Role of Spinal Nitric Oxide in the Inhibitory Effect of [D-Pen², D-Pen⁵]-Enkephalin on Ascending Dorsal Horn Neurons in Normal and Diabetic Rats

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ABSTRACT

Intrathecal [D-Pen²,D-Pen⁵]-enkephalin (DPDPE; a δ-opioid agonist) has a profound antinociceptive effect in neuropathic pain. Spinal nitric oxide (NO) has been implicated in the analgesic effect of several G protein-coupled receptor agonists. Little, however, is known about the role of spinal NO in the inhibitory effect of DPDPE on spinal dorsal horn neurons. In the present study, we determined the role of NO in the inhibitory effect of DPDPE on ascending dorsal horn neurons in normal rats and in a rat model of diabetic neuropathic pain. Single-unit activity of ascending dorsal horn neurons was recorded in anesthetized rats. The responses of dorsal horn neurons to graded mechanical stimuli and von Frey filaments were determined before and after local spinal application of 0.1 to 5 μM DPDPE. The influence of an NO synthase inhibitor, 1-(2-trifluoromethylphenyl)imidazole (TRIM; 30 μM), on the effect of DPDPE was then studied in separate groups of dorsal horn neurons in normal and diabetic rats. DPDPE inhibited the response of dorsal horn neurons in both normal and diabetic rats in a concentration-dependent fashion. The inhibitory effect of 1 μM DPDPE was abolished by 1 μM naltrindole, a δ-opioid antagonist. Furthermore, the inhibitory effect of DPDPE on the evoked response of dorsal horn neurons was largely eliminated by TRIM in normal and diabetic rats. These data suggest that DPDPE has a profound inhibitory effect on dorsal horn neurons in normal and diabetic rats. Spinal endogenous NO is essential for the inhibitory effect of DPDPE on ascending dorsal horn neurons in both normal and diabetic rats.

The spinal cord dorsal horn is an important site for transmission and modulation of nociception. Spinally administered μ- and δ-opioid receptor agonists produce potent analgesia (Heyman et al., 1987; Malmberg and Yaksh, 1992; Hurley et al., 1999). This is consistent with high levels of μ- and δ-opioid receptors in the spinal cord (Gourderes et al., 1985; Dickenson et al., 1987; Besse et al., 1991). Diabetic neuropathic pain, however, is often poorly relieved by μ-opioid receptor agonists in patients (Arner and Meyerson, 1988; Wright, 1994). Also, experiments performed in the rat model of diabetic neuropathic pain have consistently shown a reduced analgesic effect of μ-opioid agonists (Courteix et al., 1994; Maccagno and Tomlinson, 1998; Zurek et al., 2001). The underlying mechanisms of neuropathic pain are complex and probably include both the peripheral and central components. The presence of hypersensitivity of spinothalamic tract dorsal neurons has been demonstrated in a rat model of diabetic neuropathic pain (Chen and Pan, 2002). Thus, pharmacological suppression of hypersensitivity of dorsal horn neurons represents an important strategy for treatment of this neuropathic pain condition.

On the other hand, the δ-opioid receptor agonist [D-Pen²,D-Pen⁵]-enkephalin (DPDPE) probably is an important alternative for the treatment of neuropathic pain because of its increased analgesic potency, lowered abuse potential, and fewer adverse effects compared with the μ-opioids (Quock et al., 1999). In this regard, DPDPE produces an antinociceptive effect both at the spinal and supraspinal levels (Heyman et al., 1987; Kamei et al., 1992; Malmberg and Yaksh, 1992; Stewart and Hammond, 1993; Takemori and Portoghes, 1993; Hurley et al., 1999). The antinociceptive effect of intrathecal DPDPE has been demonstrated in neuropathic pain caused by diabetic neuropathy and sciatic nerve injury in rodents (Kamei et al., 1992; Mika et al., 2001). The increased analgesic potency of DPDPE in diabetic animals indicates that it has a potential for treatment of neuropathic pain in diabetic patients. It is well established that the effect of δ-opioid agonists is dependent on the coupling to inhibitory G

