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Upregulation of Spinal Muscarinic Receptors and Increased Antinociceptive Effect of Intrathecal Muscarine in Diabetic Rats

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Abstract

Spinally administered muscarinic receptor agonists or acetylcholinesterase inhibitors produce effective pain relief. Intrathecal injection of a small dose of neostigmine produces a profound antiallodynic effect in rats with diabetic neuropathy. However, the mechanisms of increased antinociceptive effect of cholinergic agents on diabetic neuropathic pain are not clear. In the present study, we tested the hypothesis that spinal muscarinic receptors are upregulated in diabetes. The withdrawal threshold of the hindpaw in response to noxious heat and pressure stimuli was determined in streptozotocin-induced diabetic and age-matched normal rats. Muscarine-stimulated [^{35}S]GTPγS binding was used to assess the change of functional muscarinic receptors in the spinal cord in diabetes. The [^{3}H]AF-DX 384 membrane binding was performed to determine the number and affinity of spinal cord M2 muscarinic receptors in normal and diabetic rats. We found that the antinociceptive effect of intrathecal 2-12 μg muscarine in diabetic animals was potentiated significantly compared to that in normal animals. The maximal muscarine-stimulated [^{35}S]GTPγS binding was 112.5 ± 8.3% in normal rats and 168.8 ± 12.1% ($P<0.05$) in diabetic rats. Although the K_D value (2.9 nM) was similar in both groups, the B_{max} of [^{3}H]AF-DX 384 membrane binding was significantly higher in diabetic than in normal rats (255.2 ± 5.9 vs. 165.9 ± 3.5 fmol/mg protein, $P<0.05$). Collectively, these data strongly suggest that the muscarinic receptor is upregulated in the dorsal spinal cord in diabetic rats. This finding probably accounts for the increased efficacy of the antinociceptive effect of intrathecal muscarinic agonists in diabetic neuropathic pain.

Neuropathic pain is an important and common complication of diabetes and remains difficult to manage (Brown and Asbury, 1984; Clark and Lee, 1995). Pain associated with diabetic neuropathy 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) (Brown and Asbury, 1984). Diabetic neuropathic pain is often chronic and difficult to treat since it is resistant to traditional analgesics such as opioids (Wright, 1994; Boulton and Malik, 1998). The mechanisms of diabetic neuropathic pain are still unclear. The dorsal horn of the spinal cord is an important site for pain transmission as well as modulation. In addition to abnormal activity from damaged primary afferent nerves (Burchiel et al., 1985; Khan et al., 2002), hypersensitivity of spinal cord dorsal horn neurons also likely contributes to the development of chronic neuropathic pain in diabetes (Chen and Pan, 2002). Thus, suppression of the hypersensitivity of dorsal horn neurons represents an important therapeutic strategy to alleviate diabetic neuropathic pain symptoms.

The spinal cholinergic system and muscarinic receptors are important for the regulation of different physiological functions including nociception. Intrinsic cholinergic innervation has been demonstrated in the spinal cord. For example, neurons and nerve terminals expressing choline acetyltransferase and acetylcholinesterase (enzymes for acetylcholine synthesis and degradation) are located in the spinal cord dorsal horn (Ribeiro-da-Silva and Cuello, 1990; Wetts and Vaughn, 1994). Also, intrathecal muscarinic receptor agonists or acetylcholinesterase inhibitors produce antinociception in both animals and humans (Naguib and Yaksh, 1994; Hood et al., 1997; Naguib and Yaksh, 1997). The analgesia produced by muscarinic receptor agonists or acetylcholinesterase inhibitors is blocked by the muscarinic antagonist atropine (Naguib and Yaksh, 1994).

Autoradiographic studies have demonstrated that the highest density of muscarinic receptors in the spinal cord is located in the superficial laminae in both rats and humans (Yamamura et al., 1983; Villiger and Faull, 1985). We recently have found that intrathecal injection of a small dose of neostigmine produces a profound antiallodynic effect in diabetic rats (Chen et al., 2001). However, the mechanisms underlying the increased antinociceptive potency of intrathecal cholinergic agents in diabetic rats are still unclear. In the present study, using a rat model of diabetic neuropathic pain, we tested the hypothesis that the spinal cord muscarinic receptors are upregulated in diabetes.

