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Contractile Hyperreactivity and Alteration of PKC Activity in Gastric Fundus Smooth Muscle of Diabetic Rats

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SAKAI, Y., M. INAZU, A. SHAMOTO, B. ZHU AND I. HOMMA. *Contractile hyperreactivity and alteration of PKC activity in gastric fundus smooth muscle of diabetic rats.* PHARMACOL BIOCHEM BEHAV **49**(3) 669–674, 1994. – Contraction dose dependently induced in gastric smooth muscle of diabetic rats by Bay K 8644 in the presence of 20 mM KCl was about two times that induced in controls, and was inhibited more than 50% by 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7). Contraction was caused in diabetics but usually not in controls by 10^{-5} M phorbol 12-myristate 13-acetate (PMA). In diabetics, this contraction was about 2.5 times that in controls. Protein kinase C (PKC) activity in the soluble fraction was depressed by H-7 or staurosporine, and depended on PMA concentration, but was greater in diabetics than in controls at any PMA concentration. PKC activity in the soluble fraction was inhibited by lower Ca^{2+} concentration, and was greater in diabetics than in controls. Affinity and density of binding sites of a Ca^{2+} channel antagonist ligand, [^{3}H]PN200-110, were the same in plasma membrane-enriched fractions isolated from either controls or diabetic preparations. Thus, hyperreactivity in diabetic fundus may depend, in part, on alteration of PKC properties, but not on the density of Ca^{2+} channels.

Diabetes Protein kinase C Gastric smooth muscle Ca^{2+} signal H-7

ALTHOUGH pathology of the gastrointestinal tract has often been recognized in diabetes mellitus (DM), it is still poorly understood. Physiological changes in animal models of DM resemble the abnormalities that develop in humans who have diabetes. Morphological and histological studies describe gastrointestinal dysfunction in diabetic animals (2,3). There are contradicting opinions about gastric emptying in diabetic rats (10,11), but current evidence places the responsibility for gastric motor disturbance on vagal autonomic neuropathy in diabetic patients with gastroparesis. We have suggested that hyperreactivity of gastric fundus smooth muscle (1), decrement of gastric fundus relaxation, and increased contraction induced by norepinephrine (NE) in diabetic rats may be results of fewer β-adrenoceptor binding sites caused by downregulation due to higher catecholamine levels (17). However, decrease of β-adrenoceptors is not the only cause of dysfunction of gastric fundus in diabetic rats. Protein kinase C (PKC), an enzyme that is activated by the receptor mediated hydrolysis of inositol phosphatides, relays information in the form of various extracellular signals across cell membranes to regulate Ca^{2+}-dependent processes (13). Phosphorylation of Ca^{2+} channels by PKC might modulate the activity of voltage-dependent Ca^{2+} channels (VDC) and Ca^{2+} influx in smooth muscle (12). The hyperreactive contraction induced by KCl and acetylcholine (ACh), and reversal of the response to NE (1,17) suggest that the contractile response system may be related to Ca^{2+} channels and PKC activity in gastric fundus smooth muscle of diabetic rats. Preliminary data has been reported to the Japanese Physiological Society (18).

METHOD

Induction of Diabetes

Male 8-week-old Wistar rats weighing 220–260 g were randomly separated into control and experimental groups. Each rat was anesthetized with diethylether, and each test rat was given one injection of streptozotocin (STZ; 60 mg/kg, IV) in a citrate buffer (diabetic group). The control animals were injected with vehicle alone. Six weeks after injection, the animals were sacrificed. Blood samples were taken, and plasma glucose was measured by enzyme assay methods (17).