ABBREVIATIONS: DPDPE, [D-Pen²,D-Pen⁵]-enkephalin; NO, nitric oxide; NOS, nitric-oxide synthase; aCSF, artificial cerebrospinal fluid; nNOS, neuronal nitric-oxide synthase; TRIM, 1-(2-trifluoromethylphenyl) imidazole; imp, impulses.
proteins, and stimulation of δ-opioid receptors reduces intracellular cAMP levels and modulates the voltage-gated calcium and potassium channels (Quock et al., 1999). The analgesic mechanisms of DPDPE in the spinal cord, however, are not yet fully known, and the effect of DPDPE on dorsal horn projection neurons in neuropathic pain has not been examined previously.

Nitric oxide (NO) is involved in the antinoceptive effect of peripherally applied δ-opioid agonists in a rat model of inflammatory pain (Nozaki-Taguchi and Yamamoto, 1999b). Administration of NO donors also can enhance the analgesic effects of peripherally administered morphine (Nozaki-Taguchi and Yamamoto, 1998a). Nitric-oxide synthase (NOS)-containing neurons are present in the superficial layers of the spinal dorsal horn in rats (Valtschanoff et al., 1992). Some inhibitory interneurons in the dorsal horn are also known to contain neuronal NOS (Valtschanoff et al., 1992). Spinal endogenous NO is an important mediator for the analgesic actions of intrathecal muscarinic and α2 receptor agonists in rats (Iwamoto and Marion, 1994; Pan et al., 1998). Furthermore, spinal NO contributes to the antinoceptive action of systemic morphine in normal rats (Song et al., 1998). Little, however, is known about the role of endogenous NO in the effect of DPDPE on dorsal horn neurons in diabetic neuropathic pain. In the present study, we first examined the effect of DPDPE on spinal dorsal horn projection neurons in normal rats and in a rat model of diabetic neuropathic pain. Subsequently, the role of endogenous NO in the inhibitory effect of DPDPE on dorsal horn neurons was investigated in normal and diabetic rats.

Materials and Methods

General Procedures

Male rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 250 g were used in all experiments. The experimental procedures and protocols were approved by the Animal Care and Use Committee of Penn State University College of Medicine. All efforts were made to minimize the suffering and number of animals used. Diabetes was induced in rats by intraperitoneal injection of streptozotocin (50 mg/kg) (Chen et al., 2001; Chen and Pan, 2002). Electrophysiological experiments were conducted in age-matched normal and diabetic rats 4 to 5 weeks after streptozotocin treatment. Each diabetic rat was tested for mechanical allodynia on the day before the experiment was conducted using von Frey filaments (Chen et al., 2001; Chen and Pan, 2002). Diabetes was confirmed in streptozotocin-injected rats by measuring plasma glucose concentrations (>300 mg/dl) in blood samples obtained from the tail vein. The glucose level was measured using Sigma diagnostic glucose reagents (Sigma-Aldrich, St. Louis, MO). Only those diabetic rats showing tactile allodynia (threshold less than 6 g) were used in the electrophysiology study. This experimental model of diabetic neuropathic pain has been described as a relevant model of chronic pain with alterations of pain sensitivity and poor responses to μ-opioids (Courteix et al., 1994; Malcangio and Tomlinson, 1998; Zurek et al., 2001; Chen and Pan, 2002).

