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The role of nitric oxide in the development of morphine tolerance in rat hippocampal slices

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Abstract

In the present study, we investigated the effects of a nitric oxide (NO) precursor, L-arginine, on the effect of different drugs, [*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide hydrochloride] (U-50,488, a κ-opioid receptor agonist); dPTyr(Me)AVP (a vasopressin receptor antagonist); dizocilpine (MK-801, a *N*-methyl-D-aspartate (NMDA) receptor antagonist), to block the development of morphine tolerance or NO release in Sprague–Dawley rat hippocampal slices (450 μm). Slices were continuously superfused with artificial cerebrospinal fluid (ACSF) or drugs at 1 ml/min. Nichrome wire electrodes were placed in the Schaffer-collateral pathway and used to deliver biphasic 0.2-ms pulses of 5–30 V (0.033 Hz). A glass microelectrode was placed in the CA1 area to record population spikes. The amount of NO released in the superfusate was measured as nitrite formation. When the slices were superfused with 10 μM morphine, the amplitude of population spikes increased 200%–300% in 30–40 min. However, this effect of morphine decreased, i.e., tolerance developed, after continuous superfusion of morphine for 2–6 h. On the other hand, the nitrite level was increased about 250% of the control level through 6 h of morphine superfusion. Co-superfusion of L-arginine with morphine could further increase the nitrite level and also facilitate the development of morphine tolerance. On the other hand, 3-Br-7-nitroindazole (a neuronal NO synthase inhibitor) decreased the nitrite level significantly and blocked the development of morphine tolerance. When either U-50,488 (200 nM) or dPTyr(Me)AVP (500 pM) or MK-801 (500 pM) was co-superfused with morphine (10 μM), the development of morphine tolerance was blocked significantly and the nitrite level decreased to 100%–150% of the control level. L-arginine (500 nM) significantly reversed the effect of these drugs to block the development of morphine tolerance or to decrease the nitrite level through 6 h of superfusion. These data suggest that NO may play a key role in the development of morphine tolerance. Drugs which suppress the synthesis or release of NO would be expected to block the development of morphine tolerance.

Keywords: Morphine; Tolerance; U-50,488; MK-801; *N*-methyl-D-aspartate (NMDA); Vasopressin; 3-Br-7-Nitroindazole; Nitric oxide (NO)

1. Introduction

Morphine is an effective analgesic for the clinical treatment of severe pain. However, its effectiveness decreases with chronic use, i.e., tolerance develops over time. Recently, some drugs have been reported to block the development of morphine tolerance, such as κ-opioid receptor agonists, dynorphin A-(1–13) (Tulunay et al., 1981; Khazan et al., 1983; Green and Lee, 1988; Takemori et al., 1992) or U-50,488, [*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide hydrochloride] (Yamamoto et al., 1988; Takahashi et al., 1991; Tao et al., 1994); [Arg^8]vasopressin (vasopressin) antiserum (Xu et al., 1992) or vasopressin receptor antagonist (Tao et al., 1997); competitive or noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonists (Marek et al., 1991; Trujillo and Akil, 1991; Tiseo and Inturrisi, 1993), and nitric oxide (NO) synthase inhibitors (Kolesnikov et al., 1992; Majeed et al., 1994). These reports suggest that the development of morphine tolerance may involve suppression of the κ-opioid receptor, activation of the vasopressin receptor, activation of the NMDA receptor and synthesis of NO.

The aim of this project was to investigate whether NO is a common factor affected by drugs which can block the development of morphine tolerance. We have chosen to use rat hippocampal slices as our experimental model, because it not only is a good in vitro model for electro-

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physiological measurement but also contains μ and κ-opioid receptors (Mansour et al., 1987), vasopressin receptors (Phillips et al., 1988) and NMDA receptors (Greenamyre et al., 1985). Thus we could study the effects of U-50,488 (κ-opioid receptor agonist), dPTyr(Me)AVP (vasopressin receptor antagonist) and MK-801 (dizocilpine, NMDA receptor antagonist) on the development of morphine (μ-opioid receptor agonist) tolerance and NO release in this model.

