Abnormalities of Retinal Metabolism in Diabetes or Experimental Galactosemia

V. Relationship Between Protein Kinase C and ATPases

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In the retinas of diabetic animals, protein kinase C (PKC) activity is elevated, and Na^+-K^+-ATPase and calcium ATPase activities are subnormal. These abnormalities are also present in another model of diabetic retinopathy, experimental galactosemia. We have investigated the relationship between hyperglycemia-induced abnormalities of PKC and ATPases using a selective inhibitor of β isoform of PKC (LY333531). Diabetes or experimental galactosemia of 2 months' duration resulted in >50% elevation of PKC activity in the retina, and administration of LY333531 prevented the elevation. In retinas of the same rats, the LY333531 prevented hyperglycemia-induced decreases of both Na^+-K^+-ATPase and calcium ATPase activities. Retinal microvessels, the main site of lesions in diabetic retinopathy, likewise showed elevated activity of PKC and inhibition of ATPases in diabetes and in experimental galactosemia, and administration of LY333531 to diabetic animals prevented these abnormalities. PKC activity in sciatic nerves, in contrast, became subnormal in diabetes and experimental galactosemia, and LY333531 had no effect on PKC activity in the sciatic nerve. PKC activity in the cerebral cortex was not affected by diabetes or experimental galactosemia. The results suggest that diabetes-induced reductions in Na^+-K^+-ATPase and calcium ATPase in the retina are mediated in large part by PKC-β. The availability of an agent that can normalize the hyperglycemia-induced increase in PKC activity in the retina should facilitate investigation of the role of PKC in the development of diabetic retinopathy. *Diabetes* 47:464–469, 1998

Elevation of blood hexose concentration (glucose or galactose) has been found to be sufficient to initiate the development of diabetic retinopathy (1,2). Alterations in retinal metabolism induced by hyperglycemia presumably contribute to the development of this retinopathy, but which metabolic abnormalities may be critical in the etiology of diabetic retinopathy remains to be established. One sequela of hyperglycemia that has been postulated to play a role in the pathogenesis of diabetic retinopathy involves changes in activity of protein kinase C (PKC) (3). PKC helps to regulate diverse processes, including signal transduction for hormones, vascular hemodynamics, cellular proliferation and migration, and neovascularization (4–7) and regulates activities of many enzymes, including cytosolic phospholipase A_2 (8), nitric oxide synthase (9), and Na^+-K^+-ATPase (10,11). Diabetes alters PKC activity in a tissue-dependent manner: the activity is increased in retina, aorta, heart, glomeruli in diabetes, and vascular cells cultured in high glucose or galactose (3,12–15), but is decreased in peripheral nerves (16) and remains unchanged in the brain (3,17).

The effect of experimental galactosemia on PKC activity in retina and other tissues is not well studied. Because diabetes and experimental galactosemia have been postulated to cause retinopathy by final common pathway, measurement of PKC activity in retinas of galactose-fed animals is a valuable test of the postulated role of PKC in the pathogenesis of diabetic retinopathy. To date, exposure of cultured rat aortic smooth muscle cells to high galactose concentration has been found to increase PKC activity, and galactose-fed dogs have increased levels of diacylglycerol (DAG) (a regulator of PKC) in retina and aorta (18).

PKC agonists and antagonists have been used in vitro to demonstrate the many actions of PKC, but these agents are not isoform specific and are undesirable for in vivo studies because of the possible nonspecificity of their actions and toxicity during prolonged administration (19,20). In vitro, PKC agonists have been reported to normalize diabetes-induced abnormalities of Na^+-K^+-ATPase in erythrocytes (21) and sciatic nerves (22).

