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Possible involvement of the locus coeruleus in inhibition by prostanoid EP_3 receptor-selective agonists of morphine withdrawal syndrome in rats

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Abstract

We examined the mechanism of the inhibitory effect of prostanoid EP_3 receptor agonists on naloxone-precipitated withdrawal syndrome in morphine-dependent rats. Rats were rendered morphine dependent by subcutaneous (s.c.) implantation of two pellets containing 75 mg morphine for 5 days. Morphine withdrawal syndrome was precipitated by i.p. injection of naloxone (3 mg/kg). Intracerebroventricular (i.c.v.) administration of (±)-15α-hydroxy-9-oxo-16-phenoxy-17,18,19,20-tetranorprost-13-*trans*-enoic acid (M&B28,767: prostanoid EP_3 receptor agonist) or sulprostone (prostanoid EP_1/EP_3 receptor agonist) significantly suppressed many withdrawal signs. Northern blotting and in situ hybridization studies revealed that i.c.v. administration of M&B28,767 (1 pg/rat) attenuated the elevation of c-*fos* mRNA during naloxone-precipitated withdrawal in many brain regions, including the cerebral cortex, thalamus, hypothalamus and locus coeruleus. Double in situ hybridization analysis revealed that in the locus coeruleus most of the tyrosine hydroxylase mRNA-positive neurons expressed μ-opioid receptor mRNA and more than half of these neurons were positive for prostanoid EP_3 receptor mRNA. These results indicate that the suppression by prostanoid EP_3 receptor agonists of naloxone-precipitated morphine withdrawal syndrome can be attributed to the inhibition of neuronal activity in several brain regions, including the locus coeruleus, the largest source of central noradrenergic neurons.

Keywords: Morphine withdrawal; Prostaglandin; Prostanoid EP_3 receptor; μ-Opioid receptor; c-*fos*; Locus coeruleus

1. Introduction

Morphine is widely used in the clinical management of pain. However, in addition to its analgesic effect, morphine produces a variety of side effects, including respiratory depression, nausea, obstipation and physical and psychic dependence, which limit its usefulness. The mechanisms of the development of dependence and/or expression of opioid withdrawal syndrome have been vigorously investigated, and several neurotransmitters have been shown to be involved. The central noradrenergic system has been suggested to be one of the most important systems in opioid withdrawal. Clonidine, an α_2-adrenoceptor agonist, has been used to detoxify opioid addicts (Bhargava, 1994). A number of behavioral, biochemical and electrophysiological studies have indicated that the inhibitory effect of clonidine on morphine withdrawal signs is due to a reduction of activity of noradrenergic neurons originating in the locus coeruleus (Aghajanian, 1978; DiStefano and Brown, 1985; Taylor et al., 1988). Furthermore, recent evidence supports the involvement of other neurotransmitters or neuromodulators, including excitatory amino acids (Marek et al., 1991a,b; Fundytus and Coderre, 1994; Fundytus et al., 1997), substance P (Ueda et al., 1987; Chahl and Johnston, 1993; Kreeger and Larson, 1993; Maldonado et al., 1993), neuropeptide FF (Malin et al., 1990, 1991), cannabinoids (Vela et al., 1995) and cytokines (Dafny, 1983; Okutomi et al., 1992) in morphine withdrawal syndrome. Recently, we showed that intracisternal administration of interleukin-1β, a cytokine, attenuated naloxone-precipitated jumping behavior in morphine-dependent mice (Katsumata et al., 1995), and that the inhibitory effect was blocked by pretreatment with sodium salicylate, a cyclooxygenase inhibitor (Nakagawa et al., 1995). Prostaglandin E_2 is widely distributed in the central nervous system and has numerous and diverse biological effects on a variety of physiological and pathological activities. Prostaglandin E receptors are pharmacologically classified into four sub-

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types, EP_1, EP_2, EP_3 and EP_4, on the basis of their respective responses to various agonists and antagonists (Coleman et al., 1994; Ichikawa et al., 1996). We observed that stimulation of the prostanoid EP_3 receptor, but not EP_1, EP_2, IP or FP receptor, suppressed morphine withdrawal jumping behavior (Nakagawa et al., 1995).

Since the expression of c-*fos* mRNA is rapidly and transiently induced in response to a wide variety of stimuli, the induction of c-*fos* mRNA has been used as a marker of neuronal activation in the nervous system (Sheng and Greenberg, 1990; Morgan and Curran, 1991; Zhu and Inturrisi, 1993). Indeed, morphine withdrawal has been demonstrated to produce marked increases in the level of c-*fos* mRNA in many brain regions (Hayward et al., 1990; Stornetta et al., 1993; Beckmann et al., 1995). In order to elucidate the mechanism of the inhibitory effect of prostanoid EP_3 receptor agonists on naloxone-precipitated morphine withdrawal syndrome, in this study we examined the effects of intracerebroventricular (i.c.v.) administration of prostanoid EP_3 receptor agonists on morphine withdrawal signs and the induction of c-*fos* mRNA precipitated by naloxone in morphine-dependent rats. Furthermore, to obtain a better understanding of the inhibitory effect of prostanoid EP_3 receptor agonists in the locus coeruleus, which plays an important role in the expression of morphine withdrawal syndrome, we examined the coexistence of mRNA for the prostanoid EP_3 receptor or the μ-opioid receptor, which is known to play an important role in opioid dependence and/or withdrawal syndrome, with mRNA for tyrosine hydroxylase, a marker of catecholamine-containing neurons, by means of a double in situ hybridization technique.

