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Defective phosphatidic acid–phospholipase C signaling in diabetic cardiomyopathy

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Abstract

The effects of exogenous phosphatidic acid (PA) on Ca^{2+} transients and contractile activity were studied in cardiomyocytes isolated from chronic streptozotocin-induced diabetic rats. In control cells, 25 μM PA induced a significant increase in active cell shortening and Ca^{2+} transients. PA increased IP_3 generation in the control cardiomyocytes and its inotropic effects were blocked by a phospholipase C inhibitor. In cardiomyocytes from diabetic rats, PA induced a 25% decrease in active cell shortening and no significant effect on Ca^{2+} transients. Basal and PA-induced IP_3 generation in diabetic rat cardiomyocytes was 3-fold lower as compared to control cells. Sarcolemmal membrane PLC activity was impaired. Insulin treatment of the diabetic animals resulted in a partial recovery of PA responses. Our results, therefore, identify an important defect in the PA-PLC signaling pathway in diabetic rat cardiomyocytes, which may have significant implications for heart dysfunction during diabetes.

Keywords: Phosphatidic acid; Phospholipase C; Diabetic cardiomyopathy; Cardiomyocyte; Calcium transients; Heart

Cardiac sarcolemmal (SL) membrane phosphatidic acid (PA) can be generated by hydrolysis of phosphatidylcholine by phospholipase D (PLD) or via the phosphorylation of *sn*-1,2 diacylglycerol (DAG) derived from the hydrolytic action of phospholipase C (PLC) [1]. Because of this central position linking two important pathways in lipid metabolism, PA has been suggested as a potentially important signaling molecule in cell biology [2,3]. In support of this hypothesis, PA has been shown to affect cell growth, proliferation, and migration. PA can also stimulate inositol 1,4,5-trisphosphate (IP_3) production in adult cardiomyocytes [4], which in turn modulates Ca^{2+} movements within the cardiomyocyte [5–8] and influences cardiac contractile function [6]. PA has been reported to increase intracellular concentration of free Ca^{2+} in adult cardiomyocytes and augment cardiac contractile activity of the normal heart [9], an effect considered to be mediated by activation of PLC [2,10,11].

The incidence of heart disease is greater in the diabetic population than the non-diabetic population [12,13]. The mechanism responsible for this is presently unclear, however, the presence of a primary cardiomyopathy in diabetes has been identified [14–18]. This cardiomyopathic condition is associated with defects in the capacity of cardiomyocytes from diabetic animals to regulate intracellular ionic homeostasis in a normal manner [19] resulting in abnormal Ca^{2+} transients and contractile activity [20–26]. These are critical defects that necessitate further understanding of the mechanism responsible for those lesions.

Although a diminished cardiac response to PA has been shown to occur in chronic diabetic rats [27], it is unclear if this effect is due to a direct action on cardiomyocytes or an indirect effect from an action on the cardiac vasculature. We have previously identified a

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depressed SL PA level in hearts from diabetic rats [28], and it appears possible that during diabetes the heart may adapt to the lower PA content with a change in sensitivity to this signaling molecule. It was hypothesized that a reduced sensitivity of cardiomyocytes from diabetic rats to PA may exist. This could have important implications for signal transduction pathways within the cardiomyocyte. The present study was undertaken, therefore, to directly examine the changes in cardiomyocyte contractile activity and Ca^{2+} transients in response to PA in an experimental model of streptozotocin-induced diabetes (Type 1, insulin-dependent diabetes mellitus) and to determine the reversibility of these changes upon insulin treatment.

Materials and methods

Animals. Male Sprague–Dawley rats weighing 125–145 g each were made diabetic ($n = 48$) with a single tail vein injection of streptozotocin (65 mg/kg body weight, dissolved in 0.1 M citrate buffer, pH 4.5) [16,18,28,32,33]. Age-matched control animals ($n = 48$) received citrate buffer only. All rats were provided commercial food and water ad libitum until they were sacrificed by decapitation at 8 weeks. Some randomly chosen diabetic animals ($n = 22$) were given daily subcutaneous injections of 3 U Ultralente insulin for the last 2 weeks [28,29]. Blood glucose and insulin levels were measured at the termination of the study using techniques described elsewhere [28,29]. Hearts were removed and processed for cardiomyocyte isolation. All guidelines set by the University of Manitoba were followed for the care and treatment of animals used in this study.

Preparation of cardiomyocytes. Single cardiomyocytes were isolated by collagenase digestion of perfused rat hearts as described previously [30–32]. After isolation of the left ventricular cardiomyocytes, cells were allowed to adhere to laminin-coated coverslips. Coverslips were washed to remove loose cells and were left overnight in Medium 199 supplemented with 0.02% BSA, 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco-BRL, Burlington ON, Canada). Rod-shaped quiescent cardiomyocytes as seen under the phase contrast microscope comprised greater than 90% of the final cell population. In fact, 3.7×10^6 cardiomyocytes were isolated per control heart, whereas the yield from a heart of a diabetic animal was 1.6×10^6 cardiomyocytes and that from a diabetic + insulin was 2.3×10^6 cardiomyocytes.

