiological technique. A single iontophoretic application of morphine or an antagonist alone induced weak inhibition of wide dynamic range (WDR) cell responses to iontophoretically applied NMDA and C-fiber stimulation. Although there was a little difference in the potentiating effects, the antinociceptive action of morphine was potentiated when morphine was iontophoretically applied together with Mn^{2+}, verapamil, AP_5, Mg^{2+} or H-7. However, the potentiating action between morphine and each antagonist was not apparent, when the antinociceptive action evoked by morphine or the antagonist alone was too strong. These results suggest that the potentiating effect can be caused by the interaction between morphine and each antagonist in the spinal dorsal horn.

Key Words: Dorsal horn neuron responses, Morphine-induced inhibition, Calcium channel antagonist, NMDA receptor antagonist, Protein kinase C inhibitor

INTRODUCTION

Morphine and related opioid substances are probably the most frequently used analgesics. Opioid substances increase the conductance of K^+ channels which result in the hyperpolarization of neuronal membrane (Williams et al, 1982; Cherubini & North, 1985). The amplitude and duration of Ca^{2+} current are dose-dependently reduced by opiates (Guerrero-Munoz et al, 1979; Werz & Macdonald, 1983; Attali et al, 1989). These opiate-induced increase in K^+ conductance and decrease in Ca^{2+} current reduce neurotransmitter release from presynaptic nerve terminal and inhibit a cascade of pain reactions in the dorsal horn neurons.

However, the repeated administration of opiates causes the development of tolerance characterized by their reduced ability to induce antinociceptive action (Mao et al, 1994). In neuropathic and diabetic pain, antinociceptive action of opioid substances has also been reported to be reduced as in tolerant animals (Mao et al, 1995a; Ossipov et al, 1995; Ohsawa & Kamei, 1997). Both the animals with hyperalgesia and morphine tolerance have common features such as increases in intracellular calcium concentration (Guerrero-Munoz et al, 1979), N-methyl-D-aspartate (NMDA) current (Mao et al, 1992; Neugebauer et al, 1994), NO formation (Kolesnikov et al, 1993; Thomas et al, 1996), c-fos-like immunoreactivity (Rohde et al, 1997), activities of protein kinase C(PKC) (Mao et al, 1995c; 1995d) and cAMP-dependent kinase (Nestler & Tallman, 1988) in the dorsal horn neurons. Mao et al (1995b) suggested that common intracellular mechanisms are involved in morphine tolerance and hyperalgesia such as neuropathic pain. This enhanced activity of PKC further increases neurotransmitter release, NMDA current (Chen & Mae Huang, 1992) and Ca^{2+} current (Reeve et al, 1995), whereas the efficacy of opioid substances is reduced (Mao et al, 1995d). In many behavioral studies, it was reported that coadministration of opiates with an antagonist of excitatory amino acid (EAA), calcium channel blockers, or PKC inhibitors reduced the development of tolerance (Mao et al, 1994; Mao et al, 1995d; Ohsawa & Kamei, 1997; Michaluk et al, 1998) and also increased the antinociceptive potency of opiates (Del Pozo et al, 1987; Mao et al, 1995c; Mao et al, 1995d) in the rat model of acute, neurogenic and diabetic pain. In the present study, we investigated whether an iontophoretic application of antagonist of EAA, calcium channels and PKC potentiates the antinociceptive action of morphine on the responses of rat dorsal horn neurons to NMDA and C-fiber stimulation.

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ABBREVIATIONS: WDR, wide dynamic range; NMDA, N-methyl-D-aspartate; AP_5, 2-amino-5-phosphonopentanoic acid; PKC, protein kinase C; EAA, excitatory amino acid; NO, nitric oxide.

METHODS

Sprague-Dawley male rats (300~450 gm) were anesthetized with urethane (1.2 gm/kg, i.p) and pentobarbital sodium was infused if needed. A tracheotomy was performed and the rats were artificially ventilated by a small animal ventilator (Model 683, Harvard Apparatus, U.S.A). The end-tidal CO_2 level (3.5~4.5%) and rectal temperature (37°C) were maintained within the physiological range. After exposing a lumbar enlargement between T13 and L3, and common peroneal and tibial nerves at the popliteal fossa, the rats were placed in a stereotaxic apparatus. Liquid paraffin pools were made around the exposed tissues to prevent drying. Extracellular activities of wide dynamic range (WDR) cells evoked by electrical stimulation of the afferent nerves were recorded through the central barrel of a seven-barrel microelectrode which contained a low impedance carbon filament. All evoked activities were amplified (WPI, DAM80, U.S.A) and fed into a window discriminator (Frederic Haer & Co, U.S.A) whose outputs were used for compilation of the post-stimulus time histogram.

