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Intracerebroventricular treatment of mice with pertussis toxin induces hyperalgesia and enhances ^{3}H-nitrendipine binding to synaptic membranes: Similarity with morphine tolerance

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Summary. The effect of intracerebroventricular treatment of mice with pertussis toxin (PTX) on pain perception and ^{3}H-nitrendipine binding was examined to study a possible change in the GTP-binding proteins in morphine tolerant rodents. It was observed that both PTX treatment and chronic administration of morphine cause hyperalgesia in the acetic acid-induced writhing test. Analgesic effects brought by the acute administration of morphine or nifedipine, a calcium antagonist, were not affected by PTX treatment. In synaptic membrane fractions prepared from mice treated with PTX or morphine chronically, specific binding of ^{3}H-nitrendipine was enhanced approximately 41.8% and 35.7%, respectively, without alteration in its affinity. Chronic administration of morphine followed by PTX treatment did not display further increases in ^{3}H-nitrendipine binding.

These results suggest that the PTX-sensitive GTP-binding proteins may not be involved in the manifestation of the analgesic effect of morphine in mice.

Key words: GTP-binding protein – Pertussis toxin – Morphine tolerance – ^{3}H-Nitrendipine binding

Introduction

It has been suggested that morphine exhibits an analgesic effect through the inhibition of calcium influx followed by the reduction of the release of transmitters. Thus, morphine reduces calcium contents in brain synaptosomal fraction (Cardenas and Ross 1976; Harris et al. 1977; Yamamoto et al. 1978). Furthermore, it has been shown that calcium antagonizes the analgesic effect of morphine (Kakunaga et al. 1966; Harris et al. 1975). The analgesic action of some calcium channel blockers has also been demonstrated (Del Pozo et al. 1987).

A family of the GTP-binding proteins has been shown to transduce signals from receptors to effectors. Evidence indicates that the GTP-binding proteins may be involved in the regulation of ion channels. Regarding the action of morphine, Hescheler et al. (1987) demonstrated that it regulates calcium channel through the GTP-binding proteins in neuroblastoma × glioma hybrid cells. In addition, the reduction of antinociceptive effect of morphine has been shown following pertussis toxin (PTX) treatment of rats (Parenti et al. 1986; Hoehn et al. 1988). PTX, a toxin produced by *Bordetella pertussis*, has been shown to inactivate the GTP-binding proteins, more specifically G_o and G_i, by the ADP-ribosylation reaction.

Ramkumar et al. (1984) and Saito et al. (1985) previously demonstrated an increase of ^{3}H-nitrendipine, a calcium antagonist, binding to brain membrane fractions following chronic treatment of rats with morphine. The increase of ^{3}H-nitrendipine binding to membrane fractions following chronic morphine administration could indicate enhanced influx of calcium. Assuming that morphine predominantly affects calcium dynamics in the nervous system, increasing doses of morphine would be required to suppress the sustained level of calcium influx. This was also supported by the result showing a decrease in the analgesic effect of nifedipine, a calcium antagonist, following chronic treatment of mice with morphine (Ohnishi et al. 1988).

In this context, in vivo PTX treatment would cause the reduction of the level of GTP-binding proteins and may lead to the enhancement of calcium influx which can be detected by the increase of calcium antagonist binding to membranes. Moreover, in such circumstance that chronic morphine administration causes an increase of calcium influx, in vivo PTX treatment may not affect calcium antagonist binding.

In the present study, the effect of in vivo treatment of mice with PTX on pain perception and on the binding of calcium antagonist to membrane fractions was investigated. Effect of PTX treatment on calcium channel antagonist binding in morphine tolerant mice was also examined in the aim to demonstrate reduced level/or activity of the GTP-binding proteins in those mice.