Preparation of and perfusion with phosphatidic acid. A dispersion solution of PA (bulk concentration 1 mM) was made by suspending PA (L-α-PA, from egg yolk lecithin, sodium salt, Sigma Chemical, St. Louis, MO) in Hepes perfusion buffer containing 140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 10 mM dextrose, and 6 mM Hepes (pH 7.4), and 0.02% BSA by sonication for 30 min in a Branson (model 1200) sonication bath at 4 °C and protected from direct light. Cardiomyocytes mounted on glass coverslips were mounted in a Leiden chamber heated to 37 °C with a Medical Systems PDMI-2 Open Perfusion Micro-Incubator (Greenvale, NY) [31]. Cells were perfused with the Hepes buffered solution bubbled with 100% oxygen [30] without or with PA. Myocytes were equilibrated for 10–15 min prior to treatment with PA at concentrations of 10, 25, and 50 μM. Cells were perfused with PA for a period of 15 min. In some experiments, cardiomyocytes were perfused with PLD (from *Streptomyces chromofuscus*: Sigma–Aldrich, Canada, ON) for 15 min.

It is possible that any effects we observed were due to an effect of lyso-PA (LPA) instead of PA. This may have occurred due to contamination of the PA with LPA or due to generation of LPA in the cardiomyocytes. Therefore, we measured the amount of LPA that is generated during the perfusion of cardiomyocytes with 25 μM PA. We also tested the PA for any evidence of LPA contamination. Cardiomyocytes were perfused with 25 μM [^{14}C]PA (NEN Life Sciences Products, Boston, MA) for 15 min. Cells were then harvested and following a lipid extraction with only chloroform/methanol (2:1, by volume), the amount of LPA generated was measured by thin layer chromatography [1]. LPA migration was monitored using authentic unlabeled LPA (L-3-LPA, free acid from egg yolk lecithin, Serdary Research Laboratories, Englewood Cliffs, NJ), visualized with iodine vapor, scraped, and counted for radioactivity in a Beckman LS 1701 liquid scintillation counter.

Cardiomyocyte contractile performance. Active and passive cardiomyocyte contractile activity was monitored as unloaded cell shortening with the use of a video-edge detection system (Crescent Electronics, Sandy, UT) [30,31]. This camera is capable of capturing data at a rate of 60 Hz. Cell shortening was induced at a frequency of 0.5 Hz with a duration of 200 ms at supramaximal intensity. The signal was calibrated with a microscope stage micrometer, as described [30,31].

Measurement of intracellular Ca^{2+}. Ca^{2+} transients were measured spectrofluorometrically with the Ca^{2+} sensitive dye fura-2 (Molecular Probes, Eugene, OR). Adherent cells were loaded with 1 μM fura-2 AM for 15 min at 37 °C, washed, and then mounted in a Leiden chamber heated to 37 °C with a Medical Systems PDMI-2 Open Perfusion Micro-Incubator (Greenvale, NY). This system was fixed to the stage of a Nikon Diaphot epifluorescent microscope, which was attached to a SPEX Fluorolog spectrofluorometer, as described previously [30,31]. Cardiomyocytes were excited alternatively at 340 and 380 nm wavelengths with emission monitored at 505 nm. Fluorescent signals were quantitated with photomultiplier tubes and the data were analyzed on a computer. Calibration of the fluorescent signal was completed as described previously [30,31]. The Ca^{2+} transient was calculated as an amplitude by subtracting the end-diastolic [Ca^{2+}] from the peak systolic [Ca^{2+}].

Determination of cytosolic IP_3 content and sarcolemmal phospholipase C δ_1 activity. IP_3 content of the cytosol fraction isolated from left ventricular (LV) cardiomyocytes as previously described [32,39] was measured using a Biotrak radioimmunoassay kit (Amersham Biosciences, Quebec). The procedures involved were carried out according to the manufacturer's instructions. Briefly, unlabeled IP_3 in samples competes with a fixed amount of [^{3}H]-labeled IP_3 for a limited number of sites on bovine adrenal IP_3 binding protein. Bound and free IP_3 are separated by centrifugation. D-Myo-IP_3 was used as a standard. Activity of SL PLC δ_1, the major SL PLC isozyme, was measured by immunoprecipitation, as already reported [11]. Briefly, SL membranes from the LV tissue of control, diabetic, and diabetic + insulin groups were isolated according to the method of Pitts et al. as described elsewhere [28]. Solubilized SL membrane proteins [11] were incubated overnight at 4 °C with monoclonal antibodies to PLC δ_1 (5 μg antibody to 350 μg membrane extract). The immunocomplex was captured by adding 100 μL (50 μL packed beads) of washed (three times with 30 mM Hepes; pH 6.8) protein-G–Sepharose bead slurry at 4 °C by rotation for 2 h. The agarose beads were collected by pulse centrifugation (5 s) at 10,000*g* washed with Hepes buffer, and then assayed for the activity of PLC δ_1 by measuring the hydrolysis of [^{3}H]phosphatidylinositol-4,5-bisphosphate (NEN Life Sciences Products, Boston, MA), as described previously [11]. For control experiments, immunoprecipitation and subsequent activity measurements were conducted with non-immune mouse IgG. Immunoprecipitation of the PLC isozyme is complete under the condition described here. The SL fractions under study had only minimal (2–4%) but equal amounts of contamination from other subcellular organelles. Protein concentrations were determined by the method of Lowry et al. as indicated elsewhere [11,28].

Statistical analysis. A Student's *t* test was used to assess statistical significance when two groups were being compared. When more than two groups were being compared, ANOVA analysis was used followed by a Student–Newman–Keuls post hoc test. A P level of <0.05 was considered significant.

