Contribution of Ca\(^{2+}\)-Dependent Protein Kinase C in the Spinal Cord to the Development of Mechanical Allodynia in Diabetic Mice

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In this paper, we directly demonstrate, for the first time, the activation of Ca\(^{2+}\)-dependent protein kinase C (PKC) in the spinal cord of diabetic mice. In streptozotocin (STZ)-treated (200 mg/kg, i.v.) diabetic mice, hypersensitivity (allodynia) to mechanical stimulation appeared 7 d after STZ injection. This mechanical allodynia was inhibited by intrathecal injection of the PKC inhibitors 1-(5-isouquinolinesulfonyl)-2-methylpiperazine (H-7) and calphostin C, but not the protein kinase A inhibitor N\(^{6}\)-2-(p-bromocinnamamylamo)ethyl]-5-isouquinolinesulfonamide (H-89). The activity of membrane-associated Ca\(^{2+}\)-dependent PKC in the spinal cords of STZ-induced diabetic mice was significantly higher than that observed in non-diabetic mice. These results suggest that activation of Ca\(^{2+}\)-dependent PKC in the spinal cord contributes to the mechanical allodynia in the pain associated with diabetic neuropathy.

Key words  diabetic mice; mechanical allodynia; spinal cord; Ca\(^{2+}\)-dependent protein kinase C

Patients with diabetic neuropathy suffer from various types of aberrant pain.\(^{1,2}\) This painful neuropathy develops in early stages of diabetes. Although hyperglycemia is considered to be a major pathogenic factor in the development of diabetic neuropathy, the mechanisms associated with this are not yet fully understood. Painful diabetic neuropathy can not be alleviated satisfactorily with either non-steroidal anti-inflammatory drugs or opioids.\(^{3–5}\)

Since studies have shown that neuropathic pain is caused by injured traumatic nerves in the spinal dorsal horn,\(^{6–8}\) the neuropathic pain associated with diabetes is thought to be due to hyperactivity of damaged C-fibers. Moreover, protein kinase C (PKC) appears to be involved in functional changes of spinal cord neurons during the development of neuropathic pain.\(^{9}\) In fact, an intrathecal injection of a PKC inhibitor attenuates the thermal allodynia seen in diabetic mice.\(^{10}\) Furthermore, Ahlgren and Levine\(^{11}\) have reported that both mechanical behavioral hyperalgesia and C-fiber hyperexcitability resulting from mechanical stimuli in streptozotocin (STZ)-induced diabetic rats are reduced by agents that inhibit PKC. These findings suggest that spinal PKC may have an important role in the development of neuropathic pain in diabetic patients. However, it has yet to be demonstrated that PKC activity is increased in the spinal cords of diabetic animals.

Based on structural variations and biochemical properties, the PKC family of proteins can be categorized into three types: conventional PKC, novel PKC, and atypical PKC. Conventional PKC are Ca\(^{2+}\)- and phospholipid-dependent, whereas novel PKC and atypical PKC do not require any calcium for their activities. However, we know nothing about the relative contributions of the three PKC types in diabetic neuropathic pain. Thermal and mechanical hyperalgesia were observed in STZ-induced diabetic mice\(^{4,12}\) and rats.\(^{3,13}\) Therefore, we examined to clarify the types and activation of PKC in the spinal cord in diabetic neuropathic pain using STZ-induced diabetic mice.

MATERIALS AND METHODS

Animals  Male ddY mice (Kyudo, Kumamoto, Japan) weighing 25–30 g were used in our experiments. Mice were housed at 22±2 °C with a 12/12 h light/dark cycle (lights on at 07:00 h), and were given free access to commercial food and tap water. Experimental procedures were based on the Guidelines of the Committee for Animal Care and Use of Fukuoka University.

Diabetic Mice  Diabetic mice were developed by injection of STZ (200 mg/kg body weight) dissolved in 33 mM sodium citrate buffered (pH 4.5) saline directly into the tail vein (intravenous injection, i.v.). Age-matched non-diabetic mice were injected with the same volume of saline only. Diabetes was confirmed in experimental animals 1 or 2 d after injection of STZ. This was done by measuring urinary glucose with a Test-tape A (Shionogi Pharmaceutical Co., Osaka, Japan) and by measuring the glucose concentration in a blood sample obtained from the tail vein with a GLUTEST E kit (Sanwa Chemical Co., Nagoya, Japan). Mice with blood glucose levels above 300 mg/dl were used as diabetic mice.