One outer barrel of the 7-barrel microelectrode was filled with 0.15 M NaCl and used for current balancing which made the net current of all channels zero. The other outer barrels were used for iontophoretic application of NMDA (0.05M), morphine sulfate (0.05 M, pH=5.0), Mn^{2+}(0.2 M), Mg^{2+} (0.2 M), verapamil HCl (0.03 M, pH=5.0), 2-amino-5-phosphonopentanoic acid (AP_5, 0.05 M) and H-7 dihydrochloride (0.01 M) (Neurophore BH-2 system, Medical System Corp, U.S.A) All solutions were at pH 7.5~8.0 except the verapamil and morphine. Retaining current sufficient to prevent drug leakage (3~10 nA) was used between applications of each drug. While continuously recording the control responses of the WDR cells to iontophoretically

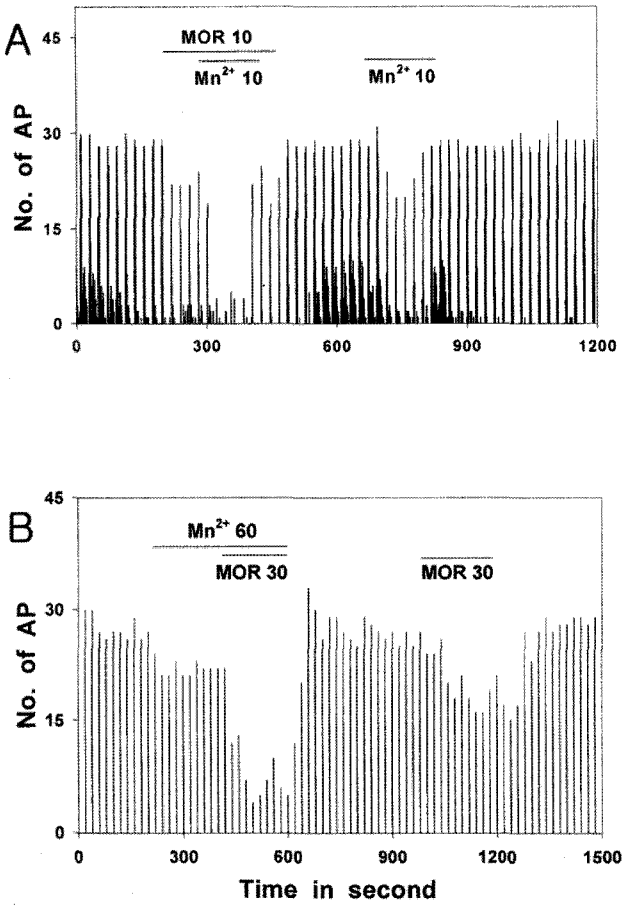

Fig. 1. Changes in the inhibitory action of morphine on responses of WDR cells to iontophoretically applied NMDA (A) and C-fiber stimulation (B) after a single or combined application of morhine with Mn^{2+}. Mophine (MOR) or Mn^{2+} alone had weak inhibitory action on the responses to NMDA (A) and C-fiber stimulation (B). However, the combined application of morhine with Mn^{2+} *producced stronger inhibitory actions on NMDA and C-fiber* responses. In each figure, the number next to or below the name of each drug shows the amount of ejection current (nA). The horizontal bars above each figure indicate the duration of iontophoretic applications, and the binwidth was 1000 msec. AP is the number of action potentials induced by iontophoretically applied NMDA or C-fiber stimulation.