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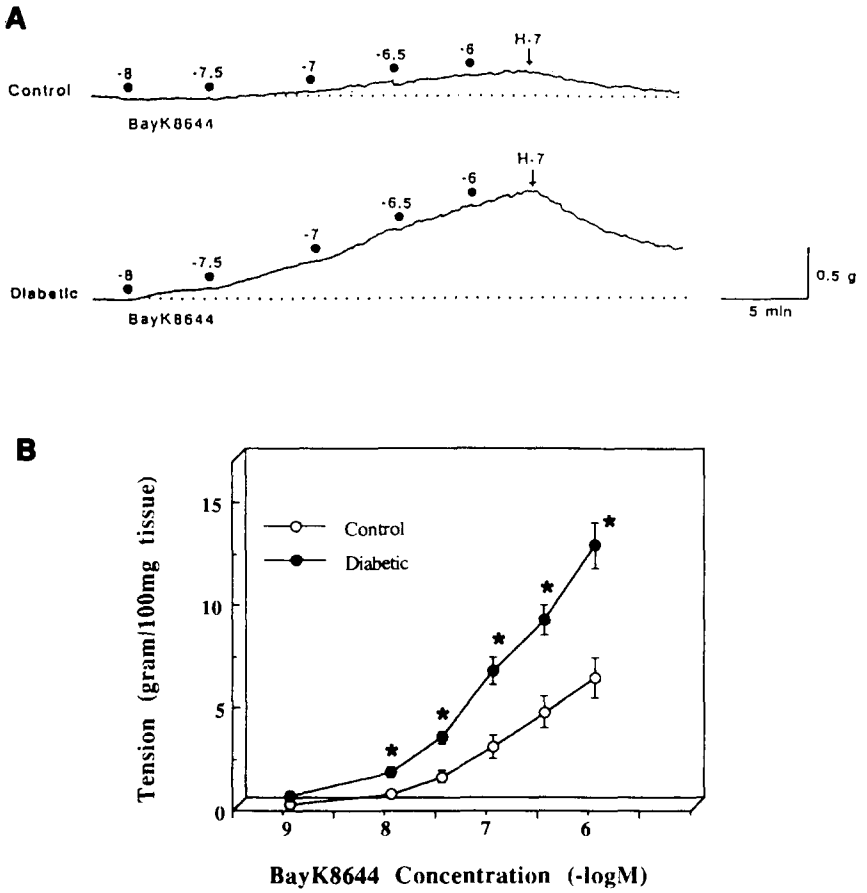

FIG. 1. Contractile responses to Bay K 8644 in the presence of 20 mM KCl in gastric fundus smooth muscle from control and diabetic rats. (A) Typical concentration responses to Bay K 8644 in the presence of 20 mM KCl. Numbers represent Bay K 8644 concentration as log (mol/l). 10^{-5} M H-7 suppressed the contraction induced by Bay K 8644. (B) Summary of force development data (g/100 mg tissue weight). Values are mean ± SE (n = 7). *Significantly different from control ($p < 0.01$).

Contraction Study

After a rat was sacrificed, the stomach was removed, dissected from surrounding tissue, and placed in a petri dish containing the following standard solution (mM): NaCl, 118; KCl, 5.8; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.4; $NaHCO_3$, 21.4; glucose, 11.5; equilibrated with 95% O_2 and 5% CO_2 to give pH 7.3 to 7.4 at room temperature. The gastric fundus region was dissected from the stomach, its mucosal layer was removed, and 2-3 mm wide by 15-18 mm long muscle strips were disected and immediately placed in 10 ml organ baths, each containing the above solution at 37°C, aerated with 95% O_2 and 5% CO_2, and maintained at pH 7.4. Isometric tension was recorded after 60 min equilibration under 1.0 g load, as previously described (17).

Preparation of Plasma Membrane-Enriched Fraction

After a rat was sacrificed, the gastric fundus was quickly removed and washed in cold 250 mM sucrose-50 mM Tris-HCl buffer (ST, pH 7.4). Mucosa and connective tissue were removed, and the remaining smooth muscle was used for preparing a plasma membrane-enriched fraction, as previously described (17). Gastric fundus material pooled from five rats was minced in 10 vol of ST buffer containing 5 mM $MgCl_2$ and homogenized in a polytron for 10 s, three times at 30-s intervals. The homogenate was centrifuged at 900 × g for 10 min, and that supernatant was centrifuged at 10,000 × g for 10 min. The supernatant from this last centrifugation was again centrifuged at 110,000 × g for 30 min. The pellet was resuspended in 50 mM Tris-HCl buffer (PH 7.4) containing 5 mM $MgCl_2$, and this suspension was centrifuged at 10,000 × g for 10 min. The supernatant was stored at −80°C until used. Protein concentration was measured by the method of Lowry et al. (9), with bovine serum albumin as a standard. Measurement of the activity of 5′-nucleotidase, a marker for plasma membrane, indicated that the plasma membrane enrichment was the same (8-10 times that in the post nuclear supernatant) in the control and diabetic preparations.