Materials and Methods

Induction of diabetes and implantation of intrathecal catheters

Male rats (Harlan Sprague-Dawley) weighing 225-250 g were used in this study. The surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine. Diabetes was induced by a single intraperitoneal injection of streptozotocin (50 mg/kg) freshly dissolved in 0.9% sterile saline. Two days later, diabetes was confirmed by measuring the blood glucose level (> 350 mg/dl) using the Sigma diagnostic glucose reagents (St. Louis, MO). Previous studies have demonstrated that after streptozotocin injection rats display a reproducible mechanical allodynia and hyperalgesia within 3 weeks, lasting for at least 7 weeks (Chen and Pan, 2002; Khan et al., 2002). 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 allodynia but not profound illness in diabetic rats (Chen et al., 2001; Chen and Pan, 2002; Khan et al., 2002). A lower dose of streptozotocin was used to reduce the severity of diabetic illness to an acceptable level. This experimental model of diabetic neuropathic pain has been considered as a relevant clinical model of chronic pain, with alterations of pain sensitivity and poor responses to μ opioids administered systemically or intrathecally (Malcangio and Tomlinson, 1998; Zurek et al., 2001).

Intrathecal catheters were inserted in diabetic and age-matched normal rats anesthetized using halothane. The catheters (polyethylene-10 tubing) were 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 catheters were externalized to the back of the neck and sutured

to the musculature and skin at the incision site. After a 5-7 day recovery following cannulation, the rats were used for the behavior testing. Only animals with no evidence of neurological deficits after catheter implantation were studied. All the pharmacologic experiments were conducted between 4 to 6 weeks after streptozotocin injection.

Behavioral testing of nociception

Thermal nociceptive test: Rats were placed on the glass surface of a thermal plantar testing apparatus (IITC Inc./Life Science Instruments, Woodland Hills, CA). The rats were allowed to acclimate for 30 min before testing. The temperature of the glass surface was maintained constant at 30°C. A mobile radiant heat source located under the glass was focused onto the hindpaw of the rats. The paw withdrawal latency was recorded by a digital timer. The withdrawal latencies for the left and right paws were averaged, and the mean value was used to indicate the sensitivity to noxious heat stimulation. The cutoff of 30 s was used to prevent potential tissue damage (Chen and Pan, 2002). The dose-response effect of intrathecal muscarine on the nociceptive paw withdrawal threshold was tested in diabetic and age-matched normal rats. After the baseline was measured, rats were treated with intrathecal 2, 6, or 12 μg muscarine. The paw withdrawal latency was determined every 15-30 min for 120 min after intrathecal injection. To ensure the specific action of muscarine, a separate group of rats was pretreated with intrathecal 30 μg atropine followed in 15 min by 12 μg muscarine. The doses of muscarine and atropine were selected based on previous studies (Naguib and Yaksh, 1997; Pan et al., 1999) and our pilot experiments. Drugs for intrathecal injections were dissolved in normal saline and administered in a volume of 5 μl followed by a 10 μl flush with normal saline.

Nociceptive pressure test: The mechanical withdrawal thresholds, expressed in grams, were measured with the Randall-Selitto test using an Ugo Basil Analgesimeter (Varese, Italy). The test was performed by applying a noxious pressure to the hindpaw. By pressing a pedal that activated a motor, the force increased at a constant rate on the linear scale. When the animal displayed pain by withdrawal of the paw or vocalization, the pedal was immediately released and the nociceptive threshold read on the scale. The cutoff of 400 g was used to avoid potential tissue injury (Chen and Pan, 2002; Chen and Pan, 2003). Both hindpaws were tested in each rat, 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 hindpaw were determined. The animals were then given intrathecal injection of muscarine, and the mechanical threshold in response to the pressure stimulus was determined every 15-30 min for 120 min. The antinociceptive effect of intrathecal muscarine (2, 6, and 12 μg) was tested in both normal and diabetic rats. Repeat intrathecal injections in the same animals were separated by 3-4 days.

Motor function was evaluated by testing the animals' ability to stand and ambulate in a normal posture and to place and step with the hindpaw (Chen et al., 2000). We assessed the motor function in a simple manner by grading the ambulation behavior of rats as the following: 2=normal; 1=limping; 0=paralyzed.

Muscarinic-stimulated [^{35}S]GTPγS binding in spinal membranes

Muscarine-stimulated [^{35}S]GTPγS binding was conducted to assess the change of functional muscarinic receptors (Maher et al., 2001; Chen et al., 2002). The [^{35}S]GTPγS assay is based on the principle that the inactive state of the G-protein α subunit has a relatively high affinity for GDP over

