Protein Kinase Cβ Selective Inhibitor LY333531 Attenuates Diabetic Hyperalgesia Through Ameliorating cGMP Level of Dorsal Root Ganglion Neurons

Hyoh Kim,[1] Teiji Sasaki,[1] Kengo Maeda,[1] Daisuke Koya,[2] Atsunori Kashiwagi,[3] and Hitoshi Yasuda[1]

Streptozocin (STZ)-induced diabetic rats show hyperalgesia that is partially attributed to altered protein kinase C (PKC) activity. Both attenuated neuronal nitric oxide synthase (nNOS)-cGMP system and tetrodotoxin-resistant (TTX-R) Na channels in dorsal root ganglion neurons may be involved in diabetic hyperalgesia. We examined whether PKCβ inhibition ameliorates diabetic hyperalgesia and, if so, whether the effect is obtained through action on neurons by testing nociceptive threshold in normal and STZ-induced diabetic rats treated with or without PKCβ-selective inhibitor LY333531 (LY) and by assessing the implication of LY in either nNOS-cGMP system or TTX-R Na channels of isolated dorsal root ganglion neurons. The decreased nociceptive threshold in diabetic rats was improved either after 4 weeks of LY treatment or with a single intradermal injection into the footpads. The treatment of LY for 6 weeks significantly decreased *p*-PKCβ and ameliorated a decrease in cGMP content in dorsal root ganglia of diabetic rats. The latter effect was confirmed in ex vivo condition. The treatment with NO donor for 4 weeks also normalized both diabetic hyperalgesia and decreased cGMP content in dorsal root ganglions. The expressions of nNOS and TTX-R Na channels were not changed with LY treatment. These results suggest that LY is effective for treating diabetic hyperalgesia through ameliorating the decrease in the nNOS-cGMP system. *Diabetes* 52:2102–2109, 2003

Diabetic neuropathy is the most common disease of peripheral neuropathy in Western countries, as well as the most frequent microangiopathic complication of diabetes (1). Some patients with diabetic neuropathy have various forms of neuropathic symptoms, including hyperalgesia and spontaneous pain, which are often developed in early stages but may occur at any stage. The painful symptoms are troublesome and reduce the patients' quality of life. Thus, the relief from painful symptoms should be a main purpose for the treatment of diabetic neuropathy. However, the mechanism by which such neuropathic symptoms develop remains unclear, and useful drugs for pain management in diabetes remain unavailable.

The underlying mechanisms of painful symptoms may be closely associated with hyperglycemia and/or the pathogenic mechanism of diabetic neuropathy itself. Various hypotheses have been proposed to explain the pathogenesis of diabetic neuropathy (2,3): polyol pathway hyperactivity, decreased nerve blood flow followed by endoneurial hypoxia, increased glycation of proteins, abnormal activity of protein kinase C (PKC), decreased neurotrophism, and the associated exaggeration of oxidative stress. Among these hypotheses, the involvement of PKC may be one of the most relevant. Hyperglycemia activates PKC, especially its βII isoform, through increased de novo synthesis of diacylglycerol in retina (4), glomeruli (5), and aorta and heart (6). This increased activity of PKCβ may impair retinal (4) and endoneurial (7) blood flow, causing renal hyperfiltration (8), resulting in the development of diabetic retinopathy, nephropathy, and neuropathy. In addition, selective PKCβ inhibitors ameliorate these abnormalities (7,9,10).

The role of PKC hyperactivity has also been well investigated with reference to pain generation, using not only phorbol esters and PKC activators (11), but also various members of the PKC superfamily. Chronic inflammation-evoked thermal hyperalgesia may involve several protein kinases, including PKCγ and protein kinase A (12). PKCε has also been shown to regulate nociceptor function in the experiments using either PKCε mutant mice or a PKCε-selective inhibitor peptide in dorsal root ganglion (DRG) neurons (13). Increase in PKCβII activity has been reported to participate in hyperalgesia caused by adjuvant-induced inflammation in the rat hind paw (14).

The important contribution of PKC to hyperalgesia has also been reported in diabetic animals. Phorbol esters enhance thermal hyperalgesia in diabetic mice. The hyperalgesia and C-fiber hyperexcitability to mechanical stimuli observed in diabetic rats are reduced by intradermal injection of agents that inhibit PKC (15). In an in vitro study using rat sensory neurons, PKC was shown to mediate release of substance P and calcitonin gene-related peptide (CGRP) from sensory neurons. This PKC-induced

From the [1]Division of Neurology, Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, Japan; the [2]Division of Nephrology and Diabetes, Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, Japan; and the [3]Division of Endocrinology and Metabolism, Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, Japan.

Address correspondence and reprint requests to Hitoshi Yasuda, PhD, Division of Neurology, Department of Medicine, Shiga University of Medical Science, Seta, Otsu, Shiga 520-2192, Japan. E-mail: hyasuda@belle.shiga-med.ac.jp.

Received for publication 20 August 2002 and accepted in revised form 1 May 2003.

CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; NMDA, *N*-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; PKC, protein kinase C; PKG, cGMP and cGMP-dependent protein kinases; RIPA, radioimmunoprecipitation assay; STZ, streptozotocin; TTX, tetrodotoxin; TTX-R, TTX-resistant.

enhancement of peptide release may be a mechanism underlying the neuronal sensitization that produces hyperalgesia (16). Thus, although the hyperactivity of PKC is thought to contribute to hyperalgesia in diabetes, the responsible mechanism has not yet been identified.

Aside from the implication of PKC in the generation of pain in diabetes, attenuated neuronal nitric oxide synthase (nNOS)-cGMP system in DRGs may play a role in the pathogenesis of hyperalgesia in streptozotocin (STZ)-induced diabetic rats (17). In addition, tetrodotoxin (TTX)-resistant (TTX-R) Na^+ currents are exaggerated in the small DRG neurons of diabetic rats (18); TTX-R Na channels have been considered to profoundly contribute to nociception (19). In spite of the accumulating data on diabetic hyperalgesia and its mechanisms, all of the data so far published have been somewhat fragmentary, so the whole story on diabetic hyperalgesia needs more clarification.

