

## Intrathecal Pertussis Toxin Treatment Attenuates Opioid Antinociception and Reduces High-affinity State of Opioid Receptors

Chih-Shung Wong, M.D.,\* Ying-Fu Su, Ph.D.,† Kwen-Jen Chang, Ph.D.,‡ W. David Watkins, M.D., Ph.D.§

The effect of pertussis toxin on opioid antinociception was studied in rats. Intrathecal injection of a single dose of pertussis toxin reduced the antinociceptive effect of PL017, a highly selective  $\mu$ -opioid agonist, in a dose- and time-dependent manner. The maximal effective dose of pertussis toxin was 1  $\mu$ g, and the maximal effect was seen at day 3. The effect of the toxin lasted for 2 weeks, and the antinociceptive response recovered partially at the third week. The dose-response curves of the antinociceptive effect of PL017 were shifted to the right with increasing doses of pertussis toxin. Three days after pertussis injection, receptor-binding activity of membranes in the lumbosacral segment of spinal cords decreased to 70% of control as assayed by  $^{125}\text{I}$ -FK33824, a highly selective  $\mu$ -receptor agonist. In experiments using [ $^3\text{H}$ ]naloxone as the radiolabeled ligand, displacement curves of FK33824 were shifted to the right after pertussis toxin treatment. The shift also was dose and time dependent. Scatchard analysis of binding data showed that, after pertussis toxin treatment, there was no significant change in the total number of binding sites, but a class of low-affinity binding sites appeared in addition to the high-affinity sites. When spinal membranes were washed in  $\text{Na}^+$  (100 mM) and guanosine diphosphate (100  $\mu\text{M}$ ) and binding was assayed in the presence of  $\text{Mg}^{2+}$  (5 mM), all the  $\mu$ -receptors were in the high-affinity state in control membranes. After the pertussis toxin treatment, the ratio of low-affinity sites to high-affinity sites increased. The emergence of low-affinity binding sites appeared to correlate with a decreased antinociceptive effect of PL017. The maximal effect of pertussis toxin was reached at 1  $\mu$ g. These results indicate that pertussis toxin treatment generated a class of low-affinity-state opioid receptors that were not found in untreated membranes. The total number of receptors was not affected by the toxin treatment. The appearance of low-affinity receptors correlates with attenuated opioid response. Thus, the authors suggest that receptor uncoupling might be a mechanism of opioid tolerance. (Key words: Analgesics, opioid: tolerance. Anesthetic techniques: intrathecal. Receptors: G protein. Spinal cord. Toxin: pertussis.)

OPIOIDS are a mainstay in the pharmacologic production of analgesia. Prolonged administration of opioids induces tolerance. One would expect receptor activity to play a major role in the responses of cells and neurons to drugs. Cellular responses can vary because of the intrinsic activities of drugs and the number of receptors on membrane surfaces. This may be demonstrated best by the studies of Stevens *et al.*<sup>1</sup> and Sosnowski and Yaksh,<sup>2</sup> which showed that opioid tolerance in rats was dependent on the timing, dose, and intrinsic activity of inducing drugs. "Spare" receptors also are speculated to play an important role. In some *in vitro* systems, receptor down-regulation is observed after prolonged incubation of cells with opioids. For example, in neuroblastoma cells<sup>3-6</sup> and hippocampal slices,<sup>7</sup>  $\delta$ -opioid receptors are down-regulated by prolonged incubation with the  $\delta$ -receptor-selective peptide DADLE ([D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin). In these systems, morphine does not produce down-regulation. In earlier animal studies, the number of brain opioid receptors was not altered in rats rendered tolerant to opioids by chronic administration of morphine.<sup>9,7,8</sup> Using an *in vivo* model<sup>9</sup> in our laboratory, we found that spinal cord  $\mu$ -receptors are down-regulated by chronic intrathecal infusion with the  $\mu$ -receptor-selective opioid peptide PL017.<sup>10</sup> Again, the receptor down-regulation is induced only by the peptide, but not by morphine. Thus, receptor down-regulation may play a role in tolerance induced by opioid peptides, but this mechanism cannot explain the phenomenon of morphine tolerance.

Incubation of neuroblastoma x glioma hybrid cells *in vitro* with opioids results in down-regulation and acute desensitization of adenylate cyclase inhibition.<sup>6,11</sup> The desensitization appears to be correlated with a shift of opioid receptor affinity for agonist from the high-affinity state to the low-affinity state. Opioid receptors, like other G protein-associated receptors, have to be in the high-affinity state to functionally express agonist activity. The low-affinity receptor, presumably in a conformation unfavorable for "coupling" to  $G_i/G_o$  proteins, is functionally less active or inactive. The shift of receptor to the non-functional low-affinity state generally is accepted as evidence of uncoupling of the receptor from the system.<sup>11</sup> In addition, desensitization of adenylate cyclase can be produced by opioid peptides and morphine, a nonpeptide agonist.<sup>5,6</sup> This suggests that the uncoupling process might relate to tolerance observed in animals. Receptor down-

\* Instructor in Anesthesiology, Tri-Service General Hospital and National Defense Medical Center, Taipei, Taiwan, Republic of China. Current position: Ph.D. Candidate in Pharmacology, Duke University Medical Center.

† Research Assistant Professor in Anesthesiology, Duke University Medical Center.

‡ Principal Scientist, Division of Cell Biology, Burroughs Wellcome Company; Adjunct Professor in Anesthesiology and Pharmacology, Duke University Medical Center.

§ Professor in Anesthesiology and Pharmacology, Duke University Medical Center.