GTP, whereas activation of a receptor by its agonist shifts the α subunit into a higher affinity for GTP versus GDP. The [^{35}S]GTPγS is a hydrolysis-resistant form of GTP, and the degree to which an agonist stimulates [^{35}S]GTPγS binding can be quantified in tissue membranes. Unlike traditional receptor binding techniques, the agonist-stimulated [^{35}S]GTPγS binding can provide functional information about the receptor (i.e., activation of G proteins). For [^{35}S]GTPγS binding in membranes, the spinal cord was quickly removed from age-matched normal and diabetic rats after being anesthetized with halothane. The dorsal half of the spinal cord was dissected and stored at -70°C until assay. Six animals in each group were used for these experiments. The tissue was thawed, quickly chopped, and suspended in ice-cold 50 mM Tris buffer containing 3 mM $MgCl_2$ and 1 mM EGTA (pH 7.4). The tissue was homogenized and disrupted by sonication. The homogenate was then centrifuged at 500 g for 10 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 48,000 g for 20 min at 4°C. The pellet was resuspended in fresh Tris buffer and was centrifuged again as described above. The final pellet was resuspended in 50 mM Tris buffer containing 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EGTA (pH 7.7), and disrupted by sonication for 5 s. The protein content was measured using Bio-Rad protein assay. The concentration-effect curve of muscarine-stimulated [^{35}S]GTPγS binding included 0.001-10 μM muscarine, 30 μM GDP, 0.05 nM [^{35}S]GTPγS, 4 mU/ml adenosine deaminase, 20 μg protein, and assay buffer in a final volume of 1 ml. The basal binding was determined in the presence of GDP and absence of muscarine, and nonspecific binding was assessed in the presence of 10 μM unlabeled GTPγS (Chen et al., 2002). After incubation at 30°C for 1 hr, the reaction was terminated by filtration through Whatman GF/B filters on a cell harvester with cold 50 mM Tris buffer (pH 7.4). Radioactivity was determined by immersion of filters in scintillation fluid, incubated for 2 hr

at room temperature, and counted by a liquid scintillation counter (LS 6500, Beckman Coulter, Inc., Fullerton, CA). Nonlinear regression analyses of concentration-effect curves were performed using Prism (GraphPad Software, San Diego, CA). Percent stimulation was calculated as: (net stimulated binding/basal binding) × 100%. [^{35}S]GTPγS (1,250 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). GDP, muscarine, atropine, and adenosine deaminase were from RBI-Sigma (St. Louis, MO). GTPγS was purchased from Boehringer Mannheim (Indianapolis, IN). All other reagent grade chemicals were obtained from Sigma (St. Louis, MO).

[^{3}H]AF-DX 384 spinal membrane bindings

The M2 subtype is the most important muscarinic receptor mediating analgesia produced by muscarinic agonists in the spinal cord (Gomeza et al., 1999; Duttaroy et al., 2002). To further determine the potential change in spinal cord M2 muscarinic receptors in diabetes, [^{3}H]AF-DX 384, a selective M2 radioligand (Aubert et al., 1992; Kitaichi et al., 1999), was used in the membrane binding of spinal cord tissues. Six diabetic and 6 age-matched normal rats were decapitated following halothane anesthesia. The spinal cord was quickly harvested, and the dorsal half was dissected and used for the binding experiments. The membrane protein isolation and quantification method was similar to that described above. Saturation radioligand binding experiments were performed using 200 μl aliquots of tissue and increasing concentrations of [^{3}H]AF-DX 384 (100 Ci/mmol, PerkinElmer Life Sciences, Boston, MA) from 0.04 to 20 nM. Nonspecific binding was obtained in the presence of 1 μM atropine (Aubert et al., 1992). 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 on a cell harvester with cold Tris buffer (pH 7.4). Radioactivity was

quantified by immersion of filters in scintillation fluid, then incubated overnight at room temperature, and counted the next day. The saturation binding data were processed using nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA) to calculate maximal specific binding (B_{max}) and dissociation constant (K_D).

Data are presented as mean ± S.E.M. The effect of muscarine on the paw withdrawal threshold was determined by repeated measures ANOVA followed by Dunnett's *post hoc* test. For computation of ED_{50}, data were converted to the percentage of the inhibitory effect of muscarine based on the following calculation: [(maximal effect - baseline)/(cutoff - baseline)] × 100%. The ED_{50} values of muscarine and their 95% confidence limits were determined by nonlinear regression analyses of the dose-response curves using Prism (GraphPad Software). The binding data between the normal control and diabetic groups were compared using the Student's t test or ANOVA followed by Tukey's *post hoc* test. $P < 0.05$ was considered to be statistically significant.