In the present study, we have attempted to examine whether PKCβ inhibition may ameliorate hyperalgesia in diabetes and, if so, whether the effect is obtained through nonvascular action on DRG neurons or nerve fibers with special reference of the nNOS-cGMP system and TTX-R Na^+channels.

RESEARCH DESIGN AND METHODS

Experimental animals and research protocols. Male Sprague-Dawley rats aged 8 weeks (250–300 g) were used in all experiments and were housed in an aseptic animal room at 20–24°C and 40–70% humidity with a 12:12 h light-dark cycle in an illumination-controlled facility. Diabetes was induced by a single injection of STZ (50 mg/kg) freshly dissolved in 50 mmol/l citrate buffer (pH 4.5) (Sigma, St. Louis, MO) into the tail vein. Citrate buffer was injected into age-matched control animals. One week after STZ administration, rats with plasma glucose concentrations >20 mmol/l were designated diabetic rats. To evaluate the effect of LY333531 on diabetic hyperalgesia, control and diabetic rats were divided into two groups, untreated and LY333531 treated. Then, four groups including control untreated ($n = 7$), control LY333531 treated ($n = 8$), diabetic untreated ($n = 8$), and diabetic LY333531 treated ($n = 7$) rats were used for this study. LY333531 (courtesy of Eli Lilly) was given at a dose of 10 $mg \cdot kg^{-1} \cdot day^{-1}$ compounded into feed every day for 6 weeks. In another study to evaluate the effect of L-arginine on diabetic hyperalgesia, L-arginine was intraperitoneally given to diabetic rats ($n = 5$) at a dose of 250 mg/kg in saline, every day for 4 weeks, starting 2 weeks after STZ injection. All experiments were conducted with the approval of the Institute for Experimental Animals at Shiga University of Medical Science and in complete compliance with the guidelines for Animal Experimentation for the Study of Pain at Shiga University of Medical Science.

Nociceptive tests. The mechanical threshold for the nociceptive flexion reflex elicited by stimulation of the dorsal surface of the hind paw was quantified using an analgesymeter (Ugo Basile). This device generates a mechanical force that increases linearly with time. The force was applied by a dome-shaped plunger (1.4 mm diameter, radius of curvature 36°). The nociceptive threshold is defined as the force, in grams, at which the rat withdraws its paw. The average threshold of three training trials constituted the baseline nociceptive threshold for that day. Rats were trained in the paw-withdrawal test at 5-min intervals for 30 min each day for 3 days. In the first experiment, withdrawal threshold was tested in untreated and LY333531-treated diabetic and control rats ($n = 9$ per group) at 0, 2, 4, and 6 weeks after STZ injection. In the second experiment to analyze the effect of L-arginine, nociceptive threshold was compared in rats ($n = 5$) treated only with saline at 0, 2, 4, and 6 weeks after STZ injection. To evaluate the possible effect of topical PKC inhibition in diabetic hyperalgesia, nociceptive threshold was assessed 6 weeks after STZ injection in the hindpaw of diabetic and age-matched control rats at intervals up to 24 h after intradermal injection of various concentrations of LY333531 into the footpads. The effect was compared with at the contralateral side, where the same volume of saline was injected. LY333531 dissolved in 0.01% DMSO in saline was injected in a volume of 2.5 μl with a 30-gauge needle.

Immunoblotting. Animals of each group were killed by decapitation under anesthesia. Bilateral L4-6 DRG samples from individual rats were dissected out and immediately homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mmol/l Tris-HCl, pH 7.4; 150 mmol/l NaCl; 1 μg/ml each of leupeptin, aprotinin, and pepstatin; 1 mmol/l EDTA; 1 mmol/l phenylmethylsulfonyl fluoride; 1 mmol/l Na_3VO_4; 1 mmol/l NaF; and 1% NP-40). Samples were centrifuged at 15,000*g* for 30 min at 4°C. Proteins in the supernatant were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with affinity-purified rabbit polyclonal antibodies: PKCα (sc-208), PKCβI (sc-209), PKCβII (sc-210) (Santa Cruz Biotechnology), nNOS (Transduction Labs), and monoclonal antibodies to TTX-R Na channel protein, SNS/PN3, and SNS2/NaN (20) (courtesy of Roche Bioscience) after blocking with 5% milk at 4°C overnight.

After standard washing, immunoreactivity was detected by enhanced chemiluminescence on film. The immunoreactive bands were measured using NIH Image.

Membrane preparation and assay for PKCβ activity. Both control and STZ rats were killed by decapitation under anesthesia. Bilateral L4-6 DRG samples from individual rats were dissected out and immediately homogenized in ice-cold buffer A (20 mmol/l Tris-HCl, pH 7.4; 0.25 mol/l sucrose; 0.15 mol/l NaCl; 25 μg/ml each of leupeptin and aprotinin A; 5 mmol/l EDTA; 2.5 mmol/l EGTA; and 2 mmol/l dithiothreitol) using a Wheaton-33 homogenizer for 30 s. Samples were centrifuged for 1 h at 100,000*g* at 4°C. The supernatant constituted the cytosolic PKC preparation. The pellet was rinsed twice in the same buffer, resuspended by a brief 30-s rehomogenization in buffer A containing 0.5% Nonidet P-40, and incubated on ice for 30 min with intermittent mixing. The extract was then centrifuged at 100,000*g* for 1 h at 4°C with the supernatant constituting the membrane-associated PKC preparation. The activity of PKCβ in both membrane and cytosol fractions was quantified by immunoprecipitation of the isozyme in the resuspended immunocomplexes. PKCβII isoforms in solubilized membrane and cytosol suspensions (100 μg protein/assay) were immunoprecipitated with protein A agarose and affinity-purified rabbit polyclonal IgG antibodies for PKCβII (Santa Cruz) at 3 μg/ml. After overnight incubation at 4°C, immunocomplexes were recovered by brief low-speed centrifugation. They were then rinsed once with a 1:1 mixture of the tissue homogenization buffer and 0.4 mol/l NaCl and recentrifuged for 20 s. The resulting pellet was resuspended in homogenization buffer (25 μl), and PKC activity was quantified using a commercial PKC enzyme detection system (RPN.77; Amersham Life Sciences, Arlington Heights, IL), based on the modification of a mixed micelle assay using a phorbol ester (21).

