Role of GABAergic Systems in the Development of Morphine Tolerance in Formalin-Treated Mice

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ABSTRACT—Since the development of tolerance to morphine antinociception in formalin-treated mice was delayed and diazepam normalized the delay, the involvement of GABAergic systems in the process was investigated. Gamma amino-n-butyric acid (GABA) at 10 mg/kg and the GABA<sub>A</sub>-receptor agonist muscimol at 0.05 mg/kg, i.p., 30 min before daily morphine injection at 10 mg/kg, s.c. completely reversed the delay in the development of morphine tolerance in the formalin-treated mice. The GABA<sub>A</sub> antagonist bicuculline at 1 mg/kg and the Cl<sup>-</sup>-channel blocker picrotoxin at 1 mg/kg extinguished the reverse effect of muscimol and GABA, respectively. In contrast, the GABA<sub>B</sub> antagonist CGP 35348 (3-aminopropane-diehtoxyz-methyl-phosphinic acid) up to 100 mg/kg, i.p. failed to abolish the GABA effect; and baclofen, a GABA<sub>B</sub>-receptor agonist, at 0.5 and 2 mg/kg, i.p., 30 min before morphine was without effect on the delay. On the other hand, bicuculline was incapable of abolishing the reverse effects of diazepam on the delay of tolerance development; and likewise, the reverse effect of muscimol was not affected by flumazenil. No appreciable influence of these GABA-related compounds was seen on morphine antinociception itself nor the development of tolerance in normal mice. These results suggest that the benzodiazepine-GABA<sub>A</sub>-Cl<sup>-</sup> channel complex is involved in the mechanism underlying the delay of the development of morphine tolerance in formalin-treated mice; however, it is deduced that benzodiazepine-receptor and GABAergic systems are not always functionally coupled to each other in the mechanisms.

Keywords: GABA receptor, Benzodiazepine receptor, Morphine, Tolerance, Formalin treatment
perature of the animal room was kept at 22 ± 1°C and maintained on a 12-hr light/dark cycle. They were used in the experiments after reaching a weight of around 25 g.

**Drugs**

Morphine-HCl and diazepam (Cercine®; Takeda, Osaka); gamma amino-n-butyric acid (GABA) (Kishida, Osaka); muscimol, a GABA<sub>A</sub>-receptor agonist, and (+)-bicuculline, a GABA<sub>A</sub>-receptor antagonist (Sigma, St. Louis, MO, USA); picrotoxin, a chloride channel blocker (Nacalai Tesque, Kyoto); CGP-35348, a GABA<sub>B</sub>-receptor antagonist (3-aminopropane-diethoxy-methyl-phosphinic acid) (a kind gift from Ciba-Geigy, Basel, Switzerland); and formalin (Katayama, Osaka) were used. All drugs were dissolved in saline except for bicuculline which was dissolved in 0.1 N HCl, and the pH was adjusted to around 4–5 with the addition of NaOH.

**Induction of inflammatory pain**

A single injection of 10 ml of 2% formalin was given into the dorsal part of the left hind paw (2). The thickness of the paw was measured with slide calipers, and the pain threshold was measured by the method of Randall and Selitto (11) using an analgesy-meter (MK-300; Muromachi, Tokyo), with a slight modification.

**Evaluation of antinociceptive effect**

The antinociceptive effect was measured by the modified Haffner's method (12), with a 6-sec cutoff time to avoid tissue damage. Measurements were made every 15 min after the administration of morphine at 10 mg/kg, s.c. for a period of 90 min, and the effect was expressed as the area under the time-response curve (AUC, min·sec), by plotting the increase in response time (sec) on the ordinate and the time interval (min) on the abscissa.

**Assessment of tolerance**

The AUC of 10 mg/kg of morphine, s.c. was measured daily; and a significant decrease of AUC, compared with that of the 1st day, indicated the development of tolerance.

**Statistical analyses**

Results are expressed as the mean ± S.E. Following analysis of variance for repeated measures of the overall data to assess statistical significance, differences between the individual mean values in various groups were analyzed by Dunnett's test or Student's t-test. Differences were considered significant at \( P < 0.05 \).

**RESULTS**

*Effects of GABA and its related compounds on the delay of the development of morphine tolerance in formalin-treated mice*

Daily injection of morphine at 10 mg/kg, s.c. developed complete tolerance to the antinociception within 5 days in normal mice, but the development was significantly delayed until 5 or 6 days in formalin-treated mice, and then a gradual decrease of antinociception led to tolerance development (2). Pretreatment with GABA at 2, 5 and 10 mg/kg did not affect the development of morphine tolerance in the normal animal (Fig. 1A). GABA at 2 and 5 mg/kg, 30 min before daily morphine, did not affect the delay in the development of tolerance, but at 10 mg/kg, it completely abolished the delay in formalin-treated mice (Fig. 1B). GABA, at doses up to 10 mg/kg, did not affect the antinociceptive effect of morphine in both the formalin-treated and untreated animals as shown on the initial day.

