

Antinociceptive Effect of Morphine, but not μ Opioid Receptor Number, Is Attenuated in the Spinal Cord of Diabetic Rats

Shao-Rui Chen, M.D.* Hui-Lin Pan, M.D., Ph.D.†

Background: The mechanisms of decreased analgesic potency of μ opioids in diabetic neuropathic pain are not fully known. The authors recently found that G protein activation stimulated by the μ opioid agonist is significantly reduced in the spinal cord dorsal horn in diabetes. In the current study, they determined potential changes in the number and binding affinity of μ opioid receptors in the spinal cord in diabetic rats.

Methods: Rats were rendered diabetic with an intraperitoneal injection of streptozotocin. The nociceptive withdrawal threshold was measured before and after intrathecal injection of morphine by applying a noxious pressure stimulus to the hind paw. The μ opioid receptor was determined with immunocytochemistry labeling and a specific μ opioid receptor radioligand, [3 H]-(*D*-Ala²,*N*-Me-Phe⁴,Gly-ol⁵)-enkephalin ([3 H]-DAMGO), in the dorsal spinal cord obtained from age-matched normal and diabetic rats 4 weeks after streptozotocin treatment.

Results: The antinociceptive effect of intrathecal morphine (2–10 μ g) was significantly reduced in diabetic rats, with an ED₅₀ about twofold higher than that in normal rats. However, both the dissociation constant (3.99 \pm 0.22 vs. 4.01 \pm 0.23 nM) and the maximal specific binding (352.78 \pm 37.26 vs. 346.88 \pm 35.23 fmol/mg protein) of [3 H]-DAMGO spinal membrane bindings were not significantly different between normal and diabetic rats. The μ opioid receptor immunoreactivity in the spinal cord dorsal horn also was similar in normal and diabetic rats.

Conclusions: The reduced analgesic effect of intrathecal morphine in diabetes is probably due to impairment of μ opioid receptor–G protein coupling rather than reduction in μ opioid receptor number in the spinal cord dorsal horn.

DIABETIC neuropathy is one of the commonest long-term complications of diabetes mellitus. Painful diabetic neuropathy poses a major medical problem.^{1–3} Diabetic neuropathic pain can occur either spontaneously or as a result of exposure to only mildly painful stimuli (hyperalgesia) or to stimuli not normally perceived as painful (allodynia).^{1,3} Although μ opioids are widely used to treat patients with acute and chronic pain, they are often ineffective in the treatment of diabetic neuropathic pain.^{3,4} In this regard, several animal and human studies have shown a decreased analgesic potency of μ opioid agonists in diabetic neuropathic pain.^{4–8} The mecha-

nisms of the reduced analgesic effect of μ opioids on diabetic neuropathic pain are still not fully known.

The spinal cord is an important site for modulation of nociception by μ opioid receptor agonists.⁹ We recently found that the inhibitory effect of systemic morphine on spinothalamic tract neurons is substantially reduced in diabetic rats,¹⁰ suggesting a reduction in or dysfunction of μ opioid receptors in the spinal cord dorsal horn in diabetes. Zurek *et al.*¹¹ also showed that intrathecal injection of fentanyl has no analgesic action in diabetic rats. Although some studies demonstrated a reduction in spinal μ opioid receptors after peripheral nerve ligation,^{12,13} changes in the number and binding property of μ opioid receptors in the spinal cord in diabetic neuropathy have not been studied previously. Using a functional assay for the μ opioid receptor-activated G proteins, we found that [35 S]-guanosine 5'-triphosphate- γ S ([35 S]GTP γ S) binding stimulated by (*D*-Ala²,*N*-Me-Phe⁴,Gly-ol⁵)-enkephalin (DAMGO), a μ opioid receptor agonist, is significantly reduced in the spinal cord dorsal horn in diabetic rats.¹⁴ However, it remains unclear whether the reduction in DAMGO-stimulated [35 S]GTP γ S binding in diabetes is due to impaired receptor–G protein coupling or changes in the receptor number and affinity of μ opioid receptors in the spinal cord in diabetes. Therefore, in the current study, we directly compared the antinociceptive effect of intrathecal morphine in normal and diabetic rats. The potential changes in the number and binding property of μ opioid receptors in the dorsal spinal cord were determined in rats with diabetic neuropathy.