Materials and methods

Intracerebroventricular (i.c.v.) injection of PTX. Male ICR mice (3–5 weeks, Charles River Japan) were employed throughout the present study. I.c.v. administration of PTX was performed according to a modification of the method of Haley and McCormick (1957) with a special reference of mouse brain atlas given by Sidman et al. (1971). Mice were

Abbreviation. PTX, Pertussis toxin

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Table 1. Effect of the i.c.v. PTX treatment on acetic acid-induced writhing

	Number of writhing			
	Vehicle	PTX		
		0.10 μg	0.25 μg	0.40 μg
Control	26.7 ± 8.6 ($n = 9$)	35.4 ± 5.7* ($n = 11$)	49.4 ± 5.3** ($n = 5$)	38.6 ± 14.2* ($n = 11$)
Morphine tolerant	43.4 ± 5.3** ($n = 7$)	–	46.3 ± 11.9** ($n = 7$)	–

Values represent means ± SD of number (n = parentheses) of determinations. Significantly different from control group receiving the i.c.v. administration of vehicle.
** $p < 0.01$, * $p < 0.05$ as determined by a one-way analysis of variance

anesthetized with ether and given i.c.v. administration of 0.05 M sodium phosphate buffer or PTX (0.10, 0.25 and 0.40 μg) dissolved in the buffer in a volume of 2 μl. Two days following PTX treatment, mice were subjected to an analgesic study. Preparation of membrane fractions for ^{3}H-nitredipine binding experiment was also performed 2 days following the PTX-treatment.

Analgesic test. Analgesia was assessed by an acetic acid-induced writhing test (Chapman and Way 1982). Ten ml/kg of 1% acetic acid was intraperitoneally (i.p.) injected in mice and the number of writhes was counted for the 10 min period starting 5 min after the administration of acetic acid. Morphine (1, 5 and 7 mg/kg) or nifedipine (2, 5 and 7 mg/kg, dissolved in 10% Tween 80) was administrated (i.p.) 30 min prior to acetic acid injection.

Preparation of membrane fractions and ^{3}H-nitrendipine binding. Mouse cerebral cortex was homogenized with 10 vol of 0.32 M sucrose. The homogenate was centrifuged at 1000 g for 10 min. The supernatant was centrifuged at 11,500 g for 30 min and the resultant pellet was disrupted by suspending it in 10 vol of 10 mM Tris-HCl buffer (pH 7.4). Crude synaptic membranes obtained after centrifugation at 11,500 g for 30 min were washed two times with 50 mM Tris-HCl buffer and employed for binding experiments. For assay of ^{3}H-nitrendipine binding, the membrane suspension (100 μg protein/tube) was incubated at 27°C for 90 min in 2 ml of 50 mM Tris-HCl buffer in the presence and absence of 10^{-6} M nifedipine. The reaction was terminated by filtering the incubation medium through Whatmann Glass filters (GF/F). The filters were washed 3 times with 5 ml of ice-cold incubation medium and the radioactivity was counted in a liquid scintillation counter. The differences in the amount of ^{3}H-nitrendipine bound in the presence and absence of 10^{-6} M nifedipine were designated as specific ^{3}H-nitrendipine binding.

In vitro PTX treatment of membranes. In vitro PTX treatment of membrane fractions was performed according the method of Katada and Ui (1982). Briefly, membrane fractions (6–7 mg protein) were incubated at 37°C for 15 min with 25 μg PTX in 1 ml Tris-HCl buffer (50 mM, pH 7.4) containing 1 mM ATP, 10 mM thymidine, 10 μM NAD and 5 mM $MgCl_2$. The reaction was terminated by adding 2.5 ml of ice-cold Tris-HCl buffer (50 mM, pH 7.4). This was immediately employed for ^{3}H-nitrendipine binding experiment.

Chronic administration of morphine. Mice were made tolerant by injecting morphine subcutaneously for seven days. Increasing doses of morphine were administered twice a day. Two and half, 5, 10, 60 and 80 mg/kg of morphine were given from the first to fifth day and additional injections of 100 mg/kg morphine were administered on days 6th and 7th. On the 8th day, mice were given either PTX or vehicle (i.c.v.).

Materials. PTX and ^{3}H-nitrendipine (77.4 Ci/mmol) were obtained from The Chemo-Sero-Therapeutic Research Institute and New England Nuclear, respectively.