2. Materials and methods

2.1. Preparation of hippocampal slices (Dunwiddie and Lynch, 1978; Dunwiddie et al., 1987)

Male Sprague–Dawley rats (200–300 g) were decapitated. The brain was rapidly removed and placed in ice-cold ACSF (NaCl, 124 mM; KCl, 3.3 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 2.4 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25.7 mM; D-glucose, 10 mM) which was pregassed with 95% O_2 and 5% CO_2. The hippocampus was dissected free of the surrounding tissue and transverse slices (450 μm) were cut on a Sorval tissue chopper. Then the slices were placed immediately on nylon nets in a recording chamber containing oxygenated ACSF maintained at 33.5 ± 1°C. Before being used for electrophysiological recording, the slices were held at the liquid medium-gas interface in this chamber for at least 60 min. During the recording, the slices were continuously superfused with ACSF at the rate of 1 ml/mn.

2.2. Electrophysiology

Schaffer and commissural fibres in the stratum radiatum near the border of CA1–CA2 were stimulated (8–30 V, 0.2-ms duration, 0.033 Hz). Extracellular population spikes (field potentials) in response to the stimulation were recorded from the CA1 cell layer with glass microelectrodes filled with 3 M NaCl. The amplitude of the population spike was taken as the average of the differences between the spike peak negativity and the preceding and following positivities (Fig. 1). The stimulation voltage was adjusted to elicit a baseline population spike between 0.7 and 1.8 mV. A period of at least 30 min of stable baseline response was allowed in each experiment before drug application.

2.3. Determination of nitrite formation

The formation of NO was determined by measuring nitrite, a stable oxidation product of NO. One ml perfusate was collected before and after drug administration for 30, 60, 120, 240 and 360 min. Nitrite levels of the perfusate were measured with Griess reagent, and then assessed in a spectrophotometer at 540 nm (Ignarro et al., 1987).

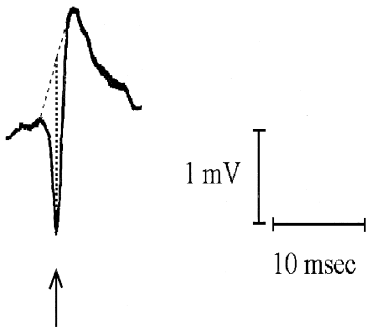

Fig. 1. Recording of population spike. The amplitude of the negative-going population spike (arrow) was determined as the length of the vertical dashed line illustrated.

2.4. Statistical analysis

Each group had at least six animals. The results are expressed as the means ± S.E.M. Student's *t*-test was used to analyze the statistical significance of differences between two groups. Analysis of variance was used to assess the statistical significance by repeated measures of the overall data. And differences between individual mean values in different groups were analyzed using the Duncan multirange test. The difference was considered to be significant at $P < 0.05$.

2.5. Materials

U-50,488 was a gift from Dr. Chen-Yu Cheng who prepared it according to the literature (Szmuszkovicz, 1979; Szmuszkovicz and Von Voigtlander, 1982) and was isolated as the hydrochloride salt, mp 221.5–222.5°C, 99.5% by high performance liquid chromatography (RP-select B; $H_2O/CH_3OH = 1{:}4$ buffered at pH 7.52; 254 nm). Morphine hydrochloride was purchased from the Narcotics Bureau of the National Health Administration, Taipei, Taiwan, ROC. MK801 was purchased from Research Biochemicals International (RBI; MA, USA). All other chemicals used were reagent grade and supplied by Sigma (St. Louis, MO, USA).