Immunocomplex assay for phospho-PKC. To examine the effect of LY333531 on PKC activity in DRGs of diabetic rats, the activities of PKCβII in DRGs were quantified by immunoprecipitation of the isozyme followed by phosphorylated PKCβ content in the resuspended immunocomplexes. PKCβII in DRG, homogenized as described above, were immunoprecipitated with protein A agarose and affinity-purified rabbit polyclonal IgG antibody for PKCβII (Santa Cruz) at 1 μg/ml. After 2-h incubation at 4°C, immunocomplexes were recovered by brief low-speed centrifugation and rinsed once with homogenized RIPA buffer. The resulting pellet was resuspended in sample buffer and expanded to SDS-PAGE. After transferring to nitrocellulose membrane, the membranes were incubated with rabbit polyclonal phospho-PKC antibody (no. 9371S; New England Biolabs). Immunoreactivity of phospho-PKCβ was detected as described above. The PKCβII protein was confirmed by reblotting the molecule of the band corresponding to phospho-PKCβII.

cGMP and cAMP assay. Bilateral L4-6 DRG samples were dissected out, immediately homogenized in 0.1N HCl, and heated at 100°C for 10 min in Eppendorf test tubes. The supernatant was obtained after centrifugation at 15,000*g* for 30 min. The cGMP content of the solution was measured using a cGMP radioimmunoassay kit (Yamasa). Protein in the solution was assayed, after neutralization, by the Bradford method (Bio-Rad). The cGMP content was corrected for protein concentration.

Effect of LY333531 on cGMP content of DRG: ex vivo assay. Bilateral L4-6 DRGs were dissected out from control and diabetic rats and immediately incubated in the oxygenized PBS medium (pH 7.4) containing 500 mmol/l isobutyl methyl xanthine and phosphodiesterase inhibitor for 30 min at 37°C. Our preliminary data suggested that the maximum effect of LY333531 on DRG cGMP content was obtained at 200 μmol/l LY333531 (data not shown). One side of L4-6 DRG for each rat was incubated in the medium without LY333531 and the other side was incubated in the presence of 200 μmol/l LY333531. After incubation, cGMP content of DRGs was measured as described above.

Immunohistochemistry. Animals were deeply anesthetized (pentobarbital, 60 mg/kg, intraperitoneally) and perfused intracardially with PBS followed by paraformaldehyde fixative. After perfusion, L4-6 DRGs were removed and fixed by immersion for 12–16 h at 4°C. Tissues were then rinsed in PBS, cryoprotected in 20% sucrose in PBS, frozen in O.C.T. compound with nitrogen liquid, and stored at –20°C until processing. Serial sections of frozen DRGs were cut in a cryostat (16 μm), mounted onto silanized slides, and used in immunohistochemical detection of individual PKC isoforms. Sections were incubated overnight at 4°C with one of the affinity-purified rabbit polyclonal antibodies listed below. Antibodies were diluted in PBS with 0.01% Triton