The reverse effect of GABA was antagonized by bicuculline given at the dose of 1 mg/kg, i.p. 15 min before the agonist; however, CGP 35348 at the dose of 100 mg/kg had no effect on the effect of GABA (Fig. 2A).

Picrotoxin at 1 mg/kg, i.p., 15 min before GABA (10 mg/kg, i.p.) antagonized the GABA reversal of delay in the development of morphine tolerance in formalin-treated mice (Fig. 2B).

Bicuculline, CGP 35348 and picrotoxin themselves at
the doses employed had no effect on morphine anti-
nociception and the development of morphine tolerance
in normal and formalin-treated mice (data not shown).

Preliminary experiments were performed to select the
appropriate doses to use.

Muscimol at 0.05 and 0.2 mg/kg, 30 min before daily
morphine did not affect the development of morphine
tolerance in the normal animal (Fig. 3A). On the con-
trary, muscimol even at 0.05 mg/kg significantly reversed
the delay in the development of morphine tolerance in
formalin-treated mice. This effect of muscimol was an-
tagonized by bicuculline at 1 mg/kg, i.p., 15 min before
muscimol (Fig. 3B). Subanalgesic doses of muscimol at
0.05 and 0.2 mg/kg when combined with morphine did
not significantly influence the antinociceptive effect of
morphine, as seen on the 1st day.

Baclofen at 0.5 and 2 mg/kg when administered daily
30 min before morphine did not affect the development of
morphine tolerance in normal mice (Fig. 4A) or the delay
in the development of morphine tolerance in formalin-
treated mice (Fig. 4B).

Effects of bicuculline and flumazenil on the reversal by
diazepam and muscimol, respectively, of the delay of
tolerance development

Daily injection of DZP at 1 mg/kg s.c., 1 hr before mor-
phine administration completely reversed the delay of the
development of morphine tolerance in the formalin-treat-

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Fig. 2. Effect of bicuculline, CGP 35348 and picrotoxin on the
GABA reversal of delay in morphine tolerance development in for-
malin-treated mice. Morphine at 10 mg/kg, s.c. was started 2 hr
after formalin injection on the 1st day. GABA (□) was administered
daily at 10 mg/kg, i.p., 30 min before daily morphine for 5 days.
Bicuculline (A: △, 1 mg/kg), CGP 35348 (A: △, 100 mg/kg) or
picrotoxin (B: ●, 1 mg/kg) was given i.p. 15 min before GABA.
The formalin (■) control group received saline instead of drugs.
The control group (○) was given saline instead of formalin on the
1st day, and this was followed by daily morphine and saline for 5
days. Each point indicates the mean ± S.E. (n = 8). *P < 0.05,
**P < 0.01, compared with the corresponding value on the 1st day.
&P < 0.05, &P < 0.01, compared with the corresponding value in the
saline control group on the respective days.

Fig. 3. Effect of muscimol and bicuculline on the delay of mor-
phine tolerance development with or without formalin treatment in
mice. A: Formalin non-treatment. Muscimol was administered daily
at 0.05 (△) and 0.2 (□) mg/kg, i.p., 30 min before morphine at 10
mg/kg, s.c. for 5 days. The control group (○) received saline instead
of muscimol. B: Formalin treatment. Morphine injection was
started 2 hr after formalin injection on the 1st day. The schedule of
muscimol treatment was similar to that shown in A. Bicuculline (△,
1 mg/kg) was given i.p. 15 min before muscimol. The formalin (■)
control group was given saline instead of drugs. The control group
(○) was given saline instead of formalin on the 1st day, and this was
followed by daily morphine and saline for 5 days. Each point indi-
cates the mean ± S.E. (n = 8). *P < 0.05, **P < 0.01, compared
with the corresponding value on the 1st day. &P < 0.05, &P < 0.01,
compared with the corresponding value in the saline control group
on the respective days.