2. Materials and methods

2.1. Materials

Morphine hydrochloride was purchased from Takeda Chemical Industries (Osaka, Japan). Morphine pellets each containing 75 mg of morphine base were prepared according to the method of Gibson and Tingstad (1970). (±)-15α-Hydroxy-9-oxo-16-phenoxy-17,18,19,20-tetranorprost-13-*trans*-enoic acid (M&B28,767) and sulprostone were gifts from Rhone-Poulenc Rorer (Dagenham, UK) and Schering (Berlin, FRG), respectively. M&B28,767 and sulprostone were dissolved in ethanol and stored at −20°C. For i.c.v. injection of these drugs, ethanol was removed by evaporation under nitrogen gas and the drugs were dissolved in phosphate-buffered saline (PBS). Salmon sperm DNA, polyadenylic acid, ribonuclease A and naloxone hydrochloride were purchased from Sigma (St. Louis, USA). Naloxone hydrochloride was dissolved in saline. Proteinase K was from Merck (Darmstadt, FRG). 11-Digoxigenin-uridine triphosphate and yeast tRNA were from Boehringer Mannheim (Mannheim, FRG). The cDNA for the probe of c-*fos* was purchased from Takara (Kyoto, Japan). Other chemicals were from Nacalai Tesque (Kyoto, Japan). Prostanoid EP_3 receptor cDNA was a gift from Prof. M. Negishi (Sugimoto et al., 1992).

2.2. Animals

Male Sprague–Dawley rats weighing 200–250 g were used. They were kept at a constant ambient temperature of 24 ± 1°C under a 12-h light/dark cycle with free access to food and water. After arrival, rats were individually kept in plastic cages with chips for at least 1 day until implantation of the guide cannula.

2.3. Implantation of the guide cannula

Under pentobarbitone (50 mg/kg, i.p.) anesthesia, a stainless steel guide cannula (o.d. 0.7 mm) was stereotactically (P 0.8, L 1.5, H 2.0) implanted according to the atlas of Paxinos and Watson (1986). After surgery, the animals were returned to the cages until recovery.

2.4. Development of morphine dependence

Rats were rendered morphine dependent by subcutaneous (s.c.) implantation of morphine pellets. Under light ether anesthesia, rats had a morphine or placebo pellet implanted in the back of the neck (day 1). Twenty-four hours later (day 2), the rats received a second morphine or placebo pellet. Seventy-two hours after the second pellet was implanted (day 5), each rat was placed in a Plexiglass cylinder 30 cm in diameter and 50 cm in height for 30 min to acclimatize it to the experimental environment. We checked that the above procedure produced tolerance to morphine by the paw-pressure test (data not shown).

2.5. Drug administration, precipitation of withdrawal and behavioral observations

After the 30-min habituation period, M&B28,767 (0.01–100 pg/rat), sulprostone (0.01–10 ng/rat) or vehicle (5 μl/rat) was administered via the injection cannula which reached the lateral ventricle (P 0.8, L 1.5, H 4.0) when attached to the guide cannula, and then the rats were returned to the Plexiglass cylinder. The drugs were administered i.c.v. in a volume of 5 μl at a constant rate of 10 μl/min. Thirty minutes after i.c.v. administration of the drugs, naloxone (3 mg/kg) was administered i.p. Then, the rats were immediately returned to the cylinder and behavior was observed for 1 h. The numbers of occurrences of stretching, wet-dog shake, teeth chattering, jumping, paw shake, head shake, backwards walking and ejaculation were counted, and the occurrence of diarrhea, salivation, ptosis, lacrimation and rhinorrhea was checked. Body weight was measured just before and 1 h after administration of naloxone. These experiments were performed be-

tween 1300 and 1700 h. Counts of each behavior are presented as means ± S.E.M. of total numbers during a period of 1 h, and the data were analyzed by the Mann–Whitney *U*-test. The occurrence of a behavior is presented as the number of rats showing positive signs over the total number of rats tested, and the data were compared by the Fisher's Exact Probability test. Differences with $P < 0.05$ were considered significant. The rats were killed by decapitation immediately after the behavioral observation and the brains were removed for Northern blotting analysis and in situ hybridization histochemistry.

2.6. Preparation of antisense RNA probe

c-*fos* cDNA (about 1.0 kbp) was subcloned into pBluescript II SK (−) (Stratagene, La Jolla, USA), and the plasmid linearized at *Bam*HI site was used as a template to generate an antisense RNA probe. ^{32}P-Labeled antisense RNA probe for c-*fos* was synthesized in the presence of [α-^{32}P]uridine triphosphate (15 TBq/mmol, Amersham, Buckinghamshire, UK) using T3 RNA polymerase (Promega, Madison, USA). ^{35}S-Labeled antisense RNA probe for c-*fos* was synthesized in the presence of [α-^{35}S]uridine triphosphate (30 TBq/mmol, Amersham) with the same template and transcription system as used for the synthesis of the ^{32}P-labeled probe. The RNA probe was alkaline hydrolyzed to about 250 bases.

Rat tyrosine hydroxylase cDNA was subcloned into pCR II (Invitrogen, San Diego, USA) and linearized with *Hin*dIII for use as a template for an antisense probe. Digoxigenin-labeled antisense RNA probes for tyrosine hydroxylase mRNA were synthesized in the presence of 11-digoxigenin-uridine triphosphate with a rat tyrosine hydroxylase cDNA as a template using T7 RNA polymerase. As the coding regions of μ-, δ- and κ-opioid receptor mRNAs are highly homologous with one another, the 3′-non-coding region of μ-opioid receptor cDNA, including 104 bp of the coding region, in a length of about 1200 bp was used as a template for the synthesis of an antisense RNA probe as described previously (Minami et al., 1994, 1995). The cDNAs for the μ-opioid receptor and the prostanoid EP_3 receptor were subcloned into pBluescript II SK (−), and each was linearized with *Xho*I and *Bam*HI, respectively, for use as a template for an antisense probe. ^{35}S-labeled antisense RNA probes for the μ-opioid receptor and the prostanoid EP_3 receptor were synthesized in the presence of [α-^{35}S]uridine triphosphate as described above using T3 and T7 RNA polymerase, respectively.

