CONDITIONED SUPPRESSION AND OPIOID KAPPA RECEPTOR IN MICE

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(Received May 29, 1985)

Mice exhibited a marked suppression of motility (conditioned suppression) when placed in the same environment in which they had previously received an electric footshock. Furthermore, chronic morphine (mu agonist)-pretreated mice, as well as chronic vehicle-pretreated mice, exhibited the conditioned suppression but chronic ethylketocyclazocine (kappa agonist)- and pentazocine (kappa agonist)-pretreated mice did not. On the other hand, in the synaptic membranes of the chronic vehicle- and morphine-pretreated mice showing conditioned suppression, the specific binding of [3H]phencyclidine (sigma agonist) and [3H]naloxone (mu antagonist) significantly increased, while the specific binding of [3H]ethylketocyclazocine did not change compared to those of the corresponding control groups. However, in the chronic pentazocine- and ethylketocyclazocine-pretreated groups showing non-conditioned suppression, the specific binding of [3H]phencyclidine and [3H]naloxone were not altered. Moreover, a decrease of [3H]ethylketocyclazocine binding was observed in the chronic pentazocine-pretreated group. These results suggest that the binding function of different opioid receptor subtypes may be altered differently by stress, and that the kappa receptor may be important for the conditioned suppression of motility in mice.

Keywords — conditioned suppression; kappa receptor; opioid receptor subtype; sigma receptor; stress

INTRODUCTION

Rats and mice exhibit a marked suppression in motility when they are placed in the same environment in which they have previously received an electric footshock. This suppression is considered to be a conditioned emotional response to the environment (conditioned suppression). Furthermore, the conditioned suppression is attenuated by morphine and cyclazocine. The effect of these drugs is completely antagonized by pretreatment with naloxone. Thus, it is possible that the opiate-induced reduction of conditioned suppression is mediated by opioid receptor sites. Pharmacologically, opiate receptors have been assigned to one of three major groups named after prototypic drugs: mu for morphine, kappa for ketocyclazocine and sigma for SKF-10,047. The discovery of enkephalins has led to the fourth classification for peptides, delta. These observations have led to numerous studies attempting to elucidate each physiological function of different opioid receptors. In addition, it has been reported that the attenuation of conditioned suppression in motility may be mediated through the opioid receptor subtypes, sigma and mu receptors, since phencyclidine, cyclazocine and morphine attenuate the conditioned suppression, but not ethylketocyclazocine and methionine-enkephalin. Furthermore, in the synaptic membranes of mice showing conditioned suppression, the binding capacity of [3H]naloxone and [3H]phen-
cyclidine increased, but not that of \(^{3}H\)ethylketocyclazocine.\(^9\) In the present experiments, we attempted to investigate which opioid receptor subtypes correlate with the development of conditioned suppression using the opioid binding assay technique and mice tolerant to different opiate agonists.

**METHODS**

*Subject* — Male ddY mice, 8 weeks of age, were housed in a group of 12–20 in a controlled environment (23 ± 1 °C, 50±5% humidity) and were allowed food and water *ad libitum*. The room lights were off between 8:00 p.m. and 8:00 a.m.

*Test Procedure* — Experiments were carried out as previously described using a transparent acrylic rectangular cage (23 × 28 × 12 cm) equipped with a metal wire floor.\(^8\) The test cage was located in a sound-attenuated room and lit with a 20 W bulb. Each mouse was placed in the test cage and received electric shocks (0.1 Hz, 200 ms, 300 V DC) for 6 min through an isolated stimulator (Nihon Koden, Tokyo, Japan). When an animal was placed in the test cage, the current resistance varied between 100 and 250 kΩ. Therefore, each animal received electric shocks in a range of 1.2–3 mA. The animal was again placed in the same cage 24 h after the shock treatment, but no electric footshock was given [conditioned suppression (CS) group]. The motility of the CS group was measured for 6 min in the test cage surrounded by an Opto-Varimex (Columbus Instruments, Ohio, U.S.A.), a locomotor activity meter, 24 h after the shock treatment. The control group was treated exactly the same way as the CS group except for the absence of the electric footshock treatment.