PKC Determination in Soluble Fractions

The PKC activity was determined by a slightly modified, previously described procedure (8). Smooth muscle preparations isolated from control and diabetic rats were incubated in normal modified Krebs solution for 60-70 min, then washed with ice-cold 20 mM Tris-HCl buffer containing 2 mM EDTA,

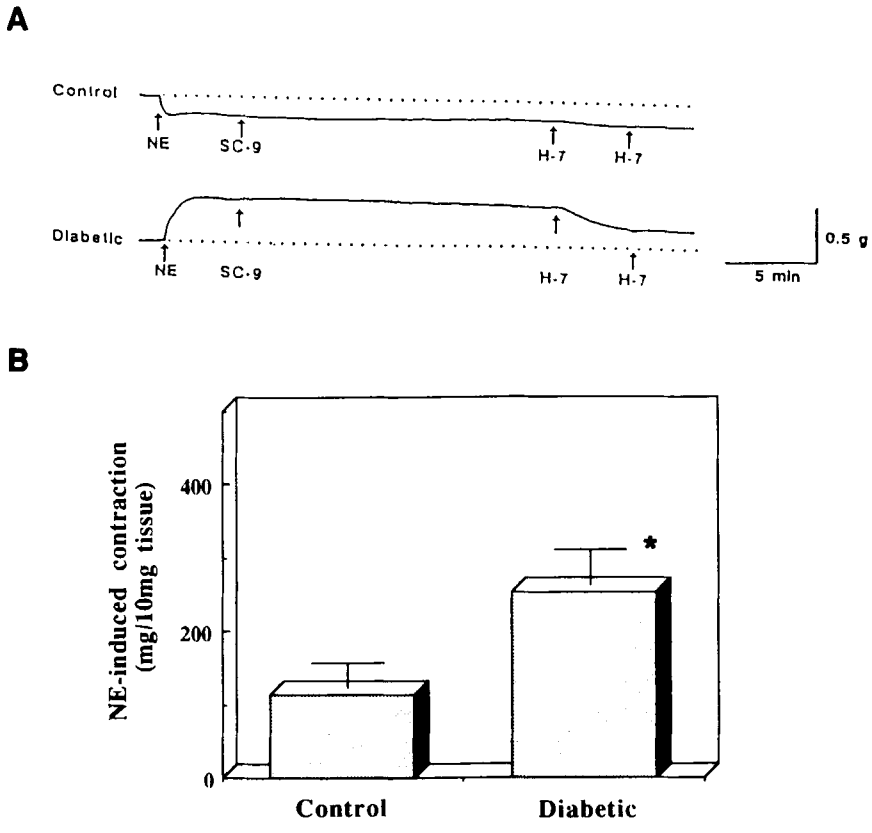

FIG. 2. (A) Contractile responses to norepinephrine (NE), SC-9, and H-7 in gastric fundus smooth muscle from control and diabetic rats. All drugs applied at 10^{-5} M concentration. (B) NE-induced contraction in gastric fundus smooth muscle isolated from control and diabetic rats. Values are mean ± SE (n = 11). *Significantly different from control ($p < 0.01$).