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Address reprint requests to Dr. Wong: Department of Anesthesiology, P.O. Box 3094, Duke University Medical Center, Durham, North Carolina 27710.

regulation and desensitization are two separate mechanisms of cellular adaptation to long-term agonist exposure, and they are studied especially well in the  $\beta$ -adrenergic receptor system.<sup>12</sup> We hypothesize that receptor uncoupling could be one of the *in vivo* mechanisms involved in the opioid tolerance.

Experimentally, one can chemically modify  $G_i/G_o$  proteins *in vitro* to uncouple the receptor system. Pertussis toxin has been shown to uncouple  $\alpha$ -adrenergic receptors from adenylate cyclase by adenosine diphosphate ribosylation of the  $G_i/G_o$  proteins.<sup>13-15</sup> In an opioid receptor system, incubation of cultured cells with pertussis toxin also results in covalent modification of the  $\alpha$ -subunit of  $G_i/G_o$  proteins and uncoupling of opioid receptors.<sup>16</sup> Werling *et al.* further demonstrated *in vitro* receptor uncoupling by showing that pertussis toxin treatment of brain membranes shifted  $\mu$ -opioid receptors to the low-affinity state with concomitant loss of the guanine nucleotide regulatory effect on receptor binding.<sup>17</sup> These are examples of pertussis toxin-induced receptor uncoupling leading to loss of effector function or receptor affinity alteration. In animal studies, administration of pertussis toxin by the intracerebral ventricular or intrathecal route results in attenuation of antinociceptive action of opioids.<sup>18-21</sup> Thus, pertussis toxin is able to induce a tolerance-like state in rats that is behaviorally similar to opioid tolerance. However, the receptor status in the pertussis toxin-induced reduction of opioid response is not known. In the current study, we used the rat spinal model and uncoupled the opioid receptor from G proteins with pertussis toxin. Then we examined the effect of the pertussis toxin treatment by measuring both the antinociceptive response *in vivo* and the receptor affinity *in vitro*.

## Materials and Methods

### MATERIALS

Pertussis toxin was purchased from LIST Biological Laboratories (Campbell, CA). The highly selective  $\mu$ -opioid receptor agonists, [NMe<sup>3</sup>Phe, D-Pro<sup>4</sup>] morphiceptin (PL017)<sup>22</sup> and [D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Met-(o)<sup>5</sup>-ol]-enkephalin (FK33824),<sup>23</sup> and highly selective  $\delta$ -opioid receptor agonist, [D-Pen<sup>2,5</sup>] cyclic enkephalin (DPDPE),<sup>24,25</sup> were purchased from Peninsula Laboratories, Inc. (Belmont, CA). [<sup>3</sup>H]Naloxone, a competitive, relatively selective  $\mu$ -opioid antagonist,<sup>26,27</sup> was purchased from New England Nuclear (Wilmington, DE); (5 $\alpha$ ,7 $\alpha$ ,8 $\beta$ )-N-methyl-N-(7-[1-pyrrolidinyl]-l-oxaspiro[4,5] dec-8yl) benzeneacetamide (U-69593),<sup>28-30</sup> a highly selective  $\kappa$ -opioid receptor agonist, was purchased from Upjohn Company (Kalamazoo, MI). All other chemicals were reagent grade and purchased from Sigma Chemical Company (St. Louis, MO).

### ANIMALS

Male Sprague-Dawley rats (400–450 g) were purchased from Hilltop Laboratory Animals, Inc. (Scottsdale, PA). Two PE-10 lumbar intrathecal catheters were inserted *via* a slit in the atlantooccipital membrane, approximately 8–10 cm down to the lumbar enlargement. One catheter was used for intrathecal pertussis toxin injection and the other for the PL017 antinociceptive test. Rats with gross neurologic injury or fresh blood in their cerebrospinal fluid were eliminated from the study. The animals were housed individually and maintained on a 12-h light/night cycle. Food and water were freely available. All tests were performed during the light cycle. All experimental studies on animals were performed according to the Guiding Principles in the Care and Use of Animals, as accepted by the Council of the American Physiologic Society, and were approved by the Duke University Animal Care and Use Committee.

### PERTUSSIS TOXIN TREATMENT AND ANTINOCICEPTION ASSAY

Pertussis toxin was dissolved in sterile normal saline and stored at 4° C before use. The tail-flick test was used for antinociception evaluation. The heat intensity of the lamp was adjusted so that the mean latency response is near 4.0 s. For a group of 40 unmedicated rats, 3 days after delivery, the actual mean latency response was 3.7  $\pm$  0.6 s. This heat-intensity setting was used as the thermal stimulation for all additional work. An automatic cutoff time for the lamp at 10 s allows multiple tests without excessive tissue injury.<sup>31</sup> Rats were placed in plastic restrainers for drug injection and the antinociception assay.

The dose-response effect of pertussis toxin was established by intrathecal injection with various doses of pertussis toxin (0.125, 0.25, 0.5, 1, and 2  $\mu$ g/10  $\mu$ l), which were flushed with 10  $\mu$ l normal saline. Control animals received 20  $\mu$ l normal saline. One week after the toxin administration, the antinociceptive effect of PL017 (1  $\mu$ g/10  $\mu$ l/rat, intrathecally) was measured. In the time course study, 1  $\mu$ g pertussis toxin was injected as described. Control animals received an equal amount of normal saline. The basal threshold of tail-flick latency was measured in control and treated groups at 1, 3, 7, 14, and 21 days after pertussis toxin injection. The antinociceptive potency of intrathecally administered PL017 also was evaluated at 1, 3, 7, 14, and 21 days after injection of pertussis toxin.

