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Hyper-reactivity of diacylglycerol kinase is involved in the dysfunction of aortic smooth muscle contractility in streptozotocin-induced diabetic rats

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1 Dysfunction of vascular contraction in diabetes has been reported; however, the mechanisms are poorly understood. In this study, calcium sensitization involving increases in contraction in streptozotocin-induced diabetic rat aorta was detected. We hypothesize that an alteration in the intracellular signalling system plays a role in the dysfunction of vascular contractility in diabetes. Therefore, diacylglycerol (DG) kinase as a key enzyme of phosphatidylinositol (PI) turnover was investigated.

2 Treatment with norepinephrine (NE) caused time- and dose-dependent activation of DG kinase in control rats. This activation required simultaneous increases in intracellular calcium concentration ($[Ca^{2+}]_i$) and protein kinase C (PKC) activation.

3 In diabetic rats, hyper-reactivity of DG kinase involving inactivation in the resting state and over-activation in NE stimulation was observed. During hyper-reactivity, $[Ca^{2+}]_i$ dependency of DG kinase was enhanced. Treatment with 50 mM KCl induced significant escalation in activity; moreover, basal activation of PKC was detected only in diabetes. These results suggested that PKC had been activated in the resting state. In contrast, these conditions were insufficient for DG kinase activation due to the absence of $[Ca^{2+}]_i$ elevation.

4 During NE-stimulation, PKC activation was maintained and $[Ca^{2+}]_i$ increased. Therefore, DG kinase was activated and an elevation in calcium dependency enhanced this activation.

5 The present study suggested that DG kinase hyper-reactivity in diabetes involved both an increase in $[Ca^{2+}]_i$ and basal activation of PKC. This phenomenon may be associated with increased vascular contraction in diabetes mediated by acceleration of PI-turnover.

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Abbreviations: 18:0/20:4-DG, stearoyl-arachidonyl-*sn*-glycerol; [^{32}P]Pi, inorganic phosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; DG, diacylglycerol; diC8, dioctanoyl-*sn*-glycerol; DMSO, dimethyl-sulphoxide, DTT, dithiothreitol; EGTA, glycoletherdiaminetetraacetic acid; fura-2/AM, 1-(2-(5′-carboxyoxazol-2′-yl)-6-amino-benzofuran-5-oxy)-2-(2′-amino-5′-methyl-phenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid penta-acetoxymethyl ester; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo-(2, 3-α)pyrrolo(3,4-c)-carbazole; IDDM, insulin-dependent diabetes mellitus; IP_3, inositol trisphosphate; MOPS, 3-(N-morpholino)propanesulphonic acid; NE, norepinephrine; Neo, neomycin; PA, phosphatidic acid; Phen, phenoxybenzamine; PIP_2, phosphatidylinositol-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate, 13-acetate; R59022, 6-[2-(4-[(4-fluorophenyl)phenyl-methylene]-1-piperidinyl)ethyl]-7-methyl-5H-thiazolo [3,2-α] pyrimidine-5-one; STZ, streptozotocin

Introduction

Insulin-dependent diabetes mellitus (IDDM) results as a consequence of decreased insulin secretion and/or desensitization of insulin receptors, leading to complicating dysfunctions in many types of tissue (Ozturk *et al*., 1996). Diabetic patients appear to be particularly prone to disorders of the neurological, cardiovascular, gastrointestinal and reproductive systems (Raccah, 1998). It has been suggested that these complications are due to abnormalities in the nervous system. Recently, correlations between changes in intracellular signalling pathways and complications in many cell types have also been demonstrated (Ozturk *et al*., 1996; Schmidt *et al*., 1999). Clarification of this relationship may provide new information regarding the development of a treatment for diabetes.

Phosphatidylinositol turnover (PI-turnover) occurs in numerous cell types; moreover, this process is involved in the intracellular signalling pathway. Changes in PI-turnover activity are believed to influence cellular functions. In PI-turnover, diacylglycerol (DG) plays an important role in cellular function as an endogenous activator of protein kinase

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C (PKC). Diacylglycerol kinase (DG kinase) phosphorylates DG, generating phosphatidic acid (PA) (Flores *et al*., 1996). Consequently, endogenous DG levels can be altered by DG kinase activity. We previously found that the carbachol (CCh)-induced muscarinic receptor mediated DG kinase activation in guinea-pig taenia coli (Nobe *et al*., 1994). Furthermore, we examined the mechanisms of regulation of DG kinase activation and discovered that both an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and PKC activation were required (Nobe *et al*., 1995; 1997).

These observations suggested that regulation of DG kinase by PKC was an important feedback mechanism in PI-turnover. On the basis of these data, alterations in DG kinase activity in some tissues isolated from streptozotocin (STZ)-induced diabetic rats were detected (Nobe *et al*., 1998). In that investigation, DG kinase activity in diabetic aortic smooth muscle indicated marked changes. However, the mechanism(s) has not been clarified.

The objective of this study was to clarify the mechanism(s) of DG kinase activity change in diabetic rat aorta in order to understand the relation between alterations in the intracellular signalling pathway and dysfunctions in vascular smooth muscle contractility. The present findings suggest that both increases in DG kinase calcium dependency and basal activation of PKC are involved in the hyper-reactivity of DG kinase, which may lead to enhancement of aortic contractility in diabetes.

Methods

Preparation of diabetic rats

Experimental diabetes was induced in rats by treatment with STZ (Rakieten *et al*., 1976). STZ at 60 mg (kg body weight)$^{-1}$ in citrate buffer was injected into the lateral vein of 8-week-old Wistar rats (male, 220–260 g body weight). Age-matched controls were injected with citrate buffer. Rats were decapitated 6–7 weeks after injection. Randomly selected diabetic rats received insulin subcutaneously daily following confirmation of the development of diabetes 35 days after injection of STZ (insulin-treated diabetics) as described below. On each of the first 4 days, 9.6 units kg^{-1} day^{-1} of insulin was injected; subsequently, treatment dosage was increased to 12 units kg^{-1} day^{-1}. Blood glucose level (70–100 (mg glucose) dl^{-1} in control, 400$\leqslant$ (mg glucose) dl^{-1} in diabetic rats) was determined on a Tidex glucose analyser (Bayer-Sankyo, Tokyo, Japan). Following resection, fat adhesion and connective tissues were removed from the aorta. Internal surfaces of the aorta were gently rubbed with a wooden rod to eliminate the inhibitory influence of vascular endothelial cells. Tissues were then equilibrated in physiological salt solution (PSS) supplemented with (mM) NaCl 118, KCl 5.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.4, $NaHCO_3$ 21.4 and glucose 11.5 aerated with 95% O_2 and 5% CO_2 at 37°C.

Fura-2 loading for measurement of $[Ca^{2+}]_i$ and force development

Tissues were initially incubated in tubes filled with 2.0 ml of PSS containing 5 μM of fura-2/AM dissolved in dimethylsulphoxide (DMSO; final concentration was 1.0%) (Nobe *et al*., 1993). The noncytotoxic detergent pluronic F_{127} (final concentration, 0.5%) was added in order to increase the solubility of fura-2/AM. Loading was conducted at 18°C for 4 h. Tissues were subsequently transferred from the loading solution to PSS.

These experiments were performed within 30 min after rinsing. Changes in fluorescent intensities were monitored utilizing a fluorometer specifically designed to measure surface fluorescence of living tissues (CAF-100, Japan Spectroscopic, Tokyo, Japan). The ratio of fluorescence (500 nm) attributable to excitations at 340 nm (F_{340}) and 380 nm (F_{380}) was calculated from successive illumination periods. This value is referred to as $R_{340/380}$. The tissue was positioned horizontally in a temperature-controlled 3 ml organ bath. One end of the tissue was connected to a strain gauge transducer (Type T-7-8-240, Orienteck, Tokyo, Japan) to monitor the contractile responses. Aortic contractions were normalized to vascular cross-sectional area employing the following equation

$$\text{Cross-sectional area (mm}^2\text{)} = (2 \times \text{wet weight (mg)})/(1.06 \times \text{circumference (mm)}),$$

where 1.06 is vascular tissue density (mg mm^{-3}).