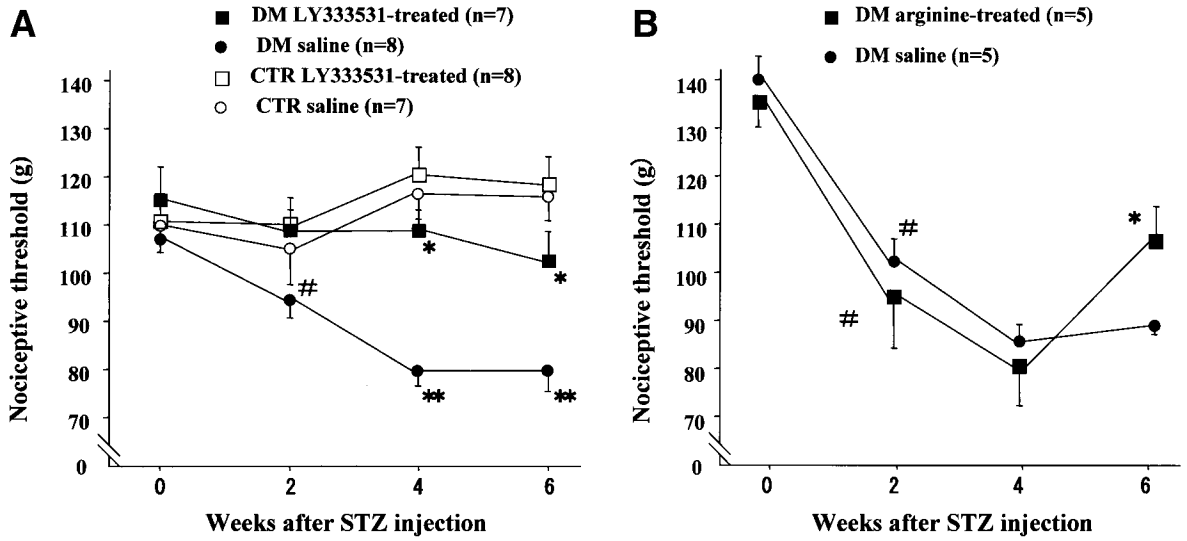

FIG. 1. ***A*: Time course of changes in mechanical nociceptive threshold in untreated control (○), LY333531-treated control (□), untreated diabetic (●), and LY333531-treated diabetic (■) rats. Values are means ± SE. The threshold was significantly decreased in untreated diabetic rats at 2 weeks (#$P < 0.05$) and later up to 6 weeks after STZ injection (**$P < 0.01$) compared with both groups of control rats. LY333531 significantly increased the threshold at 4 (*$P < 0.01$) and 6 (*$P < 0.01$) weeks after the start of the treatment. *B*: Time course of changes in mechanical nociceptive threshold in untreated diabetic (●) and L-arginine–treated diabetic (■) rats. Values are means ± SE. The threshold was significantly decreased at 2 weeks and later after STZ injection in both groups of diabetic rats versus before STZ injection (#$P < 0.05$). L-Arginine, which was given intraperitoneally every day for 4 weeks from 2 weeks after STZ injection, significantly increased threshold after 4 weeks of treatment (*$P < 0.05$).**

X-100 (PBS-TX) to enhance penetration of antiserum into the tissue. The antibodies were anti-PKCα (1:400), anti-PKCβI (1:250), anti-PKCβII (1:250), and anti-PKCγ (1:250) (Santa Cruz). After brief rinses in PBS and 1-h incubation in diluted biotinylated goat anti-rabbit IgG, the sections were incubated for 1 h with Vectastain Elite ABC Reagent (Vector Laboratories). The antigen-antibody complexes were visualized by incubation in 0.1% 3,3′-diaminobenzidine in 0.1 mol/l PBS (pH 7.4) containing 0.001% H_2O_2. Sections were dehydrated through ascending concentrations of ethanol and cover-slipped with Entellan (Merck).

Statistical analysis. All data are expressed as means ± SE. Treatment effects were analyzed by one-way or two-way ANOVA with post hoc analysis by the Scheffé test. $P < 0.05$ was considered statistically significant.

RESULTS

Nociceptive threshold in experimental rats: effects of LY333531 and L-arginine. In untreated diabetic rats, a significant decrease in nociceptive threshold developed at 2 weeks and lasted at least up to 4 weeks after the injection of STZ, compared with control rats (Fig. 1*A*). The hyperalgesia observed in untreated diabetic rats was significantly prevented with LY333531 treatment (Fig. 1*A*). To confirm the direct effect of LY333531 on DRG neurons or nerve fibers, the compound was intradermally injected into the foodpads of the rats. In control rats, the nociceptive threshold was not changed up to 24 h after the injection of either saline or LY333531 (20 μmol/l [data not shown] and 200 μmol/l). In diabetic rats, the nociceptive threshold peaked only 1 h after the injection of 200 μmol/l LY333531 and returned to the basal level by 24 h. Treatment with 20 μm LY333531 had no effect (data not shown). Thus, since the significant effect of LY333531 on nociceptive threshold was obtained only 1 h after its injection into diabetic rats, the dose-response curve of nociceptive threshold in diabetic rats was tested 1 h after the injection, demonstrating significant effects at >200 μmol/l of LY333531 (Fig. 2*B*).

In the next experiment to assess the effect of L-arginine on diabetic hyperalgesia and a possible implication of decreased NO-cGMP pathway, decreased nociceptive threshold developed 2 weeks after STZ injection. There was a significant reversal of the effect 4 weeks after the start of the treatment (Fig. 1*B*).

Immunoblotting of PKCs, nNOS, and SNS proteins. By immunoblotting, the expression of PKCβII, nNOS, and SNS/PN3 proteins was significantly decreased by 39, 64, and 90% in DRGs of diabetic rats compared with control DRGs (Fig. 3). Other isozymes were also decreased in diabetic rats: PKCα, 26%; PKCβI, 34%; and PKCγ, 43%. TTX-R Na channel protein SNS2/NaN was not detected in all groups of DRGs. Treatment with LY333531 did not change the expression of proteins

PKCβII activity and effect of LY333531 on phospho-PKCβII. The membrane and cytosolic fractions of PKCβII protein from DRGs were measured in control and diabetic rats. The cytosolic fraction was significantly larger than the membrane fraction in both types of rats ($P < 0.01$) (Fig. 4*A*). The cytosolic fraction was significantly larger in control than diabetic rats ($P < 0.01$) (Fig. 4*A*). The ratio of membrane to cytosol PKCβII was significantly larger in diabetic than in control rats (Fig. 4). To evaluate the effect of LY333531 on enzyme activity, phospho-PKCβII of DRGs was determined in diabetic rats. LY333531 treatment significantly reversed the increase of phospho-PKCβII protein in diabetic rats (Fig. 5). This was also true when phospho-PKCβII protein was normalized (data not shown)

cGMP content of DRGs. Cyclic GMP content of DRGs was significantly decreased in diabetic rats 6 weeks after STZ injection, compared with control rats (Table 1). Six weeks of LY333531 treatment completely reversed this decrease in diabetic rats, but it did not change cGMP content in control rats. In an in vivo study, 200 μmol/l LY333531 significantly increased cGMP content of DRGs from diabetic rats. Four weeks of intraperitoneal L-arginine injections also significantly restored decreased cGMP content of DRGs in diabetic rats.