Intrathecal (i.t.) Injection  For the i.t. injection of drugs, a 28 gauge needle was connected to a 25 µl Hamilton microsyringe, and inserted into the intervertebral space between lumbar 5 and 6 vertebrae as previously described.\(^{12,14}\) The accurate placement of the needle was confirmed by a quick “flick” of the mouse’s tail.

Drugs for i.t. injection were given slowly in a total volume of 5 µl. Control mice received the same volume of only artificial cerebrospinal fluid (aCSF), saline or vehicle.

Mechanical Allodynia  The von Frey filament (Semmes-Weinstein monofilaments, Stoelting, IL, U.S.A.) with a strength of 0.407 g was used to assess mechanical allodynia.
Mechanical allodynia was characterized as the response frequency of paw withdrawal to stimulation with a 0.407 g von Frey filament. Further details are given in the Materials and Methods. Results are mean±S.E.M. for 7—10 mice in each group. *p<0.05, **p<0.01 compared to vehicle-treated mice (non-diabetic mice).

Mechanical allodynia in Mice after the Injection of STZ

Fig. 1. Time Course of Mechanical Allodynia in Mice after the Injection of STZ

Mechanical allodynia was characterized as the response frequency of paw withdrawal to stimulation with a 0.407 g von Frey filament. Further details are given in the Materials and Methods. Results are mean±S.E.M. for 7—10 mice in each group. *p<0.05, **p<0.01 compared to vehicle-treated mice (non-diabetic mice).

As shown in Fig. 1, the frequency of paw withdrawal response was shown in about 10% when the filament was applied 10 times to the planter surface. Therefore, we considered it to be an adequate value for the measurement of mechanical allodynia.

Briefly, mice were placed individually in a glass cage with a wire mesh bottom. After mice had adapted to the testing environment for 60 min, the von Frey filaments were applied perpendicularly to the mid-planter surface of the left hind paw through the mesh floor and held for 3—5 s with the filament slightly buckled. The degree of mechanical allodynia was measured as the frequency of paw withdrawal when the filament was applied 10 times to the planter surface of the left hind paw with intervals of 5 s between applications. Results were expressed as the percent response frequency of paw withdrawals (100×number of withdrawal/10).

PKC Assay On the 7th day after i.v. injection of STZ, the lumbar part of the spinal cord was rapidly dissected out and then frozen. Samples were homogenized in ice-cold homogenization buffer containing 250 mM sucrose, 150 mM NaCl, 2.5 mM EGTA, 5 mM EDTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin (pH 7.5) and were centrifuged for 1 h at 100000×g at 4 °C. The pellet was homogenized in homogenization buffer containing 1% Nonidet P-40, and was then incubated on ice for 30 min. The homogenate was then centrifuged at 100000×g for 1 h at 4 °C. The resultant supernatant (membrane-associated PKC containing plasma membrane and nuclear membrane) was used in a PKC activity assay.

Protein levels were assayed according to the method of Lowry et al.15) The activity of PKC was assayed using a PKC enzyme assay system (Amersham Pharmacia Biotech Buckinghamshire, U.K.). The principle and the protocol have been described in the manual of the PKC enzyme assay system kit (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). Briefly, samples (22—24 μg) were incubated with the reaction medium-A (synthetic peptide (VRKRTLRRL) substrate, 30 mM dithiothreitol, [γ-32P] adenosine triphosphate, magnesium ATP buffer (1.2 mM ATP, 30 mM HEPES and 72 mM MgCl₂, pH 7.5), phosphatidyl serine, phorbol 12-myristate acetate, and 12 mM calcium acetate in 50 mM Tris–HCl, pH 7.5). Ca²⁺-independent PKC activation was obtained by replacing 12 mM calcium acetate in the reaction medium-A with 12 mM EGTA, and the medium is called reaction medium-B in this study. Basal activation (background) was determined by omitting phorbol 12-myristate acetate further from the reaction medium-B. After the addition of stop solution to all reactions, the solution was mixed for 3 s. The mixture was then transferred onto peptide binding paper. These papers were washed three times with 75 mM H₃PO₄ and the incorporation of [32P] into the peptide was determined by the use of a liquid scintillation counter. The values obtained by incubation with reaction medium-A represented total PKC activity. Ca²⁺-dependent PKC activity was determined by subtracting the values obtained by incubation of sample with reaction medium-B and background from the total PKC activity. Ca²⁺-independent PKC activity was determined by subtracting values of background from the values obtained by incubation with reaction medium-B.