2.7. Northern blotting analysis for c-fos mRNA

The brains were rapidly dissected into six regions; that is, the cerebral cortex, hippocampus, striatum, thalamus, hypothalamus and pons–medulla. Then, they were frozen in liquid nitrogen and stored at −80°C until use. Total RNA was extracted from tissues of each brain region pooled from three rats using ISOGEN (Nippon gene, Tokyo, Japan). Total RNA samples (15 μg each) were fractionated by electrophoresis in 1.2% agarose gels containing 6% formaldehyde, transferred onto nylon membranes (Biodyne, Pall, Glen Cove, USA) and baked at 80°C for 2 h. The membranes were prehybridized and then hybridized to ^{32}P-labeled antisense RNA probe for c-*fos* at 68°C. The membranes were washed twice in 2× saline sodium citrate (SSC)/0.1% sodium lauryl sulfate (SDS) for 5 min at 68°C, incubated with ribonuclease A (50 μg/ml in 0.5 M NaCl/10 mM Tris/1 mM ethylendiaminetetraacetic acid, pH 8.0) for 5 min at 37°C, and then washed twice in 0.1× SSC/0.1% SDS for 30 min at 68°C. The membranes were exposed to X-ray film at −80°C with an intensifying screen.

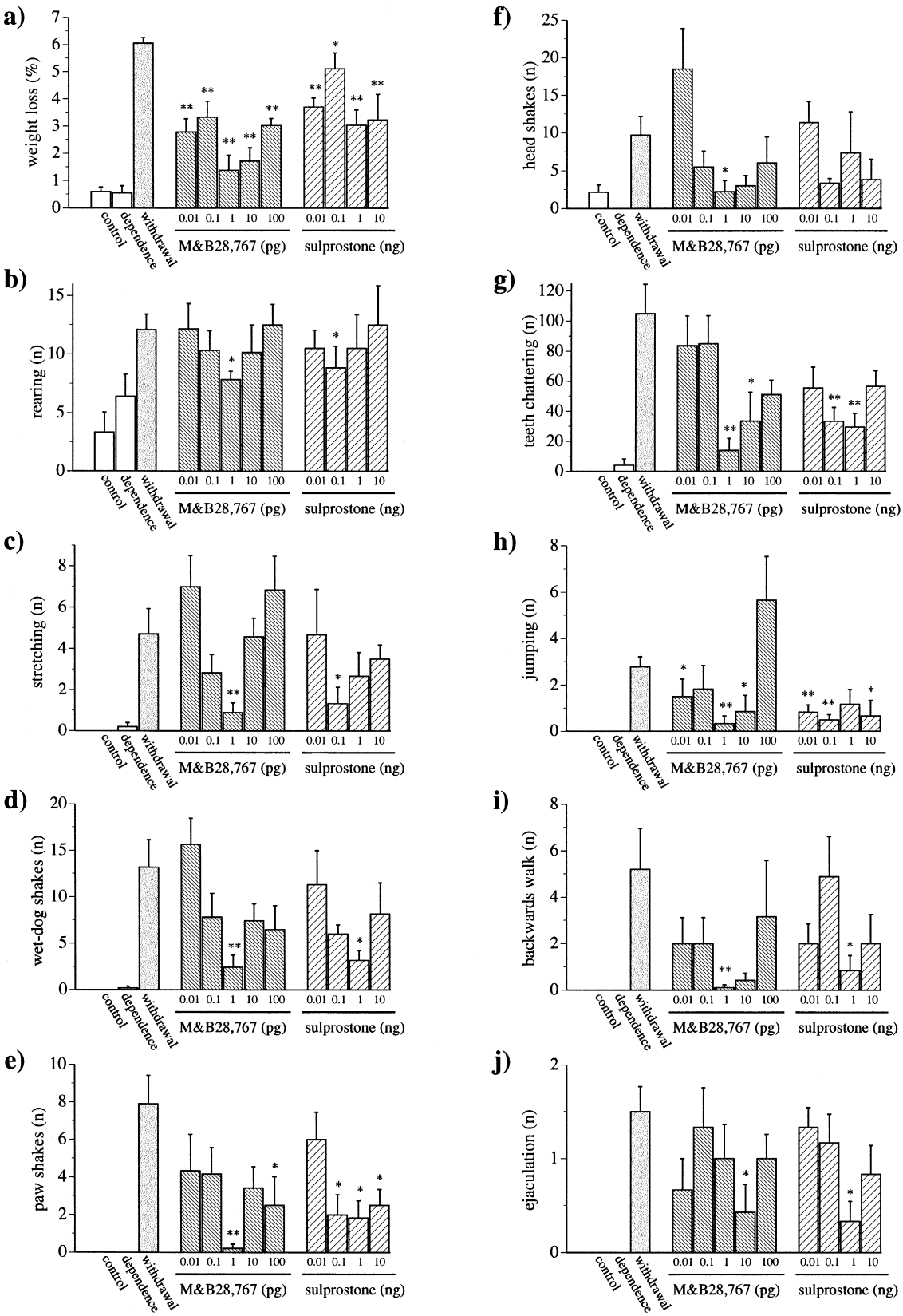
2.8. In situ hybridization for c-fos mRNA