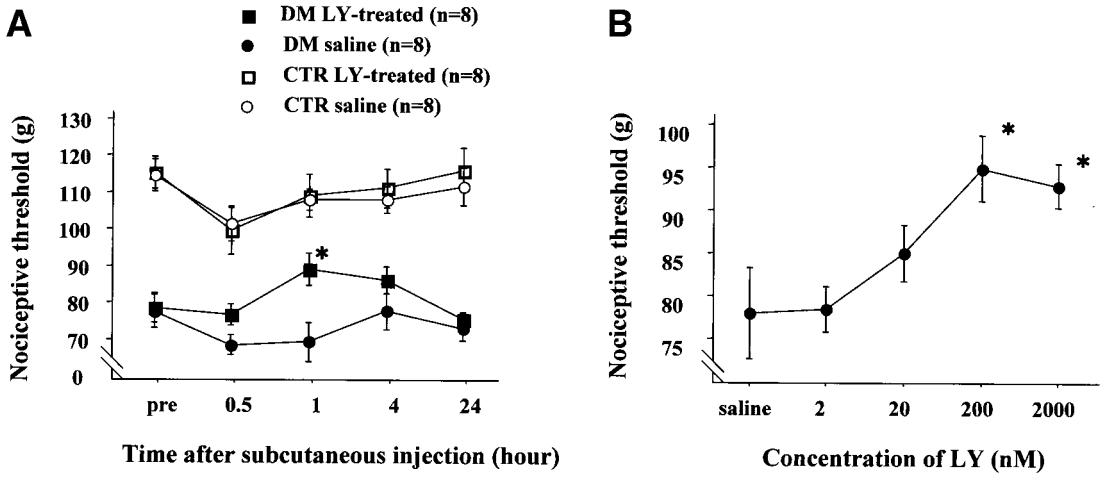

FIG. 2. *A*: **Time course of the effect of intradermal injection of saline (○, ●) and LY333531 (□, ■) on mechanical nociceptive threshold in control (○, □) and diabetic (●, ■) rats. Baseline mechanical nociceptive threshold was significantly lower in STZ diabetic rats than in control rats. The injection with 200 μmol/l LY333531 did not change the threshold in control rats. By contrast, 200 μmol/l LY333531gradually increased the threshold up to 1 h after the injection following its decline in diabetic rats, although the increase reached the significant level only at 1 h (*P < 0.05 vs. saline injection)** *B*: **Dose-response curve for the effect of intradermal injection of LY333531 on mechanical nociceptive threshold in STZ diabetic rats, assessed 1 h after the injection. The threshold was maximally increased with the doses >200 μmol/l LY333531 (*P < 0.05 vs. saline injection at 200 and 2000 μmol/l LY333531; n = 8 for each concentration).**

Immunohistochemistry of PKC isoforms in DRGs (Fig. 6). DRG neurons were immunostained by antibodies for PKCα, -βII, and -γ but not by an antibody for PKCβI. By contrast, satellite cells immunostained for all these isoforms, but the staining was rather weak for PKCα antibody. Among DRG neurons, small neurons were immunostained mainly by PKCβII antibody. Endoneurial vascular cells were also stained by this antibody. Indeed, half of the small neurons showed intense immunostaining, whereas other small neurons only showed slight or negative stain. In contrast, PKCβI was detected mainly in satellite cells and endoneurial vascular cells. There was no significant difference in intensity and distribution of immunostaining for PKCβI and βII between control and diabetic rats.

DISCUSSION

In the present study, STZ rats showed an exaggerated response to painful stimuli (hyperalgesia), which confirms our previous reports (17,18). It has been established that hyperalgesic behavior develops in diabetic animals (22,23),

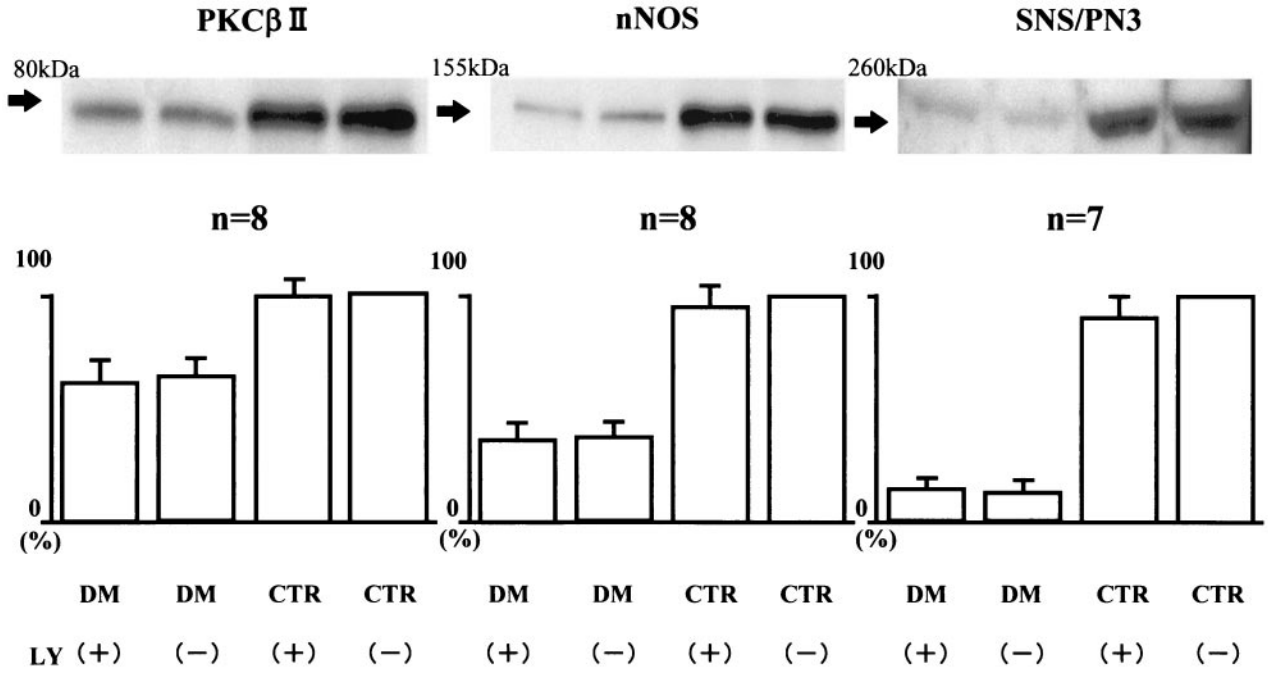

FIG. 3. Immunoblotting with anti-PKCβII, anti-nNOS, and anti-SNS/PN3 antibodies in DRG of control and diabetic groups with and without LY333531 treatment. In the expression of all proteins, diabetic rats showed significantly decreased expression compared with control rats without any effect of LY333531 treatment (P < 0.001, control group with or without LY333531 treatment versus diabetic group with or without LY333531 treatment, respectively).