Results

Effects of PTX treatment of mice on acetic acid-induced writhing

The significance of PTX effects was confirmed with a one-way analysis of variance (ANOVA) in acetic acid-induced writhing test. Treatment of mice with PTX (i.c.v., 0.10, 0.25 and 0.40 μg) didn't cause any loss of food intake, body weight and others. They became hypersensitive to the audiogenic stimulation. Mice receiving the i.c.v. administration of vehicle displayed 26.7 ± 8.6 responses for a 10 min period in the acetic acid-induced writhing test (Table 1). Following the i.c.v. PTX treatment of mice, was observed a significant increase in writhing behavior. The significant increase of writhing behavior was also observed in the morphine tolerant group. Further increase of writhing behavior, however, was not observed when morphine tolerant mice were treated with PTX.

Effect of PTX treatment of mice on morphine analgesia

Acetic acid-induced writhing behavior was reduced by morphine in a dose-dependent manner in control mice (Fig. 1a). Although i.c.v. treatment of mice with PTX caused hyperalgesia, acute administration of morphine was still effective in inhibiting writhing behavior. Dose-inhibition curve of morphine on writhing behavior shifted upwards in PTX-treated mice leaving the responses equivalent to hyperalgesic reactions unaffected. It was also observed that acutely administered morphine is equipotent in analgesic test in PTX-treated, morphine tolerant and PTX-treated morphine tolerant mice (Fig. 1a, b). As previously shown (Ohnishi et al. 1989) nifedipine also displayed the analgesic effect (Fig. 2). The analgesic effect of nifedipine was also observed following i.c.v. treatment with PTX. In this case, however, the hyperalgesic response was also reduced by nifedipine.

^{3}H-Nitrendipine binding to cortical membranes

When specific binding of ^{3}H-nitrendipine was measured in mouse cortical membranes, a single component having K_d and B_{max} values of 0.23 nM and 76.1 fmol/mg protein, respectively, was observed (Fig. 3a, b). In synaptic membrane fractions prepared from mice receiving i.c.v. administration

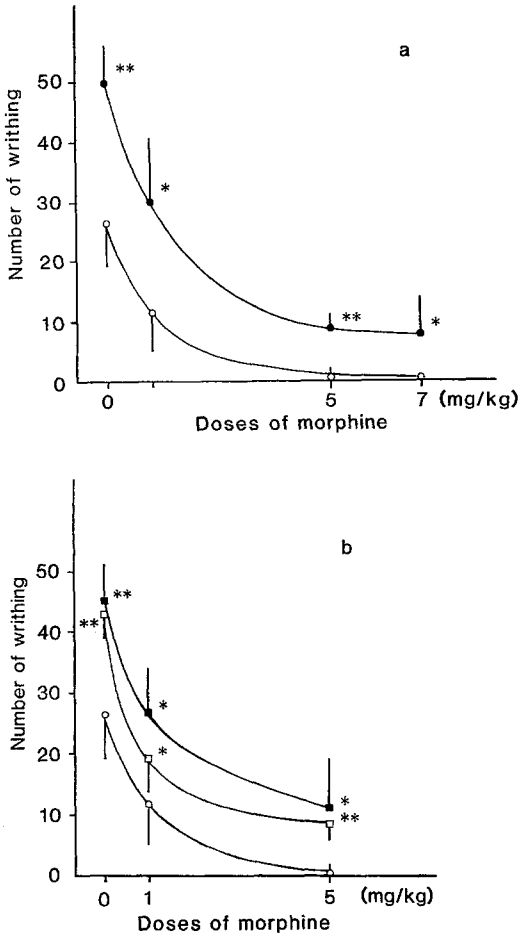

Fig. 1a, b. Effect of morphine on the acetic acid-induced writhing (**a**); in the control mice receiving the i.c.v. administration of vehicle (○) or PTX (●), (**b**); in the morphine tolerant mice receiving the i.c.v. administration of vehicle (□) or PTX (■). *Vertical lines* indicated SD of the means of 5–11 experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ as determined by a one-way analysis of variance