The antinociceptive test was performed 30 min after the PL017 intrathecal injection, an interval in which maximal agonist effect was achieved. Rats then were killed, and the spinal cords were harvested for opioid receptor binding studies. Tail-flick test data were converted from latency response into maximum percent effect (MPE), with the following formula:

$$\text{MPE} = \frac{\text{response time unit} - \text{basal time unit}}{\text{cutoff time unit} - \text{basal time unit}} \times 100\%$$

Responses below the baseline or above the cutoff time were assigned values of 0% and 100% of MPE, respectively.

#### MEMBRANE PREPARATION

Rats were killed by decapitation, and the spinal cords were removed quickly and stored in a  $-70^{\circ}\text{C}$  freezer. Spinal cords were cut into cervicothoracic and lumbosacral segments, and membranes were prepared by homogenization with a PT20 Polytron (Kinematica, Luzern, Switzerland) (setting at 6) in 50 mM Tris-hydrochloric acid (pH 7.4) containing 50  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitor, 1 mM EDTA, 10  $\mu\text{g}/\text{ml}$  leupeptin, and 100  $\mu\text{M}$  phenylmethylsulfonyl fluoride. This standard buffer also was used as the binding assay buffer. The total membrane particulates were obtained by centrifugation at  $40,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ . The pellets were resuspended with the 5 mM Tris-hydrochloric acid buffer containing 50  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitor, 1 mM EDTA, 10  $\mu\text{g}/\text{ml}$  leupeptin, and 100  $\mu\text{M}$  phenylmethylsulfonyl fluoride and incubated three times with 100  $\mu\text{M}$  GDP and 100 mM NaCl for 30 min at room temperature to dissociate endogenous and exogenous opioids from the membranes. The membrane pellets then were washed three times with the standard buffer to remove NaCl and guanosine diphosphate (GDP), and the final membranes were resuspended in the standard buffer for binding studies. Previous work showed that this washing procedure removes almost all the endogenous and exogenous opioid peptides.<sup>10</sup>

#### OPIOID RECEPTOR BINDING STUDIES

Binding assays were performed in the standard buffer described above. We have found that, in control membranes washed with  $\text{Na}^+$  plus GDP and assayed in the presence of 5 mM  $\text{Mg}^{2+}$ , only one class of high-affinity  $\mu$ -receptor is observed.<sup>32</sup> This is the binding condition used in this study. All binding incubations were performed at room temperature for 90 min. A rapid filtration method was used to terminate the binding reaction.  $^{125}\text{I}$ -FK33824 ( $\sim 0.1$  nM) was the radiolabeled ligand used to measure the high-affinity state of  $\mu$ -opioid receptor, and nonspecific binding was determined by 10  $\mu\text{M}$  unlabeled FK33824. The incubation volume was 0.5 ml, and the protein concentration was approximately 500  $\mu\text{g}$ . In the displacement studies, [ $^3\text{H}$ ]naloxone ( $\sim 1.2$  nM) was used as the radiolabeled ligand. DPDPE (100 nM) and U-69593 (100 nM) were added to block  $\delta$ - and  $\kappa$ -opioid receptor sites. Unlabeled FK33824 (10  $\mu\text{M}$ ) was used to measure nonspecific binding. The protein concentration was approximately

500  $\mu\text{g}/\text{tube}$ , and the incubation volume was 1 ml. All binding assays were determined in triplicate. Data were analyzed with EBDA<sup>33</sup> and LIGAND<sup>34</sup> data analysis programs. The protein concentration was determined by the method of Lowry *et al.*,<sup>35</sup> with crystalline bovine serum albumin as the standard.

#### STATISTICAL ANALYSIS

Data are presented as mean  $\pm$  standard error of the mean for the given number of rats. Data were analyzed by analysis of variance. Significant differences of data between treatment and control groups were analyzed by Dunnett's test. A significant difference was defined as  $P < 0.05$ .

#### Results

##### EFFECT OF PERTUSSIS TOXIN ON PL017 ANTINOCICEPTION

Similar to observations by others,<sup>18-21</sup> pertussis toxin attenuated the efficacy of opioids to produce spinal antinociception in rats. Figure 1 shows that the antinociceptive effect of PL017 was decreased by pertussis toxin in a dose-dependent manner 1 week after treatment. The range of dose-response effects was rather narrow, and the maximum effect was found at 1  $\mu\text{g}$ . The intrathecal injection of a single 1- $\mu\text{g}$  dose of pertussis toxin did not produce any apparent behavioral deficit during the period of the experiment. At 2  $\mu\text{g}/\text{rat}$ , the toxin caused neurologic effects in some rats; the dose was not used in other experiments. We, as well as others,<sup>18-21</sup> did not observe any significant change in baseline latency during the period of pertussis toxin treatment ( $3.2 \pm 0.5$  s for control and

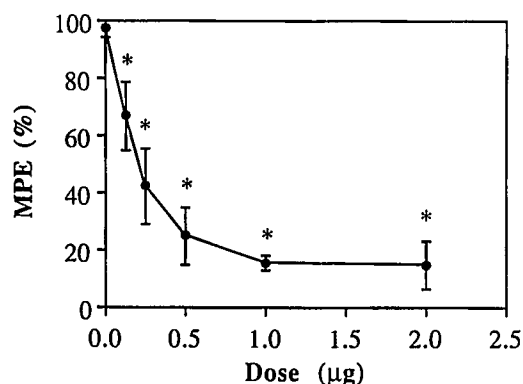


FIG. 1. Dose-dependent effect of pertussis toxin on PL017 antinociception. Rats were injected i.t. with different amounts of toxin as indicated on the graph. One week after administration of pertussis toxin the antinociceptive effect of PL017 (1  $\mu\text{g}/\text{rat}$ , i.t.) was measured by the tail-flick test and the data were converted from latency responses into MPE. Each point represents the mean  $\pm$  SEM of six rats. \* $P < 0.05$  as compared to control.