3. Results

3.1. Effect of L-arginine or NO synthase inhibitor on the development of morphine tolerance or the release of NO in hippocampal slices

The population spikes were recorded from stratum pyramidal of the hippocampal CA1 region as shown in Fig. 1. The drugs (except morphine) used in this study (such as L-arginine, 3-Br-7-nitroindazole, U-50,488, MK-801, dPTyr(Me)AVP) had no effect on population spikes by themselves or in combination with L-arginine. Representative

data are shown in Fig. 2. However, of the drugs, only L-arginine alone could increase the release of NO (Fig. 3). When either 3-Br-7-nitroindazole, U-50,488, MK-801 or dPTyr(Me)AVP was co-superfused with L-arginine for 30 min, the nitrite level did not increase (Fig. 3). Superfusion of morphine increased the amplitude of population spikes. The maximum effect was obtained by superfusion with 10 μM of morphine for 30–60 min and the amplitude of population spikes usually rose to 200%–300% of the control value. When the slices were continuously superfused with 10 μM morphine for 6 h, the amplitude of population spikes gradually declined, which meant this effect had become tolerant to 10 μM morphine (Fig. 4A). When 500 nM L-arginine (a NO precursor) was co-superfused with morphine, the development of morphine tolerance was facilitated. However, no morphine tolerance developed when 1 nM 3-Br-7-nitroindazole (a neuronal NO synthase inhibitor) was co-superfused with morphine (Fig. 4A). The amount of NO released was measured as nitrite formation and is shown in Fig. 4B. It was found that morphine could increase nitrite to about 250% of the control level through 6 h superfusion of morphine. Co-superfusion of L-arginine with morphine could further increase the nitrite level but co-superfusion of 3-Br-7-nitroindazole decreased the nitrite level significantly.

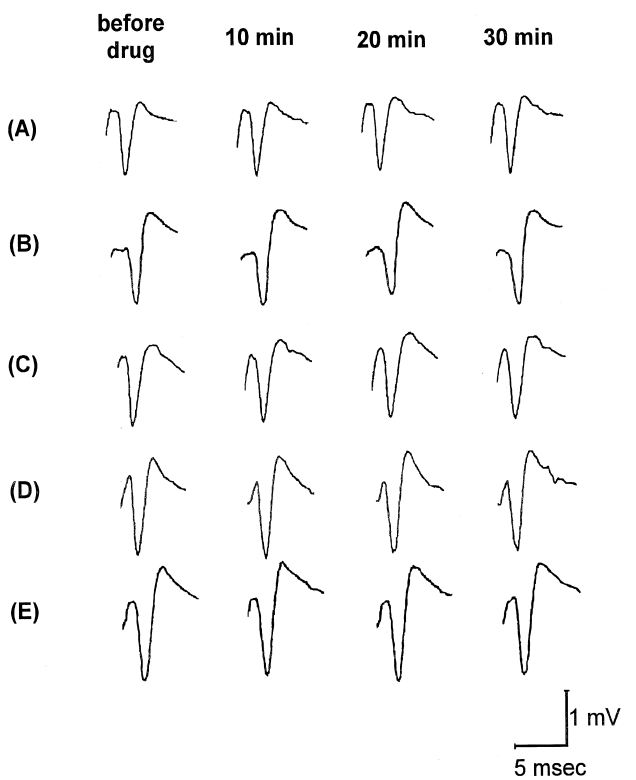

Fig. 2. Representative recordings of (A) L-arginine (500 nM), (B) 3-Br-7-nitroindazole (1 nM), (C) L-arginine + U-50,488 (200 nM), (D) L-arginine + MK-801 (500 pM), (E) L-arginine + dPTyr(Me)AVP (500 pM) on population spikes 10, 20 or 30 min after superfusion of each drugs.