Assay of DG kinase activity utilizing diC8 in tissue

DG kinase activity in tissue was determined *via* measurement of [^{32}P]-dioctanoyl-phosphatidic acid ([^{32}P]diC8-PA) accumulation from diC8 in radioactive inorganic phosphate ([^{32}P]-Pi) and diC8-pre-labelled tissues. Simultaneously, the endogenous PA level was established by determination of [^{32}P]-PA accumulation in each tissue. For this assay, diC8 was dissolved in chloroform and stored at -40°C as a stock solution. Prior to use, the stock solution was dried under Ar gas at room temperature and dissolved in a 50% ethanol solution (final concentration of 0.03%). The diC8 ethanol solution was added to PSS supplemented with 2.7 mg ml^{-1} BSA (diC8 solution). Tissues (25–50 mg wet weight tissue per tube) were initially incubated with 2.22 MBq ml^{-1} of [^{32}P]-Pi in 1 ml of diC8 solution for 90 min at 37°C, followed by washing (two repetitions) with 10 ml PSS. The reaction was initiated by the addition of 0.8 ml PSS containing various compounds at 37°C. Introduction of 3 ml of ice-cold chloroform/methanol/10 M HCl (100 : 200 : 1, v v v^{-1}) terminated the reaction. Subsequently, specimens were homogenized with a glass homogenizer in ice-cold water. Excitation and quantitation of [^{32}P]-diC8-PA and [^{32}P]-PA were performed as described previously (Nobe *et al*., 1994). Results were expressed as c.p.m. (mg wet weight tissue)$^{-1}$.

Measurement of PKC activity

Aortae treated under various conditions were homogenized with a Polytron homogenizer (Iuchi Co. Osaka, Japan) in 3 ml of ice-cold solution consisting of 20 mM 3-(N-morpholino)propanesulphonic acid (MOPS) (pH 7.2), 250 mM sucrose, 1 mM dithiothreitol (DTT), 1 mM glycoletherdiaminetetraacetic acid (EGTA), 1 μg ml^{-1} pepstatin, 1 μg ml^{-1} leupeptin and 50 μg ml^{-1} trypsin inhibitor (Buffer

A). The homogenates were centrifuged ($1000 \times g$ for 5 min) to remove the nuclei. Supernatants were decanted and pellets were washed once with buffer B (sucrose-free buffer A). The combined supernatants were centrifuged again ($2000 \times g$ for 30 min). Finally, the membrane and cytosol fractions were collected by centrifugation ($100{,}000 \times g$ for 60 min). PKC activity in the membrane (pellet) and cytosolic (supernatant) fractions were determined utilizing an Amersham protein kinase C assay kit.

Measurement of total mass of DG

Isolated tissues were incubated in PSS containing various compounds at 37°C. The total mass of DG in each tissue was measured as described previously (Nobe *et al*., 1993). Diorelin was used as a standard. Results were expressed as ng (mg wet weight tissue)$^{-1}$.

Measurement of [^{3}H]-myo-*inositol incorporation*

Measurement of [^{3}H]-*myo*-inositol incorporation was conducted employing the method of Conrad *et al*. (1991). [^{3}H]-*myo*-inositol pre-labelled tissues were pre-incubated in the presence or absence of each reagent for 10 min. Ten μM NE was subsequently added for 15 min. Following termination of the treatment, [^{3}H]-phosphoinositide incorporation was analysed.

Materials

Carrier- and HCl-free radioactive inorganic phosphate ([^{32}P]-Pi) and [^{3}H]-*myo*-inositol were purchased from Du Pont-New England Nuclear (Boston, MA, U.S.A.). Bovine serum albumin (BSA), STZ, NE and phenoxybenzamine (Phen) were acquired from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DiC8 and diorelin were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). 6-[2-(4[(4-Fluorophenyl)phenyl-methylene]-1-piperidinyl)ethyl]-7-methyl-5H-thiazolo [3,2-α] pyrimidine-5-one (R59022) and 1-(2-(5′-carboxyoxazol-2′-yl)-6-amino-benzofuran-5-oxy)-2-(2′-amino-5′-methyl-phenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid penta-acetoxymethyl ester (fura-2/AM) were purchased from Janssen Life Science Products (Beerse, Belgium) and Dojindo Laboratories (Kumamoto, Japan), respectively. Neomycin and ionomycin were obtained from Funakoshi Co. (Tokyo, Japan). Gö6976 was purchased from Calbiochem Co. (San Diego, CA, U.S.A.). Thin layer chromatography (TLC) plates (silica gel 60 with a concentrating zone) were manufactured by Merck Inc. (Darmstadt, Germany). Biologically synthesized human insulin (insulin) was acquired from Novo Nordisk Pharma (Tokyo, Japan). All other chemicals and materials were of reagent grade quality. R59022 was dissolved in a 10% ethanol solution containing 5 mM HCl as a 2.5 mM stock solution.

Data analysis

Values are presented as means ± s.e.m. derived from at least 5–10 animals. The significance of differences between the values was assessed by one-way analysis of variance (ANOVA), followed by Bonferroni *t*-test for multiple comparisons.

Results

Basic tests in experimental diabetic rats

Basic conditions were checked in diabetic rats at 7 weeks following STZ injection (Table 1). Body weight decreased to 77.8% of that of controls; moreover, blood glucose levels increased to 352.9% relative to that of controls. Urine volume also increased in diabetic rats (1115.4% in comparison to the levels observed in control animals). These changes in diabetic rats were partially recovered by treatment with insulin. Aortic wet weight was not significantly different from readings obtained in control rats.

NE-induced changes in [Ca^{2+}]$_i$ and force development in diabetic rat aorta

NF-induced changes in $[Ca^{2+}]_i$ and force development were measured in fura-2-loaded control and diabetic rat aortae (Figure 1). Typical records of force development (upper trace) and $R_{340/380}$ (lower trace) are presented as an indicator of $[Ca^{2+}]_i$.

In both control and diabetic rat aortae, the increase in $R_{340/380}$ following 10 μM NE treatment preceded the increase in force development; moreover, both parameters were sustained. A positive correlation was detected between $R_{340/380}$ and force development induced by NE in control and diabetic rats. However, for a given value of $R_{340/380}$, force development in diabetic rats was consistently larger than that of controls (171.0 ± 1.8%).

Changes in [^{32}P]-diC8-PA and [^{32}P]-PA accumulation induced by NE

Recently, findings were reported regarding DG kinase employing biochemical methods (Pettitt & Wakelam, 1999). However, several difficulties exist which are associated with the examination of DG kinase activity in intact tissue. Problems arising from measurement of cellular DG kinase activity are as follows. First, PA is formed *via* several routes, including hydrolysis of phosphatidylcholine by phospholipase D, *de novo* synthesis from lysophosphatidic acid and phosphorylation of DG by DG kinase; consequently, DG kinase activity cannot be estimated from endogenous PA levels. Secondly, DG does not penetrate the cell membrane. As a result, DG kinase activity cannot be measured using radiolabelled DG as an exogenous substrate. Previously, diC8, a cell-permeable species of short-chain DG, was utilized as an exogenous substrate to measure the level of DG kinase activity in tissues. DiC8 penetrated the cell membrane; subsequently, [^{32}P]-diC8-PA was formed by DG kinase in [^{32}P]-Pi-pre-labelled tissue. Since diC8 was not originally present in the tissue, we considered the changes in [^{32}P]-diC8-PA accumulation to reflect changes in DG kinase activity.