Results

General characteristics of diabetic animals

Eight weeks after STZ injection, the diabetic animals showed elevated levels of plasma glucose and decreased levels of serum insulin. The diabetic animals also exhibited a reduction in body weight and heart weight as compared to age-matched controls (Table 1). Such characteristics of the diabetic animals are similar to those reported earlier [28,29,33]. Treatment with insulin normalized the blood glucose and insulin levels, while only partially correcting body weight and heart weight (Table 1).

Effect of phosphatidic acid on cardiomyocyte contractile activity and Ca^{2+} transients

The concentration-dependent effect of PA on active cell shortening of cardiomyocytes was examined. At 25 μM, PA induced a sustained, significant increase in active cell shortening. This effect took 6 min to develop with a maximal effect at 9 min. Conversely, 50 μM PA had a small but sustained depressive effect (Fig. 1A). Ten micromolars of PA had no significant effect on the contractile performance of control cardiomyocytes, although a gradual increase in contractile activity began to emerge after 9 min. Post-perfusion wash-out reversed the contractile effects of PA (Fig. 1B). Values returned to control levels by 20 min of wash-out. The effects of PA on active cell shortening could be inhibited by pre-perfusing the cells for 15 min with 50 μM of 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbonate (NCDC) (Fig. 2A), a known blocker of PLC [2]. NCDC had no effect on the cell shortening in the absence of PA (Fig. 2B).

Lyso-PA (LPA) has also been reported to produce a positive inotropic effect [42]. Therefore, in order to exclude the possibility [44] of the increase in inotropy being due to LPA rather than PA, we measured the amount of LPA that is generated during the perfusion of cardiomyocytes with 25 μM PA. Approximately 0.26 μM LPA was produced (representing about 1% conversion of the exogenous PA to LPA). At this concentration of LPA, no stimulation of the contractile or PLC activities was observed (data not shown). LPA contamination of the PA stock was not detected.

To examine if the PA-mediated positive inotropic effect was due to a release of Ca^{2+} from the sarcoplasmic reticulum (SR), cardiomyocytes were treated with 10 μM thapsigargin to deplete SR Ca^{2+} [34]. This attenuated the contractile activity in response to PA by 53% (Fig. 3B). Furthermore, in cardiomyocytes exposed to

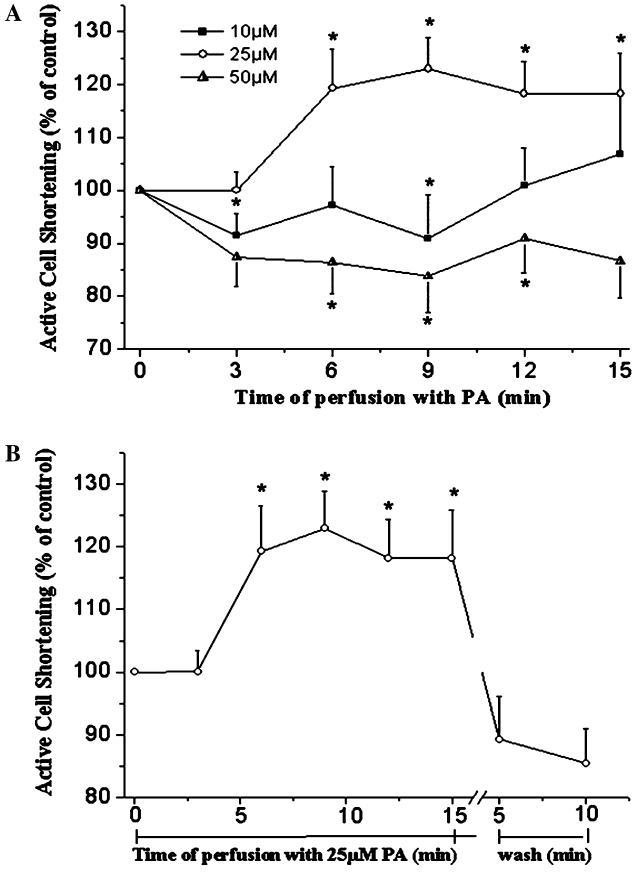

Fig. 1. Contractile response of control cardiomyocytes to varying concentrations of phosphatidic acid. (A) Active cell shortening was determined during 15 min perfusion with 10, 25, and 50 μM PA by video edge detection during electrical pacing at a rate of 0.5 Hz. (B) The effect of a 10 min wash-out. Active cell shortening at time 0 was 4.2 ± 0.03 microns. For each concentration of PA tested (10, 25, and 50 μM), measurements were conducted in three different cardiomyocytes isolated from each of the four different experimental animals. The means from each set of three measurements from each of the different animals were then used for statistical analysis. *$P < 0.05$ vs. control values. PA: phosphatidic acid.

Table 1
General characteristics of the diabetic animals

	Age-matched controls	Diabetic	Diabetic + insulin
Body weight (g)	467 ± 4	258 ± 6*	342 ± 2*
Ventricular weight (g)	1.23 ± 0.04	0.81 ± 0.01*	0.97 ± 0.01*
Plasma glucose (mg/dL)	180 ± 2	546 ± 12*	181 ± 8
Serum insulin (pmol/L)	157 ± 6	45 ± 2*	167 ± 9

Values are expressed as means ± SE from 10 to 12 experiments. Plasma glucose and serum insulin were determined as indicated in the Materials and methods.