Anesthesia was initially induced with 2% halothane in 100% oxygen. The jugular vein and the common carotid artery were cannulated for intravenous drug administration and blood pressure monitoring, respectively. Following cannulation, sodium pentobarbital (40–50 mg/kg) was given intravenously, and the injection was repeated when necessary. The level of anesthesia was maintained at a sufficient level as judged by the absence of corneal reflexes, withdrawal reflexes to noxious pinch, and spontaneous blood pressure fluctuations. The trachea was cannulated, and the rat was ventilated mechanically. The respirator was adjusted to keep the end-tidal CO₂ concentration at 3 to 4%, monitored by a Capstar-100 CO₂ Analyzer (ITC, Inc./Life Science Instruments, Woodland Hills, CA). Laminectomies were performed to expose the spinal cord at the C1–2 and L2–5 levels. Around the exposed lumbar spinal cord, a small pool (~0.2 ml) was formed by the surrounding muscle and soft tissues to serve as a reservoir for application of drugs (Hyden and Wilcox, 1986). After the dura was removed at both sites, the spinal cord was covered with artificial cerebrospinal fluid (aCSF) solution. Mineral oil was then added on top of the aCSF to minimize evaporation. A bipolar, concentric metal stimulating electrode was inserted into the ventrolateral quadrant of the first cervical segment (Vandermeulen and Brennan, 2000). Dorsal horn neurons in the contralateral side of the lumbar enlargement were recorded with a glass electrode filled with 5% KCl solution (resistance, 4–6 MΩ). A hydraulic manipulator was used to gradually descend the recording electrode until an individual dorsal horn neuron was identified (Chen and Pan, 2002). The electrode was descended up to 1 mm in depth from the dorsal surface of the spinal cord.

Individual ascending dorsal horn neurons in the lumbar enlargement were antidromically identified and characterized. The search stimulus was 0.5 to 1.0 mA, 0.2 ms, and 0.8 to 1 Hz (S48 stimulator; Grass Instruments, Quincy, MA). The dorsal horn neurons were considered to be antidromically activated if the following criteria were met (Chen and Pan, 2002): 1) the antidromically evoked spikes occurred at a constant latency; 2) the antidromically evoked spikes followed a high-frequency (400 Hz) stimulation; and 3) the antidromic action potential collided with the orthodromic spike within the critical interval. Single-unit activity of the dorsal horn neuron was isolated using a software window discriminator (DataWave Technology, Longmont, CO). The action potential of the neuron was amplified, filtered with a band-pass filter (DAM 80; World Precision Instruments, Sarasota, FL), processed through an audioamplifier (model AM8; Grass Instruments, West Warwick, RI), and monitored on a storage oscilloscope (Tektronix, Inc., Beaverton, OR). The neuronal impulse activity also was recorded into a computer through an A/D interface board for subsequent off-line quantitative analysis. Discharge frequency was quantified by using a data acquisition and analysis software (Experimental Workbench; DataWave Technology, Inc.). After the cutaneous receptive field was located, responses of dorsal horn neurons to touch, pressure, and pinch applied to the receptive field were then determined, as we described previously (Chen and Pan, 2002). The touch stimulus was applied with a cotton tip for two to three back-and-forth cycles. The wooden tip of a cotton-tipped applicator was used to apply the pressure stimulus. The tip was applied perpendicularly to the skin for 5 to 6 s to generate an intense pressure (~200 g/mm²), which was perceived by the investigator as mildly painful. The pinch stimulus was applied by means of a small forceps with a strong grip (~560 g/mm²) that produces intense pressure (~60 g/mm²) that produces distinct pain when applied to human skin without causing tissue damage (Chen and Pan, 2002). Three types of dorsal horn neurons were identified according to their differential responses to mechanical stimulation applied to the receptive field: 1) low-threshold neurons, i.e., those which responded maximally to touch; 2) high-threshold neurons, i.e., those which responded only to noxious pinch; and 3) wide-dynamic-range neurons, i.e., those which responded to mechanical stimuli of touch, pressure, and pinch with an increasing order of magnitude (pinch > pressure > touch). Low-threshold neurons were not included in this study. In addition, responses to calibrated von Frey filaments of different bending forces (4, 15, 26, and 30 g; Stoeltig, Wood Dale, IL) applied to the receptive field of dorsal horn neurons were also examined (Chen and Pan, 2002). The filaments were applied in an ascending order, starting with the lowest bending force, each being applied for 5 s. Only one ascending dorsal horn neuron was studied in each rat.
Experimental Protocols