Materials and Methods

Induction of Diabetes and Surgical Preparations

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) initially weighing 220–240 g were used in this study. The Animal Care and Use Committee of the Pennsylvania State University College of Medicine approved the experimental protocols. Diabetes was induced by a single intraperitoneal injection of 50 mg/kg streptozotocin (Sigma, St. Louis, MO) freshly dissolved in sterile saline, 0.9%.^{10,15} One week later, diabetes was confirmed in streptozotocin-injected rats by measuring plasma glucose concentrations (> 350 mg/dl) in blood samples obtained from the tail vein. The glucose concentration was assayed enzymatically using diagnostic glucose reagents (Sigma). This experimental model of diabetes has been used as a relevant model of chronic neuropathic

* Research Associate, Department of Anesthesiology. † Professor and Director of Basic Research in Anesthesiology, Department of Anesthesiology and Department of Neural and Behavioral Sciences.

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Address reprint requests to Dr. Pan: Department of Anesthesiology, H187, Pennsylvania State University College of Medicine, 500 University Drive, Hershey, Pennsylvania 17033-0850. Address electronic mail to: hpan@psu.edu. Individual reprint requests may be purchased through the Journal Web site, www.anesthesiology.org.

pain.^{7,10,16} The dose of streptozotocin used and the body weight of the rats before streptozotocin treatment are two major factors to be considered when attempting to induce neuropathic pain symptoms but not profound illness in diabetic rats.^{7,10,16} A lower dose of streptozotocin was used to reduce the severity of diabetic illness to an acceptable level. It has been demonstrated that this method induces reproducible and reliable mechanical hyperalgesia and allodynia within 3 weeks, which last for at least 6 weeks after streptozotocin injection. Both age-matched normal rats (controls) and diabetic rats were used in this study.

For surgical implantation of intrathecal catheters, controls and diabetic rats were anesthetized with 2% halothane in O₂. The catheter was inserted through an incision in the cisternal membrane and advanced 8 cm caudal so that the tip of each catheter was positioned at the lumbar spinal level. The intrathecal catheter was externalized to the back of the neck and sutured to the musculature and skin at the incision site. The rats were used for behavior testing after a 5- to 7-day recovery following cannulation. All the behavioral experiments for diabetic rats were conducted between 3 and 5 weeks after streptozotocin injection.

Effect of Intrathecal Morphine on Nociception

Behavioral Measurement of Nociceptive Threshold. The mechanical withdrawal thresholds (expressed in grams) were measured by the Randall-Selitto test using an Analgesimeter (Ugo Basil, Varese, Italy). The test was performed by applying a noxious pressure to the hind paw. By pressing a pedal that activated a motor, the force increased at a constant rate on the linear scale. When the animal withdrew the paw or vocalized, the pedal was immediately released, and the nociceptive threshold was read on the scale. The cutoff of 400 g was used to avoid potential tissue injury.^{6,10} Both hind paws of each rat were tested, and the mean value was used as the withdrawal threshold in response to the noxious pressure.

After acclimation for 30 min, baseline withdrawal thresholds in response to the pressure applied to the hind paw were determined. The animals were then given an intrathecal injection of morphine (Astra Pharmaceuticals, Westborough, MA), and the mechanical threshold in response to the pressure stimulus was determined every 15–30 min for 4 h. The antinociceptive effect of intrathecal morphine (2, 5, and 10 μ g) was tested in both controls and diabetic rats. Each rat randomly received two intrathecal injections that were each separated by at least 4 days. The behavioral testing was conducted between 8:30 AM and 1:30 PM. The final catheter location was verified by transient paralysis of the hind limbs produced by intrathecal injection of 5 μ l lidocaine, 2%, before killing the animals with sodium pentobarbital (200 mg/kg, intraperitoneally).

