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Phorbol esters elicit Ca^{2+}-dependent delayed contractions in diabetic rat aorta

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Abstract

To determine whether diabetes alters vascular effects mediated by activation of protein kinase C, the contractions induced by phorbol esters were examined in aortic rings from rats with 8- to 12-week streptozotocin-induced diabetes and compared with those from age-matched control rats. In diabetic rat aorta, phorbol 12,13-dibutyrate (PDB) (≥ 30 nM) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (300 nM) elicited a delayed, sharply developing rise in tension following an initial gradually developing contraction. In control rat aorta, these agents produced only an initial slowly developing contraction. Both the initial and the delayed contractile responses observed in diabetic aorta were completely abolished by pretreatment with 20 nM staurosporine, and the delayed phase of contraction was not seen in Ca^{2+}-free medium or in the presence of 1 μM nifedipine. The concentration-response curves for the contractions induced by PDB revealed that PDB at concentrations ≥ 30 nM produced significantly greater responses in diabetic aorta than in control aorta. In control aorta, exposure to Ca^{2+}-free medium and pretreatment with 1 μM nifedipine shifted the concentration-response curves for PDB to the right without changing the maximal response. Under these conditions, there were no differences in the curves for PDB in control and diabetic aortas. These results suggest that the appearance of the delayed phase of contraction, possibly due to a delayed opening of Ca^{2+} channels, during activation of protein kinase C may be responsible for the enhanced contractile responses to phorbol esters in diabetic rat aorta.

Keywords: Diabetes mellitus; Phorbol ester; Protein kinase C; Ca^{2+}, extracellular; Ca^{2+} channel; Aorta, rat

1. Introduction

Cardiovascular disorders are the major cause of morbidity and mortality in diabetes mellitus (Garcia et al., 1974; Kannel and McGee, 1979). The deterioration of blood vessels has been suggested to be partly a consequence of alteration in the reactivity of vascular smooth muscles to neurotransmitters and circulating hormones (Christilieb et al., 1976; Weidmann et al., 1979). Based on this suggestion, the vascular responsiveness of various vasoactive agents has been widely investigated in experimental animal models of diabetes mellitus (see Tomlinson et al., 1992 for review). Although the results of these studies are not always consistent, enhanced responsiveness to some contractile agents such as noradrenaline has been reported in different vascular tissues from diabetic animals (Owen and Carrier, 1980; Scarborough and Carrier, 1984; MacLeod and McNeill, 1985; Agrawal et al., 1987; Harris and MacLeod, 1988).

Many contractile agonists have been proposed to stimulate the hydrolysis of phosphatidylinositol, resulting in the formation of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (Berridge and Irvine, 1984). The latter is a putative intracellular activator of the Ca^{2+}- and phospholipid-dependent enzyme protein kinase C. Increased basal protein kinase C activity has been shown in the liver (Pugezhenthi et al., 1990), heart (Tanaka et al., 1991; Xiang and McNeill, 1992), renal glomeruli (Craven and DeRubertis, 1989) and prostate (Garcia-Paramio et al., 1993) from streptozotocin-induced diabetic rats. It has been suggested that activation of protein kinase C plays an important role in the development of tonic smooth muscle contraction (DeFeo and Morgan, 1985; Park and Rasmussen, 1985; Chatterjee and Tejada, 1986) and that agonist-induced

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smooth muscle contractions are partly due to activation of protein kinase C (Rasmussen et al., 1987). Thus, if protein kinase C activity is also increased in diabetic vessels, it might be, at least in part, responsible for the increased responsiveness of diabetic vessels to the contractile agonists such as noradrenaline.

The purpose of the present study was to assess the alteration of protein kinase C-mediated contractile activity in diabetic vessels. Tumor-promoting phorbol esters stimulate protein kinase C by acting at the same site as 1,2-diacylglycerol (Blumberg et al., 1984) and cause sustained contractions in various blood vessels (Rasmussen et al., 1984; Forder et al., 1985; Khalil and Van Breemen, 1988). Abebe and MacLeod (1990) demonstrated that the maximum contractile responses to phorbol 12,13-dibutyrate (PDB) are enhanced in mesenteric artery from diabetic rats, and also indicated that the enhanced responsiveness is dependent on the availability or entry of extracellular Ca^{2+}. Thus, the present study was designed to characterize in more detail alterations of the contractile responses to phorbol esters in aorta from streptozotocin-induced diabetic rats.