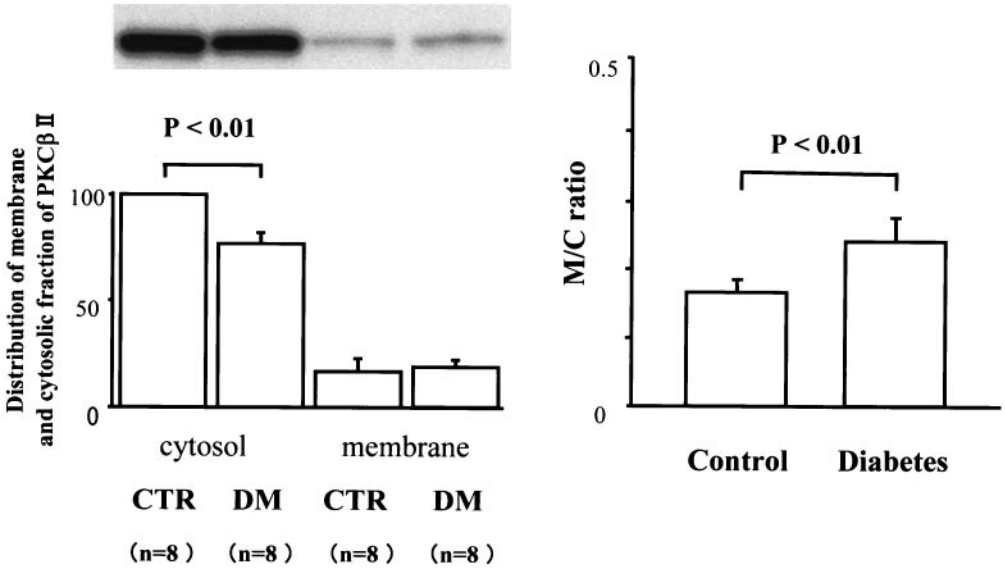

FIG. 4. ***Upper left*****: Immunoblotting with anti-PKCβII antibody of membrane and cytosol fraction of DRGs in control and diabetic rats.** ***Lower left*****: Each band of PKCβII was densitometrically and quantitatively analyzed by NIH Image. Values are means ± SE.** $P < 0.01$**: membrane vs. cytosol in CTR (control rats;** $n = 8$ **for each group) and DM (diabetic rats;** $n = 8$ **for each group);** $P < 0.01$**: CTR vs. DM for cytosol by Scheffé test.** ***Right*****: The ratio of membrane to cytosol (M/C) of PKCβII protein of DRGs was significantly higher in diabetic than control rats.**

and this decreased nociception has also been described in patients with diabetes (24). Our study clearly shows that the PKCβ-selective inhibitor LY333531 ameliorated hyperalgesia in diabetic rats. Since ischemia may be implicated in the development of diabetic hyperalgesia, and the data on the effects of LY333531 indicate a profound effect on nerve blood flow as well as retinal and renal blood flow, the possibility that LY333531 has an beneficial effect on diabetic hyperalgesia through ameliorating nerve blood flow may not be excluded. However, since this ameliorative effect was obtained by intradermal injection of LY333531 into the footpad of diabetic rats and occurred shortly after the injection, it may be that at least part of the effect is obtained through direct action on cutaneous nerve fibers.

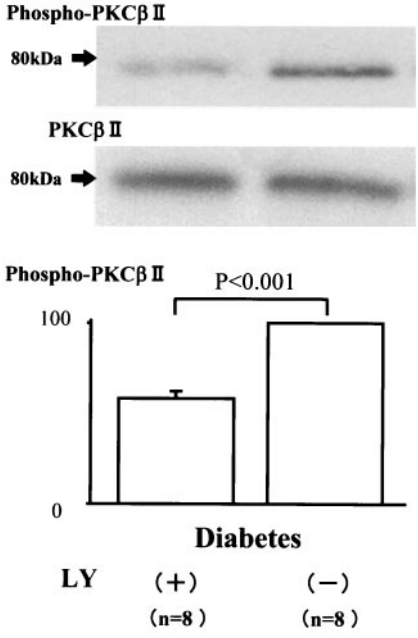

FIG. 5. Immunoblotting with anti-PKCβII and anti-phospho-PKCβII antibodies in DRGs of diabetic rats with and without LY333531 treatment. Phospho-PKCβII bands were densitometrically and quantitatively analyzed by NIH Image. The phospho-PKCβII was significantly decreased in LY333531-treated diabetic rats compared with untreated diabetic rats ($P < 0.01$**).**

The mechanism of diabetic hyperalgesia remains unknown. Although STZ-diabetic animals show cachexia, malnourished condition, and ketosis from the uncontrolled hyperglycemia, there is no report that each of these conditions per se relates to hyperalgesia. However, the irritability due to general ill-health in STZ-induced diabetic animals may partly contribute to higher response to nociceptive stimuli (25). Aside from the latter point, many researchers have noticed the presence of mechanical hyperalgesia in STZ-induced diabetic animals. In fact, high glucose per se (26), changes of neurotransmitters (27), alterations of opioid metabolism and receptors (28,29), or physiologically increased responsiveness or abnormalities of ion channels of neurons (15,18,30,31,32) have been proposed as contributing factors to hyperalgesia. Many reports have supported the significant effect of PKC modulators on the generation of pain; PKC may contribute to primary afferent C-fiber excitability, because phorbol esters can depolarize cultured DRG neurons with C-fiber properties (33). Primary cultured DRG neurons in STZ-induced diabetic rats depolarize because of altered TTX-R Na^+ channel activity (18). Ahlgren and Levine (15) reported the intradermal effect of PKC inhibitors on the reduced mechanical nociceptive threshold and on the increased C-fiber excitability in STZ-induced diabetic rats. The relief by intradermal injections of PKC inhibitors is consistent with our observation of the effect of LY333531 on neurons or nerve fibers.