Drugs H-7 and H-89 were purchased from Seikagaku Co. (Tokyo, Japan). STZ and calphostin C were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). H-7 was dissolved in aCSF. Calphostin C was dissolved in saline containing 1.1% ethanol. H-89 was dissolved in saline containing 10% dimethyl sulfoxide (DMSO).

Statistical Analysis Statistical analysis of data were performed by the Student’s t-test for comparison between the two groups, and by using analysis of variance (ANOVA) followed by Dunnett’s test or Turkey’s test for multiple comparisons. Results are expressed as means±S.E.M. The level of significance was set at p<0.05.

RESULTS AND DISCUSSION

Mechanical Allodynia STZ-injected mice had glucosuria, an increase in blood glucose level and a decrease in body weight on 3 d after the STZ injection. Seven days after injection of STZ, the mean blood glucose level of STZ-injected mice (348.0±18.2 mg/dl, n=7) was significantly (p<0.01) elevated when compared with aged-matched non-diabetic mice (152.3±3.8 mg/dl, n=7). STZ-induced diabetes produced an approximately 3-fold increase in blood glucose level as compared to the level in age-matched non-diabetic control mice, for up to 2 weeks following STZ injection. However, the body weight of STZ-induced diabetic mice (30.8±0.3 g, n=8) was significantly (p<0.001) reduced as compared to aged-matched non-diabetic mice (35.94±0.7 g, n=8).

As shown in Fig. 1, the paw-withdrawal-frequency stimulated by a von Frey filament with a strength of 0.407 g was significantly increased in diabetic mice as compared with age-matched non-diabetic mice at 7 d and lasted for 14 d after the injection of STZ. These results indicate the development of mechanical allodynia in diabetic mice.

Effects of PKC Inhibitors on Mechanical Allodynia in Diabetic Mice To examine the involvement of spinal PKC in the development of mechanical allodynia, H-7, a non-specific PKC inhibitor, was administered intrathecally into diabetic mice 7 d after STZ injection. As shown in Fig. 2A, diabetic mice exhibited considerable reduction in paw-withdrawal frequency following i.t. injection of H-7 (10 pmol). Moreover, intrathecal H-7 inhibited paw-withdrawal-frequency in a dose-dependent manner.

H-7 is known to be a non-selective inhibitor of PKC that
inhibits a wide range of different protein kinases. On the other hand, calphostin C is more selective than H-7, is highly potent, and is a specific inhibitor of the conventional and novel PKC isoforms.\textsuperscript{16,17} I.t. injection of calphostin C (20 pmol) in diabetic mice resulted in a significant reduction in paw-withdrawal-frequency (Fig. 2B). Intrathecal calphostin C also showed dose-dependent inhibitory effects on paw-withdrawal-frequency. In contrast, i.t. injections of H-7 (10 pmol) and calphostin C (20 pmol) had no significant effect on the paw-withdrawal-frequency in non-diabetic mice (data not shown).

Effects of the Selective Protein Kinase A Inhibitor H-89 on Mechanical Alldynia in Diabetic Mice
I.t. injection of H-89 (2, 5 nmol) in diabetic mice 7 d after STZ injection did not result in any significant change in paw-withdrawal-frequency (Fig. 3).

Activity of Membrane-Associated PKC in the Spinal Cord
The activity of membrane-associated PKC in the spinal cords of both age-matched non-diabetic and diabetic mice was examined 7 d after the injection of STZ. As shown in Fig. 4A, Ca\textsuperscript{2+}-dependent PKC activity was significantly increased in diabetic mice but Ca\textsuperscript{2+}-independent PKC activity remained unchanged. On the other hand, basal activity (non-protein kinase C activity) remained at the same levels in both non-diabetic and diabetic mice (Fig. 4B).