In addition, to assess if the antinociceptive effect of morphine in this study was limited to the caudal spinal cord, we measured the effect of intrathecal morphine (5 μ g) in four separate controls on the paw withdrawal latency of the forelimb and hind limb in response to a radiant heat stimulus.¹⁰ The paw withdrawal latency of both forelimb and hind limb was measured every 30 min for 120 min after morphine injection.

Immunocytochemistry Labeling of μ Opioid Receptors in the Spinal Cord Dorsal Horn

Immunocytochemistry labeling of μ opioid receptors in the spinal cord dorsal horn was performed for three controls and three diabetic rats 4 weeks after streptozotocin injection. The hyperalgesia in all diabetic rats was first confirmed before the spinal cord tissue was removed. The rats were deeply anesthetized with intraperitoneal injection of 60 mg/kg sodium pentobarbital and perfused transcardially with paraformaldehyde, 4%, in 0.1 M phosphate-buffered saline (PBS) followed by sucrose, 10%, in 0.1 M PBS. The lumbar (L4–L6) spinal segment was quickly removed, postfixed for 2 h in the same fixative solution, and cryoprotected in sucrose, 30%, in 0.1 M PBS for 48 h at 4°C. The sections were cut to 25 μ m and collected freely floating in 0.1 M PBS. For μ opioid receptor immunofluorescent labeling, the sections were rinsed in 0.1 M PBS and blocked in normal goat serum, 4%, in PBS for 1 h. The sections were then incubated with the primary antibody (rabbit antibody to μ opioid receptor; dilution, 1:1,000; Neuromics, Inc., Minneapolis, MN)¹⁷ diluted in PBS containing 2% normal goat serum, 0.3% TX-100, and 0.05% Tween-20 for 2 h at room temperature and overnight at 4°C. Subsequently, sections were rinsed in PBS and incubated with the secondary antibody (Alexa Fluor-488 conjugated to goat antibody to rabbit IgG; dilution, 1:400; Molecular Probes, Eugene, OR). Finally, the sections were rinsed for 40 min, mounted on slides, and dried, and coverslips were applied. Control sections for μ opioid receptor immunolabeling were obtained by omission of the primary antibody in the reaction sequence. Sections were imaged using a charge-coupled device camera (Optronics, Goleta, CA) attached to a microscope (BX50; Olympus, Tokyo, Japan) and digitized using a computer. The absorbance of the immunoreactivity of μ opioid receptors in the superficial laminae of the dorsal horn was measured by a computer-based imaging analysis program (AIS; Imaging Research, Inc., St. Catharines, Ontario, Canada). The absorbance in the sections was analyzed by drawing regional boundaries to select the appropriate area of interest (laminae I–III), as we described previously.¹⁸ The area of immunoreactivity was first visualized and defined in the normal spinal cord section, and then it was applied to the corresponding sections from the diabetic rats. Measurements (relative absorbance) were

taken from 15–20 coronal sections of the lumbar spinal cord from each animal.