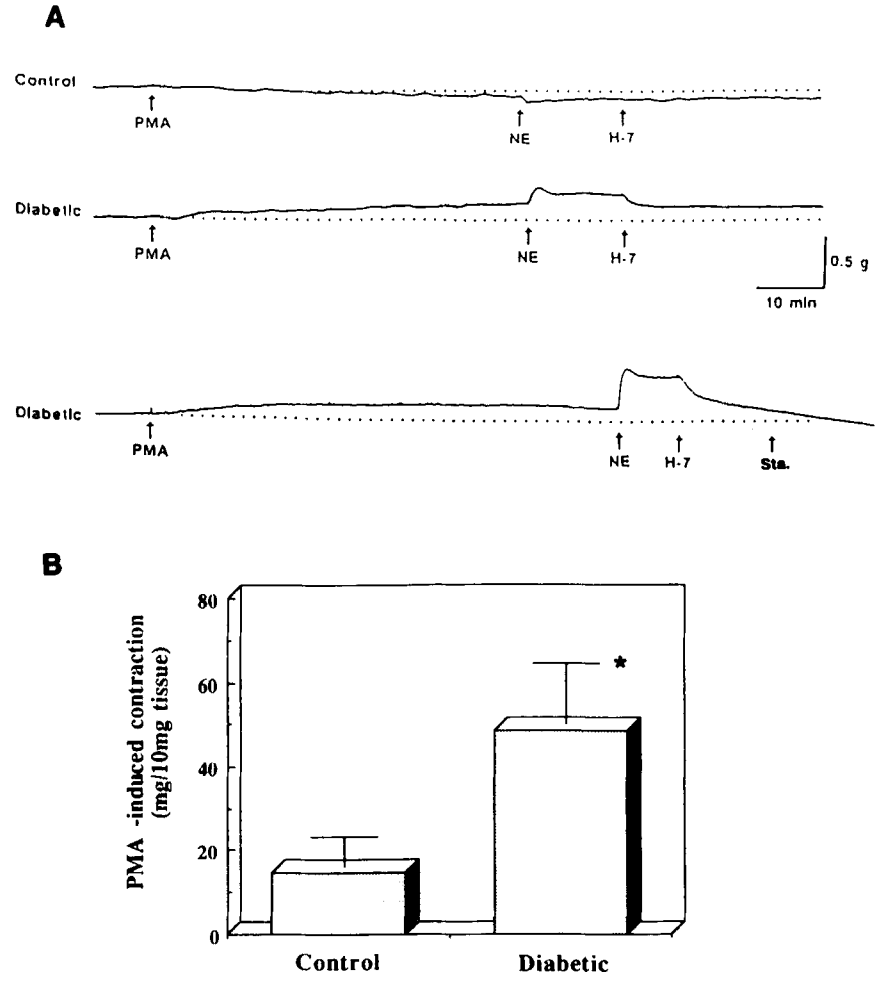

FIG. 3. (A) Typical contractile responses to phorbol 12-myristate 13-acetate (PMA), NE, and H-7 in gastric fundus smooth muscle isolated from control and diabetic rats. All drugs were applied at 10^{-5} M except staurosprine (Sta. 10^{-6} M). (B) PMA-induced contraction 10 min after 10^{-5} M PMA application. Values are mean ± SE (n = 8). *Significantly different from control ($p < 0.01$).

0.5 mM EGTA, and 2 mM phenylmethylsulphonyl fluoride (PMSF) pH 7.4 for 1 min. The washed tissue was homogenized with 50 mM Tris-HCl buffer containing 5 mM EDTA, 10 mM EGTA, 0.3% w/v β-mercaptoethanol, 10 mM benzamidine and 50 μg/ml PMSF, pH 7.5. After the tissue homogenization, the mentioned above procedure for isolation of soluble fraction was performed. We determined the PKC activity in the soluble fractions from gastric fundus smooth muscle of the control and diabetic rats by PKC assay kits (Amersham).

[^{3}H]PN200-110 Binding

For [^{3}H]PN200-110 [(methyl-^{3}H)PN200-110, 37 MBq/ml, Amersham] saturation and competitive binding analysis, 40 to 70 μg of microsomal protein per 1.0 ml of assay was incubated with ligand at 37°C for 60 min. After incubation, the bound and free radioligands were separated by rapid filtration through a Whatman GF/B filter followed by three washings with 4.0 ml Tris buffer. Nonspecific binding was defined as the amount of radioligand bound in the presence of 10^{-6} M unlabeled nifedipine. The radioactivity was counted in a Packard liquid scintillation spectrometer.

RESULTS

Contraction Induced by Bay K 8644, NE, or PMA

Bay K 8644 concentration dependently induced contraction in the presence of 20 mM KCl in gastric fundus smooth muscle isolated from control and diabetic rats. This contractile response at each concentration was significantly greater in diabetics than in controls, as shown in Fig. 1. These contractions were inhibited by 10^{-5} M H-7 (Fig. 1A). Responses to NE were mainly relaxation in gastric fundus isolated from controls, and contraction in fundus from diabetics, which agreed with previously reported results (17). Even if we could find NE-induced contraction in the controls, the contraction was about half of that in the diabetics (Fig. 2). SC-9, a PKC activator, did not affect NE-induced contraction in diabetics, but NE-induced contraction was inhibited by 10^{-5} M H-7 (Fig. 2B). PMA (10^{-5} M) caused contraction in gastric fundus smooth muscle from diabetics, but not in all (four of eight preparations) fundus from controls. The PMA-induced contraction in diabetics was about 2.5 times that in controls, as shown in Fig. 3. The NE-induced contraction was completely inhibited by 10^{-5} M H-7, and the residual contraction induced by PMA was completely inhibited by 10^{-6} M staurosporine (Fig. 3A).