2.9 ± 0.8 s for the toxin-treated animals). Figure 2 shows the time course of the effect of 1 μg pertussis toxin on PL017 antinociception. This effect was evident 1 day after intrathecal injection. The maximum effect was reached at day 3 and lasted for 2 weeks. The antinociceptive effectiveness of PL017 recovered partially on the third week. These results led us to use 1 μg pertussis toxin in later experiments and to harvest spinal cords 1 week after pertussis toxin treatment to get the maximum effect for our opioid receptor binding studies.

The dose-response curves of the antinociceptive effect of PL017 shifted progressively to the right with increasing doses of pertussis toxin (fig. 3). The doses of PL017 that produce 50% MPE (ED<sub>50</sub>) were 0.23 μg for saline-control animals and 0.91, 1.59, and 4.06 μg after pertussis toxin treatment at 0.125, 0.25, and 0.5 μg, respectively. An ED<sub>50</sub> cannot be determined for animals treated with 1 μg pertussis toxin because PL017 did not produce 100% MPE even at the dose of 50 μg. At a higher dose (100 μg), PL017 produced muscle rigidity in animals, and the tail-flick response could not be measured.

#### EFFECT OF PERTUSSIS TOXIN ON <sup>125</sup>I-FK33824 BINDING

Next, we examined the receptor-binding activity of spinal cord membranes to determine the effect of pertussis toxin treatment. We previously showed that the surgical procedure and catheter implantation did not affect opioid receptor binding and that a bolus intrathecal injection of 1 μg/10 μl PL017 for the purpose of tail-flick testing did not affect the total number of binding sites in spinal cord membranes.<sup>10</sup> The membranes were washed extensively to remove exogenous and endogenous opioids that might interfere with the binding assay. The binding incubation

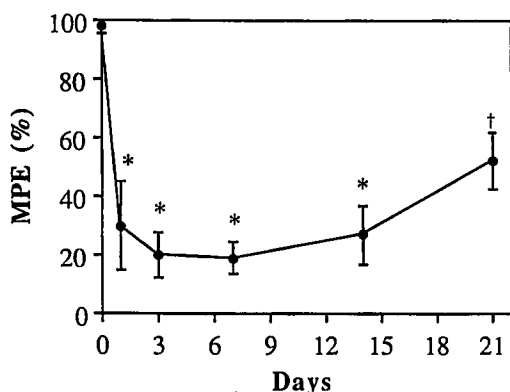


FIG. 2. Time course of pertussis toxin effect on PL017 antinociception. Rats received a single injection of pertussis toxin (1 μg/rat, i.t.) and the antinociceptive effect of PL017 (1 μg/rat, i.t.) was measured at different days after the toxin treatment. Each point represents the mean ± SEM of six rats. \**P* < 0.05.

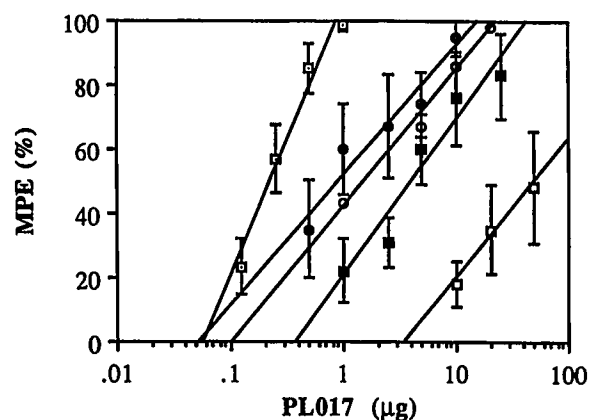


FIG. 3. The dose-response curves of PL017 antinociceptive effect after pertussis toxin treatment. Rats were i.t. treated with different doses of pertussis toxin: filled circle = 0.125 μg; open circle = 0.25 μg; filled square = 0.5 μg; open square = 1 μg; open square with dot = control. Control animals were treated with an equal amount of saline. The antinociceptive responses of PL017 were measured 1 week after the toxin administration. Each point represents the mean ± SEM of six rats.

was performed in the presence of 5 mM Mg<sup>2+</sup> to ensure that native receptors in control membranes remained in the high-affinity state.<sup>32</sup>

When the spinal cords from the animals injected with pertussis toxin were examined for receptor-binding activity, we found that the number of binding sites decreased to 70% of control (fig. 4B). The spinal cord membranes were prepared 3 days after toxin injection, when the rats were clearly tolerant by the MPE measurement (fig. 4A). The binding was performed with <sup>125</sup>I-FK33824, which measures only the high-affinity state of μ-opioid receptor with the binding conditions we used. The decrease in the high-affinity binding site was found only in the lumbosacral segment of spinal cords; there was no change in the membranes from the cervicothoracic segment of spinal cords (fig. 4B).

#### EFFECT OF PERTUSSIS TOXIN ON [<sup>3</sup>H]NALOXONE BINDING

The total number of opioid receptor-binding sites in the spinal cord membranes was examined with [<sup>3</sup>H]naloxone, which labels both high- and low-affinity receptor sites. One week after intrathecal injection of pertussis toxin (0, 0.125, 0.25, 0.50, and 1 μg), the displacement curves showed progressive right-hand shifts in a dose-dependent manner (fig. 5A), indicating alteration of receptor affinity for the μ-agonist FK33824. Scatchard analysis of the binding data showed that pertussis toxin treatment induced a class of low-affinity binding sites that was absent in the control membranes. In the control membranes, only one class of high-affinity binding sites