*Drugs Treatment* — In order to assess the involvement of opioid receptor subtypes in the CS, we investigated whether the degree of conditioned suppression decreased in the mice chronically pretreated with three different opiates: morphine hydrochloride (MOR, Shionogi Pharmaceutical Co., Osaka, Japan), ethylketocyclazocine methanesulfonate (EKC, Sterling-Winthrop Research Institute, Rensselaer, NY) and pentazocine (PTZ, Pentagin Inj., Sankyo Pharmaceutical Co., Tokyo, Japan). Four groups each of 26–35 mice were chronically treated with one of the above drugs or saline following various schedules: MOR (the initial dose of MOR 25 mg/kg was injected s.c. twice a day, after which the dose of MOR was doubled every 2 d until the 6th day), EKC (4 mg/kg/d for 6 d s.c.) and PTZ (50 and 100 mg/kg/d for the first and the second 3 d, respectively, s.c.). Chronic vehicle-pretreated groups received the physiological saline (10 ml/kg/d for 6 d s.c.). It was confirmed that chronic administration of these opiate agonists to mice on previous schedules resulted in a marked tolerance development to their antinociceptive effects. Tolerance development to MOR/EKC and PTZ was assessed by the method of tail-flick and acetic acid-induced writhing, respectively. In addition, the mice chronically treated with opiate agonists showed the same basal pain threshold as control mice on antinociceptive response.\(^4\) The chronic vehicle- and chronic opiate-pretreated mice were divided into two groups. First (CS) groups of 12–15 mice received footshocks for 6 min 24 h after the last administration of these opiates and the motility was measured as described above, 24 h after the shock treatment. Second (control) groups of 13–20 mice were subjected to the same procedure as the first groups except for the absence of electric shock.

*Preparation of Synaptic Membrane* — The mice of the CS and control groups were decapitated immediately after measurement of motility as described above. The whole brains excluding cerebellum were rapidly removed and synaptic membranes were prepared by the method of Sivam *et al.*\(^13\) The pooled brains were homogenized with 10 volumes of 0.32 M sucrose in 50 mM Tris-HCl buffer (pH 7.4 at 4 °C) and centrifuged at 1000 × *g* for 10 min. The supernatant fraction was collected and centrifuged at 20000 × *g* for 20 min to obtain a pellet containing the crude mitochondrial fraction (P2 fraction). The P2 fraction was subjected to osmotic
shock by adding 10 ml of distilled, deionized water. The suspension was centrifuged at 12000 × g for 20 min. The carefully decanted supernatant fluid, properly divided, was layered over a discontinuous gradient consisting of 0.6 and 1.0 M sucrose in 5 mM Tris-HCl buffer (pH 7.4 at 4 °C), and centrifuged at 100000 × g for 60 min. The band between 0.6 and 1.0 M sucrose (P2B fraction) was collected. The pooled P2B fraction was divided into proper aliquots and stored at −70 °C.

The aliquots were diluted with 10 volumes of standard buffer (25 mM Tris-HCl, pH 7.4 at 4 °C) and centrifuged at 25000 × g for 20 min, to obtain a pellet. The pellet was resuspended with a proper volume of standard buffer and incubated for 30 min at 37 °C to remove endogenous opioids and residues of administrated opiates. After the incubation, the suspension was re-centrifuged at 25000 × g for 20 min, to obtain a pellet. This pellet was resuspended in fresh standard buffer to obtain the final membrane preparation for the binding assay; this preparation contained 0.15–0.25 mg protein/ml.

**Binding Assay** — Opiate receptor binding was initiated by the addition of 0.2 ml of the membrane preparation to a mixture containing 1 nM (final concentration) of [3H]phencyclidine ([3H]PCP: sigma agonist), [3H]naloxone ([3H]NLX: mu antagonist) or [3H]ethyketocyclazocine ([3H]EKC: kappa agonist) in a total volume of 1 ml. Incubations were carried out at 25 °C for 30 min. The binding reaction was stopped by rapid filtering through Whatman GF/B filters and the filters were washed twice with 5 ml of ice-cold 25 mM Tris-HCl buffer (pH 7.6 at 4 °C). The filters were transferred to scintillation counting vials shaken for 60 min and the radioactivity was measured using a Model 3255 Tri-Carb Liquid Scintillation Spectrometer System. Specific opiate receptor binding was defined as the difference between the binding of the labeled ligand in the presence and the absence of 5 μM non-radioactive PCP, NLX and EKC in the cases of [3H]PCP, [3H]NLX and [3H]EKC, respectively. Presoaking filters in 0.01% poly-L-lysine at 4 °C for 120 min was used for the binding experiments of [3H]PCP to reduce “specific” ligand binding to the filters.16)

The protein content of each membrane preparation was determined by the method of Lowry et al.17)

**Statistics** — Where applicable, statistical significance was analyzed by Student's t-test for biochemical data and by Mann-Whitney U-test for behavioral data.