PKC Activity and Effects of PMA

The PKC activity in the soluble fraction of gastric fundus smooth muscle isolated from the diabetic rats was significantly greater at all concentrations than that in controls (Fig. 4). Figure 4B shows the PMA concentration dependence of PKC activity in soluble fractions isolated from controls and diabetics. At low concentration the PKC activity in diabetic preparations 1.5 times that in controls. This difference in activity become smaller at higher concentration of PMA. Characteristics of PKC activity in controls and diabetics.

Figure 5 shows characteristics of PKC activity in soluble fractions isolated from gastric fundus smooth muscle from controls and diabetic rats. There was no effect on PKC activity in either group by 10^{-5} M SC-9. PKC activity in each group was slightly but significantly inhibited by lowering the Ca^{2+} concentration to one-fifth of the normal assay; this inhibited

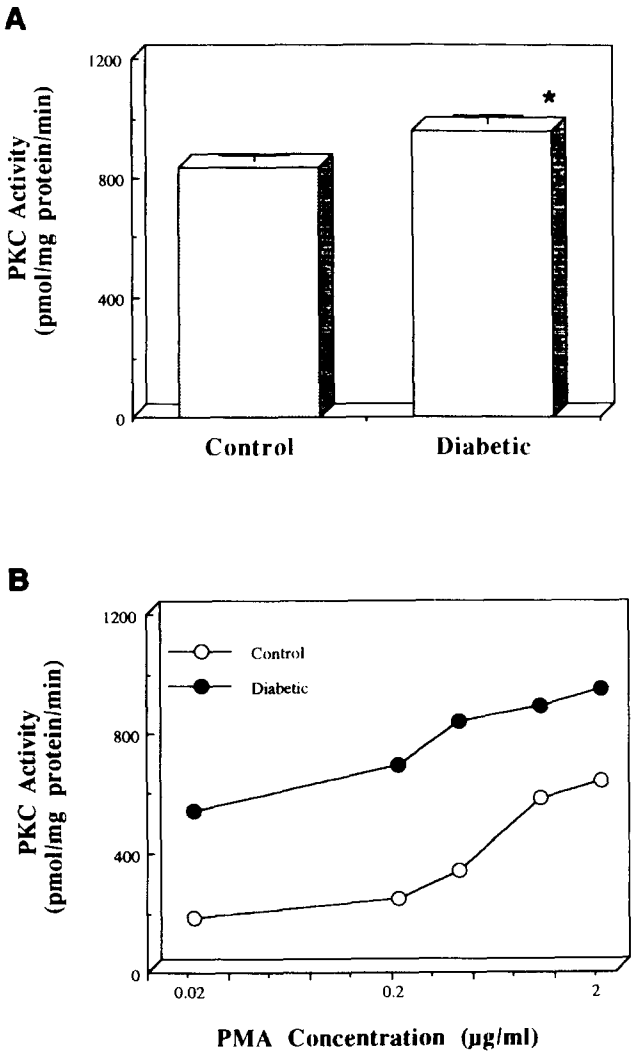

FIG. 4. Protein kinase C (PKC) activity and PMA dependence in soluble fractions isolated from gastric fundus smooth muscle in control and diabetic rats. (A) PKC activity in soluble fractions. Values are mean ± SE (n = 9). *Significantly different from control ($p < 0.05$). (B) PMA dependence of PKC activity in soluble fractions isolated from gastric fundus smooth muscle in control and diabetic rats. Each point is the mean of duplicate measurements in a typical experiment.

PKC activity slightly more in diabetics than in controls. H-7 (10^{-5} M) and staurosporine (10^{-6} M) both depressed PKC activity greatly in both the control and diabetic groups. Maximum depression by 10^{-5} M H-7 was about 50%, and maximum depression by 10^{-6} M staurosporine was about 90%.