Previous studies have demonstrated that STZ-induced diabetes in animals leads to a reduction in the mechanical tactile threshold.\textsuperscript{3,5,7,12,18} In addition, it has been reported that i.t. injection of a PKC inhibitor leads to a reduction in the degree of thermal alldynia and hyperalgesia in STZ-induced diabetic mice.\textsuperscript{10} However, direct evidence that relates activation of spinal PKC to the development of mechanical hyperalgesia and alldynia has yet to be demonstrated in an animal model of diabetes mellitus.

In the present study, STZ-induced diabetic mice exhibited an increase in mechanical non-nociceptive response, as determined by the application of von Frey filaments. The mechanical alldynia in diabetic mice was alleviated by the i.t. injection of PKC inhibitors, but not the i.t. injection of a protein kinase A inhibitor. We have demonstrated, for the first time, that there is a marked increase of membrane-bound Ca\textsuperscript{2+}-dependent PKC activity in the spinal cords of diabetic mice.

Based on biochemical and molecular cloning studies, Ca\textsuperscript{2+}-dependent PKC has been classified into four isoforms: PKC\textgreekalpha, PKC\textgreekbeta, PKC\textgreekbetaII, and PKC\textgreekgamma. In the rat spinal cord, four Ca\textsuperscript{2+}-dependent PKC isoforms (\textgreekalpha, \textgreekbeta, \textgreekbetaII, and \textgreekgamma) have been detected.\textsuperscript{9,10} Several lines of evidence indicate that spinal PKC\textgreekgamma plays an important role in the development of alldynia and hyperalgesia in animal models of peripheral nerve injury.\textsuperscript{9,10,20} Malmberg et al.\textsuperscript{21} reported that neuropathic pain could not be detected in PKC\textgreekgamma knockout mice following peripheral nerve injury. On the other hand, another study reported that the activity of PKC and the expression of PKC\textgreekalpha in the sciatic nerve and the brachial plexus were re-
duced in diabetic mice.\(^{22}\) These findings support the hypothesis that spinal Ca\(^{2+}\)-dependent PKC\(\gamma\) may contribute to neuropathic pain.

In addition, various kinds of neurotransmitters found in the spinal cord are significantly upregulated following nerve injury. These include: glutamate, substance P, cholecystokinin, galanin, vasoactive intestinal peptides and neuropeptide Y.\(^{23}\) Using an \textit{in vitro} slice preparation of spinal cord dorsal horn, Gerber \textit{et al.}\(^{24}\) showed that the phorbol esters, which activate PKC, enhance the depolarization-evoked release of excitatory amino acids in the rat and increase the amplitude and duration of depolarization produced by N-methyl-D-aspartate (NMDA) and glutamate. Chen and Huang\(^{8}\) have demonstrated that PKC stimulates NMDA-activated currents in isolated sensory neurons by increasing the openings of ion channels, and by reducing the voltage-dependent Mg\(^{2+}\) block of NMDA receptors. In addition, PKC-mediated phosphorylation in the NMDA receptors has been implicated in spinal hyperexcitability.\(^{25,26}\) More recently, Sluka and Audette\(^ {27}\) showed that activation of PKC in the spinal cord produces mechanical hyperalgesia by activating glutamate receptors. Therefore, these findings suggest that activation of PKC causes abnormal activation in sensory neurons in laminae II and III of the spinal cord and is involved in the development of mechanical hyperalgesia and allodynia. Moreover, our findings indicate that the mechanical allodynia observed in diabetic mice is due to an increase in membrane-bound Ca\(^{2+}\)-dependent PKC in the spinal cord.

Thus, the present findings strongly suggest that the activation of Ca\(^{2+}\)-dependent PKC isoforms, notably protein kinase C\(\gamma\) in the spinal cord, contributes to the mechanical allodynia associated with painful diabetic neuropathy. However, further study to clarify the spinal Ca\(^{2+}\)-dependent PKC isoforms in the diabetic neuropathic pain will be needed.

In conclusion, the present study suggests that intrathecally injected PKC inhibitors inhibit mechanical allodynia in diabetic mice. Furthermore, the activity of Ca\(^{2+}\)-dependent PKC in the spinal cords of diabetic mice was found to be increased. The increase in the level of spinal PKC activity was thereby contributing to the mechanical allodynia associated with painful diabetic neuropathy.

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