Inhibitory Effect of DPDPE on Dorsal Horn Neurons. The effect of DPDPE (0.1, 0.5, 1.0, and 5.0 μM) on identified ascending dorsal horn neurons was studied in 10 normal and 10 diabetic rats. After recording the background activity for 2 to 3 min, responses of dorsal horn neurons to touch, pressure, pinch, and von Frey filaments were examined. DPDPE, starting with the lowest concentration, was applied topically onto the recording site of the spinal cord after careful removal of aCSF from the pool (Hylden and Wilcox, 1986). Five minutes following DPDPE application, the response of neurons to mechanical stimuli was re-examined. The drug solution was then carefully removed, and the spinal cord was washed with aCSF. The procedure was then repeated to test the other concentrations of DPDPE. Adequate recovery time (15–20 min) was given between applications to allow the discharge activity of neurons to return to baseline control.

To ensure that the effect of DPDPE on dorsal horn neurons was through activation of δ-opioid receptors, the inhibitory effect of 1 μM DPDPE on dorsal horn neurons was further tested in the presence of 1 μM naltrindole, a selective δ-opioid receptor antagonist (Malmberg and Yaksh, 1992; Kohno et al., 1999). We chose a 1 μM concentration of DPDPE since this concentration of DPDPE yielded a consistent inhibitory effect on dorsal horn neurons in both normal and diabetic rats (see Results). The response of another six dorsal horn neurons to mechanical stimulation was determined before and 5 to 10 min after application of 1 μM naltrindole plus 1 μM DPDPE in normal rats. Naltrindole was applied to the spinal recording site 10 min before application of 1 μM DPDPE.

Role of Endogenous NO in the Inhibitory Effect of DPDPE. The influence of a specific nNOS inhibitor, 1-(2-trifluoromethylphenyl) imidazole (TRIM; 30 μM) (Handy et al., 1995; Pan et al., 1998), on the effects of 1 μM DPDPE was investigated in separate groups of normal (n = 6) and diabetic rats (n = 6). The response of dorsal horn neurons to graded stimuli and von Frey filaments was examined during control and 5 to 10 min after spinal application of DPDPE plus TRIM. DPDPE was applied 5 min after local application of TRIM. Responses of dorsal horn neurons to mechanical stimuli were determined using the same protocol as described above.

Streptozotocin, DPDPE, naltrindole, and TRIM were obtained from Sigma/RBI (Natick MA). Streptozotocin was prepared freshly by dissolving it in 0.9% sterile saline. All other drugs were dissolved in aCSF solution (126 mM NaCl, 2.5 mM KCl, 2.4 mM CaCl2·2H2O, 1.3 mM MgCl2·6H2O, 1.2 mM NaH2PO4, 11 mM glucose, 25 mM NaHCO3).

Statistical Analysis. Data are presented as mean ± S.E.M. The effects of various concentrations of DPDPE on dorsal horn neurons were compared using repeated measure analysis of variance followed by Dunnett’s post hoc test. Data involving a comparison between two groups were analyzed by Student’s t test. The baseline discharge rate of dorsal horn neurons was averaged during a 2-min control period and the evoked responses were quantified as the mean discharge rate over the duration of the stimulus after subtracting the background activity of the neurons. For calculation of ED50, data were converted to the percentage of the inhibitory effect of DPDPE based on the following calculation: [(evoked response during control – evoked response during DPDPE/evoked response during control) × 100%. The ED50 values of DPDPE and their 95% confidence limits were determined by nonlinear regression analyses of the concentration-response curves using GraphPad Prism (GraphPad Software, San Diego, CA). Differences were considered to be statistically significant when P < 0.05.