An inhibitor selective for the β isoform of PKC has been developed (LY333531, Eli Lilly, Indianapolis, IN), and dietary supplementation with this drug has been found to inhibit diabetes-induced abnormalities of retinal blood flow, glomerular filtration rate, and albuminuria in rats (13,23). LY333531 has a concentration for 50% inhibition (IC_{50}) of 5 nmol/l for PKC-β, which is at least 100 times lower than that for α, γ, or δ isoforms of PKC (13). In the present study, the relationship between diabetes-induced alterations in retinal PKC-β and ATPases has been investigated by studying the effect of LY333531 on these enzymes in whole retina and isolated retinal microvessels. Effects of this inhibitor on PKC activity in sciatic nerve and cerebral cortex of diabetic animals

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Received for publication 29 November 1996 and accepted in revised form 4 November 1997.

DAG, diacylglycerol; PKC, protein kinase C; TCA, trichloroacetic acid; TPA, tetradecanoylphorbol acetate.

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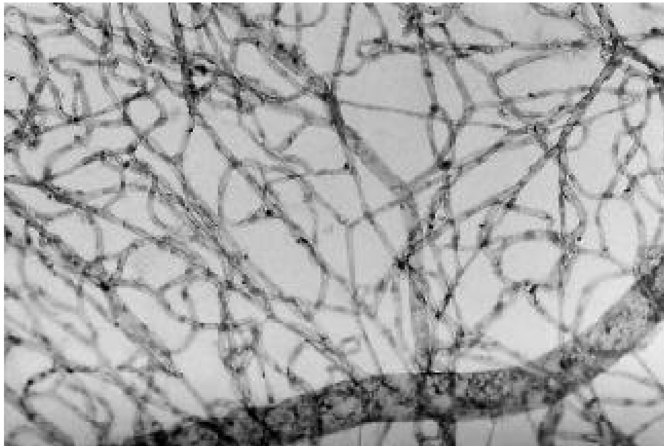

FIG. 1. Retinal microvessels isolated by the osmotic shock method (periodic acid and hematoxylin).

were also examined for comparison. In addition, this study demonstrates the effect of experimental galactosemia on PKC activity in the retina, retinal microvessels, sciatic nerve, and brain.

RESEARCH DESIGN AND METHODS

Sprague-Dawley rats (200–220 g) were assigned to remain normal or made diabetic or experimentally galactosemic. Diabetes was induced by an injection of alloxan monohydrate (45 mg/kg i.v.) after a 24-h fast. Insulin was given as needed to diabetic rats to allow slow weight gain while still allowing polyuria, hyperphagia, and hyperglycemia. Experimental galactosemia was induced in normal rats by feeding 30% galactose in the rat food. These experiments conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. Diabetic rats were randomized among four groups: diabetic control rats, and diabetic rats receiving LY333531 in powdered diet at doses anticipated to be 0.1, 1, and 10 mg · kg^{-1} body wt · day^{-1}. LY333531 was administered at an anticipated dose of 10 mg · kg^{-1} body wt · day^{-1} also to normal and galactose-fed animals in another experiment. Animals were weighed, and their food consumption was measured weekly to calculate the actual LY333531 intake. The dose of 0.1 mg · kg^{-1} body wt · day^{-1} was found to have little effect under the present experimental conditions, and the data from this group are not included. Nonenzymatically glycated hemoglobin (GHb) was measured at 2 months of diabetes using affinity columns (Glyc-Affin; Pierce, Rockford, IL). Plasma drug levels in fasted and nonfasted rats were measured using gas chromatography–mass spectroscopy.

Retina, sciatic nerve, and cerebral cortex were collected at 2 months of diabetes or experimental galactosemia, a duration at which biochemical abnormalities are observed in the retina without apparent histological changes. Tissues were obtained in the morning from anesthetized animals from which food had been removed 16–18 h before death. Because rats eat predominantly at night, the duration since last intake of LY333531 in food may have been as long as 24 h before death. Retinas were isolated by gently separating sensory retina from the pigment epithelium and choroid using a microspatula under a dissecting microscope and were kept on ice before being assayed. Sciatic nerves from both legs were isolated, cleaned, and frozen on dry ice. Cerebral cortex was exposed, and a small piece was frozen on dry ice. Initial studies showed that freezing of retina resulted in >50% loss of PKC activity, whereas freezing of sciatic nerve or cerebral cortex had no effect on their respective PKC activities. Thus, fresh retina and retinal microvessels and frozen samples of sciatic nerve and cerebral cortex were used for the PKC assay.