After decapitation, the brain was rapidly removed and frozen in powdered dry ice. Coronal sections (16 μm) were prepared in a cryostat, thaw-mounted onto gelatin-coated slides and stored at −80°C until use. In situ hybridization histochemistry was conducted as previously described (Yabuuchi et al., 1993). Sections were fixed in 4% formalin/10 mM PBS (pH 7.4) for 15 min. To reduce the background signal and to increase the accessibility of probes to the target mRNA, sections were treated with 1 μg/ml proteinase K at 37°C for 5 min and then immersed in 0.25% acetic anhydride in 0.1 M triethanolamine/saline for 10 min. After dehydration in an ethanol series (70%, 85%, 95% and 100%), sections were immersed in chloroform for 3 min, returned to 100% ethanol and then dried in air. Prehybridization was carried out at 55°C for 1 h in buffer containing 50% formamide, 4× SSC, 5× Denhardt's solution, 10 mM ethylendiaminetetraacetic acid, 33 μg/ml polyadenylic acid, 250 μg/ml yeast tRNA, 20 mM dithiothreitol and 500 μg/ml heat-denatured salmon sperm DNA. For hybridization, antisense RNA probe (about 2×10^7 cpm/ml) and 10% dextran sulfate were added to the buffer. After prehybridization, sections were hybridized to RNA probe at 55°C for 18 h. They were washed four times in 2× SSC containing 10 mM dithiothreitol for 10 min at 55°C, incubated with ribonuclease A (50 μg/ml in 0.5 M NaCl, 10 mM Tris, 1 mM ethylendiaminetetraacetic acid, pH 8.0) for 30 min at 37°C and then washed twice in 50% formamide, 2× SSC, 10 mM dithiothreitol for 30 min at 55°C. They were dehydrated in an ethanol series and dried in air. The slides were exposed to Hyper film-β max (Amersham) for 7 days at room temperature, and the film was developed in D-19 (Kodak, New York, USA).

2.9. Quantitative analysis of c-fos mRNA expression in the locus coeruleus

Quantitative analysis of film autoradiograms for c-*fos* mRNA expression in the locus coeruleus was conducted

with a computer-assisted image analysis system (NEXUS, Tokyo, Japan) as previously described (Maekawa et al., 1995). For each group, the images of the sections including the locus coeruleus from five animals were input from film autoradiograms into a computer using a video camera linked to a microscope. The optical density of pixels over



the locus coeruleus was summed for each animal and the obtained total value was divided by the value of the area of locus coeruleus to yield averaged area optical density for each animal. Background area optical density, which was the average of the area optical densities in three areas surrounding the sections, was subtracted from each area optical density of the region of interest. The corrected area optical density values for five animals were averaged to provide a mean ± S.E.M. of area optical densities for each group. Statistical analysis was performed using the Mann–Whitney *U*-test.

2.10. Double in situ hybridization

Coronal sections were prepared from non-treated rats and pretreated for in situ hybridization as described above. Double in situ hybridization histochemistry was conducted as previously described (Minami et al., 1995). Hybridization was carried out with the mixture of the radiolabeled probe for the μ-opioid receptor or the prostanoid EP_3 receptor mRNA and the digoxigenin-labeled probe for tyrosine hydroxylase mRNA at 55°C for 16 h. After hybridization, sections were washed four times in 2× SSC/10 mM dithiothreitol for 10 min each time at 55°C, incubated with ribonuclease A for 30 min at 37°C, and then washed twice in 50% formamide, 2× SSC, 10 mM dithiothreitol for 30 min at 55°C. They were dehydrated in an ethanol series and dried in air. Digoxigenin-labeled RNA probe hybridized to tyrosine hydroxylase mRNA was detected as follows. Sections were rehydrated in 0.1 M Tris-buffered saline (TBS, pH 7.4), blocked with 1% normal sheep serum (Dako, USA) for 30 min and incubated with alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody (0.1% in TBS containing 1% normal sheep serum) for 2 h at room temperature. After being washed in TBS, they were immersed in coloring buffer (0.1 M Tris, 0.15 M NaCl and 50 mM $MgCl_2$, pH 9.5) twice for 5 min each time. Visualization of the antibody that bound the probe was carried out in coloring buffer containing nitroblue tetrazolium salt (337.5 μg/ml) and 5-bromo-4-chloro-3-indolylphosphate (1.75 μg/ml) for 15 h. The coloring reaction was terminated by washing in 10 mM Tris/1 mM ethylendiaminetetraacetic acid (pH 8.0) twice for 2 h. Then, the sections were dehydrated in an ethanol series and coated with 2.5% collodion. For microautoradiography, the slides were dipped in autoradiographic emulsion NTB-3 diluted 1:1 with water. After 4 weeks of exposure, they were developed in D-19, fixed, and lightly counterstained.

Table 1
Effects of i.c.v. administration of prostanoid EP_3 receptor agonists on naloxone-precipitated withdrawal syndrome in morphine-dependent rats. Numbers denote the number of rats showing positive signs relative to the total number of rats tested

	Withdrawal signs				
	Diarrhea	Salivation	Ptosis	Lacrimation	Rhinorrhea
Control	0/8	0/8	0/8	0/8	0/8
Dependence	0/5	0/5	0/5	0/5	0/5
Withdrawal	8/9	9/9	9/9	8/9	8/9
M&B28,767 (pg)					
0.01	6/6	2/6[a]	3/6[a]	4/6	4/6
0.1	6/6	3/6[a]	5/6	3/6	2/6
1	6/9	1/9[b]	3/9[b]	2/9[a]	2/9[a]
10	5/7	1/7[b]	3/7[a]	3/7	4/7
100	6/6	4/6	5/6	3/6	1/6[a]
Sulprostone (ng)					
0.01	6/6	4/6	6/6	5/6	4/6
0.1	5/6	6/6	6/6	4/6	3/6
1	6/6	3/6[a]	4/6	2/6	1/6[a]
10	4/6	5/6	5/6	2/6	2/6

[a] $P < 0.05$ compared with the morphine withdrawal group by the Fisher's exact test.
[b] $P < 0.01$ compared with the morphine withdrawal group by the Fisher's exact test.