^3H -DAMGO Membrane Bindings in the Dorsal Spinal Cord

To determine the potential change in spinal cord μ opioid receptors in diabetes, [^3H]-DAMGO, a specific radioligand for μ opioid receptors,¹⁹ was used in the membrane binding of spinal cord tissues. Six diabetic rats (4 weeks after streptozotocin injection) and six controls were decapitated after being anesthetized with halothane. The spinal cord was quickly removed, and the dorsal half was dissected and used for the binding experiment. The tissue was thawed, quickly chopped, and suspended in ice-cold 50 mM Tris buffer containing 3 mM MgCl₂ and 1 mM EGTA (pH 7.4). The tissue was homogenized and disrupted by sonication. The homogenate was then centrifuged at 500g for 10 min at 4°C. The pellet was discarded, and the supernatant was centrifuged at 48,000g for 20 min at 4°C. The pellet was resuspended in fresh Tris buffer and centrifuged again as described above. The final pellet was resuspended in 50 mM Tris buffer containing 3 mM MgCl₂, 100 mM NaCl, and 0.2 mM EGTA (pH 7.7) and disrupted by sonication for 5 s. The protein content was measured based on the method of Bradford using bovine serum albumin as the standard (Protein Assay Kit II; Bio-Rad Laboratories, Hercules, CA). Saturation radioligand binding experiments were performed using 200- μl aliquots of tissue and increasing concentrations of [^3H]-DAMGO (2.81 TBq/mmol; Amersham Bioscience, Piscataway, NJ) from 0.2 to 13.5 nM. Nonspecific binding was determined with 1 μM naloxone (Sigma). Incubation was performed in duplicate in Tris buffer at 25°C for 60 min. The reaction was terminated by filtration through Whatman GF/B filters (Brandel, Gaithersburg, MD) on a cell harvester with cold Tris buffer (pH 7.4). Radioactivity was quantified by immersion of filters in the scintillation fluid, incubation overnight at room temperature, and measurement the next day using a liquid scintillation counter (LS 6500; Beckman Coulter, Inc., Fullerton, CA). The saturation binding data were processed using nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA) to calculate the maximal specific binding and the dissociation constant.

Data are presented as mean \pm SEM. The effect of intrathecal morphine on the paw withdrawal threshold was determined by repeated-measures ANOVA followed by Dunnett *post hoc* testing. To compare the dose-response effect of intrathecal morphine, data were converted to the percent maximal possible effect of morphine based on the following calculation: $[(\text{maximal effect} - \text{baseline}) / (\text{cutoff} - \text{baseline})] \times 100\%$. The Hill slope and ED₅₀ of morphine and their 95% confidence limits were determined by nonlinear regression analyses of the dose-response curves using Prism (GraphPad Soft-

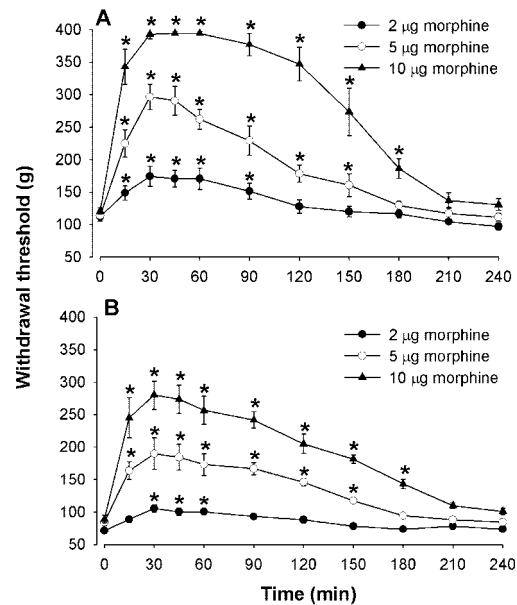


Fig. 1. (A) Time course of the effect of intrathecal injection of 2 (n = 8), 5 (n = 7), and 10 (n = 8) μg morphine on the nociceptive withdrawal threshold in normal rats. (B) Time course of the effect of intrathecal injection of 2 (n = 8), 5 (n = 9), and 10 (n = 8) μg morphine on the nociceptive withdrawal threshold in diabetic rats. The nociceptive threshold was determined by the withdrawal response of the hind paw to a noxious pressure stimulus. Data are mean \pm SEM. * $P < 0.05$ compared with the respective baseline control.

ware). The percent maximal possible effect data were analyzed using two-way ANOVA. The μ opioid receptor immunoreactivity and binding data for the normal control and diabetic groups were compared using the Student *t* test. $P < 0.05$ was considered to be statistically significant.

Results

All diabetic rats developed hyperglycemia within 1 week after streptozotocin injection; these rats had polyuria, a reduced growth rate, and a marked increase in food and water intake. The paw withdrawal threshold in response to the pressure stimulus was 112.3 ± 5.9 g before streptozotocin treatment for all diabetic rats in this study. The nociceptive mechanical threshold decreased significantly (74.5 ± 4.2 g; $P < 0.05$) 4 weeks after streptozotocin injection.