In order to measure DG kinase activity *via* this approach, it was necessary to check whether changes in [^{32}P]-diC8-PA accumulation reflected DG kinase activity. Incorporation rate of a diC8 as an extracellular substrate was increased preincubation time-dependently. Submaximal value was detected at 90 min. At this time, it was confirmed that 65.0% of diC8 was free form, 27.3% was a diC8-PA and 7.7% was another phospholipids involving phosphatidylino-

Table 1 Basic characteristics of experimental diabetic rats

Experimental Groups	*Animals*	*Body weight* (g)	*Blood glucose* (mg d l^{-1})	*Urine volume* (ml day^{-1})	*Aortic weight* (mg wet weight cm^{-1})
Control	16	388.6±6.3	122.1±11.2	13.0±0.5	2.78±0.5
Diabetic	10	302.6±10.2*	431.0±28.1*	145.0±2.6*	2.19±0.9
Diabetic + insulin	5	351.0±9.75	158.2±14.6#	20.8±1.2*#	2.58±0.7

Experimental diabetic rats were prepared as described in Methods. Basic factors were measured in control, diabetic and insulin-treated diabetic rats. *$P<0.05$ vs control rats and #$P<0.05$ vs diabetic rats.

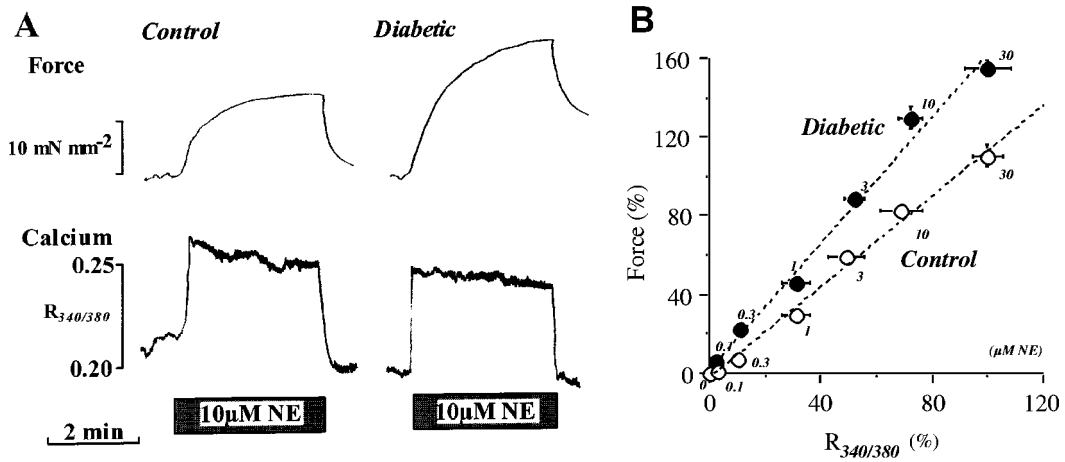

Figure 1 $[Ca^{2+}]_i$ and force development responses in diabetic rat aorta. Fresh tissue isolated from control and diabetic rats was pre-incubated with 5 μM fura-2/AM at 18°C for 4 h. Force development in these tissues was recorded and $[Ca^{2+}]_i$ determined *via* measurement of fura-2 fluorescence at 500 nm due to excitation at either 340 nm or 380 nm and are presented as the ratio of these fluorescence values ($R_{340/380}$). Typical values of force development and $R_{340/380}$ stimulated by 10 μM NE are shown (A). The relationship between $R_{340/380}$ and force development in the presence of differing concentrations of NE in each control and diabetic rat is displayed in the figure (B). Values of 100% represent the 10 μM ionomycin-induced maximal response of $R_{340/380}$ and force development measured at the end of the experiments. Each value represents the mean±s.e.mean of at least six independent determinations.

sitols and phosphatidylcholine (data not shown). Confirmation that unusual species of DG, i.e., diC8, and typical intracellular species of DG, i.e., 18:0/20:4-DG, were competitively bound and phosphorylated by DG kinase extracted from rat aorta employing identical protocols from our previous study was obtained (data not shown). Several groups have used diC8 as a cell-permeable PKC activator (Jensen, 1996). In our assay system, diC8 was used as an extracellular substrate of DG kinase, we had to avoid an effect of diC8 as a PKC activator. Therefore, we checked the effect of diC8 incorporation on PKC activity and we considered an experimental condition that the diC8 incorporated sufficiently as a DG kinase substrate without affecting the PKC activity. Although high concentrations and/or long-term treatment with diC8 induced an increase in PKC activity in the rat aorta, no significant increases in PKC activity were detected under the present experimental conditions (10 μM, diC8, 90 min) (data not shown).

The effects of both DG kinase and phospholipase C inhibitors (Phillippe, 1994) in control rats were then measured (Figure 2). Treatment with 10 μM NE for 5 min induced a significant increase in [^{32}P]-diC8-PA accumulation in control rats (5.56±0.23 c.p.m. (mg wet weight tissue)$^{-1}$ in resting and 10.28±0.53 c.p.m. (mg wet weight tissue)$^{-1}$ in NE stimulation). The DG kinase inhibitor, R59022, at 1 μM suppressed not only the increase in [^{32}P]-diC8-PA accumulation induced by NE treatment (3.22±0.23 c.p.m. (mg wet weight tissue)$^{-1}$) but also reduced the resting level (2.86±0.52 c.p.m. (mg wet weight tissue)$^{-1}$). Moreover, the phospholipase C inhibitor, neomycin (Neo), at 3 μM, did not affect resting and NE-induced [^{32}P]-diC8-PA accumulation (5.89±0.34 and 9.86±1.02 c.p.m. (mg wet weight tissue)$^{-1}$, respectively). Simultaneously, [^{32}P]-PA accumulation as an indicator of the endogenous PA level was measured. The NE induced significant increase in the [^{32}P]-PA accumulation (5.83±0.21 c.p.m. (mg wet weight tissue)$^{-1}$ in resting and 12.18±1.23 c.p.m. (mg wet weight tissue)$^{-1}$ in NE stimulation). Treatment with R59022 decreased the NE-induced [^{32}P]-PA accumulation (4.44±0.99 c.p.m. (mg wet weight tissue)$^{-1}$) without affecting resting level (4.21±0.58 c.p.m. (mg wet weight tissue)$^{-1}$). In contrast, Neo decreased both resting and NE stimulated [^{32}P]-PA accumulation (2.01±0.12 and 2.69±0.33 c.p.m. (mg wet weight tissue)$^{-1}$, respectively). These findings suggested that the accumulation of [^{32}P]-diC8-PA was a result of phosphorylation of diC8 with no contribution from endogenous PA formation. Therefore, the changes in [^{32}P]-diC8-PA accumulation reflected cellular DG kinase activity changes in rat aorta.

NE-induced increase in [^{32}P]-diC8-PA and [^{32}P]-PA accumulation in STZ rat aorta

Changes in the time course of NE-induced [^{32}P]-diC8-PA and [^{32}P]-PA accumulation were measured in [^{32}P]-Pi- and diC8-

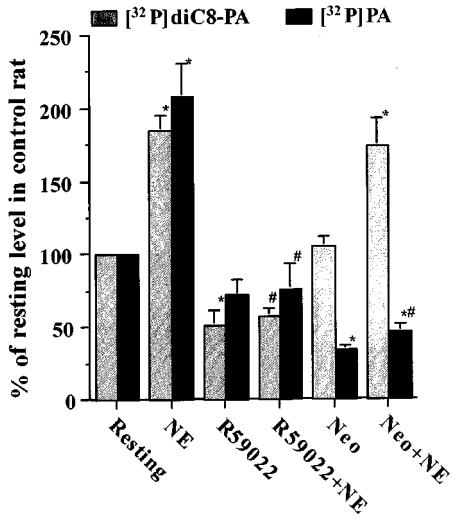

Figure 2 Effects of R59022 and Neo on [^{32}P]-diC8-PA and [^{32}P]-PA accumulation. Tissues isolated from control rats were pre-incubated with 2220 Gbq ml^{-1} [^{32}P]-Pi and 100 μM diC8 at 37°C for 90 min. Following washing, tissues were pre-incubated with 1 μM R59022 or 1 μM Neo for 5 min. Subsequently, 10 μM NE was added for 5 min. [^{32}P]-diC8-PA and [^{32}P]-PA accumulation was quantified as described in Methods. Each value represents the percentage of the resting level of at least seven independent determination. $*P<0.05$ vs resting levels, and $\#P<0.05$ vs NE-treated values.