2. Materials and methods

This study was approved by the Hokkaido University School of Medicine Animal Care and Use Committee. Male Wistar rats, 8 weeks old and 180–200 g in body weight, received a single injection of streptozotocin (45 mg/kg) in citrate buffer into the tail vein under light anesthesia with diethyl ether, as previously described (Hattori et al., 1991). Age-matched control rats received an equivalent volume of citrate buffer alone. Control and diabetic rats were caged separately but housed under identical conditions. Both groups of animals were given free access to food and water.

Eight to 12 weeks after the injection, diabetic rats and age-matched control animals were weighed and anesthetized with diethyl ether. Blood was collected from the renal vein and its glucose level was measured by means of a Rapid Blood Analyzer Super using a Uni-Kit (Chugai, Tokyo, Japan). A section of the thoracic aorta between the aortic arch and the diaphragm was carefully excised and placed in oxygenated normal physiological salt solution at room temperature. The aorta was cleaned of adhering fat and connective tissue and cut into rings of 4 mm length. The intimal surface of the aortic rings was gently rubbed with a wooden rod to remove the inhibitory influence of the vascular endothelium. The effectiveness of endothelium removal was confirmed by the absence of the characteristic relaxation induced by 1 μM acetylcholine in aorta precontracted with 1 μM noradrenaline. Each ring was suspended from a pair of stainless steel hooks under a resting tension of 2 g in a water-jacketed bath filled with 25 ml of normal physiological salt solution. The composition of the solution was (in mM): NaCl 118.2, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 10.0. The solution in the bath was gassed with 95% O_2 and 5% CO_2 and its temperature was maintained at 37° C. Force generation was monitored using an isometric transducer (Sanei-Sokki 45196, Tokyo, Japan) and a carrier amplifier (Sanei-Sokki 1236). The output of the force transducer was registered on a pen recorder (TOA Electronics ERP-241A, Tokyo, Japan) through a polygraph recorder (Sanei-Sokki 142-8).

Following the equilibration period, the rings were exposed several times to 1 μM noradrenaline until reproducible contractile responses were obtained. The pattern of the contractile response to PDB (1 nM–1 μM) or 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (300 nM) was determined by exposing the preparations to a single concentration of these agents. When staurosporine was used, the rings were incubated for 30 min with staurosporine (20 nM), which remained in the bath solution during the succeeding exposure to PDB. In some experiments, the contractile response to PDB was obtained in the presence of 1 μM nifedipine or in Ca^{2+}-free medium. Ca^{2+}-free medium was made by omitting $CaCl_2$ and adding 1 mM EGTA. The influence of nifedipine or extracellular Ca^{2+} removal on the PDB-induced contraction was assessed by adding nifedipine or by exposure to Ca^{2+}-free medium 30 min before application of PDB. Concentration-response curves for PDB were made by cumulatively increasing the concentration of the agent in the tissue chamber, in normal physiological salt solution without or with 1 μM nifedipine, or in Ca^{2+}-free medium.

At the completion of each experiment, the rings were carefully blotted dry and weighed. Contractile responses of each preparation to the agents were expressed as milligrams of developed tension per milligram of tissue wet weight.

The following compounds were used: streptozotocin, PDB, TPA and 4α-phorbol (Sigma Chemical, St. Louis, MO, USA), staurosporine (Kyowa Hakko, Tokyo, Japan), nifedipine (Bayer, Leverkusen, Germany), noradrenaline bitartrate and acetylcholine chloride (Wako Junyaku, Osaka, Japan). PDB, TPA, 4α-phorbol and staurosporine were prepared in dimethyl sulfoxide. Nifedipine was prepared in ethanol. Noradrenaline and acetylcholine were dissolved in distilled water. Further dilutions to the desired concentrations were made with the suitable buffer solution. The experiments with nifedipine were performed in the dark and the solution bottles and tubing were covered with aluminum foil for further protection against degradation.