Retinal microvessels were isolated from whole retina of rats in various experimental groups by the osmotic shock method of Cuthbertson and Mandel (24). Freshly removed whole retina was incubated with distilled water for 1 h, followed by a 5-min incubation with DNAse (2 mg/ml). The retinal vasculature was then isolated under microscopy by repetitive inspiration and ejection through Pasteur pipettes with sequentially narrower tips. Microvessels isolated by this method showed a normal complement of nuclei and were generally devoid of nonvascular contaminants (Fig. 1). The osmotic shock method used to isolate retinal microvessels might result in a loss of cytoplasmic materials, but nevertheless was found to be suitable to measure membrane-bound enzymes such as ATPases and activated PKC. Sufficient amounts of microvessels were isolated from one and two rat retinas, respectively, to quantitate PKC and Na^+-K^+-ATPase activities. Because of the limited availability of microvessels (30–35 µg protein per two retinas), calcium ATPase was not measured in microvessels.

Enzyme assays. PKC was measured in fresh retina (one-half retina per tube), isolated retinal microvessels, sciatic nerve, or cerebral cortex, using an in situ assay (25), as modified by Ishii et al. (13). Briefly, the samples were incubated at 30°C for 10 min with a buffered salt solution containing 50 µg/ml digitonin to permeabilize the tissues, and 137 mmol/l NaCl, 5.4 mmol/l KCl, 10 mmol/l $MgCl_2$, 25 mmol/l β-glycerophosphate, 5.5 mmol/l glucose, 5.0 mmol/l EGTA, 1 mmol/l $CaCl_2$, 20 mmol/l HEPES, 100 µmol/l γ-[^{32}P]ATP containing 1,000–1,400 cpm/pmol, and 100 µmol/l of PKC-specific peptide substrate. The substrate used was the octapeptide ([RKRTLRRL] corresponding to the threonine phosphorylation site of the epidermal growth factor receptor [residue 691–698]). The reaction was terminated by adding 25% trichloroacetic acid (TCA), and the samples were centrifuged and the supernatant spotted onto phosphocellulose paper (2.5-cm circles). Filter papers were washed three times with 75 mmol/l phosphoric acid for 30 min each and then counted. PKC activity was assessed based on the transfer of ^{32}P from γ-[^{32}P]ATP to the octapeptide and represents the sum of PKC activity of various isoforms present in the tissue. For measuring total protein in the assay system, the TCA pellet was solubilized by SDS. Validation of PKC activity was performed by using phorbol esters (100 nmol/l tetradecanoylphorbol acetate [TPA]) and staurosporine (50 nmol/l). TPA stimulated PKC activity in the retina, retinal microvessels, and sciatic nerve by two- to threefold, and staurosporine inhibited the enzyme activity by >95%.

ATPase and calcium ATPase were measured by methods described previously (26). Retinal microvessels were permeabilized with 0.01% SDS before measurement of Na^+-K^+-ATPase. Protein was measured by the Bradford method (27) using bovine serum albumin as standard.

Experimental groups were compared statistically using the nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U* test for multiple group comparisons. Analysis of variance with Fisher's or Tukey's group comparisons gave similar results.