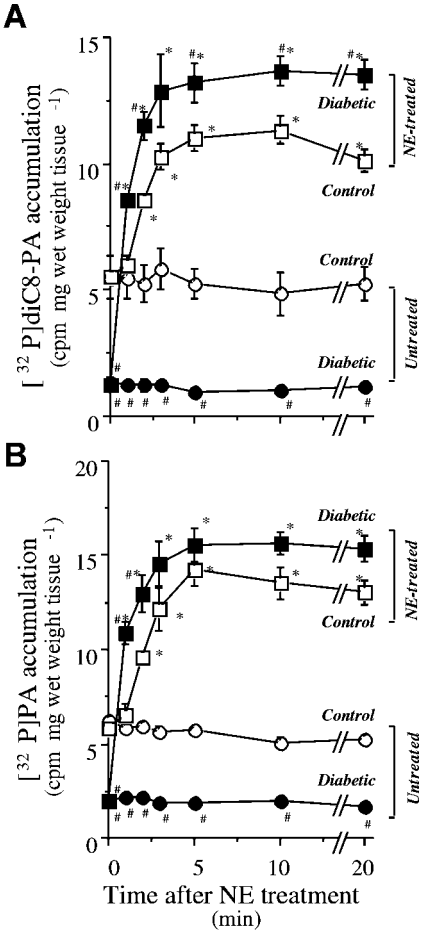

Figure 3 Time course of NE-induced [^{32}P]-diC8-PA and [^{32}P]-PA accumulation in diabetic rats. [^{32}P]-Pi and diC8-pre-labelled aortae isolated from control and diabetic rats were incubated in the presence or absence of 10 μM NE at 37°C. [^{32}P]-diC8-PA (A) and [^{32}P]-PA (B) accumulation was quantified as described in Methods. Each value represents the mean ± s.e.mean of at least 10 independent determinations. $*P<0.05$ vs non-stimulated tissue and $\#P<0.05$ vs control rats.

prelabelled rat aortae (Figure 3). Accumulation of [^{32}P]-diC8-PA was not altered in the absence of 10 μM NE for a period of 20 min in the control rat aorta. In diabetic rats, the resting level of [^{32}P]-diC8-PA accumulation declined significantly. Resting values in control and diabetic rat aortae following a 5-min incubation were 5.25 ± 0.56 and 0.98 ± 0.15 c.p.m. (mg wet weight tissue)$^{-1}$, respectively. In the presence of 10 μM NE, however, a significant increase in the accumulation of [^{32}P]-diC8-PA (8.58 ± 0.24 c.p.m. (mg wet weight tissue)$^{-1}$) was detected after a 2-min incubation in control rats. A maximal level, which was 2 fold greater than the resting values (11.02 ± 0.54 c.p.m. (mg wet weight tissue)$^{-1}$), was attained after 5 min of stimulation, which was sustained for the remainder of the 20-min incubation period. In diabetic rats, [^{32}P]-diC8-PA accumulation increased rapidly in response to NE stimulation, reaching levels higher than those in controls within the first min of stimulation (from the resting value of 1.22 ± 0.07 to 8.59 ± 0.22 c.p.m. (mg wet weight tissue)$^{-1}$. The maximal value in NE-stimulated diabetic rats was 13.7 ± 0.58 c.p.m. (mg wet weight tissue)$^{-1}$ after 10 min of stimulation. Moreover, this value was significantly higher in comparison with the controls at the identical point in time.

Similarly, [^{32}P]-PA accumulation due to endogenous DG increased in a time-dependent fashion upon NE treatment in control rats, reaching a maximal level 3 fold greater than the resting level (from a resting value of 5.74 ± 0.13 to 14.25 ± 0.90 c.p.m. (mg wet weight tissue)$^{-1}$ after a 5-min incubation). Although accumulation of [^{32}P]-PA was lower than that in controls in the absence of NE, the value rose quickly following NE stimulation. The time course of NE-induced changes in [^{32}P]-diC8-PA accumulation in control and diabetic rats corresponded well to changes in [^{32}P]-PA accumulation.

Accumulation of [^{32}P]-diC8-PA was dependent on NE concentration in control and diabetic rats (Figure 4). Incubation for 5 min with increasing NE concentrations caused maximal accumulation of [^{32}P]-diC8-PA in both control and diabetic rats; values were 10.2 ± 0.45 to 13.1 ± 0.58 c.p.m. (mg wet weight tissue)$^{-1}$ when the tissues were incubated with 10 μM NE. At NE concentrations <0.1 μM, accumulation of [^{32}P]-diC8-PA in diabetic rats was lower than that in controls, whereas at >0.1 μM, NE-induced [^{32}P]-diC8-PA accumulation was higher than that in controls. Calculated EC_{50} values of NE for accumulation of [^{32}P]-diC8-PA in control and diabetic rats were 0.90 and 0.23 μM, respectively. The dose-response curve of [^{32}P]-PA was similar to that of [^{32}P]-diC8-PA; however, it was shifted to the right in comparison with [^{32}P]-diC8-PA accumulation. EC_{50} values of control and diabetic rats were 1.70 and 0.65 μM, respectively. Increases in [^{32}P]-diC8-PA and [^{32}P]-PA accumulation in control and diabetic rats were nearly completely inhibited by 1 μM Phen, a specific antagonist of the adrenergic α-receptor (Figure 3-inset). Phen did not affect resting levels.

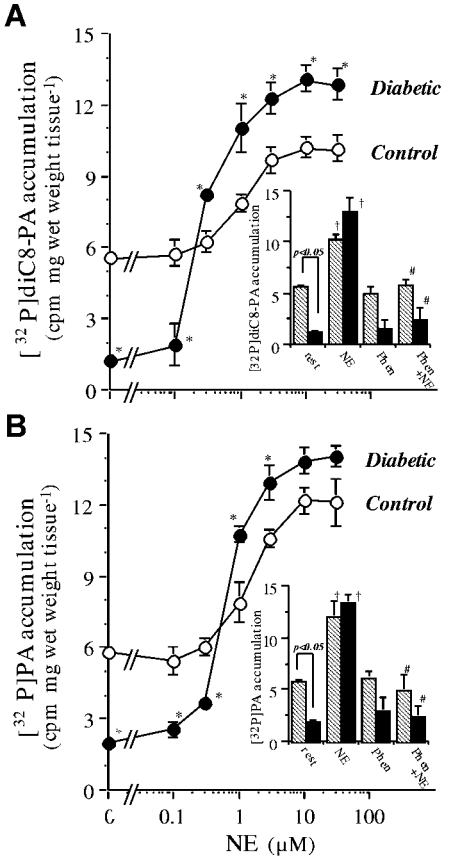

Figure 4 Dose-response curve for NE-induced α-receptor-mediated [^{32}P]-diC8-PA and [^{32}P]-PA accumulation in diabetic rat aorta. Following isolation from control and diabetic rats, tissues were pre-labelled with [^{32}P]-Pi and diC8. Several specimens were incubated with various concentrations of NE at 37°C for 5 min. Other tissues were incubated in the presence or absence of 1 μM Phen at 37°C for 5 min, followed by the addition of 10 μM NE (inset). [^{32}P]-diC8-PA (A) and [^{32}P]-PA (B) accumulation was quantified as described in Methods. Each value represents the mean±s.e.mean of at least seven independent determinations. *$P<0.05$ vs control rats, †$P<0.05$ vs resting levels and #$P<0.05$ vs NE-treated values.