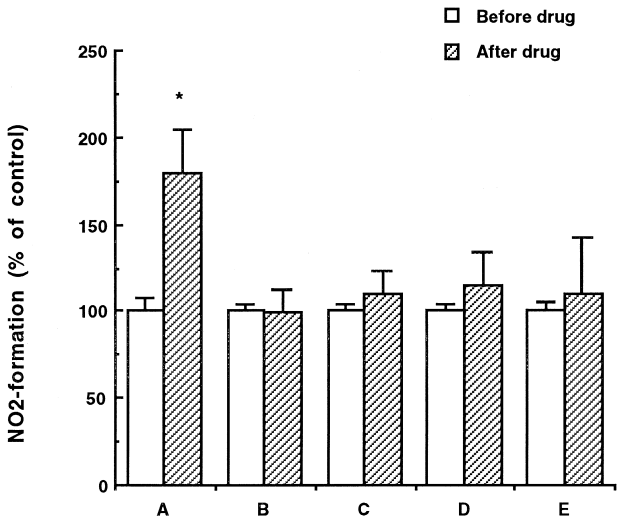

Fig. 3. Effects of (A) L-arginine (500 nM), (B) 3-Br-7-nitroindazole (1 nM), (C) L-arginine + U-50,488 (200 nM), (D) L-arginine + MK-801 (500 pM), (E) L-arginine + dPTyr(Me)AVP (500 pM) on the release of NO in hippocampal slices after 30 min of drug superfusion. The basal nitrite concentration was about 30–100 nM. Significant difference after drug compared to before drug ($^{*}P < 0.05$) by Student's *t*-test.

3.2. Effect of L-arginine on the effect of U-50,488 to block the development of morphine tolerance and the release of NO in hippocampal slices

When 200 nM U-50,488 (a κ-opioid receptor agonist which had no effect at this concentration by itself and did not affect the acute response to 10 μM morphine) was co-superfused with 10 μM morphine, the effect of morphine was maintained for at least 6 h (Fig. 5A) and the nitrite level was decreased to 120%–150% of the control (Fig. 5B). When 500 nM L-arginine was co-superfused with morphine and U-50,488 for 6 h, the effect of morphine still decreased gradually (Fig. 5A) and the nitrite level was increased to about 190%–230% of the control level through 6 h (Fig. 5B).

These experiments indicated that co-superfusion of U-50,488 (200 nM) with morphine could block the development of morphine tolerance and NO release, and that these effects of U-50,488 could be reversed by a NO precursor, L-arginine.

3.3. Effect of L-arginine on the effect of dPTyr(Me)AVP to block the development of morphine tolerance and the release of NO in hippocampal slices

When 500 pM of dPTyr(Me)AVP (a vasopressin receptor antagonist which had no effect at this concentration by itself and did not affect the acute response to 10 μM morphine) was co-superfused with 10 μM morphine, it significantly blocked the development of morphine tolerance (Fig. 6A) and the nitrite level was decreased to