Results

Effect of intrathecal muscarine in normal and diabetic rats

All the diabetic rats displayed polyuria, a reduced growth rate, and a marked increase in food and water intake. The body weight for the 28 normal and 34 diabetic rats at the end of the behavioral testing was 385±8 and 287±9 g ($P<0.05$), respectively. The plasma glucose values for the age-matched normal (104±6 mg/dl) and diabetic (337±10 mg/dl) rats at sacrifice differed significantly. The paw withdrawal threshold in response to noxious pressure in normal rats was 108.4 ± 3.2 g, which was significantly higher than that (74.4 ± 3.3 g, $P<0.05$) in diabetic rats 4 weeks after streptozotocin injection. On the other hand, the withdrawal threshold in response to noxious heat was not significantly different in normal (9.11 ± 0.23 s) and diabetic (8.83 ± 0.42 s) groups.

Intrathecal muscarine in both normal (n = 7-10) and diabetic (n = 8-9) rats dose-dependently increased the withdrawal threshold in response to the noxious heat stimulus (Figure 1). The maximal effect of muscarine appeared within 30 min following intrathecal injection, and the effect gradually diminished within 60-90 min. The antinociceptive effect of intrathecal 2-12 μg muscarine in diabetic rats was largely potentiated compared to that in control rats, with an ED_{50} value decreasing about 2.7-fold compared with that in normal animals. The estimated ED_{50} value (95% confidence limits) in normal and diabetic groups was 19.21 (2.46-77.54) and 7.04 (2.18-56.89) μg, respectively. In 6 separate normal rats, intrathecal 30 μg atropine completely blocked the effect of 12 μg muscarine (data not shown). Intrathecal administration of muscarine was not associated with any evident motor dysfunction. All rats tested received a score of 2 after intrathecal injection of 2,

6, or 12 μg muscarine.

Intrathecal injection of muscarine up to 12 μg slightly increased the withdrawal threshold in response to noxious pressure applied to the hindpaw of normal animal (n = 7-9, Figure 2). By contrast, intrathecal 2, 6, and 12 μg muscarine profoundly increased the pressure withdrawal threshold in diabetic rats in a dose-dependent manner (n = 8-10, Figure 2). The effect of intrathecal muscarine reached maximum within 30 min, and the withdrawal threshold gradually returned to baseline within 2 hr. The antinociceptive effect of intrathecal muscarine in diabetic animals also increased, with an ED_{50} value of muscarine about 3.5-fold lower than that in normal animals. The estimated ED_{50} (95% confidence limits) in diabetic and normal rats was 6.59 (2.05-56.30) and 22.98 (6.23-74.49) μg, respectively.

Muscarine-stimulated [^{35}S]GTPγS binding in the dorsal spinal cord

The concentration-effect curve for the muscarine-stimulated [^{35}S]GTPγS binding in the dorsal spinal cord membrane was performed in 6 normal and 6 diabetic rats. In the diabetic rats, the [^{35}S]GTPγS binding stimulated by 0.5-10 μM muscarine was increased significantly compared to that in normal rats (Figure 3). The level of maximal stimulation of [^{35}S]GTPγS binding by 10 μM muscarine was 112.8 ± 9.2% in normal rats and 162.3 ± 10% ($P < 0.05$) in diabetic rats. The basal [^{35}S]GTPγS level in the spinal cord tissue was slightly, but significantly, higher in diabetic rats than normal rats (41.2 ± 3.4 vs. 36.5 ± 2.4 fmol/mg protein, $P < 0.05$).

[^{3}H]AF-DX 384 binding in the dorsal spinal cord

We observed that [^{3}H]AF-DX 384 had a single and saturable high-affinity binding site in

the dorsal spinal cord tissue in both normal and diabetic rats (n = 6 in each group, Figure 4). The K_D value was similar in normal (2.86 ± 0.28 nM) and diabetic (2.88 ± 0.19 nM) groups. However, the B_{max} of [^{3}H]AF-DX 384 binding in diabetic rats was significantly higher than that in normal rats (255.2 ± 5.9 vs. 165.9 ± 3.5 fmol/mg protein, $P < 0.05$).

Discussion

This is the first study demonstrating upregulation of muscarinic receptors in the dorsal spinal cord in diabetes. In the present study, we found that intrathecal muscarine produced a profound antinociceptive effect in a rat model of diabetic neuropathic pain. Also, the maximal muscarine-stimulated [^{35}S]GTPγS binding in the spinal cord was significantly increased in diabetic rats. Furthermore, the B_{max}, but not K_D, of spinal [^{3}H]AF-DX 384 membrane binding was significantly higher in diabetic than in control rats. Collectively, these data provide new information that the muscarinic receptor is upregulated in the dorsal spinal cord in diabetes. Upregulation of muscarinic receptors in the spinal cord probably accounts for the increased antinociceptive effect of intrathecal muscarinic agonists in diabetic neuropathic pain.