Effects of calcium on NE-induced [^{32}P]-diC8-PA and [^{32}P]-PA accumulation in diabetic rats

To investigate the association between $[Ca^{2+}]_i$ and the differences in [^{32}P]-diC8-PA and [^{32}P]-PA accumulation between control and diabetic rats, accumulation of these radiolabelled compounds was measured in $CaCl_2$-replaced PSS (Ca^{2+}-free PSS) for 5 min (Figure 5). This condition achieved inhibition of the NE-induced $[Ca^{2+}]_i$ increase without affecting the resting level. The effect was confirmed using fura-2 loaded tissue (data not shown).

Pre-incubation of tissues isolated from control rats in Ca^{2+}-free PSS for 5 min prior to NE stimulation led to significant inhibition of the NE-induced increase in [^{32}P]-diC8-PA accumulation without affecting resting levels. In diabetic rats, the resting level of [^{32}P]-diC8-PA accumulation in normal PSS was significantly reduced in comparison with the values in controls. Moreover, substitution of Ca^{2+}-free PSS medium induced a further decline in [^{32}P]-diC8-PA accumulation (from a resting value of 1.02 ± 0.06 to 0.05 ± 0.01 c.p.m. (mg wet weight tissue)$^{-1}$); however, a response to NE stimulation was not demonstrated (0.03 ± 0.01 c.p.m. (mg wet weight tissue)$^{-1}$). Incubation of tissue with Ca^{2+}-free PSS did not influence the resting level of [^{32}P]-PA accumulation in either control or diabetic rats. The significant NE-induced increase in accumulation was also abolished by pretreatment with Ca^{2+}-free PSS.

NE-induced [^{32}P]-diC8-PA accumulation increased in a $CaCl_2$ concentration-dependent manner in both control and diabetic rats (Figure 5C). Non-stimulated resting level, [^{32}P]-diC8-PA accumulation was not influenced by extracellular $CaCl_2$ concentration in control rats. As same as Figure 5A, [^{32}P]-diC8-PA accumulation in non-stimulated diabetic tissue was significantly reduced from the values in control in each extracellular $CaCl_2$ concentration. These values slightly depended on the extracellular $CaCl_2$ concentration. Although a decrease in NE-induced [^{32}P]-diC8-PA accumulation was detected in the absence of $CaCl_2$ (Ca^{2+}-free PSS) in diabetes, accumulation was significantly higher than that observed in control rats at 1.0 and 2.5 mM $CaCl_2$. EC_{50} values in control and diabetic rats were 1.40 and 0.57 mM, respectively.

Effects of KCl and phorbol ester on [^{32}P]-diC8-PA and [^{32}P]-PA accumulation in diabetic rats

In order to increase the $[Ca^{2+}]_i$ without receptor stimulation, stimulation with 50 mM KCl was effected. Fura-2-loaded tissues isolated from control and diabetic rats were depolarized with 50 mM KCl at 37°C for 5 min; consequently, $[Ca^{2+}]_i$ was elevated (Figure 6A). At maximal $[Ca^{2+}]_i$, no significant differences were evident between control and diabetic rats. These values were $131.0\pm9.5\%$ and $125.0\pm12.2\%$ of each 10 μM NE-induced response ($n=6$). Treatment of the tissue with 1 μM PMA did not affect both resting and 50 mM KCl-induced responses in control and diabetic rats.

[^{32}P]Pi- and diC8-pre-labelled tissues isolated from control rats were incubated with 50 mM KCl (Figure 6B,C). This treatment did not increase [^{32}P]-diC8-PA or [^{32}P]-PA accumulation. However, treatment with KCl induced significant increases in [^{32}P]-diC8-PA and [^{32}P]-PA accumulation in diabetic rats. These values (11.58 ± 2.21 and 10.58 ± 0.48 c.p.m. (mg wet weight tissue)$^{-1}$, respectively) were close to those readings obtained from NE stimulation.

The possible role of PKC with respect to changes in [^{32}P]-diC8-PA accumulation in diabetic rats was investigated by testing the effect of PMA as a specific PKC activator. Single treatment with 1 μM PMA at 37°C for 5 min did not affect [^{32}P]-diC8-PA or [^{32}P]-PA accumulation in control rats. In order to examine the effects of increases in $[Ca^{2+}]_i$, this experiment was conducted in the presence of 50 mM KCl. Exposure of the control tissue to 1 μM PMA in the presence of 50 mM KCl induced significant increases in [^{32}P]-diC8-PA and [^{32}P]-PA accumulation to 9.44 ± 0.58 and 9.67 ± 0.86 c.p.m. (mg wet weight tissue)$^{-1}$, respectively.

Although a single treatment with 1 μM PMA did not affect the resting levels of [^{32}P]-diC8-PA or [^{32}P]-PA accumulation in diabetic rats, combined treatment with PMA and KCl led to significant escalation in the accumulation of these radiolabelled compounds. [^{32}P]-diC8-PA accumulation induced by NE in both control and diabetic rats was taken as 100%;

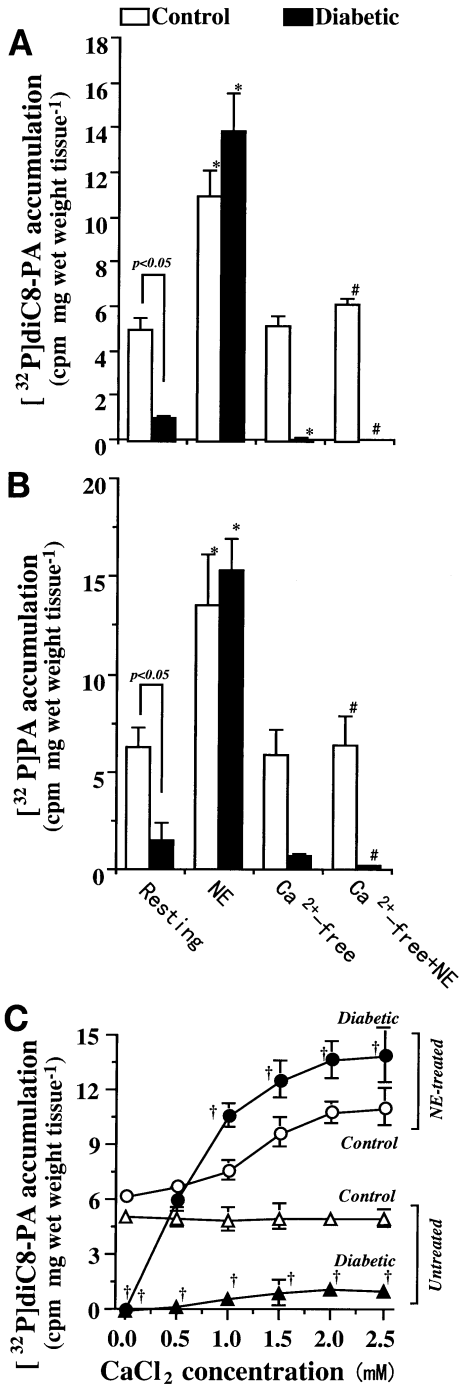

Figure 5 Effects of Ca^{2+}-free PSS on NE-induced [^{32}P]-diC8-PA and [^{32}P]-PA accumulation in diabetic rat aorta. [^{32}P]-Pi and diC8-pre-labelled aortae isolated from control and diabetic rats were incubated in the absence or presence of NE at 37°C. The extracellular medium was changed to normal or Ca^{2+}-free PSS 5 min prior to stimulation. [^{32}P]-diC8-PA (A) and [^{32}P]-PA (B) accumulation was quantified as described in Methods. Each value represents the mean ± s.e.mean of at least seven independent determinations. [^{32}P]-Pi and diC8-pre-labelled control and diabetic rat aortae were incubated in PSS containing various concentrations of $CaCl_2$ at 37°C for 5 min; subsequently, tissues were incubated in the presence or absence of 10 μM NE for 5 min (C). *$P<0.05$ vs resting levels, †$P<0.05$ vs control rats and #$P<0.05$ vs NE-treated values.

consequently, the levels of induction by PMA and KCl treatments were 73.4 and 97.1%, respectively.