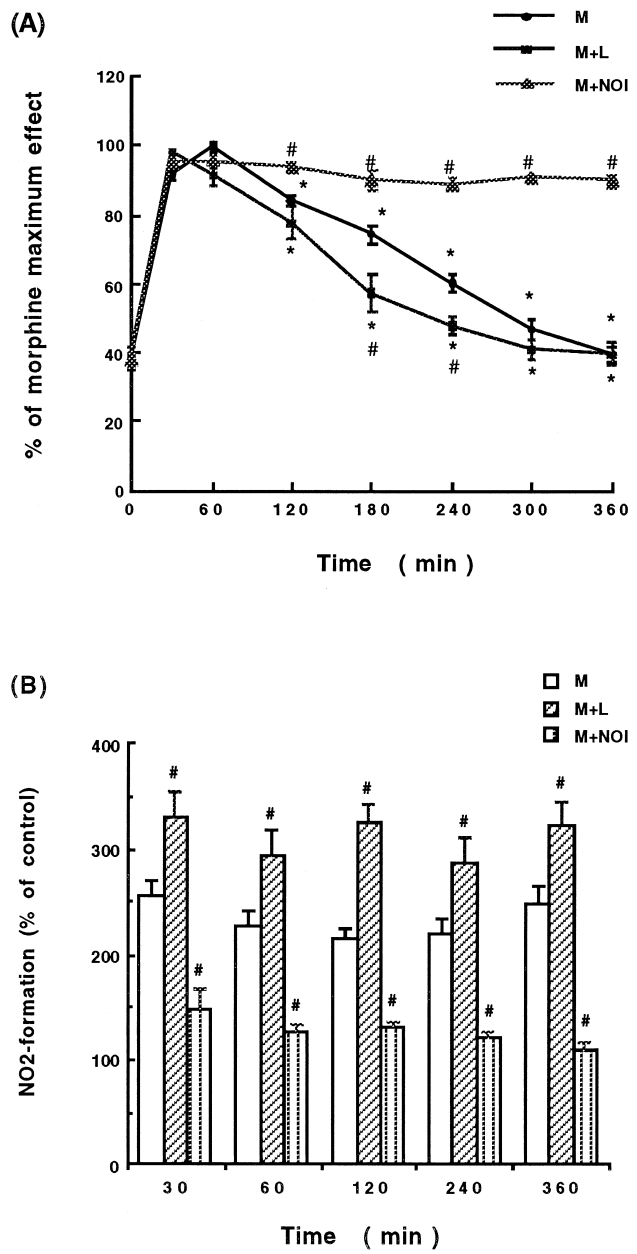

Fig. 4. The effect of L-arginine or NO synthase inhibitor on (A) the development of morphine tolerance, (B) the release of NO in hippocampal slices. **M**: superfused with 10 μM morphine; **M + L**: superfused with 10 μM morphine and 500 nM L-arginine; **M + NOI**: superfused with 10 μM morphine and 1 nM 3-Br-7-nitroindazole. Significant differences from maximum effect ($^{*}P < 0.05$); significant differences from morphine group ($^{\#}P < 0.05$) were determined with two-way ANOVA and Duncan multiple range test.

120%–140% of the control (Fig. 6B). When 500 nM L-arginine was co-superfused with morphine and dPTyr(Me)AVP for 6 h, the effect of morphine still decreased gradually (Fig. 6A) and the nitrite level was increased to about 190%–220% of the control level through 6 h (Fig. 6B).

These experiments indicated that co-superfusion of dPTyr(Me)AVP (500 pM) with morphine could block the development of morphine tolerance and NO release, and that these effects of dPTyr(Me)AVP could also be reversed by L-arginine.

3.4. Effect of L-arginine on the effect of MK-801 to block the development of morphine tolerance and the release of NO in hippocampal slices

When 500 pM MK-801 (a NMDA receptor antagonist which had no effect by itself at this concentration and did