Results

Effect of DPDPE on Dorsal Horn Neurons. In 10 ascending dorsal horn neurons examined in normal rats, four were classified as high-threshold neurons and another six were considered wide-dynamic-range neurons. The discharge activity of dorsal horn neurons increased in a graded manner in response to touch, pressure, pinch, and von Frey filaments (Fig. 1). Topical application of 0.1 to 5 μM DPDPE to the spinal cord inhibited the evoked response of neurons to pressure, pinch, and von Frey filaments in a concentration-dependent manner (Fig. 1). DPDPE had a significant effect on pinch-evoked response of dorsal horn neurons only at 0.5 μM. The effect of DPDPE appeared in less than 2 min after spinal application. The evoked neuronal response began to return to control in less than 5 min after the DPDPE solution was removed and the spinal cord was flushed with aCSF. There was no significant difference between the inhibitory effect of DPDPE on the evoked response of dorsal horn neurons measured at 5 and 10 min following drug application (data not shown).

To examine whether the inhibitory effect of DPDPE was mediated by activation of δ-opioid receptors, the evoked response of another six dorsal horn neurons in normal rats was tested before and after application of 1 μM naltrindole plus 1 μM DPDPE in normal rats. Naltrindole was applied to the spinal recording site 10 min before application of 1 μM DPDPE.

Fig. 1. The concentration-dependent effect of 0.1 (n = 6), 0.5 (n = 8), 1 (n = 9), and 5 (n = 10) μM DPDPE on responses of dorsal horn neurons to touch, pressure, pinch (upper panel), and von Frey filaments (lower panel) in 10 normal rats. *, P < 0.05 compared with the respective controls.
μM DPDPE. In the presence of 1 μM naltrindole, the inhibitory effect of 1 μM DPDPE on the evoked response of six dorsal horn neurons was completely abolished (Fig. 2).

All diabetic rats exhibited classical symptoms of diabetes, including polyuria and weight loss following streptozotocin injection. All of the 10 ascending dorsal horn neurons examined in diabetic rats were classified as wide-dynamic-range neurons. The baseline discharge activity of dorsal horn neurons was increased significantly in diabetic rats (2.25 ± 0.35 imp/s; n = 10) compared with that in normal (0.51 ± 0.09 imp/s; n = 10) rats. The evoked responses of the 10 dorsal horn neurons to touch, pressure, pinch, and von Frey filaments in diabetic rats also were significantly augmented compared with those in normal rats (Figs. 1 and 3). In diabetic rats, topical application of 0.1 to 5 μM DPDPE significantly inhibited the response of dorsal horn neurons to touch, pressure, pinch, and von Frey filaments in a concentration-dependent fashion (Fig. 3). The onset latency and time course of the inhibitory effect of DPDPE following spinal application in diabetic rats were similar to those observed in dorsal horn neurons in normal rats.

The inhibitory effect of DPDPE on evoked responses of ascending dorsal horn neurons to graded mechanical stimuli in diabetic rats increased significantly, with an ED\textsubscript{50} value decreasing at least 10-fold compared with that in normal rats (Table 1). Since the dorsal horn neurons in diabetic rats had a much greater evoked response during control than that in normal rats, we further analyzed the effect of DPDPE by normalizing the control evoked response in diabetic animals to that in normal rats. Even after correction for the difference in the control evoked response of dorsal horn neurons, the inhibitory effect of DPDPE was still potentiated in diabetic rats.