Subsequently, the influence of PKC inhibition on KCl-induced changes in [^{32}P]-diC8-PA accumulation in the diabetic rats was investigated (Figure 7). Treatment of [^{32}P]-Pi- and diC8-pre-labelled tissue with 50 mM KCl induced significant increases in [^{32}P]-diC8-PA and [^{32}P]-PA accumulation exclusively in the diabetic rats as described above. Pretreatment with Gö6976 (1 μM, 10 min), a specific PKC inhibitor, did not affect accumulation in controls under resting conditions or following KCl treatment of [^{32}P]-diC8-PA or [^{32}P]-PA. However, KCl-induced increases in [^{32}P]-diC8-PA and [^{32}P]-PA accumulation were inhibited by Gö6976 pretreatment. KCl-induced accumulation of [^{32}P]-diC8-PA and [^{32}P]-PA was taken as 100%; therefore, Gö6976 reduced accumulation to 37.0 and 23.3%, respectively. This inhibitory effect depended on a concentration of Gö6976 (data not shown). IC_{50} values was 0.65 μM. Similar results were detected using another type of PKC inhibitor, calphostin C (data not shown). Pretreatment of 1 μM calphostin C significantly reduced the KCl-induced [^{32}P]-diC8-PA accumulation in diabetes (IC_{50} values 1.42 μM).

NE-induced changes in PKC activity in diabetic rats

To investigate changes in PKC activity in diabetic rats, PKC activity in the membrane fraction prepared from diabetic rat aorta was mainly measured, because a DG was distributed in the membrane fraction and phosphorylation of DG by DG kinase also performed in membrane fraction. The resting level of PKC activity in diabetic rats was higher than that observed in controls (18.6 ± 0.50 and 5.58 ± 0.89 pmol min^{-1} mg protein^{-1}, respectively) (Figure 8A). Treatment of the tissue isolated from control rats with 10 μM NE caused time-dependent activation of PKC in the membrane fraction. Maximal activation was detected at 5 min following NE stimulation; moreover, this effect was sustained over an additional 10 min incubation period. The resting level of PKC activity was high in diabetic rats; however, no significant increase was detected following NE stimulation. The maximal level of PKC activity in diabetic rats, 24.2 ± 1.02 pmol min^{-1} mg protein^{-1}, was evident 5 min after stimulation. In a cytosolic fraction, resting level of the PKC activity was 20 fold over of the membrane fraction in control rat (122.6 ± 5.5 pmol min^{-1} mg protein^{-1}). This level was not influenced by the NE-treatment. In diabetic rats, cytosolic PKC activity was not different from the control (118.6 ± 2.8 pmol min^{-1} mg protein^{-1}). This activity was reduced time-dependently by the NE treatment. The value in 5 min NE treatment was 93.4 ± 1.0 pmol min^{-1} mg protein^{-1}.

Total mass of DG was also measured (Figure 8B). The resting level of the DG mass was 178.2 ± 11.4 ng (mg wet weight tissue)$^{-1}$) in control rat aorta. NE stimulation (10 μM, 10 min) induced a significant elevation in the DG level (255.3 ± 12.5 ng (mg wet weight tissue)$^{-1}$). The elevated DG returned to the resting level upon PSS rinse of the tissue (data not shown). In STZ rats, the resting level of the total mass of DG indicated a significant increase (244.0 ± 13.2 ng (mg wet weight tissue)$^{-1}$) compared with that of control rats. This value was close to the NE-induced maximal response in

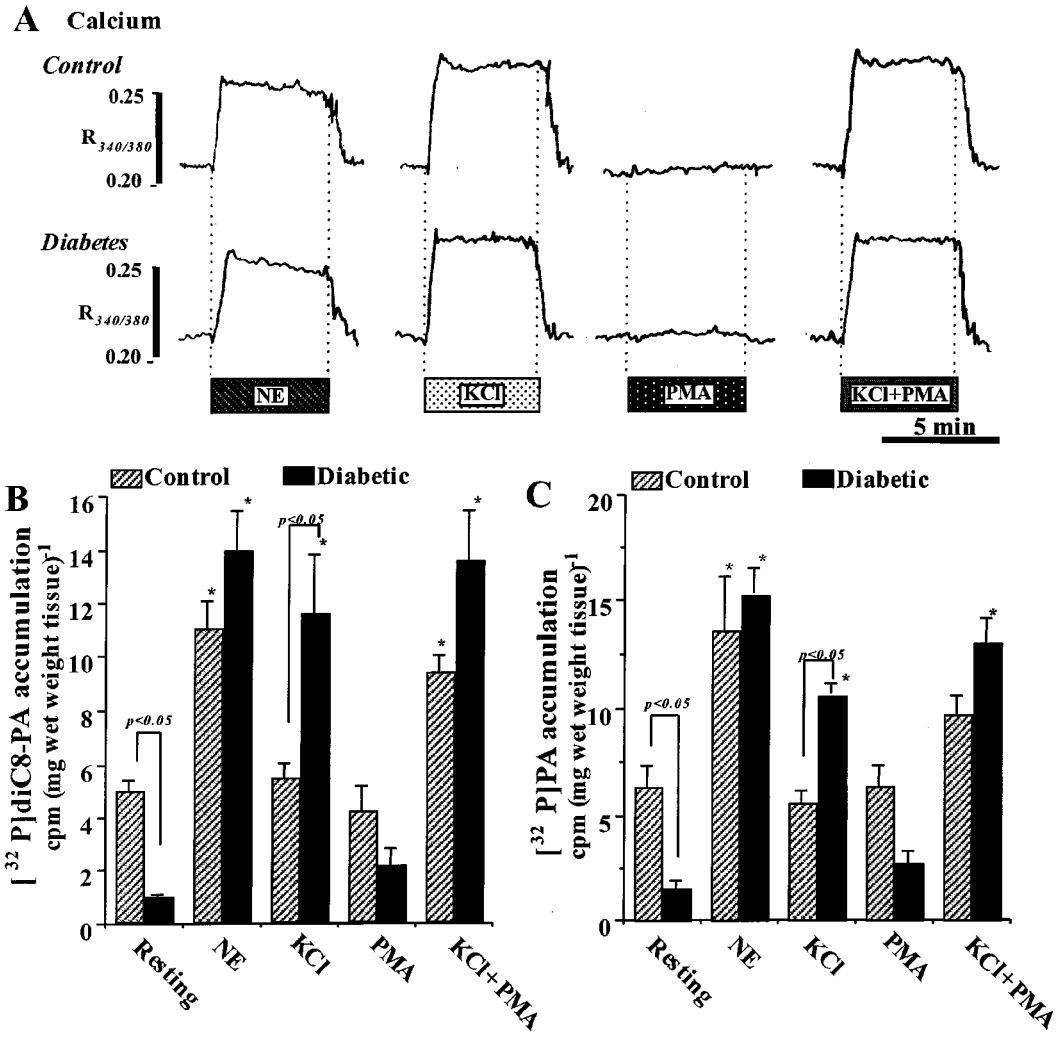

Figure 6 Effects of KCl and PMA on [^{32}P]-diC8-PA and [^{32}P]-PA accumulation in diabetic rat aortae. Changes in $[Ca^{2+}]_i$ were measured as same as Figure 1A using fura-2-loaded rat aortae. Typical changes in 1 μM NE, 50 mM KCl and/or 1 μM PMA treatments were represented (A). [^{32}P]-Pi and diC8-pre-labelled aortae isolated from control and diabetic rats were incubated in the absence or presence of 1 μM NE, 50 mM KCl and/or 1 μM PMA at 37°C for 5 min. [^{32}P]-diC8-PA (B) and [^{32}P]-PA (C) accumulation was quantified as described in Methods. Each value represents the mean ± s.e.mean of at least seven independent determinations. *$P<0.05$ vs resting levels.

control rats. Significant increases in the total mass of DG were not detected upon NE treatment in STZ rats (259.3 ± 10.7 ng (mg wet weight tissue)$^{-1}$).