RESULTS

Diabetes of 2 months' duration in rats was marked by significant hyperglycemia, as indicated by the elevation of blood glucose and GHb, and this elevation was similar in all of the three diabetic groups. Consumption of LY333531 by diabetic

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TABLE 1
LY333531 had no effect on severity of hyperglycemia or food consumption

	Body weight (g)	Food intake (g · kg^{-1} body wt · day^{-1})	Blood glucose (mg/dl)	GHb (%)
Diabetes plus:				
Control	251 ± 23*	182 ± 42*	374 ± 103*	12.6 ± 1.9*
1 mg · kg^{-1} body wt · day^{-1} LY333531	257 ± 32*	187 ± 34*	367 ± 85*	13.3 ± 1.5*
10 mg · kg^{-1} body wt · day^{-1} LY333531	248 ± 22*	184 ± 25*	385 ± 104*	13.5 ± 1.3*
Normal	365 ± 21	85 ± 19	66 ± 14	4.2 ± 0.6

Data are means ± SD of eight to ten rats in each group. *$P < 0.005$ compared with normal.

TABLE 2
Plasma LY333531 levels in fasted and fed rats

	LY333531 (ng/ml plasma)	
	Fasted	Fed
Diabetes plus LY333531		
1 mg · kg^{-1} body wt · day^{-1}	0.15 ± 0.25	1.49 ± 0.74
10 mg · kg^{-1} body wt · day^{-1}	1.49 ± 1.56	10.6 ± 4.1

Data are means ± SD of three to five animals in each group. Blood was collected at 8:00 A.M. from fed rats and from rats fasted overnight.

rats had no apparent effect on body weight, severity of hyperglycemia, or food intake (Table 1). Actual LY333531 intake was close to the intended doses, actually averaging 1.4 and 13.5 mg · kg^{-1} body wt · day^{-1} for the groups intended to receive 1 and 10 mg · kg^{-1} body wt · day^{-1}, respectively. Levels of LY333531 in plasma after an overnight fast were 7- to 10-fold lower compared with the levels measured in fed (nonfasted) rats (Table 2).

Diabetes resulted in elevation of PKC activity in whole retina by 60% above that in normal rat retina. Similarly, experimental galactosemia of 2 months' duration resulted in 50% elevation of PKC activity in retina (Fig. 2). Administration of 1 and 10 mg · kg^{-1} body wt · day^{-1} LY333531 for 2 months partially and totally, respectively, inhibited the diabetes-induced elevation of PKC activity in the retina. LY333531 likewise inhibited retinal PKC activity in normal and galactosemic animals. Our results show that the extent of inhibition of retinal PKC activity by 10 mg · kg^{-1} body wt · day^{-1} LY333531 in normal, diabetic, and galactosemic rats is similar, but the significance of these results is the fact that this PKC inhibitor can normalize elevation in retinal PKC activity induced by hyperglycemia. PKC activity was elevated by >240% in retinal microvessels of diabetic rats, and LY333531 (10 mg · kg^{-1} body wt · day^{-1}; the only dose tested with microvessels) prevented this increase (Fig. 3). In experimental galactosemia, PKC activity in retinal microvessels was elevated by 45%, an increase that approached, but did not achieve, statistical significance.

Administration of LY333531 inhibited the diabetes-induced reductions of retinal Na^+-K^+-ATPase and calcium ATPase activities, and this effect was observed at both 1 and 10 mg · kg^{-1} body wt · day^{-1} of drug (Figs. 4 and 5). The PKC inhibitor similarly corrected reductions of activities of retinal ATPases in galactosemic rats, but unlike its effect on PKC activity in retinas from normal animals, LY333531 administration had no effect on retinal ATPase activities from the same normal animals. Na^+-K^+-ATPase activity in retinas of normal, normal-plus-LY333531, galactosemic, and galactose-plus-LY333531 groups was 161 ± 29, 152 ± 8, 135 ± 34, and 151 ± 25 pmol phosphate · min^{-1} · mg^{-1} protein, respectively (n = 3 per group). These results suggest that retinal ATPases are already in activated form in normal animals and that inhibition of retinal PKC by LY333531 in normal rats has no effect on the activities of ATPases. Na^+-K^+-ATPase activity also became subnormal in retinal microvessels of diabetic and experimentally galactosemic rats. Administration of LY333531 to diabetic rats totally inhibited this abnormality (Fig. 6).