The inhibitory effect of DPDPE on evoked responses of ascending dorsal horn neurons to graded mechanical stimuli

![Fig. 2. Effect of naltrindole (1 μM) on the inhibitory effect of DPDPE (1 μM) on six dorsal horn neurons in response to touch, pressure, pinch (upper panel), and von Frey filaments (lower panel) in normal rats.](image)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>EC\textsubscript{50} of DPDPE (95% Confidence Limits)</th>
<th>μM</th>
<th>Diabetic (Normalized)</th>
</tr>
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<tbody>
<tr>
<td>Touch</td>
<td>1.08 (0.47–4.98)</td>
<td>0.09 (0.04–0.19)</td>
<td>0.23 (0.07–1.40)</td>
</tr>
<tr>
<td>Pressure</td>
<td>1.06 (0.29–4.94)</td>
<td>0.09 (0.05–0.19)</td>
<td>0.21 (0.08–1.17)</td>
</tr>
<tr>
<td>Pinch</td>
<td>1.09 (0.47–2.53)</td>
<td>0.08 (0.04–0.16)</td>
<td>0.24 (0.04–1.20)</td>
</tr>
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rats, with estimated ED$_{50}$ values of DPDPE at least 5-fold lower than those in normal rats (Table 1).

**Role of Endogenous NO in the Inhibitory Effect of DPDPE on Dorsal Horn Neurons.** In six dorsal horn neurons recorded in normal rats, 30 μM TRIM alone did not significantly alter the baseline discharge activity of these neurons at 5, 10, and 30 min following topical spinal application. The baseline discharge activity before and 5 min after application of TRIM was not significantly altered (0.77 ± 0.24 versus 0.81 ± 0.22 imp/s; $P > 0.05$; $n = 6$). The evoked responses of dorsal horn neurons to mechanical stimuli following application of TRIM also were not significantly different from those responses recorded during the control (data not shown). Considering the evoked response of dorsal horn neurons during control as 100%, data obtained during application of 1 μM DPDPE and TRIM plus DPDPE were expressed as percent inhibition of control. In the presence of 30 μM TRIM, the inhibitory effect of 1 μM DPDPE on the evoked response of six dorsal horn neurons in normal rats was significantly attenuated (Figs. 4 and 5).

Furthermore, local application of 30 μM TRIM alone failed to alter significantly the baseline activity and the evoked responses of six ascending dorsal horn neurons in diabetic rats (data not shown). In the presence of 30 μM TRIM, the inhibitory effect of 1 μM DPDPE on the response of six dorsal horn neurons to graded mechanical stimulation in diabetic rats was largely eliminated (Fig. 6).

**Discussion**

This is the first study to directly examine the effect of DPDPE on the ascending dorsal horn neurons and the role of endogenous NO in the inhibitory effect of DPDPE in normal rats and a rat model of diabetic neuropathic pain. We found that spinal application of DPDPE produced a profound inhibitory effect on the response of dorsal horn neurons to mechanical stimuli in a concentration-dependent manner in both normal and diabetic rats. The inhibitory effect of DPDPE on dorsal horn neurons was completely abolished by a specific μ-opioid receptor antagonist, naltrindole. Furthermore, pretreatment with a selective nNOS inhibitor, TRIM, largely eliminated the inhibitory effect of DPDPE on dorsal horn neurons in both normal and diabetic rats. Therefore, these data indicate that activation of spinal δ-opioid receptors produces a profound inhibitory effect on ascending dorsal horn neurons in normal rats and rats with diabetic neuropathy. This study also provides new information that endogenous NO in the spinal cord plays an important role in the inhibitory effect of DPDPE on ascending dorsal horn neurons in normal and neuropathic pain states.

Both peripheral and central mechanisms are probably re-
sponsible for the neuropathic pain syndromes in diabetic patients (Burchiel et al., 1985; Chen and Pan, 2002). Similar to what has been studied on spinothalamic tract neurons (Chen and Pan, 2002), we observed that the ascending dorsal horn neurons in diabetic rats had a higher baseline activity and an increased responsiveness to graded mechanical stimuli applied to the receptive field of neurons. These data suggest that hypersensitivity of dorsal horn neurons may contribute to the development of mechanical allodynia in diabetes. It has been shown that direct application of glutamate receptor agonists to the spinal cord can induce hyper-sensitivity of dorsal horn neurons (Dougherty and Willis, 1991). Thus, increased glutamate release may lead to an augmented excitatory tone within the spinal cord in diabetic neuropathic pain (Malencangio and Tomlinson, 1998). This augmented response of dorsal horn neurons to mechanical stimuli may be maintained by the increased excitatory glutamatergic input from primary afferents to dorsal horn neurons in diabetic rats.