*NE-induced increase in [^{3}H]-*myo*-inositol incorporation in diabetic rats*

Incorporation of [^{3}H-*myo*-inositol into phosphoinositides was measured in control and diabetic rat aorta to evaluate PI-turnover (Figure 9). In control aortae, the resting (non-stimulated) level of incorporation was 2692.5 ± 392.2 c.p.m. (mg wet weight tissue)$^{-1}$; moreover, 10 μM NE induced a significant increase in incorporation (6941.8 ± 310.6 c.p.m. (mg wet weight tissue)$^{-1}$). In diabetic rats, resting level of incorporation was slightly reduced (1581.0 ± 85.6 c.p.m. (mg wet weight tissue)$^{-1}$); furthermore, NE induced a large increase in incorporation. The value in diabetes (11461.5 ± 604.2 c.p.m. (mg wet weight tissue)$^{-1}$) was significantly elevated relative to controls.

Treatment with the DG kinase inhibitor, R59022, suppressed the increase in both control and diabetic rats. Values were 1709.5 ± 200.6 and 1641.3 ± 263.1 c.p.m. (mg wet weight tissue)$^{-1}$ for control and diabetic specimens, respectively.

Discussion

This investigation revealed a mechanism describing DG kinase hyper-reactivity in aortic smooth muscle in STZ diabetic rats. The present findings suggested that changes in calcium dependency of DG kinase and basal activation of PKC were associated with a dysfunction in the tissues in diabetes mediated by DG kinase hyper-reactivity. It is widely accepted that functional changes in the vascular system are dependent upon the stage of diabetes (Rinaldi & Cingolani, 1992). We initially evaluated basic factors (body weight, blood glucose level and urine volume) in diabetes (Table 1).

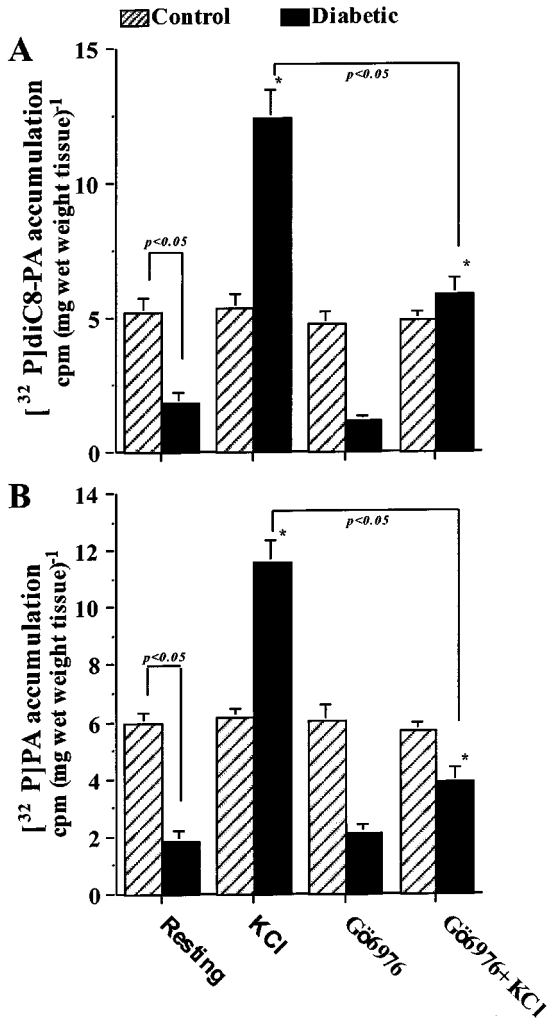

Figure 7 Effects of Gö6976 on [^{32}P]-diC8-PA and [^{32}P]-PA accumulation in diabetic rat aorta. [^{32}P]-Pi and diC8-pre-labelled aortae isolated from control and diabetic rats were incubated in the presence or absence of 1 μM Gö6976 at 37°C for 10 min. Fifty mM KCl was then added for 5 min. [^{32}P]-diC8-PA (A) and [^{32}P]-PA (B) accumulation was quantified as described in Methods. Each value represents the mean$\pm$s.e.mean of at least five independent determinations. *$P<0.05$ vs resting levels.

Alteration of these factors and effects of insulin treatment were typical diabetic parameters previously reported (Maruyama *et al*., 1999). Therefore, these STZ-induced diabetic rats served as a suitable experimental model of IDDM.

The confidence of the present DG kinase assay system was examined employing control rat aortae (Figure 2). NE stimulation increased [^{32}P]-diC8-PA and endogenous [^{32}P]-PA levels. Despite inhibition of both accumulation levels by R59022, a PLC inhibitor influenced [^{32}P]-PA exclusively. It was suggested that [^{32}P]-diC8-PA accumulation mirrored DG kinase activity; furthermore, this process proceeded in the absence of influence by endogenous PA formation and other PI-turnover-related enzymes.

The relationship between alteration in DG kinase regulation and dysfunction of aortic smooth muscle contractility in diabetes was investigated utilizing our IDDM model and DG kinase assay system. Vascular smooth muscle dysfunction has been documented previously (Abebe & Macleod, 1991; Wong, 1996); moreover, alteration in calcium sensitivity has also been suggested (Abebe *et al*., 1990). An increase in the contractile response involving enhanced intracellular calcium sensitivity was detected in diabetic rat aorta (Figure 1). The present findings suggested that increased calcium sensitivity may be involved in vascular dysfunction in diabetes.

In order to establish an association between DG kinase and changes in calcium sensitivity of contraction in diabetes, resting and NE-stimulated DG kinase activities were compared in control and diabetic rats. In control rat aortae, NE induced both time- and dose-dependent increases in DG kinase activity (Figures 3A and 4A); in addition, the response was suppressed by α-receptor antagonist (Figure 4-inset). In this receptor-mediated DG kinase activation, the requirement for activation of simultaneous increases in $[Ca^{2+}]_i$ and PKC activity was confirmed by results that activation was inhibited by Ca^{2+}-free PSS treatment (Figure 5A) and simultaneous treatment with 50 mM KCl and PMA induced a significant elevation in DG kinase activity (Figure 6B). Moreover, similar accumulation profiles of [^{32}P]-diC8-PA and [^{32}P]-PA suggested that DG kinase activation was the main route of endogenous PA formation in α-receptor stimulation (Figures 3B and 4B). A similar regulatory mechanism was previously reported in other tissue types (Nobe *et al*., 1994). Consequently, the data indicate that the mechanism may be common in receptor-mediated DG kinase activation.

In diabetic rats, DG kinase activity remained at a low level during the resting state in comparison to controls (Figure 3A). During this state, accumulation of endogenous DG (Figure 8B) and basal activation of PKC (Figure 8A) were detected. These findings suggested that inactivation of DG kinase led to accumulation of DG mediated by a reduction in metabolism. Endogenous DG functions as a PKC activator; consequently, basal activation of PKC might be due to DG accumulation. However, this interpretation appears to conflict with our discussion regarding the roles of PKC in DG kinase regulation in control rats. Activation of DG kinase requires not only PKC activation but also increased $[Ca^{2+}]_i$. Therefore, we hypothesized that $[Ca^{2+}]_i$ in the resting state maintained a low level; moreover, it could not activate DG kinase under conditions of basal PKC activation.

In order to confirm this hypothesis, the effect of 50 mM KCl and/or Gö6976 in the resting state of DG kinase activity was investigated. The KCl-induced increase in $[Ca^{2+}]_i$ in the absence of receptor-mediated stimulation significantly increased DG kinase activity exclusively in diabetic rats (Figure 6B). This KCl-induced activation was inhibited by Gö6976 (Figure 7A). These findings suggested that PKC activation was involved in the resting state of diabetes and that only the $[Ca^{2+}]_i$ increase was lacking. These results also supported our hypothesis. In the PKC activation in diabetic rat aorta, it was suggested that the PKC was a calcium dependent isoform(s), because Gö6976 has a selectivity to a cPKC (α, β-isoforms). From the results that the cytosolic PKC activity in diabetes decreased depend on an increase in the membrane fraction (Figure 8A), there is a possibility that an increase in the cPKC translocation to membrane in diabetes.