Effect of Intrathecal Morphine in Controls and Diabetic Rats

Intrathecal morphine (2–10 μg) in controls (n = 7 to 8) dose dependently increased the withdrawal threshold in response to the pressure stimulus (fig. 1A). The maximal effect of morphine appeared within 30 min after intrathecal injection, and the effect gradually diminished within 2 to 3 h. However, the antinociceptive effect of intrathecal morphine (2–10 μg) in diabetic rats (n = 8 to

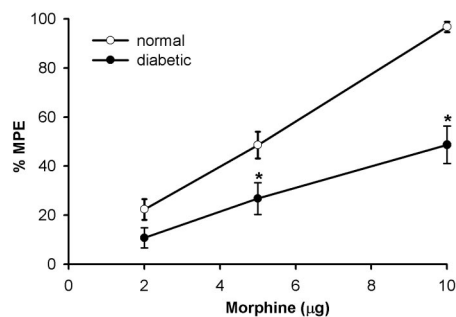


Fig. 2. Comparison of the dose–response effect of intrathecal morphine in normal and diabetic rats. Response is depicted as the percent maximal possible effect (% MPE). Values are mean \pm SEM ($n = 7$ – 9 animals). * $P < 0.05$ compared with the respective effect at the same dose in normal rats.

9) was significantly attenuated (fig. 1B) compared with that in controls. The antinociceptive effect of morphine in diabetic animals decreased significantly, with an ED_{50} increasing about twofold compared with that for controls (fig. 2). The Hill slope was 1.76 for the control group and 1.34 for the diabetic group. The estimated ED_{50} (95% confidence limits) of the effect of intrathecal morphine for the control and diabetic groups were 6.14 (2.39–24.78) and 13.37 (3.64–57.22) μg , respectively.

In four controls, intrathecal morphine (5 μg) increased significantly the withdrawal latency of the hind paw from 11.2 ± 2.3 to 22.4 ± 3.1 s in 30 min, and the effect of morphine lasted about 90 min. By contrast, morphine had no significant effect on the withdrawal latency of the forepaw within 120 min after drug injection. The withdrawal latency of the forepaw was 10.8 ± 2.5 s during the control and 10.5 ± 2.2 s 30 min after morphine injection.

Immunocytochemistry Labeling of Spinal μ Opioid Receptors in Controls and Diabetic Rats

The immunoreactivity of μ opioid receptors was mainly concentrated in the neuropil of the superficial laminae of the spinal cord dorsal horn in both controls and diabetic rats (fig. 3). The omission of the primary antibody resulted in negative labeling in the spinal cord. The density of μ opioid receptor immunoreactivity in laminae I–III of the spinal cord was not significantly different between controls and diabetic rats (relative absorbance: 0.35 ± 0.02 vs. 0.34 ± 0.01 ; $P > 0.05$).

Spinal [^3H]-DAMGO Binding in Controls and Diabetic Rats

We observed that [^3H]-DAMGO had a single and saturable high-affinity binding site in the dorsal spinal cord tissue in both controls and diabetic rats ($n = 6$ in each group; fig. 4). The dissociation constant was similar for control (3.99 ± 0.22 nM) and diabetic (4.01 ± 0.23 nM) groups. Furthermore, the maximal specific binding of [^3H]-DAMGO binding in diabetic rats ($346.88 \pm$