Many studies have demonstrated the presence of \( \mu \)- and \( \delta \)-opioid receptors in the superficial dorsal horn in rats and humans (Gouarderes et al., 1985; Besse et al., 1991). In the superficial dorsal horn, the majority of opioid receptor binding sites are \( \mu \)-opioid receptors, whereas \( \delta \)-opioid binding sites are present in moderate density (Besse et al., 1991). Several studies have shown that the antinociceptive effect of \( \mu \)-opioids are reduced in diabetic animals (Courteix et al., 1994; Malecangio and Tomlinson, 1998; Zurek et al., 2001). In support of the above behavioral findings, we have shown that the inhibitory effect of systemic morphine on spinothalamic tract neurons is diminished in diabetic rats (Chen and Pan, 2002). Although the mechanisms of diminished analgesic effect of \( \mu \)-opioids in diabetes are not fully known, we recently have shown that the functional \( \mu \)- but not \( \delta \)-opioid receptors are significantly reduced in the spinal dorsal horn of diabetic rats (Chen et al., 2002). Intrathecal administration of DPDPE produces potent analgesia in animals (Heyman et al., 1987; Malmberg and Yaksh, 1992; Stewart and Hammond, 1993), and the potent antinociceptive action of DPDPE was still retained in animal models of neuropathic pain (Kamei et al., 1992; Mika et al., 2001). The binding affinity of DPDPE for the \( \delta \)-opioid receptor is 175 times greater than that for the \( \mu \)-opioid receptor (Mosberg et al., 1983). We observed that the inhibitory effect of DPDPE on dorsal horn neurons was completely abolished by the specific \( \delta \)-opioid receptor antagonist naltrindole, suggesting that the effect of DPDPE is mediated by \( \delta \)-opioid receptors in the spinal cord. In this study, we compared the potential effect of DPDPE on ascending dorsal horn neurons in both normal and diabetic rats. We observed that the potent inhibitory effect of DPDPE on dorsal horn neurons was present in both normal and diabetic rats. In fact, the inhibitory effect of DPDPE on dorsal horn neurons appears to be enhanced in diabetic rats. We found that the effect of DPDPE on evoked responses of ascending dorsal horn neurons to graded mechanical stimuli in diabetic rats increased significantly, with an \( ED_{50} \) value decreasing at least 10-fold compared with that in normal rats. Although the increased potency of DPDPE in diabetic rats may be partially due to the potentiated responsiveness of dorsal horn neurons to mechanical stimuli, the inhibitory effect of DPDPE was still potentiated in diabetic rats, with estimated \( ED_{50} \) values of DPDPE at least 5-fold lower than those in normal rats. DPDPE can presynaptically reduce the synaptic glutamate release onto dorsal horn neurons in the spinal cord (Glaum et al., 1994; Kohno et al., 1999). Postsynaptically, DPDPE can directly inhibit the dorsal horn neurons through activation of \( \delta \)-opioid receptors and G protein-gated potassium channels (Ikeda et al., 1995). Since increased glutamate release from primary afferent central terminals to dorsal horn neurons may contribute importantly to the development of hypersensitivity of dorsal horn neurons and tactile allodynia in diabetic neuropathic pain, the potentiated effects of DPDPE on dorsal horn neurons in diabetic rats at least could be explained, in part, by the profound inhibitory effect of DPDPE on augmented glutamatergic excitatory synaptic inputs to spinal dorsal horn neurons in diabetic rats.