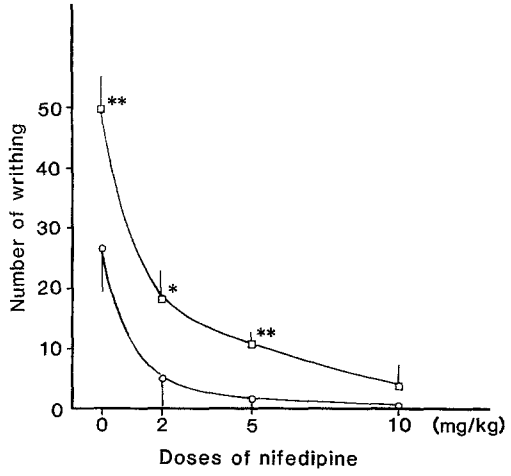

Fig. 2. Effect of nifedipine on the acetic acid-induced writhing in mice receiving the i.c.v. administration of vehicle (○) or PTX (□). *Vertical lines* indicated SD of the means of 5–9 experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ as determined by a one-way analysis of variance

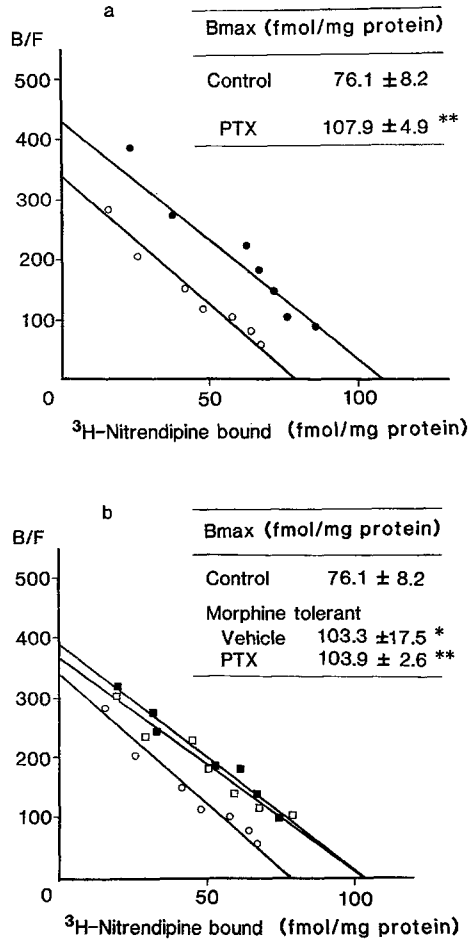

Fig. 3a, b. Scatchard analysis of ^{3}H-nitrendipine binding to cortical membranes. ^{3}H-Nitrendipine binding was measured in cortical membranes prepared from mice receiving the i.c.v. administration of vehicle (○) or PTX (●) in the control (**a**) and the i.c.v. administration of vehicle (□) or PTX (■) in the morphine tolerant mice (**b**). Membrane suspensions were incubated with increasing concentration of ^{3}H-nitrendipine (5–800 pM). *Inset:* Kinetic parameter for ^{3}H-nitrendipine binding. Values are means ± SD of 3–5 determinations. $^{**}p < 0.01$, $^{*}p < 0.05$ as determined by Student's *t*-test

of PTX, an increase of specific binding was observed approximately 42%. There was no change in the affinity. It was observed that ^{3}H-nitrendipine binding to membranes increase to the same extent following chronic treatment of mice with morphine (Fig. 3b). However, i.c.v. administration of PTX in morphine tolerant mice did not give an additive effect on ^{3}H-nitrendipine binding.

Since PTX has been shown to ADP-ribosylate the GTP-binding proteins in vitro (Katada and Ui 1982), the effect of PTX treatment of membranes on ^{3}H-nitrendipine binding was examined. When membrane fractions were incubated in the medium for ribosylation reaction, an alteration of the K_d value to 0.75 nM was observed (Fig. 4). Inclusion of PTX in the medium enhanced binding of ^{3}H-nitrendipine approximately 33% (Fig. 4).