The spinal muscarinic receptors are important for the regulation of nociception. Molecular cloning studies have revealed the existence of five molecularly distinct muscarinic receptor subtypes referred to as M1-M5 (Wess, 1996). The M1-M5 receptors are prototypical members of the superfamily of G protein-coupled receptors. The odd-numbered receptors (M1, M3, M5) couple efficiently, through $G_{q/11}$, to activate phospholipase C, which leads to IP3-mediated calcium release from the endoplasmic reticulum and to DAG-mediated activation of protein kinase C. The even-numbered muscarinic receptors (M2, M4) inhibit adenylyl cyclase activity via activation of the $G_{i/o}$ class of G proteins (McKinney, 1993; Wess, 1996). In the central nervous system, M2 and M4 receptors appear to frequently inhibit neuronal firing. Using subtype muscarinic receptor knockout mice, it has been revealed that the M2 subtype is the major muscarinic receptor present in the spinal dorsal horn (Duttaroy et al., 2002). Radioligand binding studies, using spinal cord tissues from

wild-type and muscarinic subtype knockout mice, have shown that the M2 represents ~90% of spinal cord muscarinic receptors (Duttaroy et al., 2002). This finding is consistent with immunohistochemical and radioligand binding studies showing high levels of M2 receptors in the spinal dorsal horn (Hoglund and Baghdoyan, 1997; Yung and Lo, 1997). On the other hand, pharmacological studies indicate that the M4 subtype represents only a minor fraction of the total muscarinic receptor population in the spinal cord (Hoglund and Baghdoyan, 1997). Using muscarinic subtype knockout mice, it has been demonstrated that the analgesic action of intrathecal muscarinic agonists is completely abolished in M2/M4 double-knockout mice (Duttaroy et al., 2002). Consistent with previous studies (Iwamoto and Marion, 1994; Naguib and Yaksh, 1997), we observed that intrathecal muscarine produced a profound analgesic effect in normal rats. Interestingly, we found that intrathecal muscarine had a more pronounced effect on thermal nociception in normal rats. It is not clear why the antinociceptive action of muscarine appears to be modality-specific. This may be due to the fact the muscarinic receptor is located on capsaicin-sensitive C-fiber afferent terminals. In this regard, we have shown that systemic treatment with a potent capsaicin analog, resiniferatoxin, largely depletes the M2 immunoreactivity in the rat spinal dorsal horn (Li et al., 2002).

We observed that the effect of intrathecal muscarine on the withdrawal response to both heat and pressure stimuli was largely potentiated in diabetic rats. This finding suggests that the spinal cord muscarinic receptor may be increased in diabetes. Increased glutamate availability in the spinal cord may contribute to the development of plasticity of dorsal horn neurons and hyperalgesia in diabetic neuropathic pain (Malcangio and Tomlinson, 1998). A clinical study has demonstrated that NMDA receptor antagonists produce analgesia in patients with diabetic neuropathic pain

(Nelson et al., 1997). We have shown that some M2 receptors are located presynaptically in the spinal dorsal horn since dorsal root rhizotomy markedly reduces M2 immunoreactivity in the spinal cord (Li et al., 2002). Also, acetylcholine inhibits the excitatory glutamatergic synaptic input to spinal dorsal horn neurons through muscarinic receptors (Li et al., 2002). Therefore, the increased antinociceptive action of muscarine in the diabetic rats may be due to, at least in part, inhibition of the glutamate release to spinal dorsal horn neurons through activation of presynaptic M2 muscarinic receptors.

We used agonist-stimulated [^{35}S]GTPγS binding to assess the change in functional muscarinic receptor in the spinal cord in diabetes since activation of G proteins by G protein-coupled receptors can be measured and quantified with this method. Unlike the traditional receptor binding technique, the [^{35}S]GTPγS binding is a functional assay of receptors since it measures an intracellular signal transduction system coupled to a membrane-bound receptor. Consistent with the behavioral data, we found that the muscarinic receptor in the dorsal spinal cord is significantly upregualted in diabetes. It has been shown that only receptors coupled to the $G_{i/o}$ family of G proteins can be detected with [^{35}S]GTPγS binding techniques (Maher et al., 2001; Chen et al., 2002). Thus, the M2 and M4 subtypes likely are the muscarinic receptors measured in our study since they both couple to the inhibitory $G_{i/o}$ proteins. Similar to the finding in a previous study (Chen et al., 2002), we observed a small, but consistent, increase in the basal [^{35}S]GTPγS binding in the spinal cord in diabetic rats. It is not clear what causes an increase in the basal [^{35}S]GTPγS binding in the spinal cord in diabetes, although it is possible that changes in the affinity of G proteins for GTP and GDP may contribute to the increased basal [^{35}S]GTPγS binding in the spinal cord tissue in diabetes (Chen et al., 2002).