3. Results

3.1. Effects of prostanoid EP_3 receptor agonists on naloxone-precipitated withdrawal syndrome in morphine-dependent rats

The effects of i.c.v. administration of M&B28,767 and sulprostone on naloxone-precipitated withdrawal syndrome were investigated in morphine-dependent rats. None of the rats implanted with either placebo or morphine pellets for 5 days and i.c.v. administered vehicle (PBS, 5 μl/rat) exhibited any signs characteristic of morphine withdrawal syndrome after i.p. injection of saline (control- and morphine-dependent groups, respectively). On the other hand, in the rats implanted with morphine pellets and i.c.v. administered vehicle, i.p. injection of naloxone (3 mg/kg) elicited characteristic withdrawal signs such as weight loss, rearing, stretching, wet-dog shakes, paw shakes, head

Fig. 1. Effects of i.c.v. administration of prostanoid EP_3 receptor agonists on naloxone-precipitated withdrawal signs in morphine-dependent rats: (a) weight loss; (b) rearing; (c) stretching; (d) wet-dog shakes; (e) paw shakes; (f) head shakes; (g) teeth chattering; (h) jumping; (i) backwards walking; (j) ejaculation. Animals in control and morphine-dependent groups were implanted with placebo or morphine pellets for 5 days, respectively, and i.c.v. injected with vehicle (5 μl/rat) at 30 min before the i.p. injection of saline (open bars). Morphine withdrawal group (shaded bars) or drug treatment group (hatched bars) was implanted with morphine pellets for 5 days, and i.c.v. injected with vehicle or prostanoid EP_3 receptor agonists (M&B28,767 and sulprostone) at the indicated doses, respectively, at 30 min before the i.p. injection of naloxone (3 mg/kg). Each column represents the mean ± S.E.M. of total numbers during 1 h. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the morphine withdrawal group by the Mann–Whitney *U*-test. $n = 5–9$.

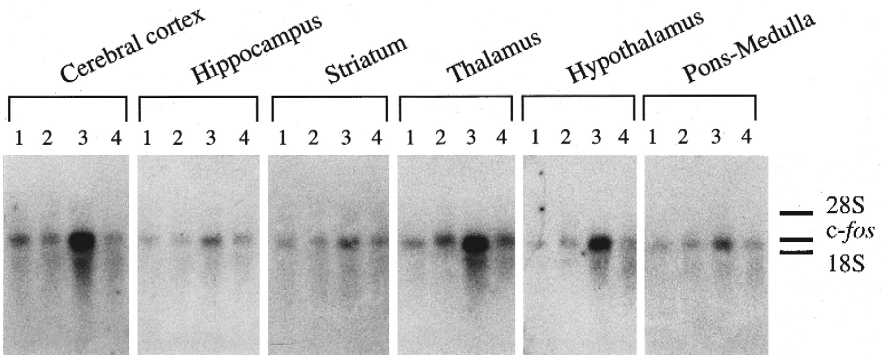


Fig. 2. Effects of M&B28,767 on the c-*fos* mRNA induction by naloxone-precipitated morphine withdrawal in the rat brain (Northern blot analysis). Brains from three rats were removed and dissected into six regions (cerebral cortex, hippocampus, striatum, thalamus, hypothalamus and pons–medulla) 1 h after i.p. injection of naloxone or saline, and total RNAs were extracted from each tissue. Fifteen micrograms of total RNA was applied to each lane. Lane 1, control; lane 2, morphine dependence; lane 3, morphine withdrawal; lane 4, M&B28,767 treatment.

shakes, teeth chattering, jumping, backwards walking, ejaculation, diarrhea, salivation, ptosis, lacrimation and rhinorrhea (morphine withdrawal group). The i.c.v. administration of M&B28,767 (0.01–100 pg/rat) suppressed all withdrawal signs except for diarrhea. The effects of M&B28,767 showed U-shaped dose–response curves, and maximal and significant effects were observed at the dose of 1 or 10 pg/rat. Sulprostone (0.01–10 ng/rat) showed similar effects on withdrawal signs, but its effects were weaker than those of M&B28,767 (Fig. 1 and Table 1). Neither M&B28,767 (1 pg/rat) nor sulprostone (1 ng/rat) affected behavior in normal rats (data not shown).

3.2. Effects of M&B28,767 on c-fos mRNA induction by naloxone-precipitated morphine withdrawal

Northern blotting analysis showed the expression of c-*fos* mRNA in the rat brain (Fig. 2). In all brain regions of control rats, c-*fos* mRNA levels were low (lane 1). In the striatum, thalamus, hypothalamus and pons–medulla, but not the cerebral cortex or hippocampus, of the morphine-dependent group, weak induction of c-*fos* mRNA was observed (lane 2) compared with that of the control group. In addition, morphine withdrawal induced widespread and pronounced c-*fos* mRNA induction. The c-*fos* mRNA levels were increased markedly in the thalamus, hypothalamus and cerebral cortex, and moderately in the hippocampus, striatum and pons–medulla in the morphine withdrawal group (lane 3), compared with those of the control and morphine-dependent groups. On the other hand, i.c.v. administration of M&B28,767 1 pg/rat, which showed maximal suppression of naloxone-precipitated withdrawal signs, attenuated the c-*fos* mRNA induction by naloxone-precipitated morphine withdrawal in all brain regions examined (lane 4), compared with that of the morphine withdrawal group (lane 3).