PKC activity in sciatic nerves from the diabetic and galactose-fed rats was decreased by >35% compared with the sciatic nerves from normal animals (P < 0.05). LY333531 failed to have any effect on diabetes-induced reduction of PKC activity in sciatic nerve. Cerebral cortex from the same rats showed no effect of either diabetes or galactosemia on PKC activity (Table 3).

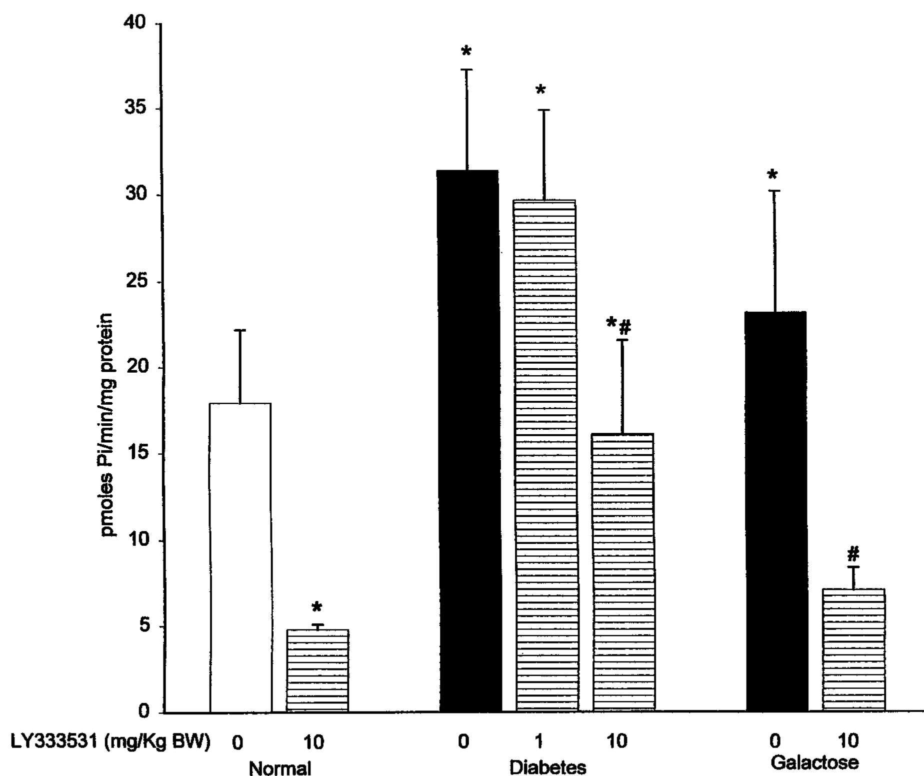

FIG. 2. Retinal PKC activity in diabetes and experimental galactosemia and effects of LY333531 on diabetes-induced elevation of PKC activity in retina. PKC activity was measured in fresh retina obtained from rats treated with or without LY333531 for 8 weeks. Data are means ± SD of 10 rats in each group. *P < 0.02 compared with normal; #P < 0.02 compared with diabetes.