* $P < 0.05$ vs. corresponding control values.

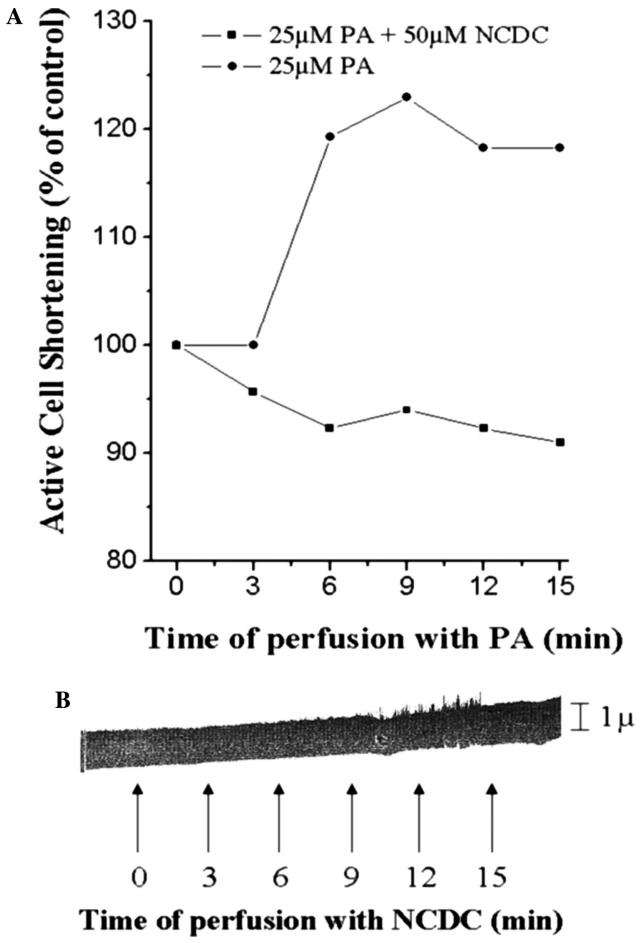

Fig. 2. Effect of NCDC on the contractile activity of control cardiomyocytes in the presence and absence of phosphatidic acid. Cardiomyocytes were pre-perfused with 50 μM NCDC for 15 min. Active cell shortening was then examined during 0–15 min perfusion with 25 μM PA. (A) Values are the means of two separate experiments with triplicate measurements conducted in each. Active cell shortening at time 0 was 4.1 ± 0.02 microns. (B) Representative trace of active cell shortening of perfusion with 50 μM NCDC for 15 min in the absence of PA. NCDC: 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbonate; PA: phosphatidic acid.

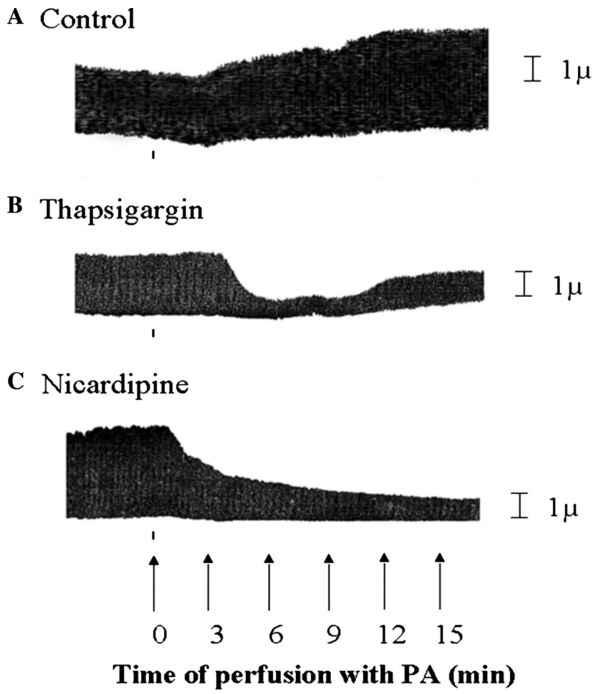

Fig. 3. Effect of thapsigargin and nicardipine on the contractile activity of control cardiomyocytes in the presence of phosphatidic acid. Representative traces of active cell shortening in control cardiomyocytes during 0–15 min perfusion with 25 μM PA (A) and 25 μM PA in the presence of 10 μM thapsigargin (B) and 4 μM nicardipine (C). Cardiomyocytes were pre-perfused with thapsigargin for 10 min.

4 μM nicardipine to block Ca^{2+} influx [35], the increase in contractile activity caused by PA was attenuated by 73% (Fig. 3C).

Although the present study was designed to examine the effects of exogenous PA, we also assessed the effects of exogenous PLD on cardiomyocyte contractile activity and measured the amount of PA generated. This would provide information that PLD can exert a positive inotropic effect directly at the cardiomyocyte level [36,37], which would be attributable to the formation of PA endogenously. As shown in Fig. 4, PLD (25 U) produced an increase in the contractile activity of 24% which was attributable to the PLD-mediated generation of ~54 μmol PA/mg cardiomyocyte protein (~34 μmol PA in the absence of PLD vs. ~88 μmol PA in the presence of PLD). An increase in the cardiomyocyte contractile activity was only observed with 25 U PLD, above which a negative inotropic effect was observed (data not shown). Taken together, the above series of experiments establish that both PLD and exogenous PA directly alter cardiomyocyte contractile activity in a similar qualitative manner and through the PLC pathway.