In situ hybridization studies showed the expression of c-*fos* mRNA. Patterns similar to those seen in Northern blotting analysis were observed in the many brain regions. It was particularly noted that in the locus coeruleus, which is known to be involved in morphine withdrawal syndrome, i.c.v. administration of M&B28,767 (1 pg/rat)

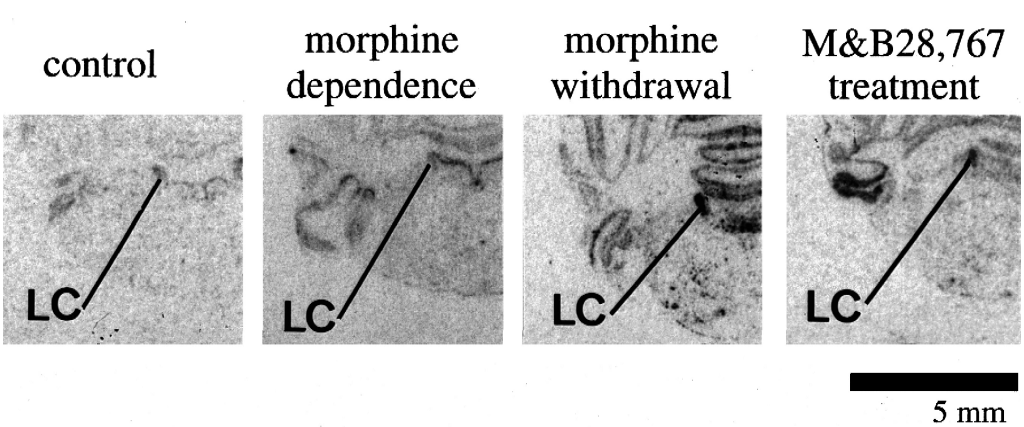


Fig. 3. Effects of M&B28,767 on the c-*fos* mRNA induction by naloxone-precipitated morphine withdrawal in the brain (in situ hybridization). Brains were removed and frozen 1 h after i.p. injection of naloxone or saline. Coronal sections (16 μm) were hybridized to ^{35}S-labeled antisense RNA probe for c-*fos*. Bar = 5 mm. *LC*, locus coeruleus.

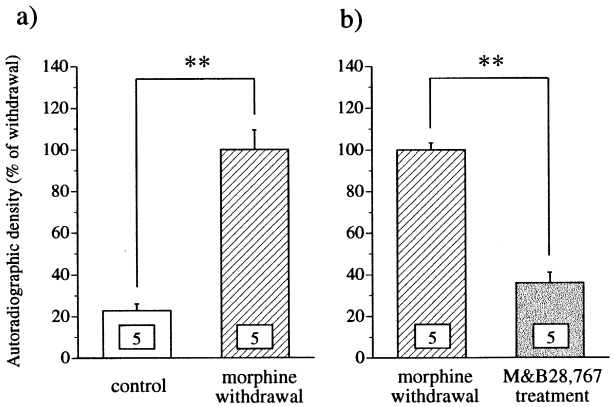


Fig. 4. Quantitative analysis of the inhibitory effect of M&B28,767 on the c-*fos* mRNA induction by naloxone-precipitated morphine withdrawal in the locus coeruleus. Quantitative analysis of film autoradiograms was conducted with a computer-assisted image analysis system (see Materials and methods). For each group, images of the sections including the locus coeruleus from five animals were input into the computer. The autoradiographic density is expressed as a percentage of that in the morphine withdrawal group. (a) and (b) were performed in separate experiments. $^{**}P < 0.01$ compared with the morphine withdrawal group by the Mann–Whitney *U*-test.

attenuated the induction of c-*fos* mRNA by naloxone-precipitated morphine withdrawal (Fig. 3).

We quantitatively analyzed the inhibitory effects of an prostanoid EP_3 receptor agonist on the induction of c-*fos* mRNA by morphine withdrawal in the locus coeruleus. Film autoradiograms were analyzed by computer-assisted image analysis system. In the morphine withdrawal group, the expression of c-*fos* mRNA was significantly elevated by 4.4-fold, compared with that of the control group (Fig. 4a). The i.c.v. administration of M&B28,767 significantly decreased the expression of c-*fos* mRNA to $36.0 \pm 5.0\%$ of that in the morphine withdrawal group (Fig. 4b).

3.3. Coexistence of μ-opioid receptor mRNA and prostanoid EP_3 receptor mRNA with tyrosine hydroxylase mRNA in the locus coeruleus

The coexistence of μ-opioid receptor or prostanoid EP_3 receptor mRNA with tyrosine hydroxylase mRNA in the locus coeruleus was examined by double in situ hybridization with ^{35}S-labeled antisense RNA probe for μ-opioid receptors or prostanoid EP_3 receptors and digoxigenin-labeled antisense RNA probe for tyrosine hydroxylase (Fig. 5). Accumulation of silver grains indicated the expression of μ-opioid receptor or prostanoid EP_3 receptor mRNA on the cells. Tyrosine hydroxylase mRNA-positive cells were stained with AP-reaction product. Most of the tyrosine hydroxylase mRNA-positive neurons in the locus coeruleus were found to express μ-opioid receptor mRNA (Fig. 5a), and more than half of these neurons were positive for prostanoid EP_3 receptor mRNA (Fig. 5b).