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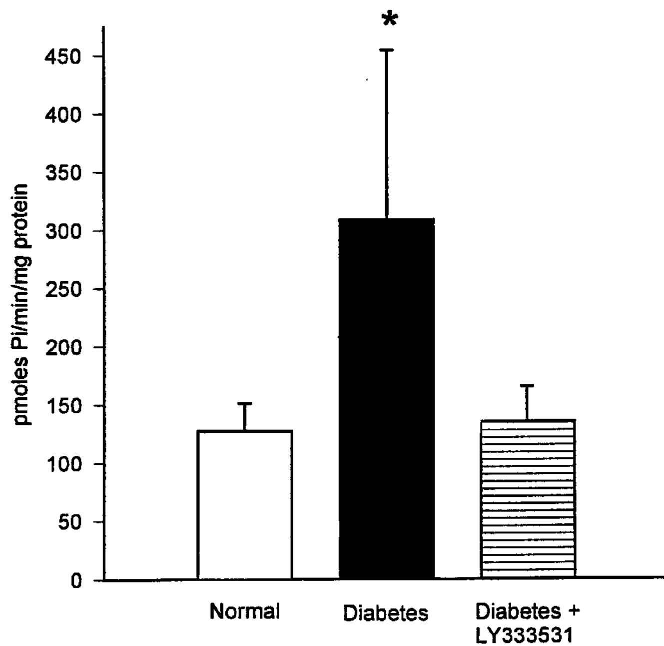

FIG. 3. PKC activity in retinal microvessels. Retinal microvessels were isolated by an osmotic shock method, and PKC activity was measured in freshly isolated microvessels. Microvessels obtained from a single retina were sufficient to measure PKC activity. Data are means ± SD of six to eight rats in each group. *P < 0.02 compared with normal.

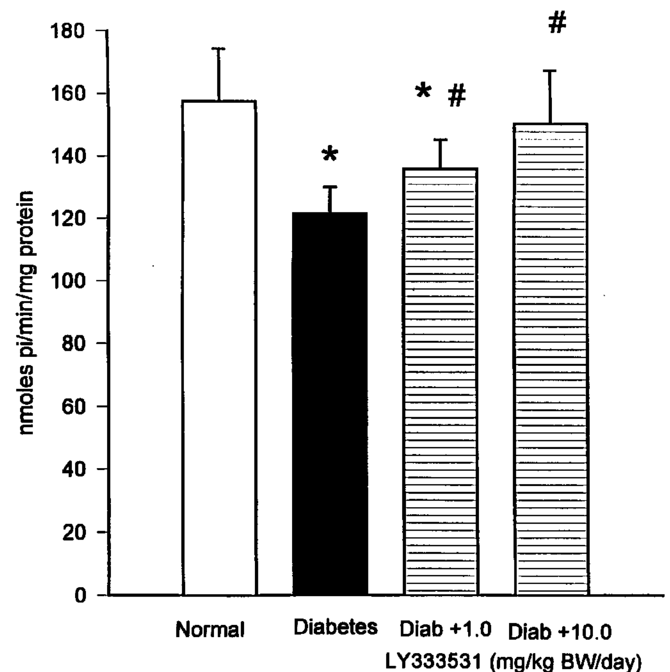

FIG. 4. Effects of LY333531 on retinal Na$^+$-K$^+$-ATPase activity. Na$^+$-K$^+$-ATPase activity was measured in one-half of the retina obtained from the same animals in which PKC activity was measured. Data are means ± SD of 8–10 rats in each group. *P < 0.02 compared with normal; #P < 0.02 compared with diabetes.

DISCUSSION

The present data demonstrate that PKC activity is increased in retinal microvessels in diabetes and confirm previous reports that PKC activity is increased in intact retina of diabetic animals (3,17). Others have shown that PKC activity of cultured endothelial cells is elevated by hyperglycemic media (15,17). Western blot analysis has shown that primarily the β isoform of PKC is increased in vascular cells and retina exposed to high glucose concentrations (3). The ability of a β isoform–selective inhibitor of PKC to effectively prevent diabetes-induced elevation of PKC in retina and in retinal microvessels likewise suggests that hyperglycemia results in activation of PKC-β in the retina. Inhibition of PKC activity might be demonstrable at even lower doses of the drug if tissues are collected from nonfasted animals in which the circulating levels of drugs are greater.