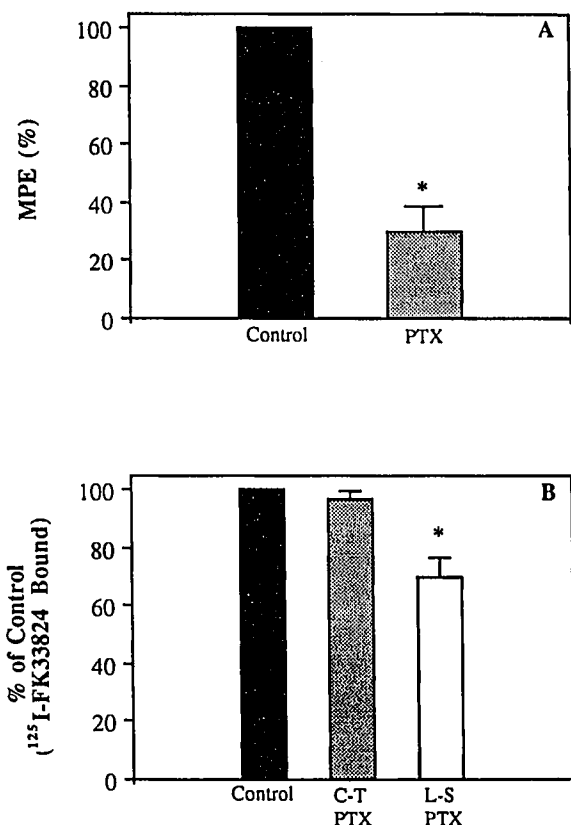


FIG. 4. The effect of pertussis toxin on PL017 antinociception and <sup>125</sup>I-FK33824 binding. A: Effect of a single injection of pertussis toxin (1  $\mu$ g/rat, i.t.) on the antinociceptive effect of PL017 (1  $\mu$ g/rat, i.t.) at day 3 after toxin injection. B: The receptor high-affinity binding sites of spinal cord membranes measured with <sup>125</sup>I-FK33824 as the radiolabeled ligand. Harvested spinal cords were divided into cervicothoracic (C-T/PTX) and lumbosacral (L-S/PTX) segments and membranes were prepared separately. Unlabeled FK33824 (10  $\mu$ M) was used to determine nonspecific binding. The results are expressed as the mean  $\pm$  SEM of six rats. \**P* < 0.05.

( $K_i = 1.5 \pm 0.9$  nM, where  $K_i$  is the antagonist dissociation constant) was found (table 1). With the toxin treatments, the low-affinity sites ( $K_i \geq 350$  nM) appeared, and the proportion of low-affinity sites increased with greater doses of pertussis toxin (table 1). The total number of binding sites remained constant. When the ratio of the low- to high-affinity sites is plotted against the pertussis toxin dose, a hyperbolic relationship is observed, with 1  $\mu$ g pertussis toxin inducing a near-maximal increase of the low-affinity sites (fig. 5B).

Because the membranes were prepared from rats that had undergone tail-flick testing, it was necessary to rule out the possibility that the shift in receptor affinity might have resulted from bolus saline or PL017 injection for the antinociception test. We found that, similar to findings in our previous study,<sup>10</sup> neither the total receptor number nor the receptor affinity was altered by saline or PL017 intrathecal bolus injections in animals before they were

killed (table 2). All the receptors in membranes from the naive animals were in the high-affinity state. The affinity of  $\mu$ -receptor for FK33824 is approximately 1–2 nM.

More important, the dose-effect of the toxin on the changes in receptor affinity correlates well with the effect on MPE changes (fig. 6). The antinociceptive efficacy of PL017 was maximal when the low-affinity binding site was undetectable in control animals, and the efficacy approached zero as the low-affinity receptors approached approximately 30% of total binding sites in pertussis toxin-treated animals. A hyperbolic curve was obtained when the  $ED_{50}$  of PL017 antinociception was plotted against the proportion of high-affinity receptors with various doses of pertussis toxin (fig. 7). Parallel to the increasing doses of pertussis toxin, the  $ED_{50}$  also increased.

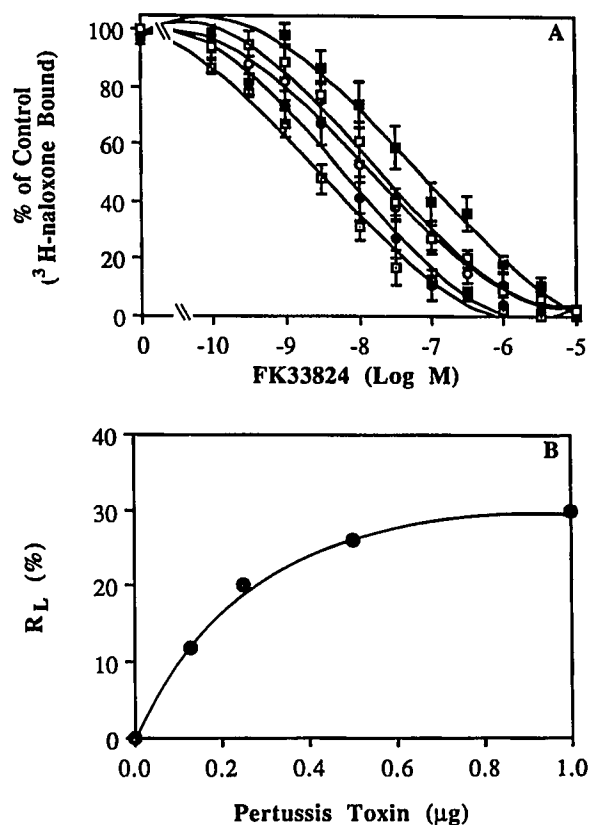


FIG. 5. Dose-dependent effect of pertussis toxin on FK33824 displacement curves. Rats received different doses of pertussis toxin intrathecally: open square with dot = control; filled circle = 0.125  $\mu$ g; open circle = 0.25  $\mu$ g; filled square = 0.5  $\mu$ g; open square = 1  $\mu$ g. One week later animals were killed by decapitation, and lumbosacral segments of the spinal cords were prepared for membranes. A: [<sup>3</sup>H]Naloxone was used as the radiolabeled ligand for the FK33824 displacement study. DPDPE (100 nM) and U-69593 (100 nM) were added in the binding buffer to block  $\delta$ - and  $\kappa$ -opioid receptor sites and 10  $\mu$ M unlabeled FK33824 was used to determine nonspecific binding. The results are expressed as the mean  $\pm$  SEM of five experiments performed in triplicate. B: The dose-dependent effect of pertussis toxin on the receptor affinity changes.