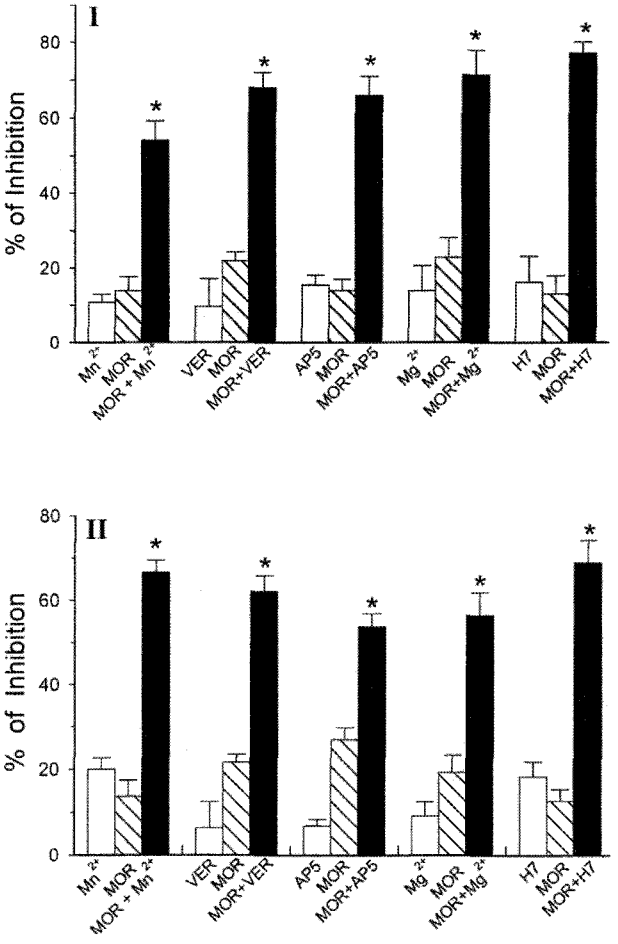

Fig. 2. *Comparision of the changes in the morphine-induced* inhibition of WDR cell responses to NMDA (I) and electrical stimulation of C-fibers (II) after single or combined application of morphine (MOR) with Mn^{2+}, verapamil (VER), 2-amino-5-phosphonopentanoic acid (AP5), magnesium (Mg^{2+}) and H-7 dihydrochloridc (H-7). *significant differences from the single application of each substance ($P<0.01$).

Table 1. Coapplication of morphine (MOR) with Mn^{2+}, verapamil (VER), 2-amino-5-phosphonopentanoic acid (AP5), Mg^{2+} and H-7 dihydrochloride (H7) enhances the analgesic activity of morphine on the responses of dorsal horn neurons to iontophoretically applied N-methyl-D-aspartate (NMDA) and C-fiber stimluation. Each value represents % inhibition induced by drugs

Drug / Response	Mn^{2+}	MOR	MOR+ Mn^{2+}	VER	MOR	MOR +VER	AP5	MOR	MOR +AP5	Mg^{2+}	MOR	MOR+ Mg^{2+}	H7	MOR	MOR +H7
NMDA Resp.	10.9 ±2.1	14.0 ±3.7	54.2 ±5.1* N=9	9.6 ±7.5	21.9 ±2.3	68.0 ±4.0* N=11	15.5 ±2.7	14.0 ±3.1	65.9 ±5.1* N=9	14.0 ±6.6	22.9 ±5.2	68.8 ±6.6* N=10	16.3 ±6.9	13.1 ±4.8	77.1 ±3.0* N=9
C-fiber Resp.	20.1 ±2.6	13.7 ±3.8	66.7 ±2.8* N=10	6.4 ±6.1	21.7 ±1.8	62.2 ±3.6* N=8	6.9 ±1.6	27.0 ±2.8	54.1 ±3.1* N=7	9.3 ±3.3	19.4 ±3.9	56.5 ±5.4* N=8	18.4 ±3.4	12.7 ±2.6	68.9 ±5.3* N=8

*; $p<0.01$, significant differences from the single application of each substance. N is number of dorsal horn neurones tested.

applied NMDA and graded electrical stimulation of the afferent nerves, the effects of the various antagonists on the inhibitory action of morphine on the WDR cell responses were investigated. NMDA was periodically ejected for 5 sec every 20 sec, and C-fibers were activated by train stimuli of three pulses (0.5 msec, 50 Hz) every 20 seconds. The intensity of the electrical stimuli was about 100~200 times the threshold for activation of Aβ fibers. The number of action potentials induced by the C-fiber stimulation was differentially sampled according to the differences in the conduction velocity of A- and C-fibers (Chung et al, 1984). All data are expressed as percentage changes in the control state. The data are expressed as mean±SE and analyzed using ANOVA followed by the Newman-Keuls test. P values less than 0.05 were considered significant. When the experiments were completed, the rats were euthanized by an overdose of pentobarbital sodium.