The data are expressed as means ± S.E.M. Statistical analysis was performed using Student's *t*-test for

unpaired observations. Differences were considered to be statistically significant when $P < 0.05$.

3. Results

The general features of the streptozotocin-treated diabetic rats and their age-matched controls are shown in Table 1. As previously reported (Hattori et al., 1991,1994), 8–12 weeks after injection, all diabetic rats exhibited severe hyperglycemia (range 499–865 mg/dl), and their serum glucose levels were elevated approximately 4.3-fold compared to those of control animals. The body weights of diabetic rats were significantly less than the weights of their corresponding controls. In addition, diabetic rats had a significantly lower aortic tissue wet weight compared to controls.

PDB initially elicited gradually developing contractions in control and diabetic rings. Fig. 1A illustrates typical examples of contractions induced by 30 nM PDB in these preparations. In control aorta, the PDB-induced contraction developed slowly, reached a maximum in 20–30 min and was maintained at this level thereafter. In diabetic aorta, the PDB-induced contraction was slower than in control aorta with respect to both onset and rate of tension development. Furthermore, there occurred an abrupt, sharp rise in tension in the course of the initial, slowly developing contraction. This delayed phase of contraction was detected in all of seven diabetic aortic rings, but was never observed in any of seven control aortic rings. The time at which the delayed phase of contraction appeared was quite variable, from 20 to 60 min after application, and its mean was 40 ± 5 min ($n = 7$). As a result, the final level which diabetic aortic rings attained in the contractile response to 30 nM PDB was significantly higher than that which the controls reached (Fig. 3). The delayed phase of contraction which characterized the response of diabetic aorta to PDB was evoked by PDB only at 30 nM and higher concentrations.

Table 1
Body weight, tissue weight and plasma glucose levels in control and diabetic rats

	Control		Diabetic		P
Body weight (g)	366±5	(24)	160±2	(24)	<0.001
Aortic wet weight (mg)	7.4±0.2	(48)	5.7±0.1	(48)	<0.001
Plasma glucose (mg/dl)	150±6	(24)	650±19	(24)	<0.001

Results are expressed as means±S.E.M. The number of preparations is given in parentheses.

Aiming to determine functionally whether the delayed phase of contraction induced by PDB in diabetic aorta is mediated by protein kinase C activation, we