4. Discussion

We previously reported that stimulation of the prostanoid EP_3 receptor, but not EP_1, EP_2, IP or FP receptor, suppressed naloxone-precipitated jumping behavior in morphine-dependent mice (Nakagawa et al., 1995). In the present study, we confirmed that i.c.v. administration of M&B28,767 (a prostanoid EP_3 receptor agonist) and sulprostone (a prostanoid EP_1/EP_3 receptor agonist) suppressed various naloxone-precipitated withdrawal signs

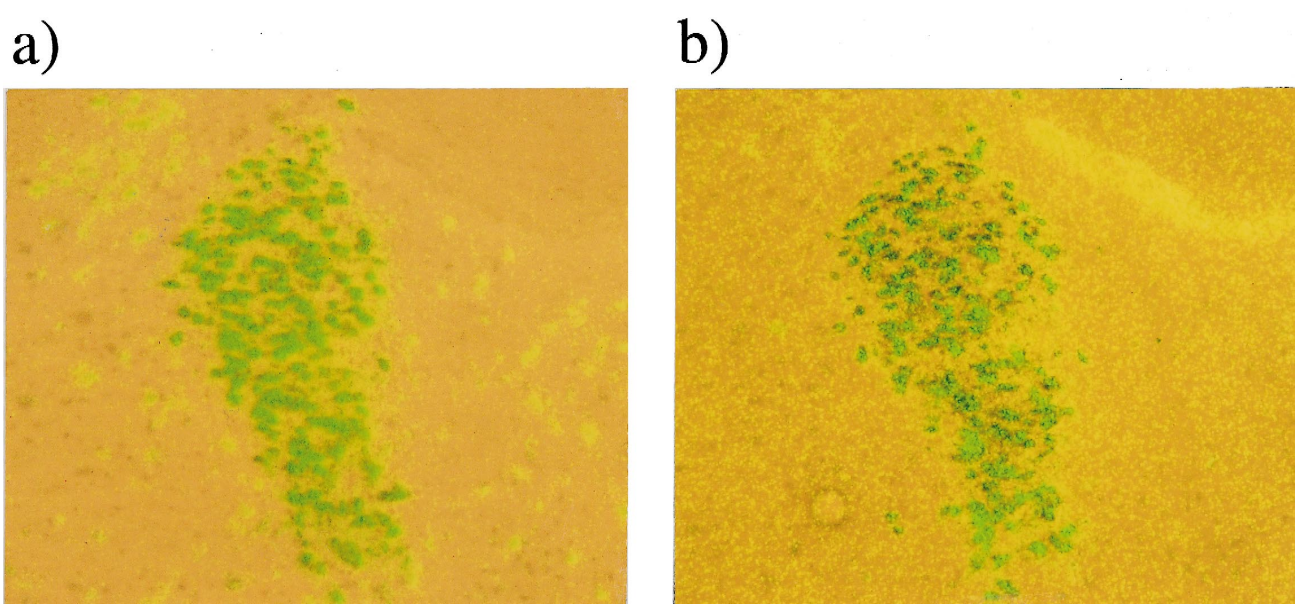


Fig. 5. Coexistence of the mRNA for μ-opioid receptors (a) or prostanoid EP_3 receptors (b) with tyrosine hydroxylase mRNA in the locus coeruleus. Autoradiographic yellow grains were accumulated on the μ-opioid receptor or prostanoid EP_3 receptor mRNA-positive cells. Tyrosine hydroxylase mRNA-positive cells were stained with AP-reaction product.

in morphine-dependent rats. The inhibitory effects of prostanoid EP_3 receptor agonists exhibited a U-shaped dose–response curve. This was consistent with our previous findings and those of other studies indicating that i.c.v. administration of prostanoid EP_3 receptor agonists induces thermal hyperalgesia with a U-shaped dose–response curve in rats (Oka et al., 1994), although the mechanism is unclear.

The i.c.v. administration of M&B28,767 and sulprostone significantly suppressed various withdrawal signs except for diarrhea. As the occurrence of diarrhea is known to be mediated by central and peripheral mechanisms (Taylor et al., 1988), it is considered that centrally injected prostanoid EP_3 receptor agonists could not suppress the expression of diarrhea mediated by peripheral mechanisms in the present experiment.

The neuroanatomical substrates for morphine withdrawal signs have been extensively investigated. It has been reported that naloxone precipitates jumping behavior when injected into the amygdala, and jumping behavior and wet-dog shakes when injected into the globus pallidus or medial thalamus (Calvino et al., 1979). In addition, Maldonado et al. (1992) demonstrated in experiments using microinjection of methylnaloxonium, a hydrophilic opioid antagonist, that withdrawal signs such as jumping behavior, rearing and locomotor activity are mainly mediated by the locus coeruleus or periaqueductal gray matter, and that wet-dog shakes involve the anterior preoptic hypothalamus and nucleus raphe magnus in morphine-dependent rats. In the present study, i.c.v. administration of prostanoid EP_3 receptor agonists suppressed almost all morphine withdrawal signs. Taken together, these observations suggest that prostanoid EP_3 receptor agonists act on widespread regions of the brain to suppress many sorts of withdrawal signs, except for diarrhea mediated by peripheral mechanisms. Indeed, Sugimoto et al. (1994) demonstrated the widespread distribution of prostanoid EP_3 receptor mRNA and its localization in neuronal cells in the mouse brain, including the amygdala, globus pallidus, medial thalamus, periaqueductal gray and locus coeruleus.