PKC was also significantly elevated in the retinas of galactose-fed animals and tended to be increased in microvessels of those galactosemics. The impression that PKC was increased less in galactosemia than in diabetes was possibly an artifact of the experimental design. All animals in the present study were killed after an overnight fast. Unlike diabetes, in which blood glucose levels remain elevated during fasting, blood levels of galactose are undetectable in galactosemic animals fasted overnight. Thus, a galactose-induced increase in retinal PKC activity likely would have been more apparent in unfasted (hypergalactosemic) animals.

Na$^+$-K$^+$-ATPase plays an important role in maintaining sodium and potassium gradients and neuronal electrical functions (28), and calcium ATPase helps regulate calcium levels in cells. These enzymes become subnormal in tissues during diabetes, including retina, sciatic nerve, and erythro-

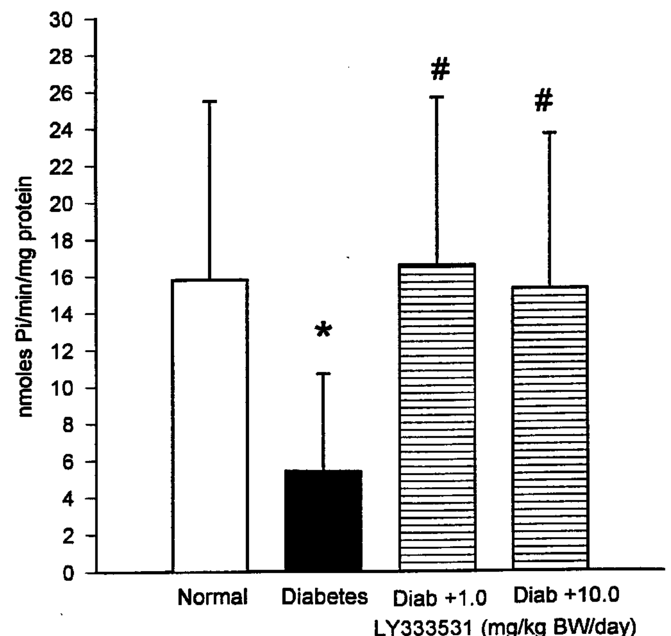

FIG. 5. Effects of LY333531 on retinal calcium ATPase activity. Data are means ± SD of 6–8 rats in each group. *P < 0.02 compared with normal; #P < 0.02 compared with diabetes.

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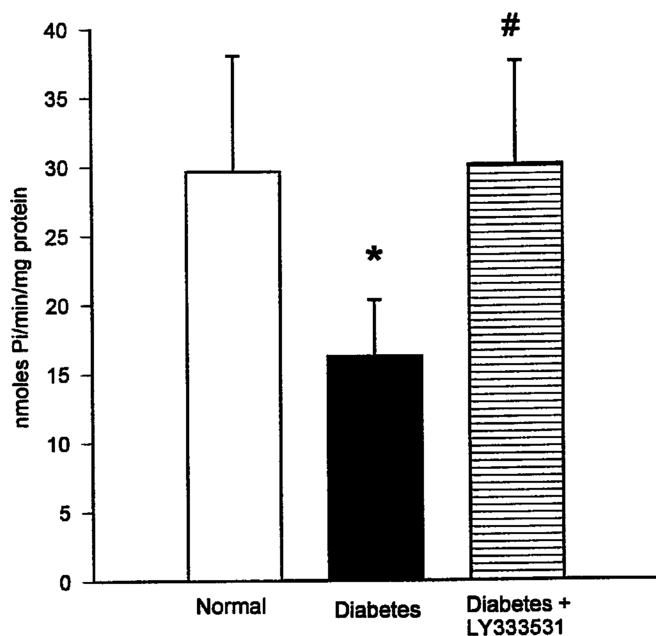

FIG. 6. Na^+-K^+-ATPase activity in retinal microvessels. Microvessels obtained from both retinas of an animal were used for each measurement of Na^+-K^+-ATPase activity, and six to eight rats were used in each group. *P < 0.02 compared with normal; #P < 0.02 compared with diabetes.