**RESULTS**

**Effects of Chronic Administration of Opiate Agonists on the CS in Motility**

As shown in Fig. 1, in the control groups which did not receive electric shock, none of the chronic administrations of opiates changed spontaneous locomotor activity. When chronic

![FIG. 1. Effect of Chronic Administration of Opiate Agonists on the Conditioned Suppression of Motility in Mice](image)

**Schedules of drug administration were described under Methods. Motility of mice was measured 24 h after footshock. Motility of mice is expressed as a percentage of the vehicle-treated control group (number of motility: 2371.7 ± 139.3).**, chronic vehicle; chronic morphine; chronic ethylketocyclazocine; chronic pentazocine. The numbers in columns show the number of mice. Values are the means ± S.E.M. from 12–20 mice. a) p< 0.01 vs. corresponding control group.
vehicle-pretreated mice were returned to the same apparatus in which they had been given electric shock, they exhibited a marked suppression of motility [42 ± 11% of the control group (conditioned suppression)]. However, the degree of motor suppression in the chronic EKC- and PTZ-pretreated mice was significantly less than that in the vehicle-pretreated shocked mice ($p < 0.01$). The levels of motility in chronic vehicle-, EKC- and PTZ-pretreated mice receiving footshocks were 42 ± 11, 98 ± 5 and 93 ± 9% of the corresponding control mice, respectively. On the other hand, the degree of motor suppression in the chronic MOR-pretreated mice was similar to that of the vehicle-pretreated shocked mice.


As shown in Fig. 2, in the synaptic membranes of chronic vehicle- or MOR-pretreated shocked mice showing CS of motility, the specific binding of [^3]H]PCP significantly increased compared to that of the corresponding control groups. On the other hand, there was no difference in the specific binding of [^8]H]PCP between the chronic EKC- or PTZ-pretreated shocked group not showing CS of motility and the corresponding control groups.


As shown in Fig. 3, in the chronic vehicle-pretreated shocked group showing CS of motility, the specific binding of [^3]H]NLX significantly increased compared to that of the control group in agreement with our previous report. Furthermore, in the chronic MOR-pretreated shocked group showing CS of motility, the specific binding of [^3]H]NLX also significantly increased compared to that of the corresponding control group. On the contrary, the binding activity of [^3]H]NLX in the chronic EKC- and PTZ-pretreated shocked groups not showing CS of motility returned to the levels of the corresponding control groups.


As shown in Fig. 4, in the chronic PTZ-

![Graph 2](image)

**FIG. 2.**[^3]H]PCP Binding to Mouse Brain Synaptic Membrane in Control and Conditioned Suppression Groups after Chronic Administration of Opiate Agonists

□, chronic vehicle; □□, chronic morphine; □□□, chronic ethylketocyclazocine; □□□□, chronic pentazocine. Values are the means ± S.E.M. The numbers in columns show the number of experiments, each in triplicate. a) $p < 0.05$, b) $p < 0.01$ vs. corresponding control group.

![Graph 3](image)

**FIG. 3.**[^3]H]NLX Binding to Mouse Brain Synaptic Membrane in Control and Conditioned Suppression Groups after Chronic Administration of Opiate Agonists

□, chronic vehicle; □□, chronic morphine; □□□, chronic ethylketocyclazocine; □□□□, chronic pentazocine. Values are the means ± S.E.M. The numbers in columns show the number of experiments, each in triplicate. a) $p < 0.05$, b) $p < 0.01$ vs. corresponding control group.
pretreated shocked group not showing CS of motility, the specific binding of [3H]EKC significantly decreased compared to that of the corresponding control group. On the other hand, in the chronic vehicle-, MOR- and EKC-pretreated shocked groups, the specific binding of [3H]-EKC was not altered.

DISCUSSION

As described in the Introduction, we have reported that the CS of motility is diminished by CLZ and PCP, both sigma agonists, and partially reduced by MOR, a mu agonist. However, the CS of motility is not affected by EKC, a kappa agonist, and methionine-enkephalin, a delta agonist. Furthermore, SKF-10047, a sigma agonist, significantly attenuated the CS of motility in a dose-dependent manner (unpublished results). Although both PTZ and CLZ are classified as opiate agonist-antagonists, it has been reported that PTZ, like EKC, elicits behavioral depression, while CLZ causes behavioral arousal and bizarre behavior as does SKF-10047. Thus, it is possible that PTZ and CLZ are relative kappa- and sigma-like agonists, respectively. Moreover, PCP binds to a sigma receptor although it does not belong to the opiates. In agreement with our previous report, chronic vehicle-pretreated shocked mice exhibited a marked suppression in motility when they were returned to the same apparatus in which they had been given electric footshock under our experimental conditions. In addition, the present experiments showed that the chronic MOR-pretreated shocked mice exhibited the CS of motility, but not the chronic PTZ- and EKC-pretreated shocked mice (Fig. 1). As described under Methods, the chronic administration of these opiates induces tolerance to their analgesic effects. CS was diminished in the chronic EKC and PTZ-pretreated mice. Therefore, with regard to our previous pharmacological study, the present results also suggest that the CS of motility may be mediated at kappa receptor. In addition, since CS was attenuated by the acute treatment of CLZ, PCP, SKF-10047 and MOR, it is suggested that sigma and/or mu receptors may be important for opiate-induced attenuation of the CS of motility.