In the animal model of diabetes, the M2 receptor is significantly upregulated in the bladder, lung, and trachea tissues in diabetes (Belmonte et al., 1997; Tong et al., 1999; Coulson et al., 2002). Since the M2 receptor is the major muscarinic subtype in the spinal cord, we also determined the change in the M2 subtype in the spinal cord in diabetes. In this study, we used [^{3}H]AF-DX 384, a more selective M2 receptor binding ligand (a specific M2 antagonist) (Aubert et al., 1992; Kitaichi et al., 1999), to determine potential changes in the number and binding affinity of the muscarinic M2 subtype in diabetes. The [^{3}H]AF-DX 384 binding data suggest that the number, but not the affinity, of the M2 subtype is significantly increased in the dorsal spinal cord in diabetes. Thus, these receptor binding data provide strong evidence to support the behavioral finding that the antinociceptive effect of intrathecal muscarinic agonists is potentiated in diabetes. Because the dorsal half of the spinal cord was used for the binding experiments, we cannot determine if the new binding sites were in the same locations or novel sites without additional localization studies.

The mechanisms of diabetes-induced increases in the functional muscarinic receptors remain to be elucidated. Although it is not clear if hyperglycemia alone affects muscarinic receptor function in the spinal cord, normal muscarinic M2 receptor function in the lung can be restored by treatment with insulin without normalization of blood glucose levels in diabetic rats (Belmonte et al., 1997). This suggests that the increase in muscarinic receptors in diabetes may be due to insulin deficiency. Also, it should be noted that hyperglycemia occurs within 24 hr following streptozotocin injection, but allodynia does not appear until 3 weeks later when the increased antiallodynic effect of intrathecal neostigmine is observed (Chen et al., 2001). Other possibilities include changes in acetylcholine metabolism, alterations of acetylcholinesterase activity, decreased availability of various neurotrophic factors, and some unknown metabolic factors associated with

diabetic neuropathy (Wahba and Soliman, 1988; Welsh and Wecker, 1991; Zhuang et al., 1997). Although the muscarinic M2 receptor is increased in the spinal cord in diabetic rats, we cannot exclude the possibility that other muscarinic subtypes, such as M4, also may be altered in diabetes. Due to lack of specific radioligands for muscarinic receptors other than the M2 subtype, we were unable to determine potential changes in other muscarinic subtypes in diabetes. It has been reported that muscarinic toxin-3 is specific for muscarinic M4 subtype (Ellis et al., 1999). Thus, this toxin may be useful to determine the potential change of the M4 subtype in the spinal cord in diabetes. Furthermore, studies using muscarinic subtype knockout mice would be valuable to establish the role of different muscarinic subtypes in enhanced muscarinic analgesia in diabetes. The limitation of using this animal model should be acknowledged. For example, the allodynia development takes about 3 weeks after streptozotocin injection in this rat model (Chen and Pan, 2002), while neuropathic pain in diabetic patients usually takes years to develop. Also, the extensive peripheral afferent neuropathy seen in diabetic patients may not be fully reflected in this short-term animal model. Therefore, further clinical studies are necessary to confirm the changes of spinal muscarinic receptors in diabetic rats.

In summary, we found in the present study that intrathecal injection of muscarine had a more pronounced antinociceptive effect in diabetic rats than in normal rats. Furthermore, data from experiments using agonist-stimulated [^{35}S]GTPγS and [^{3}H]AF-DX 384 membrane bindings provide new information that the muscarinic receptor in the dorsal spinal cord was increased in diabetic rats. Thus, this study suggests that spinally administered cholinergic agents may have some unique therapeutic value in the treatment of neuropathic pain in diabetic patients.

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Footnotes

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Figure Legend

1. Panel A: Time course of the effect of intrathecal 2 (n = 8), 6 (n = 7), and 12 (n = 10) μg muscarine on the nociceptive withdrawal threshold in normal rats. Panel B: Time course of the effect of intrathecal 2 (n = 8), 6 (n = 9), and 12 (n = 9) μg muscarine on the nociceptive withdrawal threshold in diabetic rats. The nociceptive threshold was determined by the withdrawal response of the hindpaw to a noxious heat stimulus. Data presented as mean ± SEM. * P <0.05 compared to the respective baseline control.

2. Panel A: Effect of intrathecal 2 (n = 7), 6 (n = 9), and 12 (n = 7) μg muscarine on the nociceptive withdrawal threshold in normal rats. Panel B: Effect of intrathecal 2 (n = 8), 6 (n = 10), and 12 (n = 8) μg muscarine on the nociceptive withdrawal threshold in diabetic rats. The nociceptive threshold was determined by the withdrawal response of the hindpaw to a noxious pressure stimulus. Data presented as mean ± SEM. * P <0.05 compared to the respective baseline control.