In the central nervous system, the expression of mRNA for the immediate-early gene c-*fos* is rapidly and transiently induced in response to a wide variety of stimuli. Numerous studies have successfully used the expression of c-*fos* mRNA as a marker of increased neuronal activity (Sheng and Greenberg, 1990; Morgan and Curran, 1991; Zhu and Inturrisi, 1993). In addition, it is well-known that morphine withdrawal produces marked increases in the level of c-*fos* mRNA and Fos-like immunoreactivity in various regions of the rat brain (Hayward et al., 1990; Stornetta et al., 1993; Beckmann et al., 1995). Recently, it was reported that the induction of c-*fos* mRNA by naltrexone in various brain regions of morphine-dependent rats was blocked by pretreatment with competitive and non-competitive NMDA receptor antagonists or an α_2-adrenoceptor agonist, clonidine (Rasmussen et al., 1995). In the present study, we showed by Northern blotting analysis that the expression of c-*fos* mRNA was elevated by naloxone-precipitated morphine withdrawal in various brain regions: markedly in the thalamus, hypothalamus and cerebral cortex, and moderately in the hippocampus, striatum and pons–medulla. In particular, in situ hybridization analysis revealed that the expression of c-*fos* mRNA was elevated markedly in the locus coeruleus. This confirms previous reports (Hayward et al., 1990; Stornetta et al., 1993). We found that i.c.v. administration of a prostanoid EP_3 receptor agonist attenuated the induction of c-*fos* mRNA in all brain regions examined. These results suggest that the inhibitory effects of prostanoid EP_3 receptor agonists are due to the inhibition of neuronal activity precipitated by morphine withdrawal in several brain regions, consistent with the results of the above behavioral study. Furthermore, in the present study, prostanoid EP_3 receptor agonist suppressed the morphine withdrawal-induced c-*fos* mRNA expression even in the hypothalamus and hippocampus, in which the level of expression of prostanoid EP_3 receptor mRNA was low (our preliminary data).

The central noradrenergic system is known to play an important role in the morphine withdrawal syndrome. It has been shown that the release of noradrenaline is increased during morphine withdrawal in several brain areas, including the hypothalamus and hippocampus (Silverstone et al., 1992; Maldonado, 1997; Vargas et al., 1997). Prostanoid EP_3 receptor agonists, as well as α_2-adrenoceptor agonists, have been shown to inhibit electrically evoked noradrenaline release in mouse and rat brain cortex slices (Exner and Schlicker, 1995). Thus, stimulation of prostanoid EP_3 receptors may inhibit the increased noradrenaline release from noradrenergic neuronal terminals during morphine withdrawal. Furthermore, the locus coeruleus, the largest source of noradrenergic fibers projecting to several areas of the central nervous system, has been reported to possess a high density of μ- and κ-opioid receptors and to be the most sensitive site for expression of morphine withdrawal syndrome (Koob et al., 1992; Maldonado et al., 1992). We demonstrated that i.c.v. administration of prostanoid EP_3 receptor agonists significantly attenuated the induction of c-*fos* mRNA in the locus coeruleus. Furthermore, double in situ hybridization analysis indicated that both μ-opioid receptors and prostanoid EP_3 receptors are expressed on many, probably the same, noradrenergic neurons in the locus coeruleus. These observations suggest that prostanoid EP_3 receptor agonists act on the noradrenergic locus coeruleus neurons expressing μ-opioid receptors, inhibit the increased activity of noradrenergic locus coeruleus neurons produced by morphine withdrawal, decrease noradrenaline release from the nerve terminals originating from the locus coeruleus and suppress morphine withdrawal signs.

Opioid receptors are members of the seven-transmembrane domain GTP-binding protein (G-protein)-coupled

receptor superfamily, and activation of opioid receptors inhibits adenylyl cyclase activity through the activation of $G_{i/o}$-type G-proteins (Minami and Satoh, 1995). However, chronic treatment with opioids leads to the development of supersensitivity of the adenylyl cyclase system (cyclic AMP overshoot) in cultured cells (Sharma et al., 1975; Collier, 1980; Ammer and Schulz, 1993) and in locus coeruleus neurons (Aghajanian, 1978; Rasmussen et al., 1990), although the mechanism of this effect is unclear. It has been proposed that such an explosive increase in the intracellular concentration of cyclic AMP may be involved in the expression of the withdrawal syndrome (Nestler, 1992; Nestler and Aghajanian, 1997; Nestler et al., 1993). Indeed, comparison of the results of behavioral, electrophysiological and biochemical studies indicated that intracellular changes, such as the superactivation of the G-protein/adenylate cyclase system in the locus coeruleus, are related to alterations in locus coeruleus neuronal activity during withdrawal and to the appearance of behaviors associated with opioid abstinence (Rasmussen et al., 1990). However, stimulation of prostanoid EP_3 receptors is known to inhibit adenylyl cyclase (Sugimoto et al., 1992). Taken together, these observations suggest that prostanoid EP_3 receptor agonists may inhibit the enhanced production of cyclic AMP to suppress morphine withdrawal syndrome.

In conclusion, we have shown that i.c.v. administration of prostanoid EP_3 receptor agonists suppresses both the naloxone-precipitated withdrawal syndrome and c-*fos* mRNA induction in various brain regions, including the locus coeruleus. In addition, the results of double in situ hybridization analysis suggest the possibility that prostanoid EP_3 receptor agonists may act on the locus coeruleus noradrenergic neurons expressing μ-opioid receptors. Taken together, these findings suggest that prostanoid EP_3 receptor agonists inhibit the increase in neuronal activity in several brain regions, probably via the inhibition of noradrenergic neurons in the locus coeruleus, thus suppressing naloxone-precipitated morphine withdrawal.

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