Since the maximal effect of PA in control cardiomyocytes was observed with 25 μM PA, subsequent experiments on the contractile response of diabetic cardiomyocytes to PA were examined with this concentration of PA. As shown in Fig. 5, whereas control cardiomyocytes exhibited an increase in active cell shortening and concomitant Ca^{2+} transients in response to 25 μM PA, a negative inotropic effect and blunted Ca^{2+} transients were observed in cardiomyocytes obtained from diabetic animals. Insulin treatment of the diabetic animals resulted in a significant ($P < 0.05$ vs. diabetic value) correction of the contractile activity. However, PA-evoked Ca^{2+} transients remained suppressed. Representative traces for PA-induced active cell shortening in control, diabetic, and diabetic + insulin-treated rat cardiomyocyte preparations are shown in Fig. 6. These contractility measurements were recorded during 0–15 min perfusion with 25 μM PA.

To exclude the possibility of a shift in the sensitivity of cardiomyocytes from diabetic animals to PA, the

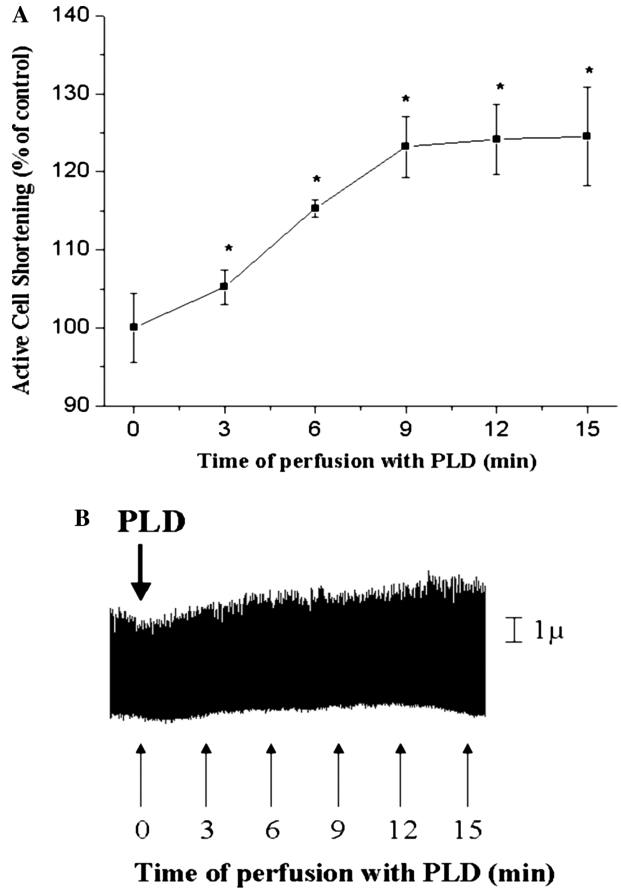

Fig. 4. Contractile response of control cardiomyocytes to exogenous phospholipase D. (A) Active cell shortening was determined during 15 min perfusion with 25 U PLD by video edge detection during electrical pacing at a rate of 0.5 Hz. Active cell shortening at time 0 was 4.1 ± 0.04 microns. Cardiomyocytes were isolated from four different experimental animals with measurements being conducted in three cardiomyocytes from each of the different animals. (B) Representative trace for PLD-induced active cell shortening in control rat cardiomyocyte preparations. $^{*}P < 0.05$ vs. control values. PLD: phospholipase D.