Spinal and supraspinal contributions to hyperalgesia have important roles by acting at a spinal *N*-methyl-D-aspartate (NMDA) receptor (34). The induction of neuropathic pain by STZ-induced diabetic rats renders spinal cord opioid systems ineffective in producing anti-nociception for noxious heat, electrical, and pressure stimuli (29). The mechanisms proposed for this opioid resistance include downregulation or destruction of opioid receptors. This is probably mediated by increased production of protein kinase C following activation of NMDA receptors in postsynaptic cells (35,36). In addition, Igwe and Chronwall (14) provided evidence for inflammation-induced upregulation of membranous PKCβII activity of the lumbar spinal cord ipsilateral to the inflammation. This indi-

TABLE 1
cGMP content in DRGs of control and diabetic rats

	In vivo				Ex vivo	
	cGMP/protein (fmol/mg)	*n*	cGMP/protein (fmol/mg)	*n*	cGMP/protein (fmol/mg)	*n*
Diabetic rats						
LY333531 treated	148.47 ± 9.84*	8			81.16 ± 1.27†	6
Untreated	91.02 ± 6.01	7	89.24 ± 7.25†	5	75.73 ± 0.84	6
L-arginine treated			113.80 ± 3.84	5		
Control rats						
LY333531 treated	123.65 ± 4.44	7			76.83 ± 0.59	6
Untreated	124.72 ± 7.81	7	116.77 ± 3.09	5	76.47 ± 1.07	6
L-arginine treated			126.69 ± 6.37	5		

Data are means ± SE. *$P < 0.01$, †$P < 0.05$ compared with untreated diabetic rats.

cates activity-dependent alterations in the regulation of translocation and activation of PKCβII, and their involvement in the initiation and maintenance of hyperalgesia. Confirmation of this was obtained by quantitative immunohistochemical analyses, time-course for increases in the intensity of PKCβII immunoreactivity as well as in the activity of membrane-associated PKCβII paralleled inflammation-mediated changes in paw withdrawal latency and paw diameter. This observation could support that the PKCβ-selective inhibitor LY333531 may have a significant effect on neurons at the spinal level.

In the peripheral nervous system, the localization of PKC isoforms has not been well examined in DRG neurons. We found that PKCβI and PKCβII were respectively