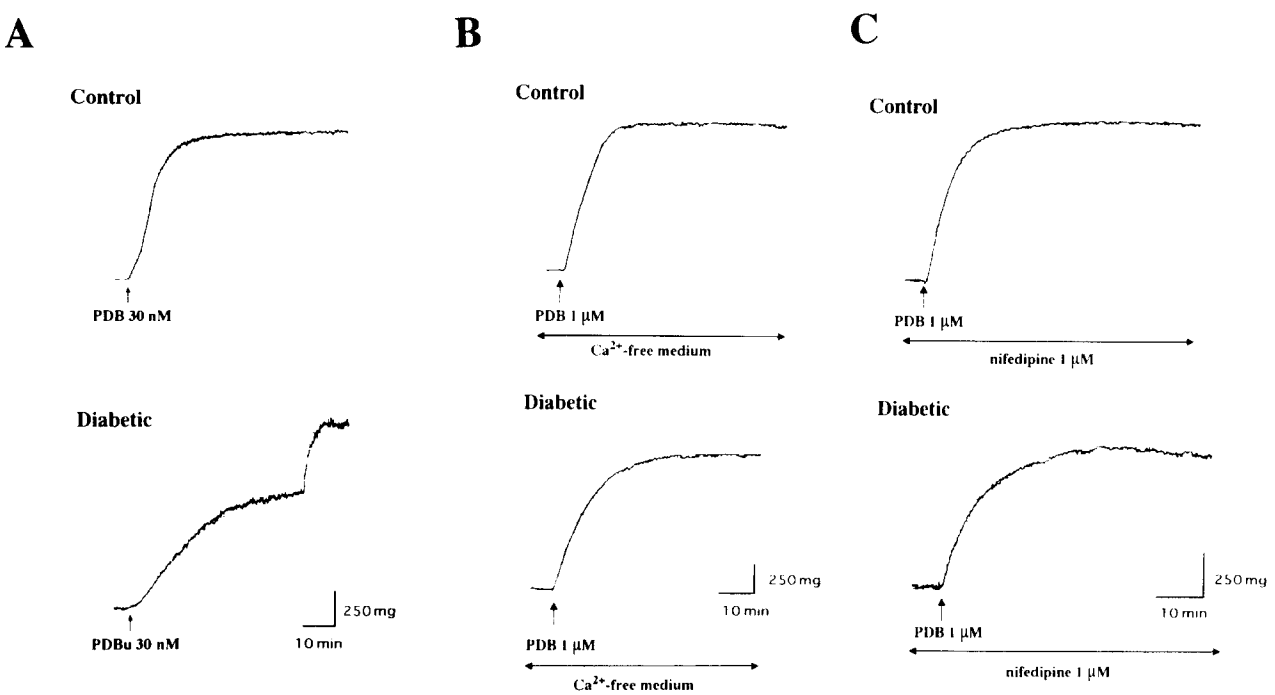

Fig. 1. Contractions induced by PDB in control and diabetic rat aortic rings in normal physiological salt solution (A), in Ca^{2+}-free solution (B) and in the presence of 1 μM nifedipine (C). In normal medium, 30 nM PDB induced slowly developing, monophasic contraction in control aorta, while producing an abrupt, sharp rise in tension following initial, slowly developing contraction in diabetic aorta. In Ca^{2+}-free medium or in the presence of 1 μM nifedipine, 1 μM PDB elicited slowly developing, monophasic contractions in both control and diabetic aortas. PDB was applied 30 min after exposure to Ca^{2+}-free medium or the addition of nifedipine.

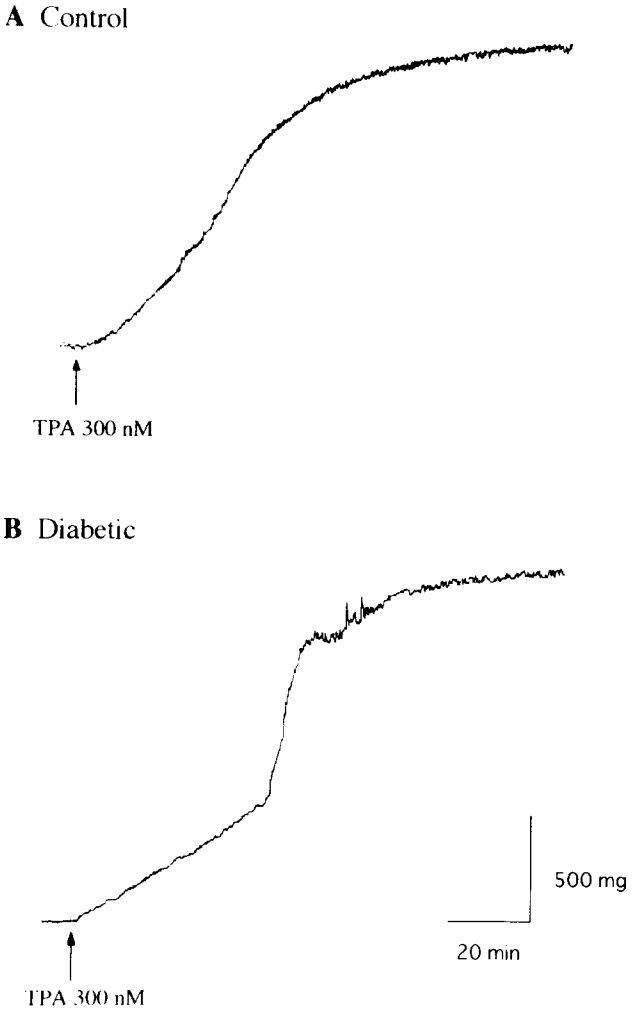

Fig. 2. Contractions induced by 300 nM TPA in control (A) and diabetic (B) rat aortic rings. Note that TPA produced a delayed phase of contraction only in diabetic aorta.