Discussion

Lines of evidence have been accumulated on the mechanism of action of morphine in which it inhibits the influx of

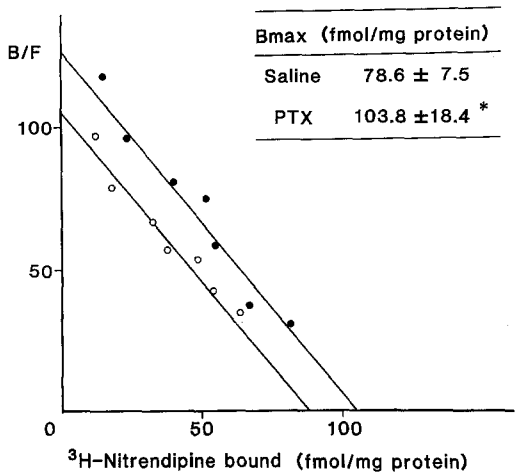

Fig. 4. Effect of the in vitro PTX treatment of cortical membranes on ^{3}H-nitrendipine binding. Membrane suspensions treated with saline (○) or PTX (●) were incubated with increasing concentration of ^{3}H-nitrendipine (5–800 pM). *Inset:* Kinetic parameter for ^{3}H-nitrendipine binding. Values are means ± SD of 5 determinations. $*p < 0.05$ as determined by Student's *t*-test

calcium followed by the reduction of the release of transmitters. Recent report has more precisely demonstrated the processes of the inhibition of calcium influx by morphine (Hescheler et al. 1987). In neuroblastoma × glioma hybrid cells, morphine inhibits calcium influx through a process in which the GTP-binding proteins (G_o) are involved. In such circumstances, PTX treatment of cells diminished the effect of morphine on calcium influx. It has been also shown that PTX treatment of rats inhibits the antinociceptive action of morphine (Parenti et al. 1986; Hoehn et al. 1988). In the present study, however, morphine displayed the analgesic effect in PTX-treated mice apparently without affecting PTX-induced hyperalgesia (Fig. 1a). Thus, the hyperalgesic responses observed in the PTX-treated rodents could be taken as a partial reversion of morphine analgesia. This could possibly explain the discrepancy observed between the present study and the report given by Parenti et al. (1986) although they didn't describe the hyperalgesic responses. In their paper, it can be seen that PTX-treatment of rats causes partial reversion of morphine analgesia. A possibility of insufficient ribosylation reaction in our in vivo experiments could be eliminated since in vitro PTX treatment of membranes under optimal conditions (Katada and Ui 1982) also enhances ^{3}H-nitrendipine binding to the same extent.

In the present study, it was clearly demonstrated that both i.c.v. treatment with PTX and chronic administration of morphine cause hyperalgesia. The fact that further enhancement of hyperalgesia was not observed in morphine tolerant mice following PTX treatment indicated a common effect of those two treatments. This was also suggested in ^{3}H-nitrendipine binding study. Combination of chronic administration of morphine and PTX treatment was not additive in enhancing ^{3}H-nitrendipine binding. Thus, the reduced level of the PTX-sensitive GTP-binding proteins following chronic treatments with morphine could be expected. This is consistent with the finding that prolonged in vivo exposure to morphine reduces the activity of GTPase associated with the GTP-binding proteins (Parenti et al. 1983).

One may have difficulties, however, to conceive that chronic administration of morphine reduces the level of the PTX-sensitive GTP-binding proteins while its effect is not mediated through the binding proteins. Continuous inhibition of calcium entry by morphine may remove/or desensitize the GTP-binding proteins which are supposed to regulate calcium channel. In this case, similar observation would be expected in morphine tolerant and PTX-treated rodents as has been shown in the present study. Direct measurement of the GTP-binding proteins should give further informations.

Ramkumar et al. (1984) and Saito et al. (1985) have already reported the increase of ^{3}H-nitrendipine binding following chronic administration of morphine. Reduced effect of nifedipine in inhibiting writhing behavior in the morphine tolerant mice further support the increase of calcium channel (Ohnishi et al. 1988). In consequence of the elevation of calcium entry, the release of transmitter would increase (Ohnishi et al. 1989). Thus, the hyperalgesic behavior observed in the morphine tolerant and the PTX treated groups should attribute to the augmentation of calcium influx.

In summarizing the results, morphine displayed the analgesic effect through the mechanism in which the PTX-sensitive GTP-binding proteins are not engaged. The chronic treatment of rodents with morphine may reduce the level of the GTP-binding proteins which would result in the increase in calcium entry.

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