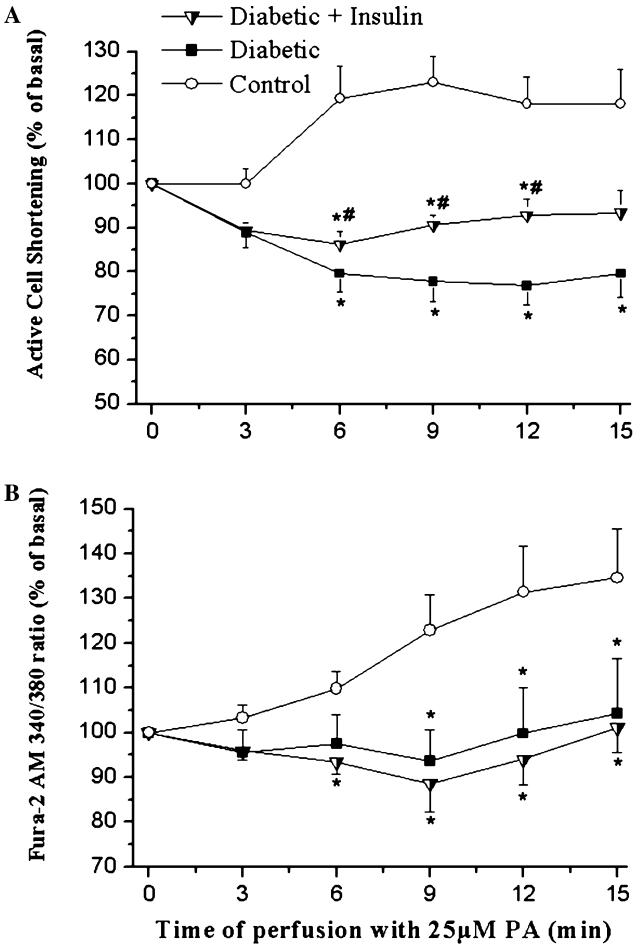

Fig. 5. Contractile response and active calcium transients in diabetic rat cardiomyocytes in response to phosphatidic acid. (A) Active cell shortening was examined in control, diabetic, and diabetic + insulin treated rat cardiomyocytes during 0–15 min perfusion with 25 μM PA. Cardiomyocytes were isolated from four different experimental animals in each of the control, diabetic, and diabetic + insulin groups. Measurements were conducted in four different cardiomyocytes isolated from each of four different experimental animals per group. The means from each set of four measurements from the different animals were then used for statistical analysis. Values were 4.3 ± 0.04, 3.1 ± 0.04, and 2.6 ± 0.05 microns at time 0. (B) Calcium transients in diabetic cardiomyocytes were examined in control, diabetic, and diabetic + insulin treated rat cardiomyocytes after 0–15 min perfusion with 25 μM phosphatidic acid (PA). Fura-2 fluorescence (340/380 nm ratio) was used as a Ca^{2+} indicator. The amplitude of the calcium transient is expressed as a percentage of values observed prior to the perfusion with PA. Measurements were conducted in four different cardiomyocytes isolated from each of four different experimental animals per group. $^{*}P < 0.05$ vs. controls, $^{\#}P < 0.05$ vs. diabetic values. PA: phosphatidic acid.

effects of different concentrations of PA on the contractile activity were tested. As shown in Table 2, 50 μM PA did not induce an increase in contractile activity. It actually generated a negative inotropic effect. Similarly, 10 μM PA elicited a negative inotropic effect. As the concentration of PA increased, a greater negative inotropy was observed. Insulin treatment of the diabetic animals removed the negative inotropic effects of PA but did not restore the stimulatory effects observed with 25 μM PA.

Effect of PA on diastolic cell length and calcium

The effects of PA on resting, diastolic cell length were also monitored. Whereas PA did not alter resting cell length in control cells in a significant manner (Table 3), it did induce a small but significant decrease in cell length in the diabetic rat cardiomyocytes. Insulin treatment partially reversed this effect. Diastolic $[Ca^{2+}]$ was not significantly different amongst the three groups (320 ± 50, 270 ± 80, and 341 ± 32 nM in control, diabetic, and insulin-treated diabetic cells, respectively). Furthermore, diastolic $[Ca^{2+}]$ did not change in any of the groups in response to PA (Table 3).

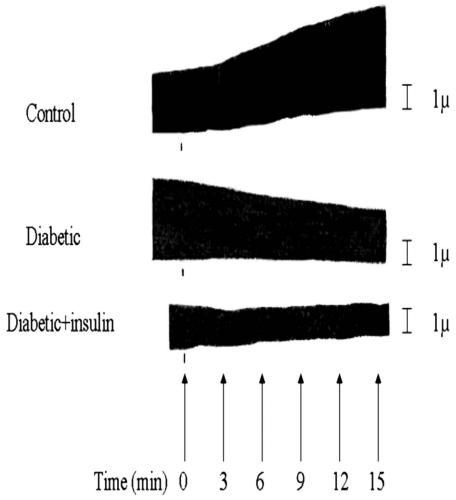

Fig. 6. Representative traces for phosphatidic acid-induced active cell shortening in control, diabetic, and diabetic + insulin treated rat cardiomyocyte preparations. Contractility measurements were measured during 0–15 min perfusion with 25 μM PA.

Table 2
Peak response of control and diabetic cardiomyocytes to varying concentrations of phosphatidic acid

	Active cell shortening (% of basal) [PA] (μM)		
	10	25	50
Control	91 ± 8	123 ± 6*	84 ± 7
Diabetic	86 ± 3	77 ± 4*	72 ± 2*
Diabetic + insulin	92 ± 3	93 ± 3*	96 ± 12

Values are expressed as means ± SE of single measurements of 10–15 experiments. Peak contractile response was observed at 9 min after the initiation of perfusion with phosphatidic acid.
* $P < 0.05$ vs. corresponding basal values. PA: phosphatidic acid.

PA-induced generation of IP_3 and PLC δ_1 activity

Since the PA-induced positive inotropic effect was blunted by NCDC, cytosolic IP_3 (a product of PLC activity) content in cardiomyocytes exposed to 25 μM PA was examined in control, diabetic, and diabetic + insulin preparations. The basal cytosolic IP_3 concentration in diabetic cardiomyocytes was significantly lower than control cardiomyocytes (Fig. 7A). Insulin treatment of the diabetic rats significantly improved basal IP_3 levels but these levels remained