3. Concentration-effect curve for the muscarine-stimulated [^{35}S]GTPγS binding in the spinal cord membrane obtained from normal and diabetic rats 4 weeks after streptozotocin injection. The data are expressed as the percentage of basal [^{35}S]GTPγS binding (mean ± SEM, n = 6 in each group). *P<0.05 compared to the value in the normal group at the same concentration of muscarine.

4. Comparison of [^{3}H]AF-DX384 saturation binding in the dorsal spinal cord homogenate from

normal and diabetic rats (n = 6 in each group).

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Fig. 1

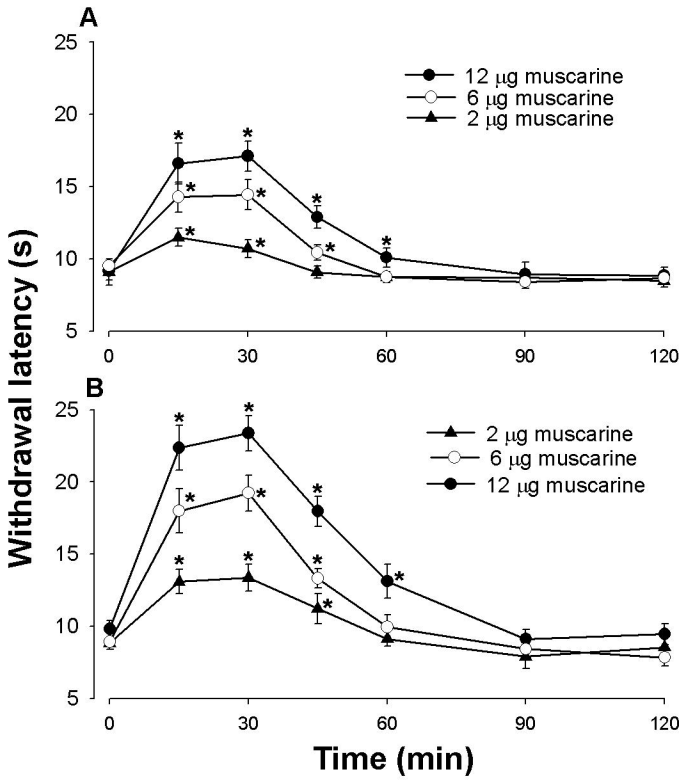

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Fig. 2

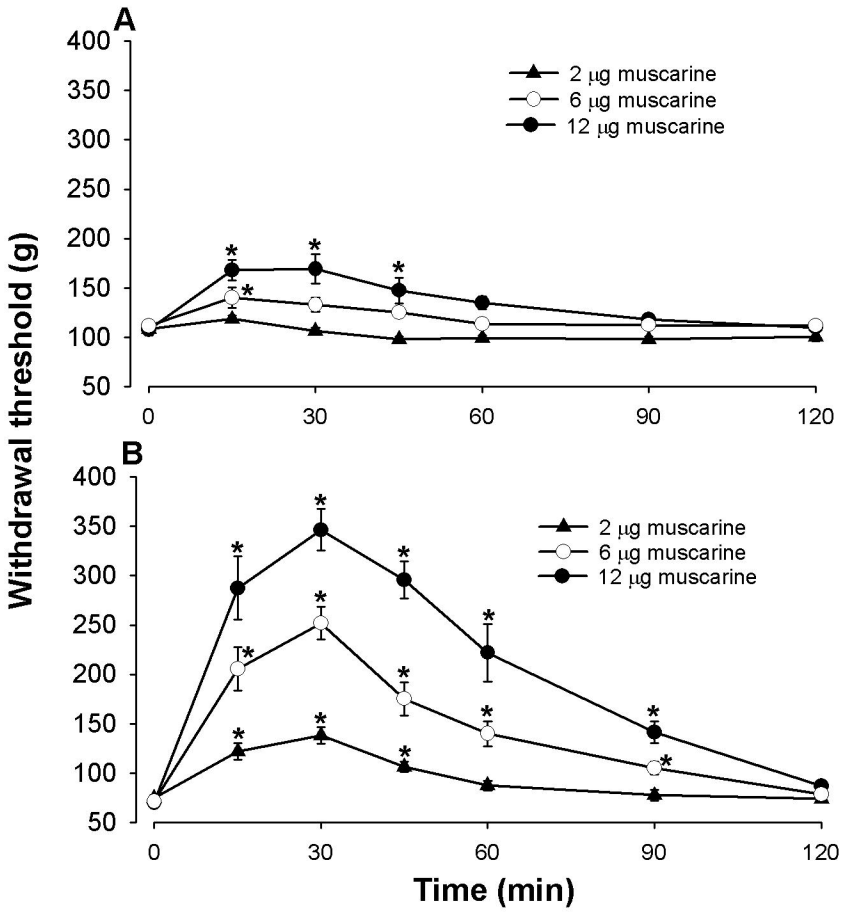

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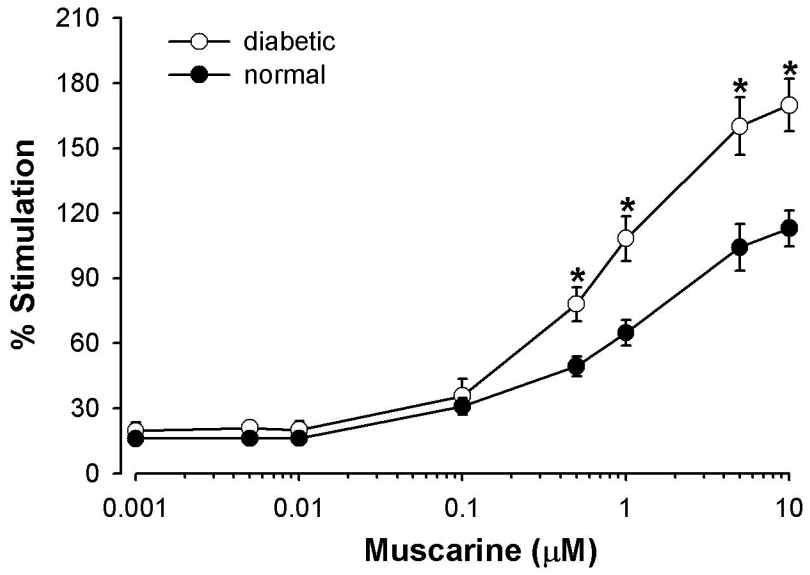
Fig. 3



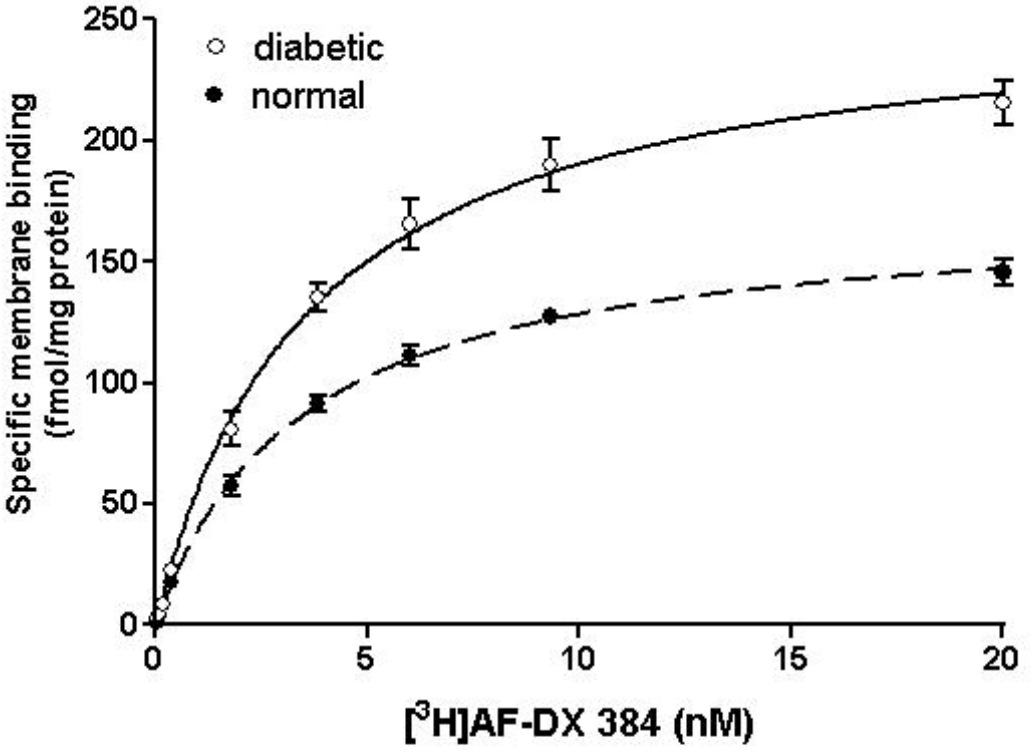

diabetic
normal
Specific membrane binding
(fmol/mg protein)
250
200
150
100
50
0
0
5
10
15
20
[3H]AF-DX 384 (nM)

Fig. 4