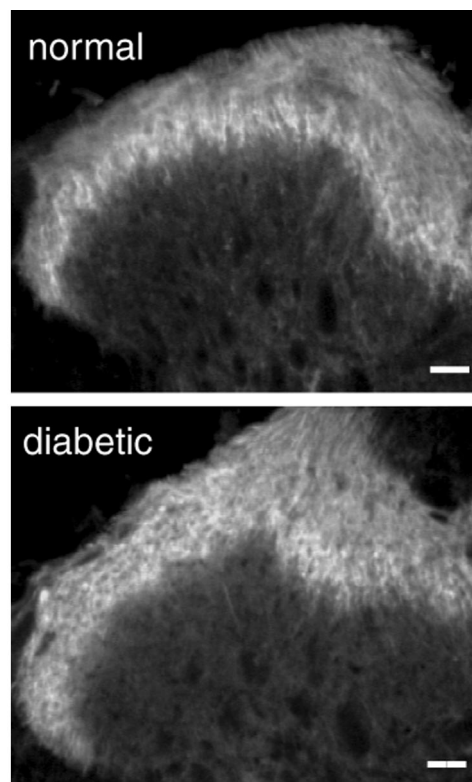


Fig. 3. Light microscopic view of μ opioid receptor immunoreactivity in the spinal cord dorsal horn in a normal (top) and a diabetic (bottom) rat. Densely labeled μ opioid receptor immunoreactivity is present in the superficial dorsal horn of the spinal cord. Scale bar = 100 μm .

35.23 fmol/mg protein) was not significantly different from that in controls (352.78 ± 37.26 fmol/mg protein; $P > 0.05$).

Discussion

In the current study, we first determined the potential changes in the antinociceptive effect of intrathecal morphine in a rat model of diabetic neuropathic pain. We found that the effect of intrathecal morphine was signif-

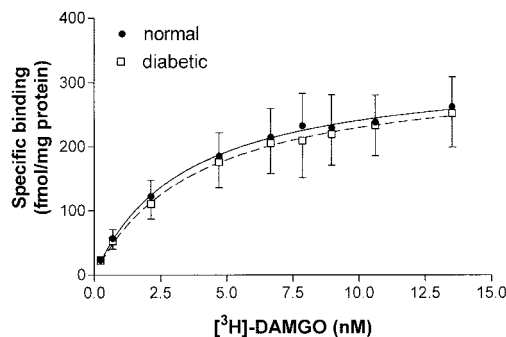


Fig. 4. Comparison of [^3H]-(D-Ala^2 , N-Me-Phe^4 , Gly-ol^5)-enkephalin ([^3H]-DAMGO) saturation binding in the dorsal spinal cord homogenate from normal and diabetic rats. Values are mean \pm SEM ($n = 6$ in each group).

icantly reduced in rats with diabetic neuropathy. In addition, we examined the density and binding affinity of μ opioid receptors in the spinal cord, using both immunocytochemistry and radioligand binding techniques for controls (age-matched normal rats) and diabetic rats. In contrast to the behavioral finding, the μ opioid receptor density and the maximal specific binding and dissociation constant of μ opioid receptors in the dorsal spinal cord were similar for controls and diabetic rats. Thus, these data suggest that the reduced analgesic action of intrathecal μ opioids in diabetic neuropathy is unlikely to be due to a decreased number of μ opioid receptors in the spinal cord. Considering the recent finding that DAMGO-stimulated [35 S]GTP γ S binding is largely attenuated in the spinal cord of diabetic rats,¹⁴ the reduced spinal analgesic action of μ opioids probably is caused by impairment of μ opioid receptor-G protein coupling. Therefore, this study provides supplemental new information about the mechanisms of reduced spinal μ opioid analgesia in diabetic neuropathic pain.

The μ opioid receptor is densely located in the superficial dorsal horn of the spinal cord,¹⁷ and spinally administered μ opioids produce potent analgesia.⁹ However, the analgesic potency of μ opioids is often reduced in patients with diabetic neuropathic pain. We found that the inhibitory effect of intravenous morphine on the evoked responses of spinal cord dorsal horn neurons to noxious stimuli is largely reduced in diabetic rats.¹⁰ Because the analgesic effect of intrathecal morphine has not been directly compared between age-matched normal and diabetic rats using the same stimulus, we conducted behavioral experiments to determine if the analgesic effect of intrathecal morphine is altered in diabetic rats. Although the percent increase in the withdrawal threshold produced by morphine appeared to be similar in the two groups, it is inappropriate to use such a measure to compare the opioid analgesia because a cutoff value was used and the baseline was different between these two groups. Thus, we used the calculated percent maximal possible effect to compare the antinociceptive effect of morphine between normal and diabetic rats in our study.