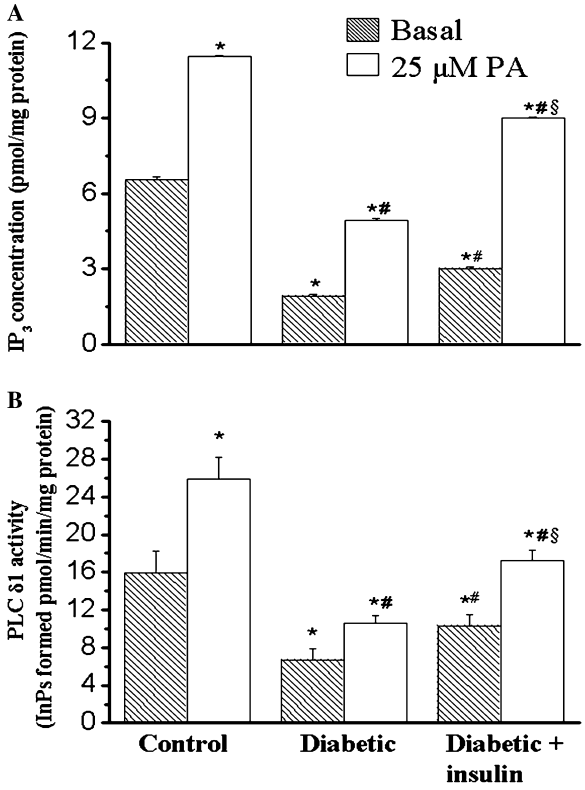

Fig. 7. Stimulation of cytosolic inositol 1,4,5-trisphosphate formation and sarcolemmal phospholipase C δ1 by phosphatidic acid in control, diabetic, and diabetic + insulin treated rats. (A) Cytosolic IP_3 was extracted from cardiomyocytes before or after the exposure to 25 μM PA. Values are means ± SE of three experiments done in triplicate. (B) PLC δ1 activity was measured in purified sarcolemmal membranes isolated from the LV tissue of hearts of control, diabetic, and diabetic + insulin groups. Values are means ± SE of five experiments and represent the sum (InPs) of the inositol phosphates (IP, IP_2, and IP_3) formed by the PLC-dependent hydrolysis of phosphatidylinositol-4,5-bisphosphate. *$P < 0.05$ vs. corresponding basal value within the same group; #$P < 0.05$ vs. corresponding control value; and §$P < 0.05$ vs. corresponding diabetic value. PA: phosphatidic acid; IP_3: inositol 1,4,5-trisphosphate.

Table 3
Diastolic [Ca^{2+}] and change in resting cell length in control and diabetic cardiomyocytes after 3, 9, and 15 min of treatment with 25 μM phosphatidic acid

	Time (min)	Control	Diabetic	Diabetic + insulin
Diastolic	3	98.0 ± 0.8	99.5 ± 0.6	100.3 ± 0.4
Calcium	9	99.6 ± 1.2	100.5 ± 1.2	100.3 ± 0.4
(% of basal)	15	101.5 ± 1.3	101.6 ± 1.8	102.7 ± 0.2
Change in	3	−0.04 ± 0.07	−0.6 ± 0.3	−0.4 ± 0.2
Resting cell	9	−0.10 ± 0.10	−1.5 ± 0.6*	−1.4 ± 0.4
Length (microns)	15	−0.04 ± 0.15	−2.2 ± 1.0*	−1.8 ± 0.6

Values are expressed as means ± SE of single measurements of 10–15 experiments.
* $P < 0.05$ vs. corresponding control values.

significantly lower than that in control. Exposure of the cardiomyocytes to PA did not correct this condition. PA induced a significant increase in IP_3 content in control cardiomyocytes. However, in the presence of PA, diabetic IP_3 formation remained significantly below control levels. Insulin treatment of the diabetic rats resulted in a significant improvement in IP_3 formation in the cardiomyocytes but, again, this did not return to control levels. To further understand the mechanism of the PA-mediated positive inotropic effect and the generation of IP_3, we also determined the activity of the major PLC isozyme, PLC δ1, in response to 25 μM PA. PLC δ1 is associated with the SL membrane [11]. PLC δ1 had a depressed responsiveness to PA in the diabetic SL preparation with a partial recovery in the diabetic + insulin treated group (Fig. 7B). A similar profile of the PA effect on the cardiomyocyte IP_3 concentration and PLC δ1 activity were observed.

Discussion

Little attention has been given to the involvement of intracellular lipid signaling pathways in diabetic cardiomyopathy despite its importance in regulating contractile performance of the heart [9,27,40,41]. The present study focused on the PA-PLC pathway as having a potential role in the cardiac contractile defects exhibited during diabetes. First, it was important to determine if PA stimulates contraction of isolated, adult cardiomyocytes and to define the mechanism whereby it exerts its inotropic effect. Twenty-five micromolars of PA induced a significant increase in contractile function in control cardiomyocytes. Similar concentrations of PA have been employed by other investigators [4,9,10,35]. Although 10 μM PA produced no increase in contractile activity during the initial 9-min perfusion, it appears that beyond this time point, there is a gradual stimulation of contraction. This may be due to a time-dependent accumulation of PA into the SL membrane and when the SL level reaches a sufficient level, an increase in the contractile activity occurs. This concentration range for PA has physiological relevance. The receptor-mediated concentration of PA in the cell has been estimated to vary from 10 to 30 μM [38] and on the basis of PA and total phospholipid content, the concentration of PA in cardiomyocytes is considered to be ~20 μM [9]. We employed PA of mixed fatty acid composition, which would also closely resemble the physiological situation. Thus, it appears that the concentrations of PA (10–25 μM) employed in this study are within the physiological range. Conversely, the negative inotropic effects of 50 μM PA are likely non-physiological and may represent non-specific interactions with the sarcolemmal membrane.