examined the contractile response of control and diabetic aortic rings to another active phorbol ester, TPA. As shown in Fig. 2A, the addition of 300 nM TPA produced a gradual increase in tension in control aortic rings. The pattern of contraction as a whole was quite similar to that with PDB, although the TPA-induced contraction developed much more slowly, and the steady level of the increased tension was attained much later (60–90 min after administration) when compared to the PDB-induced contraction. On the other hand, in diabetic aorta, 300 nM TPA evoked a sharp rise in tension springing up from a smoothly developing initial contraction 45–100 min after application (Fig. 2B). The delayed phase of contraction was observed in all of six diabetic aortic rings. Thus, the final contractile response to 300 nM TPA was significantly greater in diabetic aortic rings than that in controls (Fig. 3). The inactive phorbol ester, 4α-phorbol (1 μM), did not contract aortic rings from either control or diabetic rats (data not shown).

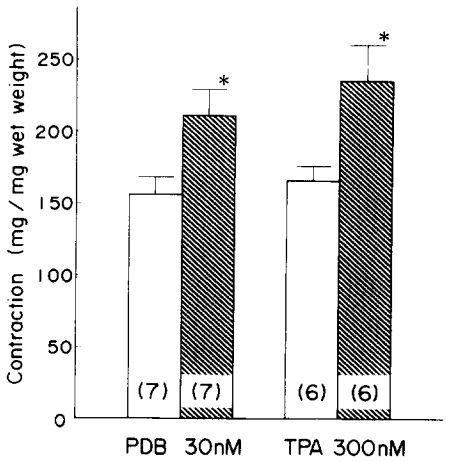

Fig. 3. Maximum contractile effects of 30 nM PDB and 300 nM TPA on aortic rings from control (open columns) and diabetic (hatched columns) rats. Numbers of experiments in parentheses; vertical bars: ±S.E.M. * $P < 0.05$, compared with corresponding control values.

Pretreatment with staurosporine, a putative protein kinase C inhibitor, changed dramatically the contractile responses to PDB. Stimulation of control aorta with 30 nM PDB after treatment with 20 nM staurosporine consistently resulted not only in a marked reduction of maximal tension development but also in a transient contraction (Fig. 4A), with the tension declining to near-basal level within 40–60 min. Interestingly, diabetic aorta pretreated with staurosporine did not ex-

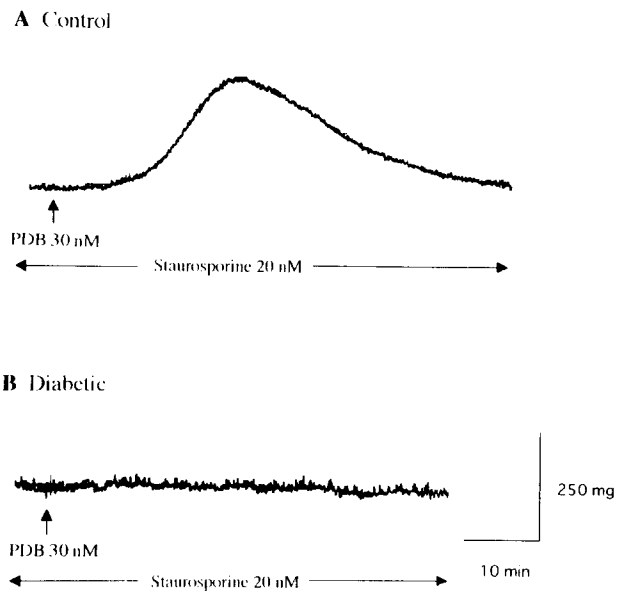

Fig. 4. Effect of staurosporine on the PDB-induced contractions in control (A) and diabetic (B) rat aortic rings. Staurosporine (20 nM) was added 30 min before application of 30 nM PDB. In control aorta, pretreatment with staurosporine caused a marked decrease in the response to PDB, which showed a transient contraction. In diabetic aorta, the PDB-induced contraction was completely abolished.

hibit any contraction in response to 30 nM PDB (Fig. 4B).