Ca^{2+} Channel Antagonist Binding

The selection of [^{3}H]PN200-110 as a ligand for L-type Ca^{2+} channels was made because of its high affinity and low nonspecific binding in both controls and diabetics; [^{3}H]PN200-110 bound in a saturable manner to the plasma membrane-enriched fraction isolated from gastric fundus smooth muscle. Saturation curves, linearly transformed in Scatchard's analysis, are shown in Fig. 6. The inset in Fig. 6 shows ligand

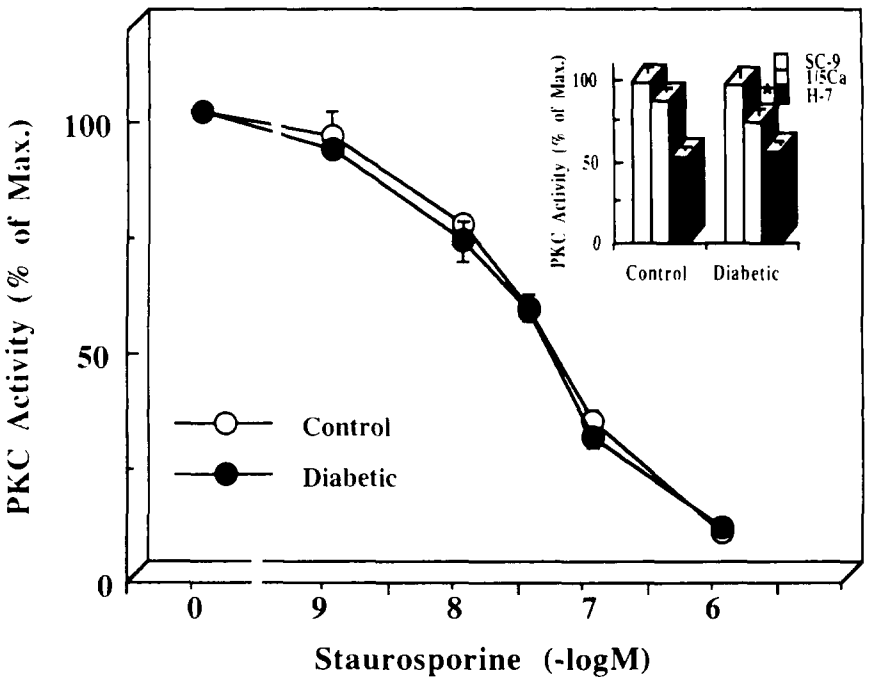

FIG. 5. Staurosporine inhibition of PKC activity in soluble fractions isolated from gastric fundus smooth muscle in control and diabetic rats. Inset: histograms of PKC activity in the presence of 10^{-5} M SC-9 (open column), 2×10^{-4} M $CaCl_2$ (shaded column), and 10^{-5} M H-7 (filled column). Basal kinase activity was determined in the presence of 10^{-3} M $CaCl_2$, 0.7 mol Lα-phosphatidyl-L-serine and 2.0 μg/ml PMA. Values are mean ± SE (n = 3–4). *Significantly different from control ($p < 0.05$).

dissociation constants (K_d) and maximum binding (B_{max}) in both the controls and diabetics. There was no significant difference of either parameter in either group. The binding affinity and density of Ca^{2+} channels were no different in diabetic preparations than in the controls.

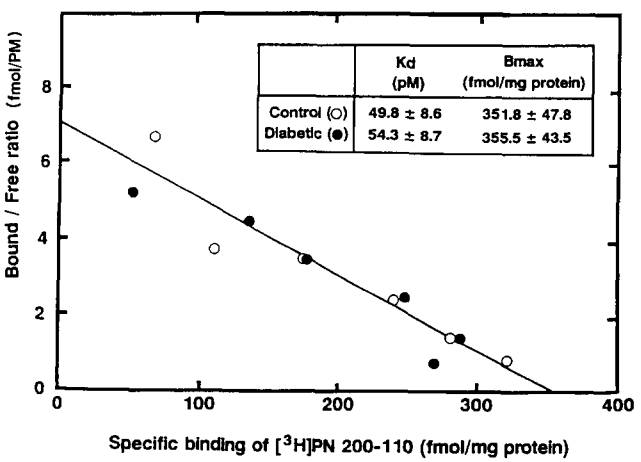

FIG. 6. Scatchard plots of specific binding of (^{3}H)PN200-110 to gastric fundus membrane isolated from control and diabetic rats. Each point is the mean of duplicate measurements in a typical experiment. Inset: Values of ligand dissociation constant (K_d) and maximum binding (B_{max}). Values are mean ± SE (n = 6).