TABLE 3
PKC activity in sciatic nerve and cerebral cortex of diabetic and galactose-fed rats

	Sciatic nerve (pmoles Pi · min^{-1} · mg^{-1} protein)	Cerebral cortex (pmoles Pi · min^{-1} · mg^{-1} protein)
Normal	34.0 ± 4.0	24.8 ± 3.4
Diabetes	21.6 ± 4.2*	22.2 ± 3.3
Diabetes plus LY333531 (mg · kg^{-1} body wt · day^{-1})	23.1 ± 5.9*	26.7 ± 6.4
Galactose	24.0 ± 4.4*	23.5 ± 3.8

Data are means ± SD of six to eight rats in each group. *P < 0.01 compared with normal.

cytes (21,26,29). Regulation of Na^+-K^+-ATPase in some tissues is reported to be mediated in part by phosphorylation/dephosphorylation of its catalytic α-subunit by PKC (11,30); in purified preparations of Na^+-K^+-ATPase, PKC has been shown to phosphorylate the α-subunit of the enzyme (31), and in sciatic nerves from diabetic rats, phorbol esters stimulate phosphorylation of α-subunits of ATPase (30). Moreover, in cultured rat vascular smooth muscle cells, PKC-mediated elevation of cytosolic phospholipase A_2 activity has been shown to regulate hyperglycemia-induced decreases in Na^+-K^+-ATPase activity (10). Our results show that diabetes-induced reductions in ATPases in retinal tissue and microvessels can be corrected by normalization of PKC-β activity, suggesting that the defect in retinal ATPases is secondary to a diabetes-induced abnormality in PKC activity. However, the exact mechanisms by which PKC and ATPases are interrelated require further investigation. Other biochemical mechanisms also may be involved in the regulation of ATPases in retina, since diabetes-induced decreases in ATPase activity can be inhibited also by aldose reductase inhibitors or supplemental antioxidants (32,33).

Our study shows that PKC activity in the sciatic nerve is decreased both in diabetes and in experimental galactosemia. The diabetes-induced reduction in sciatic nerve PKC activity is in accordance with the previous report of Kim et al. (16). Administration of β isoform–specific inhibitor, LY333531, to diabetic rats failed to have any beneficial effect on sciatic nerve PKC activity, but administration of another PKC inhibitor, WAY151003, has been shown by others to correct diabetes-induced defects in nerve conduction velocity, blood flow, and Na^+-K^+-ATPase activity (34). Which isoform of PKC is involved in the diabetes-induced abnormalities of nerve function and metabolism and which nerve cell types manifest the PKC alteration remain to be identified. Importantly, the failure of LY333531 to inhibit nerve PKC activity suggests that the drug will not further adversely effect the diabetes-induced decrease in nerve total PKC activity.

In contrast to retina and sciatic nerve, PKC activity in the cerebral cortex was not affected by diabetes or experimental galactosemia. Others, likewise, have shown that diabetes has no effect on PKC activity in the brain (3,17). Levels of DAG, a regulator of PKC activity, also are reported to remain normal in cerebral cortex of diabetic animals despite being increased in retina (3). Although retina and cerebral cortex are embryologically similar, diabetes affects these two tissues differently in other respects also: microaneurysms, acellular capillaries, and pericyte loss become common in retina in diabetes, yet remain absent from cerebral cortex (35).

Our results show that hyperglycemia-induced alteration in PKC activity plays a role in the development of abnormal retinal metabolism in diabetes. The availability of a safe and orally effective inhibitor of PKC-β should facilitate investigation of the role of PKC activation in the development of the retinopathy.

ACKNOWLEDGMENTS

This work was supported in part by grants from Eli Lilly and the American Diabetes Association (R.A.K.), PHS grant EY00300 from the National Eye Institute, and an unrestricted grant from Research to Prevent Blindness.

The authors appreciate M. Larson for his technical assistance.

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