Removal of extracellular Ca^{2+} or pretreatment with 1 μM nifedipine resulted in alterations of the contractile responses of aortic rings to PDB. First, these procedures markedly reduced the sensitivity to PDB of aortic rings from both control and diabetic rats. Increasing the concentration of PDB restored the reduced responses of control rings to PDB under Ca^{2+}-free conditions or nifedipine pretreatment. Thus, the contractile responses of control aortic rings in response to 1 μM PDB in Ca^{2+}-free medium or in the presence of 1 μM nifedipine were not different in time course or magnitude from those to 30 nM PDB in normal medium (Fig. 1). Second, exposure to Ca^{2+}-free medium or pretreatment with 1 μM nifedipine completely suppressed the appearance of a delayed sharp rise in tension in diabetic aortic rings (Fig. 1B and 1C). Thus, in Ca^{2+}-free medium or in the presence of nifedipine, diabetic aorta exhibited only a simple, monophasic increase in tension in response to 1 μM PDB.

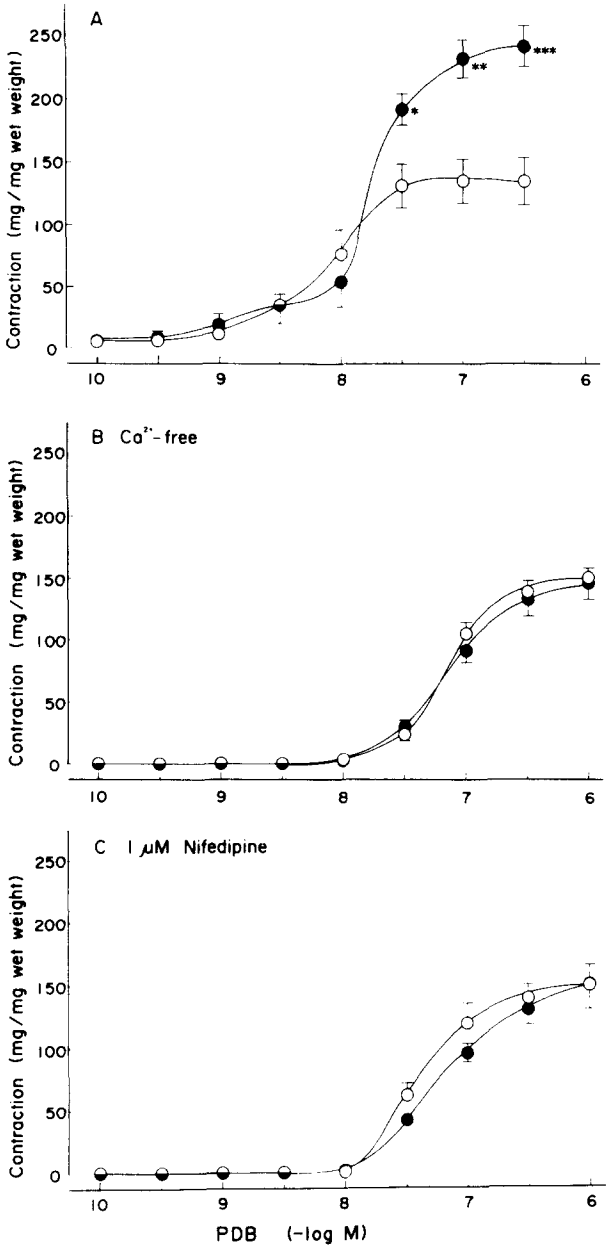

Fig. 5. Concentration-response curves for the PDB-induced contractions in control (○) and diabetic (●) rat aortic rings in normal physiological salt solution (A), in Ca^{2+}-free solution (B) and in the presence of 1 μM nifedipine (C). The preparations were exposed to Ca^{2+}-free medium containing 1 mM EGTA or incubated with 1 μM nifedipine 30 min before cumulative application of PDB. The points are means ± S.E.M. of 6–8 preparations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with corresponding control values.