DISCUSSION

Our present and previous results (17,18) provide evidence that the enhancement of contractile responses to KCl and reversal responses to NE in gastric fundus smooth muscle from rats with STZ-induced diabetes may be due partly to increased PKC activity without change in the density of Ca^{2+} channels, as well as to decreased β-adrenoceptor density.

To verify our hypothesis about the process of signal transduction involved in hyperreactive contraction of gastric fundus smooth muscle from rats with STZ-induced diabetes, we examined relations between contraction and PKC activity.

Increase of contraction induced in diabetics by Bay K 8644 has many explanations: a) increase of numbers, sensitivity, or prolongation of Ca^{2+} channel open time; b) increased sensitivity of myosin light chain kinase (MLCK) to Ca^{2+}; c) decreased Ca^{2+} pump activity in sarcoplasmic reticulum (SR) or plasma membrane (PM). Enhancement of contractile responses in diabetics may be related to: a) prolongation of open time of any single channel, because binding characteristics of Ca^{2+} channels in membrane fractions were the same whether they were isolated from controls or diabetics (Fig. 6); b) increase of sensitivity of MLCK to Ca^{2+}, because PMA caused contraction of gastric fundus from all diabetics, but not from all controls (Fig. 3), and increased the basal PKC activity level in diabetics (Fig. 4). Although many investigators have reported that phorbol ester causes contraction in vascular smooth muscle (5,6,19), there is little information about the role of PKC, after it has been activated by phorbol ester, in the control of gastrointestinal motility. Activation of PKC by phorbol ester, either directly or indirectly, causes secondary increase of slow

L-type voltage-dependent Ca^{2+} conductance. Such increase could occur through either increase in the number of channels available for voltage-dependent Ca^{2+} conductance, or increase in the open time of a single channel, or both (15). PKC activity in soluble fractions isolated from diabetic preparations was more sensitive to lowering of the concentration of Ca^{2+} than that in fraction isolated from controls (Fig. 5). The activity of PKC in diabetics was greater than that in controls at any given concentration of PMA (Fig. 4B). All PKC activity, in both groups, was inhibited by 10^{-6} M staurosporine (Fig. 5).

Activation of PKC stimulates the contractile apparatus in smooth muscle by elevating cytosolic Ca^{2+} levels, through Ca^{2+} influx from extracellular space, or through some intracellular mechanism, or both (12,19). We previously reported that the Ca^{2+} influx induced by KCl, ACh, or NE was greater in diabetics than in controls (1,18). Two protein kinases, MLCK and PKC, have been implicated, respectively, in the development and maintenance of smooth muscle contraction (4). Thus, it is possible that increase of Ca^{2+} sensitivity to MLCK and PKC are related to hyperreactivity in diabetics. Phorbol esters have been shown to elicit contraction in various tissue. Staurosporine is reported to act by inhibiting ATP binding sites, regions with a high degree of sequence homology in most kinases (16). Therefore, part of the inhibiting action of staurosporine could be mediated by inhibition of MLCK or other kinases (20). PKC was originally reported to depend on Ca^{2+} in the presence of phospholipid, but the susceptibility to Ca^{2+} varied with the PKC subspecies. These PKC subspecies have different modes of activation, kinetics, and substrate specificity. They can be subdivided into two groups, Ca^{2+}-dependent (α, β, γ), and Ca^{2+}-independent (δ, ϵ, ζ, η) (14). The calcium requirement appears to depend on the phospholipid composition of the fraction or on the enzyme property (14). The greater activity of PKC and alteration of Ca^{2+} sensitivity in diabetics (Figs. 4 and 5) may be related to these PKC subspecies or alteration of enzyme properties in diabetic preparations.