TABLE 1. The Dose-dependent Effect of Pertussis Toxin on  $\mu$ -Opioid Receptor Binding

	Control	PTX 0.125 $\mu$ g	PTX 0.25 $\mu$ g	PTX 0.5 $\mu$ g	PTX 1 $\mu$ g
IC <sub>50</sub> (nM)	3.2 $\pm$ 2.2	7.4 $\pm$ 2.5	9.0 $\pm$ 3.4	15.7 $\pm$ 6.7	22.1 $\pm$ 4.0
K <sub>i-h</sub> (nM)	1.5 $\pm$ 0.9	3.6 $\pm$ 1.6	3.0 $\pm$ 1.3	2.2 $\pm$ 2.2	3.9 $\pm$ 2.2
K <sub>i-l</sub> (nM)		350 $\pm$ 100	380 $\pm$ 90	1010 $\pm$ 570	610 $\pm$ 280
B <sub>H</sub> (fmol/mg)	27.9 $\pm$ 7.9	27.3 $\pm$ 5.2	23.4 $\pm$ 5.0	25.0 $\pm$ 7.4	15.6 $\pm$ 2.6
B <sub>L</sub> (fmol/mg)		3.9 $\pm$ 2.6	5.8 $\pm$ 1.8	8.4 $\pm$ 3.9	7.1 $\pm$ 1.7
B <sub>total</sub> (fmol/mg)	27.9 $\pm$ 7.9	31.2 $\pm$ 4.8	29.2 $\pm$ 4.1	33.4 $\pm$ 7.1	22.7 $\pm$ 2.4
B <sub>H/L</sub> (%)	100/0	88/12	80/20	76/24	70/30

Rats were injected with a single dose of 0.125, 0.25, 0.5, or 1  $\mu$ g pertussis toxin (PTX) intrathecally. Control animals were given the same amount of normal saline. One week later animals were killed and lumbosacral segments of spinal cords were harvested for membrane binding assays. The membranes were washed with Na<sup>+</sup> (100 mM) and GDP (100  $\mu$ M) three times. [<sup>3</sup>H]Naloxone was used as the radiolabeled

ligand and the nonspecific binding was determined by adding 10  $\mu$ M unlabeled FK33824. The  $\delta$ - and  $\kappa$ -opioid receptors were blocked by 100 nM of DPDPE and U-69593. Data were analyzed by EBDA and LIGAND programs. The results are expressed as mean  $\pm$  SEM of five separate experiments.

When the high-affinity sites were reduced to 76% of the total receptor sites, the ED<sub>50</sub> increased almost 20 times from the control value.

Finally, when rats were treated with 1  $\mu$ g toxin, the displacement curves also shifted progressively to the right in a time-dependent manner (fig. 8). The low-affinity sites began to appear on day 1 and reached 30% of total receptors at 1 week (table 3). The total number of binding sites remained constant. It should be observed that the emergence of low-affinity binding sites coincides with the reduction of MPE (fig. 2); both occurred on day 1 after pertussis toxin injection.

### Discussion

The current study demonstrates that intrathecal injection of pertussis toxin produces an acute and prolonged reduction of the antinociceptive effect of PL017. Our results confirm previous observations that *in vivo* pertussis toxin treatment attenuates the opioid antinociceptive effect. Parenti *et al.* reported that an intracerebroventricular injection of 0.5  $\mu$ g pertussis toxin inhibits the antinoci-

ceptive action of morphine in rats.<sup>18</sup> Similarly, pertussis toxin (0.5  $\mu$ g, intracerebroventricularly) reduces the efficacy of supraspinal opioid antinociception in mice.<sup>19</sup> Przewlocki *et al.* showed that intrathecal pertussis toxin treatment abolishes the antinociception mediated by opioid receptors in rat spinal cord in a dose- and time-dependent manner.<sup>21</sup> In *in vitro* studies, pertussis toxin has been shown to cause adenosine diphosphate ribosylation of G<sub>i</sub>/G<sub>o</sub> proteins and uncouple receptors from effectors.<sup>13-15</sup> The effect of the toxin on the antinociceptive action of morphine<sup>18-21</sup> could be the result of adenosine diphosphate ribosylation of G<sub>i</sub>/G<sub>o</sub> proteins, leading to receptors uncoupling and, thus, disruption the signal transduction. This proposed mechanism is supported by the findings of Przewlocki *et al.*,<sup>21</sup> who showed adenosine diphosphate ribosylation of spinal cord in an indirect way that appears to relate to reduced antinociceptive activity of opioids. Werling *et al.* also found evidence of receptor uncoupling in guinea pig when cortical membranes incubated with pertussis toxin *in vitro* induced a shift of  $\mu$ -

TABLE 2. The Effect of Saline and PL017 Intrathecal Injection on  $\mu$ -Opioid Receptor Binding