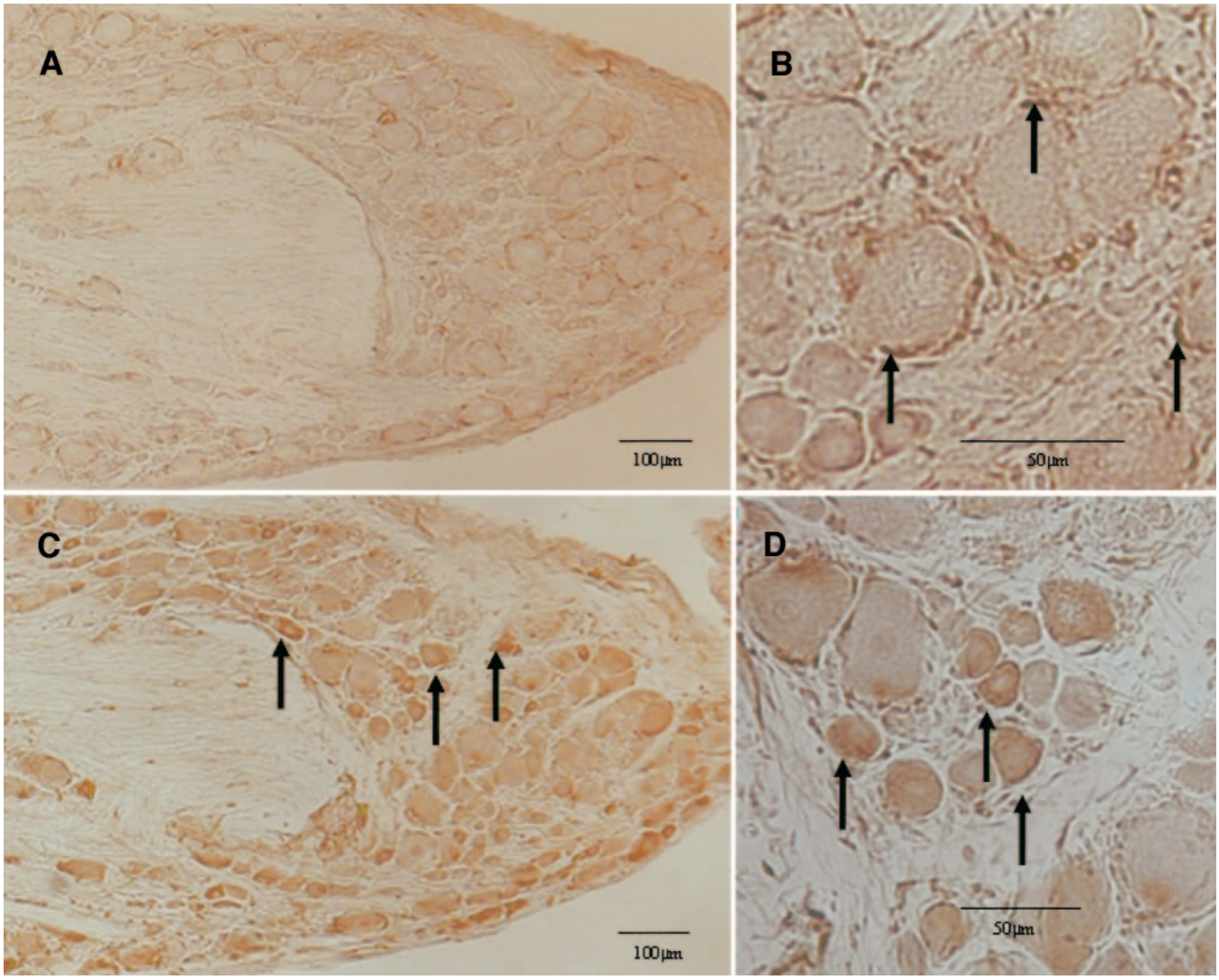

FIG. 6. Immunohistochemistry for PKCβI (*A*, *B*) and PKCβII (*C*, *D*) in the rat DRG. *A* and *B*: The immunoreactivity for PKCβI was seen in satellite cells (arrows), whereas it was not seen in neurons. *C*: The immunoreactivity for PKCβII was clearly seen in neurons. Indeed, about half of small neurons were strongly immunostained (arrows). By contrast, the immunoreactivity for satellite cells was not evident.

localized in satellite cells and neurons, especially small neurons, of DRG, the latter of whose axons, afferent Aδ- and C-fibers, conduct nociception. Together with the observation that both DRG neurons and peripheral nerves are directly modulated by PKC, these findings may be compatible with the notion that LY333531 would have a beneficial effect on hyperalgesia by directly affecting the nociceptive threshold at the peripheral nerve level. With respect to the activity of PKC and the presence of its isoforms in diabetic nerves, it remains controversial whether PKC activity in diabetic nerves is reduced (37,38), unchanged (7,39), or increased (40), and it also remains unclear which isoform is altered in diabetic nerves. Our results that the ratio of membrane to cytosol PKCβII activity was significantly higher in diabetic than in control nerves, and that LY333531significantly reduced phosphorylated PKCβII, may support increased PKCβ II activity of DRG neurons and the inhibitory effect of LY333531 on PKCβII activity of DRG neurons in diabetes.

LY333531 significantly restored the decrease in cGMP content of DRGs under ex vivo as well as in vivo conditions, which has been consistently observed in diabetic rats. Together with our observation that the treatment with L-arginine improves the cGMP content of DRG as well as hyperalgesia in diabetic rats, decreased cGMP content may underlie diabetic hyperalgesia, and amelioration of cGMP content would contribute to relief of hyperalgesia. An antinociceptive effect of L-arginine in diabetic mice has also been reported by others (41). We have previously reported the possible implication of decreased cGMP content in DRGs, as well as the decrease in nNOS expression, in the genesis of pain in diabetic rats (14). NO is a highly reactive, rapidly diffusible gas synthesized from L-arginine by tissue- and cell-specific NOS. The calcium-calmodulin-dependent constitutive nNOS produces a low level of NO, which specifically interacts with and activates heme-containing soluble guanylyl cyclase in neighboring neuronal cells in a paracrine fashion. The signal is transduced via cGMP and cGMP-dependent protein kinases (PKG). The calcium current in chick embryo DRG neuron is suppressed by NO donors and membrane-permeable cGMP analog, (42) and the calcium channel is a substrate of PKG (43). Because calcium current is closely associated with nociception (44), decreased activity of the nNOS-cGMP pathway may be involved in the genesis of hyperalgesia. In fact, the cGMP-PKG pathway was shown not only to trigger some forms of persistent pain (45), but also to be critical for the induction of long-term sensitization of nociceptive sensory neurons (46).

Although the mechanism by which LY333531 ameliorates the decrease in cGMP content remains unclear, the interrelationship between PKC and the NO-cGMP pathway has been demonstrated in the diabetic state. An impairment of NO-dependent cGMP generation in glomeruli from diabetic rats is mediated in part by an activation of PKC (47). In SH-SY5Y human neuroblastoma cells, impaired glucose-mediated NO-dependent cGMP production was corrected by PKC agonists and reproduced by PKC inhibition (48), a finding that is just opposite to ours in the contributory role of PKC for NO-cGMP metabolism.

We previously reported that TTX-R Na current was shown to be increased in DRGs of diabetic rats (18). TTX-R Na channels play an important role in nociception (19), and the inhibition of PKC activity was shown to increase nociceptive threshold in diabetic rats (15). One might speculate that the effect of LY333531 may be mediated by inhibition of the TTX-R Na current through blockage of the phosphorylation of TTX-R Na channels. An alternate explanation may include an increase in the expression of TTX-R Na channels in diabetes. Our data exclude the latter possibility. TTX-R Na channel protein of DRGs was significantly decreased in diabetic rats compared with control rats, without any effect of LY333531 on their expression. Decreased TTX-R Na channel expression in diabetic state was also reported by others (49).

In conclusion, it was clearly demonstrated that the inhibition of PKCβII activity by LY333531 ameliorated hyperalgesia in diabetic rats. Although its precise mechanism remains unclear, a restoration of cGMP content in DRG neurons, through inhibition of PKCβII activity, may contribute, at least partially, to the amelioration of hyperalgesia. Further investigations are required to clarify the mechanisms of action of LY333531 as well as the pathogenic mechanisms of diabetic hyperalgesia. However, the significant effect of LY333531 on diabetic hyperalgesia appears to indicate the potential of LY333531 as a therapeutic compound for the pain syndrome in diabetes, as well as its usefulness as a research tool for diabetic hyperalgesia.

ACKNOWLEDGMENTS

This study was supported in part by research grants from the Ministry of Education, Science, Technology, Sports and Culture of Japan and from the Ministry of Health and Welfare of Japan.

The authors are indebted to David J. Chang, Research Strategy Manager, Neurobiology Unit, Roche Bioscience, for the kind supply of anti TTX-R Na channels. The authors thank Dr. Louis “Skip” Vignati, Research Fellow, Lilly Research Laboratories, for his review of the manuscript.

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