RESULTS

The responses of 55 WDR cells were extracellulary recorded at a depth between 500μm and 1,000μm below the surface of the lumbar enlargement of the spinal cord in 35 experiments. Fig. 1 shows the potentiating effects of a calcium channel antagonist (Mn^{2+}) on the inhibitory action of morphine on the WDR cell responses to NMDA (Fig. 1A) and C-fiber stimulation (Fig. 1B). A single iontophoretic application of Mn^{2+} inhibited the WDR cell responses to NMDA and C-fiber stimulation by 10.7±3.1% (n=9) and 20.1±2.6% (n=10), respectively, and morphine by itself also suppressed the responses of the WDR cells to NMDA (13.9±3.7%) and C-fiber stimulation (13.7± 3.8%). The responses to NMDA (Fig. 1A) and C-fiber stimulation (Fig. 1B) were strongly inhibited by 54.1±5.1% and 66.7±4.7%, respectively, when morphine was co-applicated with Mn^{2+}. In Fig. 2 and Table 1, the results obtained from the experiments on the action of morphine potentiated by the coapplication of verapamil, AP_5, Mn^{2+} or H-7 are summerized. Although there were a few differences in the potentiating effects between the experimental groups, the antinociceptive action of morphine on the WDR cell responses to NMDA and C-fiber stimulation was strongly potentiated when morphine was iontophoretically applied together with Mn^{2+}, verapamil, AP_5, Mg^{2+} or H-7. However, the potentiating effect of each antagonist on morphine-induced antinociceptive action was not apparent, when the inhibitory action evoked by the morphine alone or each antagonist was excessively strong. Therefore, we adjusted the extent of inhibition by increasing or decreasing the ejection current which could produce potentiating action. The amount of ejection current for each drug which produced potentiation was very variable, as seen in Fig. 1. In general, appreciable potentiating effect between morphine and each antagonist could not be observed, when the inhibition caused by each drug was more than approximately 30% of the control responses.

DISCUSSION

The results obtained from the present *in vivo* electrophysiological experiment provide further evidence that antagonists of NMDA receptor, Ca^{2+} channel and PKC potentiate antinociceptive effect of morphine on the responses of the dorsal horn neuron to NMDA and C-fiber stimulation, in good agreement with the results of behavioral studies in which the NMDA receptor antagonists, Ca^{2+} channel antagonists and PKC inhibitors enhance the reduced efficacy of opiates in tolerant rats and in the rat model of acute, neurogenic and diabetic pain (Del Pozo et al, 1987; Mao et al, 1994; Mao et al, 1995a; Ohsawa & Kamei, 1997; Michaluk et al, 1998). However, Diaz et al. (1998) reported that Ca^{2+} channel antagonists did not potentiate antinociceptive effect on C-fiber responses of the rat dorsal horn neuron. They concluded that there was no synergistic effects between Ca^{2+} channel antagonists and morphine at the spinal level. The difference between their results and those of the present study seems to be that Diaz et al (1998) did not adjust the extent of inhibition which could produce synergistic action between morphine and the Ca^{2+} channel antagonist, because they directly applied fixed concentration of drugs onto the spinal cord. As described in the results, the extent of inhibition induced by each drug appears to be critical for the production of the potentiating effect. When the morphine- and each antagonist - induced inhibitions were too strong or weak, the potentiating effects were not observed.

In the dorsal horn neurons, activation of NMDA receptor and/or voltage-sensitive Ca^{2+}channel by excitatory amino acids and other neurotransmitters such as substance P and calcitonin gene-related peptide increase intracellular calcium concentration (Mayer et al, 1987; Womack et al, 1988), and the increased [Ca^{2+}] promotes activation of protein

kinase C, formation of eicosanoids and NO formation. All these serial reactions aggravate the development of hyperalgesia. Animals with tolerance-associated hyperalgesia show the same features as those caused by neurogenic and diabetic pain (Mao et al, 1995a; Ohsawa & Kamei, 1997). On the basis of these experimental findings, Mao et al. (1995b) suggested that morphine tolerance and hyperalgesia were caused by common intracellular mechanisms and there was an inverse relationship between the effectiveness of morphine antinociception and hyperalgesia. If this is the case, a suppression of intracellular cascades associated with the production of hyperalgesia such as NMDA receptor activation, a resultant increase in intracellular Ca^{2+} concentration and PKC activity could reduce the severity of pain and then potentiate the antinociceptive action of morphine. On the other hand, the potency of morphine antinociception were reduced in the hyperalgesic state such as neurogenic (Mao et al, 1995a), diabetic pain (Ohsawa & Kamei, 1997), and toleranе-associated hyperalgesia (Mao et al, 1994). The common feature of these hyperalgesic state is the great increase in the intracellular calcium concentration. In the behavioral studies, intrathecal administration of MK-801, verapamil, calphostin C and GM1 ganglioside is reported to increase the antinociceptive action of morphine and to attenuate hyperalgesia (Del Pozo et al, 1987; Mao et al, 1994; Mao et al, 1995c; Ohsawa & Kamei, 1997; Michaluk et al, 1998). On the other hand, the development of hyperalgesia is enhanced, and antinociceptive action of opiates is reduced by the increase in PKC activity in the spinal cord in response to noxious inputs and the development of tolerance (Mao et al, 1995c; Mao et al, 1995d). Taken together, these experimental data indicate that intrathecal administration of the antagonists of NMDA receptors, Ca^{2+} channels and PKC can reduce pain sensitivity, reduce the development of morphine tolerance and increase the antinociceptive potency of morphine.

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