	Control	Saline Injection	PL017 Injection
IC <sub>50</sub> (nM)	3.2 $\pm$ 2.3	5.2 $\pm$ 3.3	2.4 $\pm$ 1.5
K <sub>i</sub> (nM)	1.5 $\pm$ 1.8	2.2 $\pm$ 2.3	1.4 $\pm$ 2.0
B <sub>total</sub> (fmol/mg)	38.1 $\pm$ 4.5	36.8 $\pm$ 5.4	31.6 $\pm$ 8.1

Rats were i.t. injected with saline or PL017 (1  $\mu$ g). The lumbosacral segments of spinal cords were harvested 1 week after injection. Membranes were prepared for  $\mu$ -receptor binding assays. [<sup>3</sup>H]Naloxone was used as the radiolabeled ligand and nonspecific binding was determined by adding 10  $\mu$ M FK33824 to the incubation. Receptor binding was performed in 50 mM Tris-HCl buffer containing 5 mM Mg<sup>2+</sup>. The  $\delta$ - and  $\kappa$ -opioid receptor sites were blocked by adding 100 nM of DPDPE and U-69593. Data were analyzed by EBDA and LIGAND programs. The results are expressed as mean  $\pm$  SEM of five separate experiments.

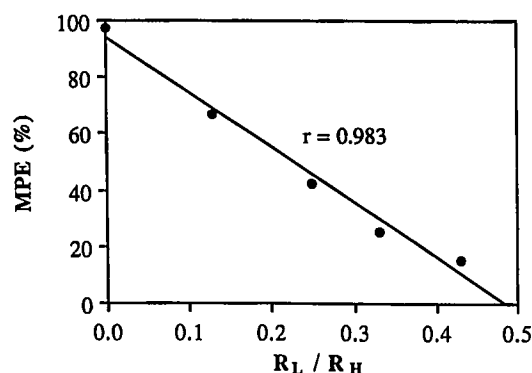


FIG. 6. The correlation between receptor affinity change and reduced antinociceptive response after pertussis toxin treatment. The antinociceptive effect of PL017 (1  $\mu$ g/rat, i.t.) was measured by the tail-flick test. The reduced antinociception (MPE) after various doses of pertussis toxin was obtained from figure 1. The ratios of low- to high-affinity states of  $\mu$ -opioid receptors were calculated from table 1.

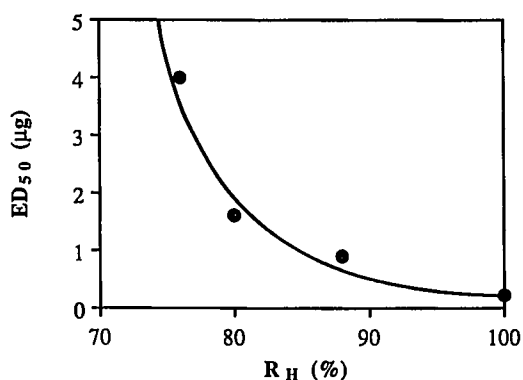


FIG. 7. The correlation between ED<sub>50</sub> of PL017 antinociception and high-affinity receptor after various doses of pertussis toxin treatment. The ED<sub>50</sub> values of PL017 antinociceptive effect were calculated from figure 3. The corresponding increases in the proportion of the high-affinity receptor were obtained from table 1. The data were from animals treated with 0.125, 0.25, and 0.5 µg of pertussis toxin and from control animals.

opioid receptor to the low-affinity state and a loss of guanine nucleotide regulatory effect on receptor agonist binding.<sup>17</sup>

In this *in vivo* study, we found that the attenuation of PL017 antinociceptive effect was accompanied by a loss of high-affinity sites as assayed with <sup>125</sup>I-FK33824. However, the total number of receptor sites was not affected by the toxin treatment, as demonstrated by binding studies performed with [<sup>3</sup>H]naloxone. Receptor binding studies of tissues from opioid-tolerant animals have yielded conflicting conclusions in the past. Results ranged from no change in the receptor density to down-regulation and up-regulation.<sup>7,8,17,36,37</sup> Certainly, factors such as tissue source, membrane preparation method, choice of radiolabeled ligand, and ionic environment in the binding buffer all affect the binding results. Therefore, a shift of receptor affinity state could be interpreted incorrectly as down-regulation where, in fact, there was no net loss of total binding site. In our study, the shift of [<sup>3</sup>H]naloxone displacement curves and Scatchard analysis of the data indicated that a shift in receptor affinity state occurred and a class of low-affinity receptors was generated; these receptors could not be converted to the high-affinity state by Mg<sup>2+</sup>, as in the control membranes. This is evidence that opioid receptors uncouple from G<sub>i</sub>/G<sub>o</sub> proteins in the presence of pertussis toxin. Consequently, the signal transduction is reduced, and the antinociceptive effect of opioids is decreased.

In the current study, only a fraction of opioid receptors were found in the low-affinity state after the toxin treatment. Werling *et al.* reported that after *in vitro* treatment with pertussis toxin, all the µ-opioid receptors were in the low-affinity state,<sup>17</sup> whereas we found a maximum of approximately 30% low-affinity receptors. The differences

between our results and those of Werling *et al.* may result, in part, from different membrane-washing methods and binding conditions. The more important factor might be that *in vitro* pertussis toxin treatment affects membrane receptors completely, whereas pertussis toxin injected intrathecally might not penetrate completely into the whole spinal cord. Przewlocki *et al.* also reported that a single dose of intrathecal pertussis toxin cannot penetrate completely into the whole lumbosacral segment of the spinal cord.<sup>21</sup> Thus, in our study, the toxin might have affected the spinal cord tissue only locally and, thus, could affect only a fraction of the G<sub>i</sub>/G<sub>o</sub> proteins at the lumbosacral segment of the spinal cord. This might explain why we found a maximum of 30% receptor conversion from the high- to low-affinity state in the lumbosacral segment, but not in the cervicothoracic segment, of the spinal cord in pertussis toxin-treated animals.