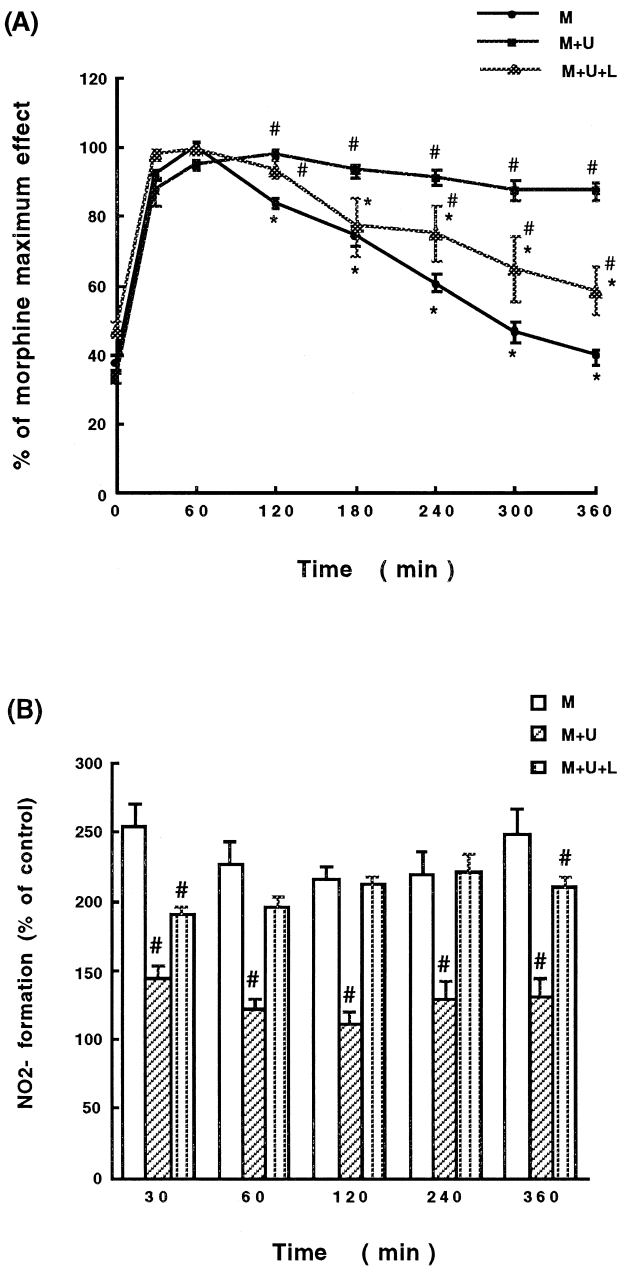

Fig. 5. The effect of L-arginine on the effect of U-50,488 to (A) block the development of morphine tolerance, (B) decrease the release of NO in hippocampal slices. **M**: superfused with 10 μM morphine; **M + U**: superfused with 10 μM morphine and 200 nM U-50,488; **M + U + L**: superfused with 10 μM morphine and 200 nM U-50,488 and 500 nM L-arginine. $^{*}P < 0.05$ represents significant differences from maximum effect; $^{\#}P < 0.05$ represents significant differences from morphine group.

not affect the acute response to 10 μM morphine) was co-superfused with 10 μM morphine, it also blocked the development of morphine tolerance as shown in Fig. 7A and the nitrite level was decreased to about 125%–165% of the control (Fig. 7B). When 500 nM L-arginine was co-superfused with morphine and MK-801 for 6 h, the effect of morphine still decreased gradually (Fig. 7A) and the nitrite level was increased to about 200%–220% of the control level through 6 h (Fig. 7B).

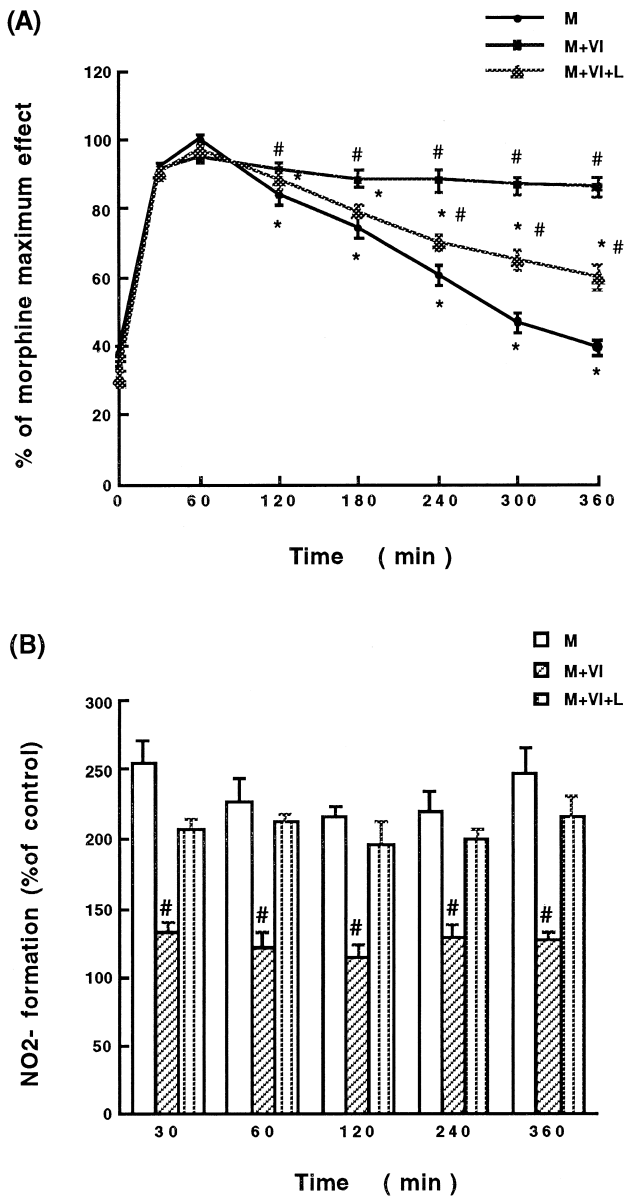

Fig. 6. The effect of L-arginine on the effect of dPTyr(Me)AVP to (A) block the development of morphine tolerance, (B) decrease the release of NO in hippocampal slices. **M**: superfused with 10 μM morphine; **M+VI**: superfused with 10 μM morphine and 500 pM dPTyr(Me)AVP; **M+VI+L**: superfused with 10 μM morphine and 500 pM dPTyr(Me)AVP and 500 nM L-arginine. $^{*}P < 0.05$ represents significant differences from maximum effect; $^{\#}P < 0.05$ represents significant differences from morphine group.