Fig. 4. Effect of baclofen on the delay of morphine tolerance
development with or without formalin treatment in mice. A: For-
malin non-treatment. Baclofen was daily administered at 0.5 (□)
and 2 mg/kg (△), i.p., 30 min before morphine at 10 mg/kg, s.c. for
5 days. The control group (○) received saline instead of baclofen.
B: Formalin treatment. Morphine injection was started 2 hr after
formalin injection on the 1st day. The schedule of baclofen treat-
ment was similar to that shown in A. The formalin (■) control group
was given saline instead of baclofen. The control group (○) was
given saline instead of formalin on the 1st day, and this was followed
by daily morphine and saline for 5 days. Each point indicates
the mean ± S.E. (n = 8). *P < 0.01, **P < 0.01, compared with the
corresponding value on the 1st day. &P < 0.05, &P < 0.01, compared
with the corresponding value in the saline control group on the
respective days.
ed mice. Bicuculline at 1 mg/kg, i.p., 15 min before DZP
failed to antagonize the reverse effect of diazepam (Fig.
5A). Likewise, flumazenil (a BZP-receptor antagonist) at
2 mg/kg had no effect on the reverse effect of muscimol
(Fig. 5B). DZP was without effect on the development of
morphine tolerance in normal mice (data not shown).

Fig. 5. Effects of bicuculline and flumazenil on the reversal in-
duced by diazepam and muscimol, respectively, in the delay of mor-
phine tolerance development in formalin-treated mice. Morphine at
10 mg/kg, s.c. was started 2 hr after formalin injection on the 1st
day. In A, diazepam (▲) was administered daily at 1 mg/kg, s.c., 60
min before daily morphine for 5 days. Bicuculline (●, 1 mg/kg) was
given i.p. 15 min before diazepam. In B, muscimol (□) was ad-
ministered daily at 0.05 mg/kg, i.p., 30 min before daily morphine
for 5 days. Flumazenil (○, 2 mg/kg) was given i.p. 15 min before
muscimol. The formalin (■) control group received saline instead of
drugs. The control group (○) was given saline instead of formalin
on the 1st day, and this was followed by daily morphine and saline for
5 days. Each point indicates the mean±S.E. (n=8). *P<0.05,
***P<0.01, compared with the corresponding value on the 1st day.
**P<0.05, ***P<0.01, compared with the corresponding value in the
saline control group on the respective days.

In agreement with our previous results that BZP-recep-
tor mediated mechanisms are involved in the delay of the
development of morphine tolerance (2), it was found that
both GABA and muscimol significantly reversed the delay
of morphine tolerance development, and bicuculline an-
tagonized the reverse effects in the formalin-treated mice.
In contrast, the GABAB-receptor antagonist CGP 35348
failed to antagonize the reverse effect of GABA and bac-
lofen itself was without effect on the delay of morphine
tolerance development, indicating that the GABAB systems are
unlikley to be involved in the mechanisms. Thus, we suggest
that the reversal of the delay by GABA is mediated
through GABA_A receptors. Supporting our assumption,
picrotoxin, a Cl^-channel blocker, likewise antagonized
the reverse effect of GABA. Thus we confirmed that the
GABA_A-BZP-Cl^- channel complex is involved in the
delay of the development of morphine tolerance in forma-
lin-treated mice.

There is considerable evidence that the BZP receptors
are a part of the BZP-GABA_A-Cl^- channel complex, and
BZP receptors act as a coupling unit between the GABA
receptor and Cl^- channel. Nevertheless, bicuculline failed
to antagonize the DZP reversal of delay in the develop-
ment of morphine tolerance in formalin treated mice, or
flumazenil did not affect the muscimol reversal of delay.
Thus, we predicted that BZP receptors and the GABA_A-
Cl^- channel system are not always cooperative in the
delay process. In fact, there are several reports indicating
that these two systems function in mutually different man-
ners (13 - 15). It is also reported that some GABA binding
sites appear not to be coupled to BZP binding sites (16).
In addition, bicuculline did not affect the anticonflict
effect of DZP (17).

GABA, as well as muscimol, resulted in the reversal of
delay in formalin-treated mice. Although the mechanism
underlying the GABA reversal of delay remains uncer-
tain, because of the low permeability of GABA across the
blood-brain barrier, the compound probably activates the
brain GABA systems due to an indirect action. Converse-
ly, it seems likely that brain penetration of GABA may be
altered by the pretreatment with formalin through a
mechanism that is not yet defined. The latter assumption
may be supported by the fact that in contrast to formalin-
treated mice, we could not discern any influence of
GABA on the development of morphine tolerance in nor-
mal animals.

The mechanism by which GABA reversed the delay is
not exactly known; however, a possibility for changes in
GABA content or its receptor sites or the implication of
other systems in the formalin-treated mice cannot be
ruled out.

In conclusion, these results suggest that the BZP-
GABA_A-receptor Cl^- channel complex plays an im-
portant role in the delay of the development of morphine
tolerance in the animal model of experimental pain/anxi-
ety, and they suggest that BZP-receptors and GABAergic
systems are not always cooperative functionally in this
process.

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