Several studies have shown that spinal NO is involved in antinociception produced by several G protein-coupled receptor agonists. In this regard, the antinociceptive effect of morphine and an \( \alpha_2 \) agonist, clonidine, is dependent on NO in the spinal cord (Pan et al., 1998; Song et al., 1998). Also, the analgesic effect of intrathecal muscarinic agonists is mediated by NO (Iwamoto and Marion, 1994). Because the \( \delta \)-opi-
iod receptors are coupled to inhibitory G proteins, we reasoned that NO may be involved in the inhibitory effect of DPDPE on dorsal horn neurons. In the present study, we found that pretreatment with a specific nNOS inhibitor, TRIM, largely abolished the inhibitory effect of DPDPE on dorsal horn neurons in normal and diabetic rats. These data suggest that spinal endogenous NO plays an essential role in mediating the inhibitory effect of δ-opioid agonists on dorsal horn neurons. This finding provides further support for the notion that generation of NO represents a common signal transduction pathway of G protein-coupled receptor agonists (Christopoulos and El-Fakahany, 1999). It should be recognized that the exact neuronal sources of NO produced by activation of δ-opioid receptors in the spinal cord and NO species involved in the inhibitory effect of DPDPE remain unclear.

We observed that treatment with TRIM alone had no effect on the baseline activity and the evoked responses of dorsal horn neurons in both normal and diabetic rats. This observation is consistent with the behavioral data that intrathecal injection of TRIM alone has no effect on the nociceptive withdrawal threshold in normal rats and animal models of neuropathic pain (Pan et al., 1998; Song et al., 1998; Chen et al., 2001). It has been reported, however, that treatment with a nonspecific NOS inhibitor, Nω-nitro-L-arginine methyl ester, increased the background activity of dorsal horn neurons in normal rats (Hoheisel et al., 2000). One of the possibilities for this discrepancy is that we selectively studied the dorsal horn projection neurons in this study. On the other hand, Hoheisel et al. (2000) did not differentiate between dorsal horn projection neurons and interneurons in their study. Several other methodological differences, including animal preparations and different NOS inhibitors used, also may account for this discrepancy. It should be acknowledged that there is some evidence suggesting that spinal NO may be involved in pain induction. In this regard, epidural injection of L-arginine produces a slowly developing thermal hyperalgesia in rats (Masue et al., 1999). Also, intrathecal administration of NOS inhibitors attenuates hyperalgesia and allodynia caused by inflammation in rats (Meller et al., 1992).

Further, it has been shown that pretreatment but not post-treatment with intrathecal NO inhibitors delays the development of thermal hyperalgesia induced by sciatic nerve constriction in rats (Yamamoto and Shimoyama, 1995). At the present time, it is difficult to reconcile the different roles of spinal NO in nociception and antinociceptive actions produced by G protein-coupled receptor agonists. Different NO species formed in the spinal cord may be involved in the opposing actions of NO mentioned in the above studies. For instance, nitrosothiol donors, but not pure NO donors, can alter synaptic neurotransmission (Pan et al., 1996). We have found that spinal NO interacts with L-cysteine to produce an antiallodynic effect through formation of S-nitrosothiols in a rat model of neuropathic pain (Chen et al., 2000). Further studies on the interaction between different redox-related NO species and G protein-coupled receptors in the spinal cord should shed light on the seemingly conflicting actions of NO.

In summary, we found that the δ-opioid receptor agonist DPDPE produced a profound inhibitory effect on ascending dorsal horn neurons in normal rats and a rat model of diabetic neuropathic pain. We also have demonstrated that spinal NO may be involved in the inhibitory effect of DPDPE on dorsal horn neurons in both normal and diabetic rats. This study provides further evidence that spinally administered δ-opioid receptor agonists may provide an important alternative therapy for patients with diabetic neuropathic pain. Furthermore, our data suggest that releasing endogenous NO is an important mechanism underlying the inhibitory effect of DPDPE on dorsal horn neurons. Thus, endogenous NO probably plays an obligatory role in the antinociceptive action of δ-opioid receptor agonists in the spinal cord during normal and neuropathic pain conditions.

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