Law *et al.* demonstrated in neuroblastoma x glioma hybrid cells that *in vitro* pertussis toxin treatment also did not completely shift all the δ-receptor to the low-affinity state<sup>38</sup> and suggested that some δ-opioid receptors still might associate with G protein and form a high-affinity-state complex. However, the complex is less effective in inducing the GDP/GTP (guanosine triphosphate) exchange and activating the effector, and this might result in reduced opioid response. In the current study, the dose-response curves of the PL017 antinociceptive effect shifted progressively to the right after treatment with increasing doses of pertussis toxin. Concurrently, the num-

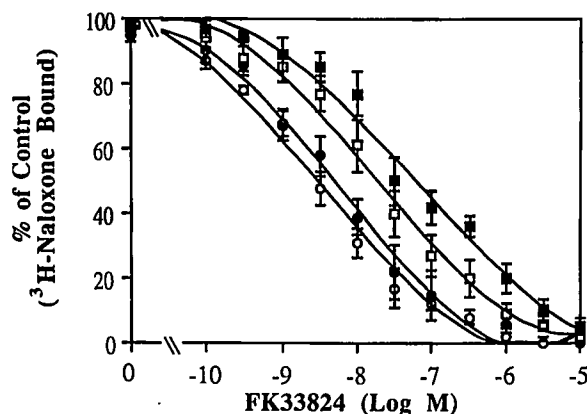


FIG. 8. Time course of pertussis toxin effect on FK33824 displacement curves. Rats received a single injection of pertussis toxin (1 µg/rat, i.t.). Spinal cords were prepared for the membrane binding assay at different times after the toxin injection. The curves represent the following: open circle = control; filled circle = 1 day after toxin injection; open square = 1 week after toxin injection; filled square = 2 weeks after toxin injection. Only the lumbosacral segments of spinal cords were used for the binding assay. [<sup>3</sup>H]Naloxone was used as the radiolabeled ligand. DPDPE (100 nM) and U-69593 (100 nM) were added to block the δ- and κ-opioid receptor sites. Unlabeled FK33824 (10 µM) was used to measure nonspecific binding. The results are expressed as the mean ± SEM of five experiments performed in triplicate.

TABLE 3. The Time Course of Pertussis Toxin Effect on  $\mu$ -Opioid Receptor Binding

	Control	PTX 1 Day	PTX 1 Week	PTX 2 Weeks
IC <sub>50</sub> (nM)	3.2 ± 2.2	5.1 ± 0.9	22.1 ± 4.0	60 ± 30
K <sub>i-h</sub> (nM)	1.5 ± 0.9	2.5 ± 0.7	3.9 ± 2.2	2.2 ± 0.7
K <sub>i-l</sub> (nM)		1330 ± 240	610 ± 280	1220 ± 420
B <sub>H</sub> (fmol/mg)	27.9 ± 7.9	23 ± 3.6	15.6 ± 2.6	15.3 ± 3.1
B <sub>L</sub> (fmol/mg)		5.1 ± 0.1	7.1 ± 1.7	7.1 ± 2.2
B <sub>total</sub> (fmol/mg)	27.9 ± 7.9	28.1 ± 2.5	22.7 ± 2.4	22.4 ± 2.6
B <sub>H/L</sub> (%)	100/0	79/21	70/30	68/32

Rats received a single injection of pertussis toxin (1  $\mu$ g/rat, intrathecal). The lumbosacral segment of spinal cords were harvested at different times and prepared for membrane binding studies. [<sup>3</sup>H]Naloxone was used as the radiolabeled ligand, and nonspecific binding was determined by adding 10  $\mu$ M FK33824 in the assay. The receptor binding

was performed in 50 mM Tris-HCl buffer containing 5 mM Mg<sup>2+</sup>. The  $\delta$ - and  $\kappa$ -opioid receptor sites were blocked by adding 100 nM of DPDPE and U-69593. Data were analyzed by EBDA and LIGAND programs. The results are expressed as mean  $\pm$  SEM of five separate experiments.

ber of high-affinity sites tended to decrease and the number of low-affinity sites tended to increase. The maximum of low-affinity sites was approximately 30% of the total receptor number. The correlation between ED<sub>50</sub>s and proportions of high-affinity receptors suggests a complex relationship that requires additional investigation. It is conceivable that the remaining high-affinity receptors may function differently than the original high-affinity receptors, as proposed by Law *et al.*<sup>38</sup>

Our previous study showed that opioid tolerance can be induced without apparent receptor down-regulation in the rat spinal cord.<sup>10</sup> Recently, we showed that receptor down-regulation can be induced with the opioid peptide PL017 and not with morphine.<sup>39</sup> However, in morphine-treated animals, approximately 30% of receptor sites appear to be low-affinity that were not found in control membranes. The guanine nucleotide regulatory effect on  $\mu$ -receptor agonist binding also is lost. The results of morphine-tolerant membranes are qualitatively and quantitatively similar to those of the membranes from pertussis toxin-treated animals and suggest that receptor uncoupling might be a mechanism of morphine-induced tolerance.<sup>39</sup>

In conclusion, pertussis toxin treatment caused a decrease in the PL017 antinociceptive effect and a reduction of high-affinity sites without changes in the total receptor number. These results are consistent with the concept that pertussis toxin treatment uncouples opioid receptors from G<sub>i</sub>/G<sub>o</sub> proteins and shifts some opioid receptors from the high-affinity state to the low-affinity state. This alteration of opioid affinity state correlates with a decrease in the opioid antinociceptive effect. Our study suggests that receptor uncoupling might be one of the mechanisms of opioid tolerance.

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