To clarify this point, the binding assay of [3H]PCP, [3H]NLX or [3H]EKC to synaptic membrane prepared from CS groups chronically pretreated with opiate agonists have been performed. As shown in Table I, in the chronic vehicle-pretreated shocked group showing CS of motility, the significant increase of [3H]PCP and [3H]NLX bindings was observed when compared to the control group. These results confirmed our previous report that the binding capacity of [3H]PCP and [3H]NLX in the CS group significantly increases when compared to the control group. Furthermore, [3H]PCP and [3H]NLX bindings in the chronic MOR-pretreated shocked group showing CS of motility indicated similar increases as seen in the chronic vehicle-pretreated shocked group (Table I). Although MOR shows the highest affinity for mu receptor, the MOR-induced attenuation of the

FIG. 4. [3H]-EKC Binding to Mouse Brain Synaptic Membrane in Control and Conditioned Suppression Groups after Chronic Administration of Opiate Agonists

□, chronic vehicle; □, chronic morphine; □, chronic ethylketocyclazocine; □, chronic penta-zocine. Values are the means ± S.E.M. The numbers in columns show the number of experiments, each in triplicate. a) p < 0.05 vs. corresponding control group.
CS may also be mediated by the sigma receptor since the dose-response curve for the effect of MOR on the CS is parallel to those of CLZ, PCP\(^9\) and SKF-10047 (unpublished result). When the present and previous results\(^8\) are taken together, we consider that the binding activities of the sigma and mu receptors might increase to compensate for the decrease in the neuronal function mediated by these receptors in the chronic vehicle- and MOR-pretreated CS groups. In addition, the kappa receptor might maintain a normal function, since the specific \(^{[3}\)H]EKC binding did not alter in the chronic vehicle- and MOR-pretreated shocked groups showing CS of motility (Table I) which is in agreement with our previous paper that there is no difference between the CS and control groups in the binding capacity and the affinity of \(^{[3}\)H]EKC.\(^8\)

In the chronic PTZ- and EKC-pretreated shocked groups not showing CS of motility, the changes of \(^{[3}\)H]PCP and \(^{[3}\)H]NLX bindings were not observed (Table I). On the other hand, a significant decrease of \(^{[3}\)H]EKC binding was observed in the chronic PTZ-pretreated shocked group when compared to the corresponding control group (Fig. 4). It is suggested that the functional degeneracy of the kappa receptor may be caused by the chronic administration of PTZ, a kappa agonist. However, in the chronic EKC-pretreated shocked group, the change in \(^{[3}\)H]EKC binding was not observed, though the CS of motility was not developed like the chronic PTZ-pretreated shocked group. These results suggest that the behavioral change is more sensitive than the biochemical change in the chronic EKC-pretreated shocked group.

The present behavioral and receptor binding experiments suggest that the CS of motility may be due to the activation of the kappa receptor, and that opiate-induced attenuation of CS may be due to the functional facilitation of the nervous system mediated by sigma and/or mu receptors.

**Acknowledgment**  We thank Mr. Y. Noda for excellent technical assistance and Sterling-Winthrop Research Institute and Endo Laboratories for supplying ethylketocyclazocine methanesulfonate and naloxone hydrochloride, respectively.

**REFERENCES**

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**TABLE I. Summary of the Effects of Chronic Administration of Opiate Agonists on the Motility and \(^{[3}\)H]Opiate Binding in Mice of Conditioned Suppression Groups**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Motility</th>
<th>PCP (sigma)</th>
<th>NLX (mu)</th>
<th>EKC (kappa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>±</td>
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<tr>
<td>MOR (mu)</td>
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<tr>
<td>EKC (kappa)</td>
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<tr>
<td>PTZ (kappa)</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>↓</td>
</tr>
</tbody>
</table>

↑: increased, ↓: decrease, ±: non-change.