We observed that the antinociceptive effect of intrathecal morphine was significantly attenuated in diabetic rats. Because the withdrawal threshold in controls reached the cutoff after injection of 10 μ g morphine, the actual effect of morphine in these rats could have been underestimated based on the calculated percent maximal possible effect. Previous studies also showed that the analgesic potency of systemic μ opioid agonists is diminished in diabetic animals.^{6,8,10} The mechanisms of reduced spinal morphine analgesia in diabetes remain unclear. The μ opioid receptor signal transduction system requires activation of pertussis toxin-sensitive G_i and G_o proteins.^{20,21} After coupling to the inhibitory G proteins, μ opioid agonists elicit biologic actions

through decreased production of cyclic adenosine monophosphate, opening of G protein-gated inwardly rectifying potassium channels, and closing of voltage-gated calcium channels.^{22,23} In a recent study, we observed a significant reduction (37%) in DAMGO-stimulated [35 S]GTP γ S binding in the spinal cord dorsal horn in diabetic rats.¹⁴ Because the DAMGO-stimulated [35 S]GTP γ S binding is considered a functional assay to measure G protein activation by the μ opioid receptor, these data suggest that the attenuated analgesic effect of intrathecal morphine in diabetic rats may be due to reduced functional μ opioid receptors in the spinal cord dorsal horn.

However, the attenuated analgesic effect of intrathecal morphine in diabetes may be due to the altered number and binding property of μ opioid receptors or their signal transduction system in the spinal cord. In this regard, it is not clear if the decreased [35 S]GTP γ S binding stimulated by DAMGO in diabetes is caused by impairment of μ receptor-G protein coupling and/or alteration of μ opioid receptor density and affinity. Thus, in the current study, we further determined the potential change in the spinal cord μ opioid receptor in diabetes. Using the immunocytochemistry technique, we found that the opioid receptor immunoreactivity in the spinal cord dorsal horn was not significantly different between controls and diabetic rats. Furthermore, using a specific radioligand for the μ opioid receptor, [3 H]-DAMGO, we found that both the maximal specific binding site and the affinity of μ opioid receptors were not significantly altered in the dorsal spinal cord of diabetic rats. Thus, data from both immunocytochemistry and radioligand binding experiments strongly suggest that the reduced antinociceptive effect of intrathecal morphine on diabetic neuropathic pain is less likely due to a reduction of available μ opioid receptors in the spinal cord dorsal horn. The current study provides further support that the impaired functional coupling between μ opioid receptors and the G protein in the spinal cord in diabetes may contribute, at least in part, to the reduced analgesic action of spinally administered μ opioid agonists in diabetic neuropathic pain.

The mechanisms responsible for the impaired coupling between G proteins and the μ opioid receptor in the spinal cord of diabetic rats are not clear. We previously showed that the expression level of α subunits of inhibitory G proteins (G_i and G_o) in the lumbar spinal cord is similar in normal and diabetic rats.¹⁴ The dysfunction of G proteins is reflected by an increase in the basal [35 S]GTP γ S binding in the spinal cord dorsal horn in diabetic rats,¹⁴ probably caused by the altered affinity of G proteins for guanosine 5'-triphosphate and guanosine 5'-diphosphate in the spinal cord dorsal horn in diabetes. Interestingly, impaired functional coupling of adenosine receptors and G proteins also has been demonstrated in adipocytes in diabetes.²⁴ Some mecha-

nisms such as impaired regulation of cytosolic calcium may be involved in the loss of the action of μ opioids in diabetes.²⁵ In addition, insulin can affect phosphorylation of μ opioid receptors and G protein coupling efficacy.²⁶ In this regard, alteration of the tyrosine phosphorylation state of μ opioid receptors due to insulin deficiency in diabetes could constitute another mechanism of the reduced analgesic efficacy of μ opioids. Further studies are warranted to determine the mechanisms of impaired functional coupling between μ opioid receptors and G proteins in the spinal cord in diabetes.

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