As shown in Fig. 5A, PDB caused concentration-dependent contractions in aortic rings from both control and diabetic rats. The magnitudes of the contractions induced by PDB at low concentrations ($\leq$ 10 nM) were undistinguishable between control and diabetic aortas. However, the contractile responses to PDB at high concentrations ($\geq$ 30 nM) were significantly enhanced in diabetic aortic rings compared to controls. In control aortic rings, the concentration-response curves for PDB were shifted 12-fold and 7.3-fold to the right (assessed by EC_{50} values) with no change in the maximal response in Ca^{2+}-free medium and in the presence of 1 μM nifedipine, respectively. On the other hand, with diabetic aortic rings, removal of extracellular Ca^{2+} or pretreatment with 1 μM nifedipine significantly reduced the maximal contractile response to PDB. As a result, in Ca^{2+}-free medium, no differences were observed in the magnitudes of contractile responses or sensitivities of control and diabetic aortas to PDB (Fig. 5B). Similarly, pretreatment with nifedipine eliminated the difference between control and diabetic aortas in the contractile responses to PDB (Fig. 5C).

4. Discussion

The results of the present study demonstrate that aortas from streptozotocin-induced diabetic rats exhibit enhanced contractile responses to PDB at higher concentrations ($\geq$ 30 nM). This observation is in agreement with the reports of other investigators who used mesenteric arteries from streptozotocin-induced diabetic rats (White and Carrier, 1990; Abebe and MacLeod, 1990). The key finding in this study is that the enhanced contractile responses to PDB resulted from the appearance of the delayed phase of contraction elicited by PDB, and observed only in diabetic aorta. This provides important insights into the mechanisms underlying the contractile responses of diabetic vessels to PDB.

In diabetic aorta, PDB ($\geq$ 30 nM) generated a delayed, sharply developing rise in tension that distinctly emerged from an initial gradual increase in tension. A delayed phase of contraction following initial tension development was also induced by another active phorbol ester, TPA, at a relatively high concentration (300 nM) only in diabetic aorta. Although TPA has been reported to be similarly or slightly more potent than PDB as an activator of protein kinase C in vitro (Castagna et al., 1982), TPA caused contractions of rat aorta with a much slower onset and with a lower potency than those obtained with PDB. This may be due to the difference in their relative hydrophobicity, thus affecting rate of penetration into the cell membrane and access to the cell interior. In contrast to PDB and TPA, the inactive phorbol ester, 4α-phorbol, did not cause contractions of aortas from either control or diabetic rats. In addition, the initial and delayed phases of contraction induced by PDB in diabetic aorta were completely abolished by staurosporine, a putative protein kinase C inhibitor (Tamaoki et al., 1986), at a concentration of 20 nM. At this concentration, staurosporine acts as a relatively specific inhibitor of the protein kinase C-mediated contractions (Kageyama et al., 1991). These data suggest that the delayed phase of contraction as well as the initial phase induced by PDB and seen in diabetic aorta are mediated by activation of protein kinase C. In control aorta, pretreatment with staurosporine did abolish the sustained part of the PDB-induced contractions as illustrated by the finding that the contractions after staurosporine pretreatment were transient. A similar inhibitory action of staurosporine on the PDB-induced contractions has been reported in canine femoral arteries (Merkel et al., 1991). Thus, staurosporine appeared to exert a greater inhibitory effect on the protein kinase C-mediated contractile responses in diabetic vessels.