Basal activation of PKC and DG accumulation in diabetes occurs in the literature (Lee *et al*., 1999; Inoguchi *et al*., 1992). These alterations may explain basal inactivation of DG kinase in a manner similar to the present model of diabetic rats. In NE stimulation, responses in diabetic rats

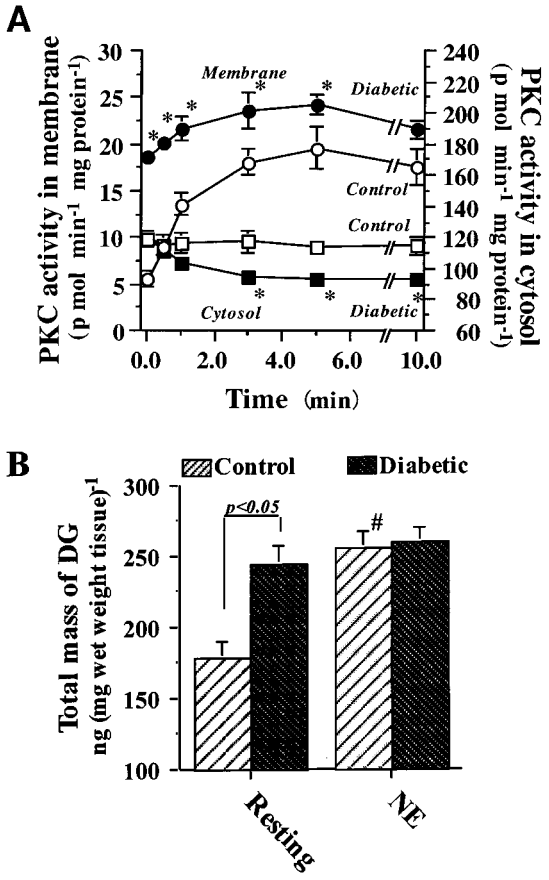

Figure 8 Time course of PKC activity and total mass of DG in diabetic rat aorta. Fresh aortae isolated from control and diabetic rats were pre-incubated in the presence of 10 μM NE for the indicated periods. These treatments were terminated and membrane and cytosol fractions were collected as described in Methods. PKC activity in each sample was assayed using an Amersham PKC-assay kit. Results are expressed as pmol min^{-1} mg $protein^{-1}$ (A). For detection of the total mass of DG, aortae isolated from control and diabetic rats were incubated in the presence or absence of 10 μM NE at 37°C for 10 min (B). Total masses of DG were quantified. Results are presented as ng mg wet weight $tissue^{-1}$. Each value represents the mean ± s.e.mean of at least five independent determinations. $*P<0.05$ vs control rats and $\#P<0.05$ vs resting levels.

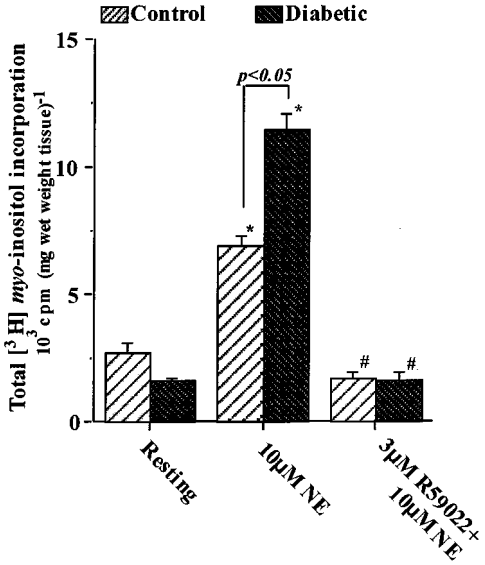

Figure 9 Effect of R59022 on NE-induced [^{3}H]-*myo*-inositol incorporation in control and diabetic rats. Aortae were isolated from control and diabetic rats. [^{3}H]-*myo*-inositol pre-labelled tissues were incubated in the presence or absence of 3 μM R59022 for 5 min, followed by the addition of 10 μM NE for 5 min. These treatments were terminated; subsequently, the radioactivity of [^{3}H]-phosphoinositides was measured as described in Methods. Each value represents the mean ± s.e.mean of at least five independent determinations. $*P<0.05$; values significantly different from resting level. $\#P<0.05$; values significantly different from maximal NE responses.

were significantly elevated relative to controls (Figures 3A and 4A). Reduced resting activity and NE-induced over-activity suggested that DG kinase hyper-reactivity plays an important role in vascular contractility dysfunction in diabetes. The enhanced calcium sensitivity of force development, detected in diabetic aortae (Figure 1), led us to investigate the effects of $[Ca^{2+}]_i$ on the hyper-reactivity of DG kinase. $[Ca^{2+}]_i$ dependency of DG kinase activity was also confirmed in control and diabetic rats; moreover, replace of extracellular calcium reduced a resting level of the DG kinase activity only in diabetes (Figure 5A), and calcium-dependent increase in the DG kinase activity in the presence of NE was detected at the low extracellular calcium concentration compared from controls (Figure 5C). In non-stimulated tissue, $CaCl_2$-dependent slight increase in DG kinase activation was detected only in diabetes. These results indicated that the DG kinase activity in diabetes was highly susceptible to the changes in calcium level. Significant increases in force development and DG kinase activation were detected under low $[Ca^{2+}]_i$ conditions. These findings suggested that this identical tendency possesses important implications with respect to the understanding of the mechanisms involved in vascular contractility dysfunction in diabetes.

Over-activation of DG kinase was maintained during NE stimulation (Figure 3A). Over-activation was predicted to increase DG levels an to return PKC activity to the resting level during this period; however, both factors maintained submaximal levels (Figure 8A). These findings suggested that DG formation might also be increased by acceleration of PI-turnover, which is accepted as an essential factor in cellular function. Moreover, acceleration of PI-turnover leads to enhanced function. The presence of this phenomenon in diabetes would strongly support our hypothesis.

Incorporation of *myo*-inositol was measured as total PI-turnover activity. In this assay system, increases in *myo*-inositol incorporation were indicative of PI-turnover acceleration (Conrad *et al*., 1991). *Myo*-inositol incorporation was significantly enhanced in diabetics (Figure 9), which suggested over-acceleration of PI-turnover. This acceleration may lead to increased vascular contractility in diabetes. Treatment with the DG kinase inhibitor, R59022, suppressed the increase in both control and diabetic rats. These results also indicated that inhibition of DG kinase influences the termination of the intracellular signalling system.

In diabetic models, changes in portions of the signal transduction system have been reported. α-adreno-receptor (Kobayashi & Kamata, 1999) and Gqα-protein (Hattori *et*

al., 1995) have been described. These changes were believed to influence DG kinase activity in diabetes. Hyper-reactivity was not a single-phase response; additionally, both inactivation in resting and over-activation in NE-stimulated states were involved. Consequently, we postulated that DG kinase hyper-reactivity was not a secondary effect of these changes. Moreover, preliminary results indicated that alternations of contractile response and DG kinase hyper-reactivity in diabetic rat aorta were partially recovered upon treatment with insulin (data is being prepared for publication). These data also indicated that these alternations were caused by an IDDM.

In the present study, we found DG kinase hyper-reactivity in STZ-induced diabetic rat aorta. This hyper-reactivity involved increases in both calcium dependency of DG kinase and basal activation of PKC. These experiments comprise the first investigation to reveal mechanisms regarding DG kinase alteration in diabetes. This mechanism may lead to over-activation as a complication in aortic smooth muscle contractility.

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