The mechanism through which PA acts was studied. The PA-induced increase in Ca^{2+}-transient and positive inotropy may be elicited through a selective inhibition of protein kinase C (PKC). The PLC generated diacylglycerol (DAG) that is stimulated by PA can activate PKC [38]. However, PKC does not appear to be involved because pretreatment of cardiomyocytes with staurosporine, an inhibitor of PKC [41] failed to alter the PA-induced response [9]. Instead, several observations would support the contention that the PA-induced positive inotropic response is mediated by PLC. First, NCDC blocked the inotropic effects of PA. NCDC blocks PLC activity [40]. Second, PA induced a significant increase in intracellular IP_3, which may be associated with the observed PA-evoked activation of SL PLC δ1. Third, as an increase in intracellular [IP_3] should stimulate Ca^{2+} transients [48], the PA-induced increase in Ca^{2+} observed in the present study is consistent with an IP_3-mediated response.

The intracellular mechanism for the inotropic action of PA was also considered. It is known that PA can diffuse across the membrane by a phosphorylation–dephosphorylation cycle [46] that can occur in cardiomyocytes. It is also known that exogenous PA induces phosphoinositide turnover [43]. PA can reach the cytoplasmic membrane leaflet and activate PLC with subsequent formation of IP_3 and release of Ca^{2+} from SR [47]. It has been suggested, however, that PA induces a biphasic Ca^{2+} mobilization, where the initial increase is due to the mobilization of Ca^{2+} from intracellular stores, whereas the secondary increase is due to influx of Ca^{2+} from extracellular sources [50]. PA has been reported to stimulate Ca^{2+} influx in fetal heart and neuroblastoma cells [45] as well as to activate the L-type Ca^{2+} channels in the atrium [49,51]. Our results are consistent with an effect at both intracellular and extracellular sites. The PA-mediated positive inotropy was attenuated by thapsigargin and nicardipine. This observation suggests that the PA-induced positive inotropic effect may be due to stimulation of Ca^{2+} influx through the L-type Ca^{2+} channels and due to Ca^{2+} release from the SR stores. Although the association of IP_3 with $[Ca^{2+}]_i$ and contraction may be a contentious issue in the heart, our results are consistent with the postulate [4] that this is an important pathway in modulating cardiac contractile function.

The mechanisms responsible for the altered responses to PA in the diabetic myocardium have also been investigated in the present study. PA can be converted to DAG by *ecto*-PA phosphohydrolase. Sarcolemmal phosphatidate phosphohydrolase type 2 (*ecto*-PA phosphohydrolase [52]) activity was measured with 25 μM PA as the substrate. No differences in the activities in the three experimental groups were detected (control: 1.81 ± 0.2; diabetic: 1.93 ± 0.3; and insulin-treated diabetic: 1.62 ± 0.3 nmol/min/mg protein). Thus,

the reduced responsiveness of diabetic rat cardiomyocytes to PA was not due to an increased conversion of the exogenous PA to DAG by *ecto*-PA phosphohydrolase.

Both direct and indirect evidence support the assertion that the IP_3 signaling pathway is defective in diabetic rat cardiomyocytes. First, if IP_3 stimulates contraction by increasing intracellular [Ca^{2+}] [4], then one would expect depressed IP_3 signaling to elicit smaller Ca^{2+} transients. Both basal and PA-stimulated Ca^{2+} transients were significantly lower in the diabetic preparations than in the control cells. Second, PA did not induce an increase in active cell shortening in the diabetic rat cells. In view of the capacity of IP_3 to induce contractile activity [4], this difference in contractile performance (>40%) is consistent with a lesion in IP_3 signaling. Third, both basal and PA-induced IP_3 levels in diabetic rat cardiomyocytes were significantly depressed in comparison to control preparations. Fourth, these data are consistent with another report that α_1-adrenergic stimulation of the diabetic heart generated less IP_3 than normal [53]. These data argue strongly that the PA-induced stimulation of contractile activity via IP_3/Ca^{2+} is defective in diabetic cardiomyocytes. Because the IP_3 receptor content was unaffected by the diabetic state (data not shown), this further emphasizes the importance of PLC as a critical defect in this pathway. The impairment in PLC signaling may also participate in the defective adrenergic responses observed during diabetes [54–56].

A 14-day treatment with insulin partially restored PA-induced cardiomyocyte contractility. This was consistent with an earlier report that insulin treatment results in a partial recovery of the sarcolemmal level of PA [28]. This is also in agreement with a partial recovery of both basal as well as PA-induced IP_3 production in the present study. Other studies [21,57–59] have also reported an incomplete recovery of cardiac function in insulin-treated diabetic animals. Although the insulin dose (3 U/day) used in this study was selected on the basis of our previous experience [28,29,60], it is possible that a higher dose of insulin and a longer treatment duration may induce a full recovery of all the changes in the diabetic heart.

In summary, our results demonstrate for the first time that a defect in PA evoked PLC-mediated IP_3 generation occurs in diabetic rat cardiomyocytes, which is associated with a depressed PA-induced stimulation of active cell shortening and Ca^{2+} transients in diabetes. This impairment of the PA-PLC signaling pathway may constitute a novel mechanism that contributes to the defective cardiac contractile performance during diabetes. We have earlier reported the occurrence of a similar signaling defect in congestive heart failure [30]. This strongly suggests that an impairment of the PA-PLC signaling pathway may contribute to depressed cardiomyocyte contractility in many different types of cardiac pathologies.

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