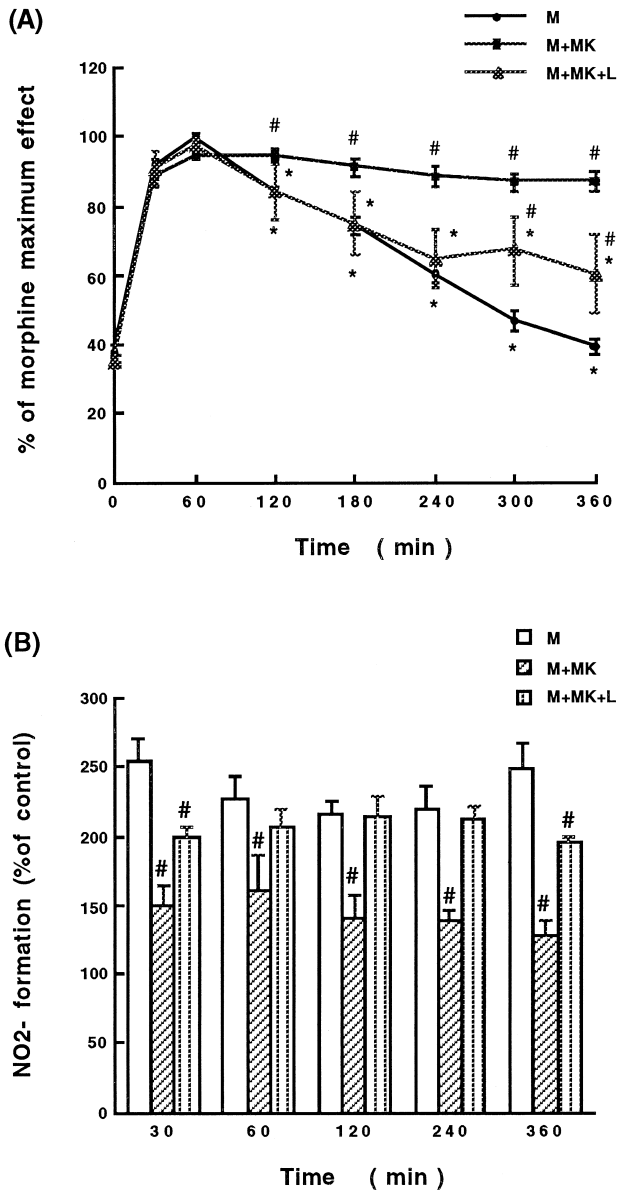

Fig. 7. The effect of L-arginine on the effect of MK-801 to (A) block the development of morphine tolerance, (B) decrease the release of NO in hippocampal slices. **M**: superfused with 10 μM morphine; **M+MK**: superfused with 10 μM morphine and 500 pM MK-801; **M+MK+L**: superfused with 10 μM morphine and 500 pM MK-801 and 500 nM L-arginine. $^{*}P < 0.05$ represents significant differences from maximum effect; $^{\#}P < 0.05$ represents significant differences from morphine group.

These experiments indicated that co-superfusion of 500 pM MK-801 with morphine also could block the development of morphine tolerance and NO release, and that these effects could also be reversed by L-arginine.