In rat aorta, the contractions induced by phorbol esters have been reported to be dependent on extracellular Ca^{2+} (Danthuluri and Deth, 1984; Chiu et al., 1987). Indeed, phorbol esters increase intracellular Ca^{2+} levels in rat aorta, and removal of extracellular Ca^{2+} or pretreatment with verapamil abolishes the increase in intracellular Ca^{2+} levels (Sato et al., 1992). Thus, activation of protein kinase C with phorbol esters may lead to opening Ca^{2+} channels allowing the influx of extracellular Ca^{2+} into smooth muscle cells of rat aorta. However, we found that, in Ca^{2+}-free medium, the concentration-response curve for PDB was shifted parallel to the right with no change in the maximal response in control rat aorta. The results obtained with nifedipine were quantitatively and qualitatively similar to those obtained under Ca^{2+}-free conditions. Our data indicate that PDB-induced contractions in control rat aorta appear to only partially require Ca^{2+} influx through Ca^{2+} channels. As Ca^{2+} is required to activate phospholipid-dependent protein kinase C (Nishizuka, 1986), the entry of Ca^{2+} may play a role in supplying the Ca^{2+} required for protein kinase C activation. It has been suggested that the sensitization of contractile proteins to Ca^{2+} seems to be most important as a mechanism of action of phorbol esters to induce contractions in rat aorta (Sato et al., 1992).

The delayed phase of contraction induced by PDB and TPA at higher concentrations in diabetic rat aorta appeared to be completely dependent on the entry of extracellular Ca^{2+}. In the absence of extracellular Ca^{2+} or in the presence of nifedipine, PDB produced a monophasic contraction without the delayed phase in diabetic rat aorta. This suggests that phorbol esters, at least in diabetic rat aorta, may be involved in the modulation of dihydropyridine-sensitive Ca^{2+} channels. There is evidence that phorbol esters activate dihydropyridine-sensitive Ca^{2+} channels in a vascular smooth muscle cell line possibly by protein kinase C-dependent phosphorylation (Fish et al., 1988). As stated above, the contribution of Ca^{2+} influx via Ca^{2+} channels, if any, to the contractions induced by phorbol esters seems to be relatively small in control rat aorta. On the other hand, in diabetic rat aorta, a greater influx of Ca^{2+} through Ca^{2+} channels may be promoted during activation of protein kinase C by phorbol esters. Hence, we assume that activation of protein kinase C may lead to delayed opening of Ca^{2+} channels, which may be responsible for the delayed phase of contraction in diabetic rat aorta. However, it is uncertain why only diabetic aorta exhibits delayed but marked contractions, possibly through phosphorylation of Ca^{2+} channels during activation of protein kinase C. Possible explanations for this question may include a greater activity of protein kinase C in diabetic rat aorta as reported for other diabetic tissues (Pugezhenthi et al., 1990; Tanaka et al., 1991; Xiang and McNeill, 1992; Craven and DeRubertis, 1989; Garcia-Paramio et al., 1993). Alternatively, diabetes may alter the number and/or nature of Ca^{2+} channels phosphorylated by protein kinase C activation.

The concentration-response curves for the PDB-induced contractions showed that the lower part of the curve obtained from diabetic aorta was similar to that from control aorta, while the upper part of the curve was significantly higher and steeper for diabetic aorta. It should be noted that the concentrations of PDB needed to reveal the enhanced contractile responses of diabetic aorta are consistent with those needed to elicit the delayed phase of contraction in diabetic aorta. Thus, it is reasonable to conclude that the enhanced contractile responses of diabetic rat aorta to phorbol esters result from the delayed phase of contraction, possibly due to increased Ca^{2+} influx via Ca^{2+} channels. In agreement with this view are our findings that,

in the absence of Ca^{2+} or in the presence of nifedipine, the concentration-response curves for PDB were similar in control and diabetic aortas.

In conclusion, we found that phorbol esters elicited a delayed, sharply developing rise in tension in diabetic aorta. The delayed phase of contraction observed in diabetic aorta was completely dependent on extracellular Ca^{2+} and was eliminated by nifedipine. Thus, we propose that activation of protein kinase C in diabetic rat aorta may result in a delayed opening of dihydropyridine-sensitive Ca^{2+} channels which may allow an increased influx of extracellular Ca^{2+} and thereby lead to the delayed phase of contraction. However, it remains to be clarified whether such altered responses to protein kinase C activation contribute to the enhanced contractile responses to vasoactive agents including noradrenaline in diabetic blood vessels.

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