It is possible that NE-induced contraction is a consequence of PKC-mediated phosphorylation of L-type channels. PKC-mediated Ca^{2+} channel activation in response to NE could represent a positive feedback for supplying the Ca^{2+} required to activate PKC. These possibilities suggest that PKC is important in mediating hyperreactivity of gastric fundus smooth muscle in diabetics. Intracellular Ca^{2+} increase has been shown to stimulate PKC translocation from cytosol to plasma membrane (7). Feedback control or downregulation by PKC also extends to receptors of other signal systems. Further study of effects of diabetes requires information about PKC translocation.

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REFERENCES

1. Aihara, K.; Sakai, Y. Hyperreactivity of contractile response in gastric fundus smooth muscle from rats with diabetes induced by streptozotocin. Arch. Int. Pharmacodyn. 302:220-230; 1989.
2. Belai, A.; Lincoln, J.; Milner, P.; Burnstock, G. Differential effect of streptozotocin-induced diabetes on the innervation of the ileum and distal colon. Gastroenterology 100:1024-1032; 1991.
3. Britland, S. T.; Young, R. J.; Sharma, A. K.; Lee, D.; Ahsee, A. K.; Clarke, B. F. Vagus nerve morphology in diabetic gastropathy. Diabetic Med. 7:780-787; 1990.
4. Fish, R. D.; Sperti, G.; Colucci, W. S.; Clapham, D. E. Phorbol ester increases the dihydropyridine-sensitive calcium conductance in a vascular smooth muscle cell line. Cir. Res. 62:1049-1054; 1988.
5. Forder, J.; Scriabine, A.; Rasmussen, H. Plasma membrane calcium flux, protein kinase C activation and smooth muscle contraction. J. Pharmacol. Exp. Ther. 235:267-273; 1985.
6. Gleason, M. M.; Flaim, S. F. Phorbol ester contracts rabbit thoracic aorta by increasing intracellular calcium and by activating calcium influx. Biochem. Biophys. Res. Commun. 138:1362-1369; 1986.
7. Haller, H.; Smallwood, J. I.; Rasmussen, H. Protein kinase C translocation in intact vascular smooth muscle strips. Biochem. J. 270:375-381; 1990.
8. Hannun, Y. A.; Loomis, C. R.; Bell, R. M. Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine J. Biol. Chem. 260:10039-10043; 1985.
9. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin reagent. J. Biol. Chem. 193: 265-275; 1951.
10. Malagelada, J. R.; Rees, W. D. W.; Mazzota, L. T. L.; Go, V. L. W. Gastric motor abnormalities in diabetic and postvagotomy gastroparesis: Effect of metoclopramide and bethanechol. Gastroenterology 78:286-293; 1980.
11. Mearin, F.; Camilleri, M.; Malagelada, J. R. Pyloric dysfunction in diabetics with recurrent nausea and vomiting. Gastroenterology 90:1919-1925; 1986.
12. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308:693-697; 1984.
13. Nishizuka, Y. Studies and perspectives of protein kinase C. Science 233:305-312; 1986.
14. Nishizuka, Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661-665; 1988.
15. Orton, E. C.; Raffestin, B.; McMurtry, I. F. Protein kinase C influences rat pulmonary vascular reactivity. Am. Rev. Respir. Dis. 141:654-658; 1990.
16. Ruegg, U. T.; Burgess, G. M. Staurosporine, K-252 and VCN-01: Potent but nonspecific inhibitors of protein kinases. Trends Int. Pharmacol. Sci. 10:218-220; 1989.
17. Sakai, Y.; Inazu, M.; Aihara, K.; Inoue, K.; Homma, I. Relations between contractile responses and β-adrenoceptors in gastric fundus of diabetic rats. Life Sci. 48:1043-1049; 1991.
18. Sakai, Y.; Inazu, M.; Homma, I. Changes of protein kinase C activity in gastric smooth muscle of experimental diabetic rats. Jpn. J. Pharmacol. 58:301p; 1992.
19. Silver, P. J.; Lepore, R. E.; Cumiskey, W. R.; Kiefer, D.; Harris, A. L. Protein kinase C activity and reactivity to phorbol ester in vascular smooth muscle from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) Biochem. Biophys. Res. Commun. 154:272-277; 1988.
20. Singer, H. A. Phorbol ester-induced stress and myosin light chain phosphorylation in swine carotid medial smooth muscle. J. Pharmacol. Exp. Ther. 252:1068-1074; 1990.