4. Discussion

U-50,488, a selective κ-opioid receptor agonist, has been reported to suppress the development of antinocicep-

tive tolerance to morphine in rats (Yamamoto et al., 1988), mice (Takahashi et al., 1991) and guinea pigs (Tao et al., 1994). In a rat hippocampal slice model, we also found that U-50,488 could effectively block the development of morphine tolerance and that this effect could be antagonized by a κ-opioid receptor antagonist, nor-BNI, indicating that this is an opioid effect (Su et al., 1998). Xu et al. (1992) reported that concomitant intracerebroventricular injection of AVP antiserum dose dependently suppressed the development of analgesic tolerance to daily morphine (10 mg/kg, s.c.) in mice. Recent studies in our laboratory have also shown that chronic co-administration of a vasopressin receptor antagonist (dPTyr(Me)AVP) (i.p. or i.c.v.) with morphine (i.p.) blocked the development of morphine tolerance in rats (Tao et al., 1997). Both competitive and noncompetitive NMDA receptor antagonists (Marek et al., 1991; Trujillo and Akil, 1991; Tiseo and Inturrisi, 1993) have recently been reported to prevent the development of morphine tolerance, suggesting that the development of morphine tolerance may also involve activation of the NMDA receptor. The activation of NMDA receptors can induce the synthesis of NO through the activation of NO synthase (Garthwaite et al., 1989; Bredt and Snyder, 1992). It has also been reported that co-administration of NO synthase inhibitors, such as N^G-nitro-arginine, along with morphine prevents morphine tolerance (Kolesnikov et al., 1992; Majeed et al., 1994).

In the present studies, rat hippocampal slices were used. In this brain region, the enhancing effect of morphine on population spikes has been reported to be a disinhibition mechanism, involving inhibition of the firing of inhibitory interneurones, i.e., blockade of the γ-amino butyric acid (GABA)-mediated inhibitory postsynaptic potential (IPSP) (Zieglgänsberger et al., 1979; Dunwiddie et al., 1980; Lee et al., 1980). The tolerance observed in our model occurs within hours of continuous superfusion of a high concentration of morphine. Tolerance could be due to a decline in the glutamate-mediated response or restoration of the GABA-mediated response. The nitrite level we measured is mostly produced by nonelectrostimulated hippocampal slices in the recording chamber. We found that L-arginine (500 nM) by itself could increase the nitrite level (Fig. 3) but had no effect on population spikes after superfusion of L-arginine for 30 min (Fig. 2). On the other hand, the nitrite level was increased to about 250% of the control level through 6 h of morphine superfusion. However it was noted that morphine-stimulated nitrite levels are maximal at a time when there is no electrophysiological tolerance (30–60 min) (Fig. 4). This apparent discrepancy might be explained by the fact that NO mediates some cellular changes to cause the development of morphine tolerance but does not itself have an effect on population spikes. As long as the NO level is maintained, the processes that mediate morphine tolerance continue on and cause further development of morphine tolerance. Co-superfusion of L-arginine (a NO precursor) with morphine could further increase the nitrite level and also facilitate the development of morphine tolerance. On the other hand, when 3-Br-7-nitroindazole (a neuronal NO synthase inhibitor) was co-superfused with morphine, it decreased the nitrite level significantly and blocked the development of morphine tolerance (Fig. 4). When either U-50,488 (200 nM) or dPTyr(Me)AVP (500 pM) or MK-801 (500 pM) was co-superfused with morphine (10 μM), the development of morphine tolerance was significantly blocked and the nitrite level decreased to 100%–150% of the control level, as shown in Figs. 5–7. L-arginine (500 nM) significantly reversed the effect of these drugs to block the development of morphine tolerance or decrease the nitrite level through 6 h of superfusion. We also found that when either 3-Br-7-nitroindazole, U-50,488, MK-801 or dPTyr(Me)AVP was co-superfused with L-arginine for 30 min, the nitrite level did not increase significantly as it did on superfusion of L-arginine alone (Fig. 3). This finding further supports the possibility that all the drugs we used in the present study (3-Br-7-nitroindazole, U-50,488, MK-801 and dPTyr(Me)AVP) may inhibit the NO synthesis stimulated by morphine through certain mechanisms that are not yet known, suggesting that NO may play a key role in the development of morphine tolerance. The NO level enhanced by chronic morphine may increase the levels of cGMP (Bredt and Snyder, 1989; Garthwaite et al., 1989) that causes phosphorylation of some key proteins and alters the physiological responses, finally inducing morphine tolerance. Drugs which suppress the synthesis or release of NO would be expected to block the development of morphine tolerance.

Acknowledgements

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