Hexosamine Pathway Is Responsible for Inhibition by Diabetes of Phenylephrine-Induced Inotropy

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Hyperglycemia diminishes positive inotropic responses to agonists that activate phospholipase C (PLC) and generate inositol trisphosphate (1,4,5). The mechanisms underlying both the inotropic responses and hyperglycemia's effects on them remain undetermined, but data from isolated cardiomyocytes suggest the involvement of capacitative Ca^{2+} entry (CCE), the influx of Ca^{2+} through plasma membrane channels activated in response to depletion of endoplasmic or sarcoplasmic reticulum Ca^{2+} stores. In neonatal rat cardiomyocytes, hyperglycemia decreased CCE induced by PLC-mediated agonists. The attenuation of CCE was also seen with glucosamine, and the inhibition by hyperglycemia was prevented by azaserine, thereby implicating hexosamine biosynthesis as the responsible metabolic pathway. In the current study, the importance of hexosamine metabolites to hyperglycemia's effects on inotropic responses was examined in isolated perfused rat hearts. The inhibition by hyperglycemia of phenylephrine-induced inotropy was reversed with azaserine and mimicked by glucosamine. An independent inhibitor of CCE, SKF96365, was also effective in blunting inotropy. These treatments did not inhibit inotropy induced by activation of adenylate cyclase through β-adrenergic receptors. These data thus implicate CCE in responses to PLC-mediated agonists in the intact heart and point to the hexosamine pathway's negative effect on CCE as being central to the inhibition seen with hyperglycemia.
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Diabetes gives rise to a spectrum of altered pathophysiological responses in the heart, including a specific diabetic cardiomyopathy, a propensity to failure, and poor outcomes after ischemia (1). Several of these conditions are influenced by aberrant responses of cardiomyocytes to agonists present in the extracellular milieu that alter the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Such agonists affect cardiac output (2–4), gene expression (5), arrhythmogenesis (6,7), remodeling (8), apoptotic cell death (9,10), and postischemia damage (1). The focus of this study was the positive inotropy that results from some of these agonists and that has previously been shown to be affected by diabetes (11,12).

The best of the agonist-mediated inotropic signaling pathways studied are those initiated by the engagement of β-adrenergic receptors and mediated by the Gα protein–induced activation of adenylate cyclase (2). Other G protein–mediated inotropic signaling pathways, initiated by the engagement of α-adrenergic, thrombin, or neuropeptide receptors, lead to the activation of phospholipase C (PLC) and the generation of inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (13). In contrast to the well-understood pathway downstream from adenylate cyclase (2), the mechanism that links PLC activation to positive inotropy remains undetermined. Although changes in myofilament responsiveness may contribute to this process (14), the preponderance of data, using both Ca^{2+}-sensitive dyes (15,16) and electrophysiological approaches (3,4), support the concept that altered handling of $[Ca^{2+}]_i$ is central to the changes. Recently, Vila Petroff et al. (3) and Liu and Kennedy (4) found that PLC-activating agonists doubled the current entering the cardiomyocyte through L-type channels, but only when the perforated patch technique was used. When the interior of the cell was more disrupted using whole-cell approaches, the increase was not seen. How PLC activation leads to this enhanced L-type current was not addressed. In light of our recent reports that capacitative Ca^{2+} entry (CCE) characterizes both neonatal (16) and adult (17) rat ventricular myocytes, we propose that this process is critical to linking PLC activation to enhanced L-type calcium current.

CCE refers to the entry of extracellular Ca^{2+} activated by depletion of endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) Ca^{2+} stores (18). CCE is typically initiated by agonist-receptor interactions that lead to the generation of IP_3 (13). This second messenger facilitates the depletion of ER/SR Ca^{2+} stores and, via a still undetermined coupling mechanism, the activation of the plasma membrane store-operated channels (SOCs) responsible for CCE. CCE contributes to increases in $[Ca^{2+}]_i$ in response to IP_3-generating agonists in nearly all nonexcitable cells but erythrocytes and has now been shown to coexist with voltage-gated Ca^{2+} channels in smooth (19), skeletal (20,21), and cardiac (16,17) muscle cells. A recent report has shown that an SOC is responsible for the $[Ca^{2+}]_i$ increases implicated in the cell death of skeletal

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ANG II, angiotensin II; CCE, capacitative Ca^{2+} entry; +dP/dt, left ventricular maximal dP/dt; −dp/dt, left ventricular minimum dP/dt; EDP, left ventricular end diastolic pressure; ER, endoplasmic reticulum; GlcNAc, *N*-acetylglucosamine; HR, heart rate; IP_3, inositol 1,4,5 trisphosphate; LVDP, left ventricular developed pressure; PE, phenylephrine; PLC, phospholipase C; RPP, rate-pressure product; SOC, store-operated channel; SR, sarcoplasmic reticulum; STZ, streptozotocin; UDP-GalNAc, uridine diphosphate-*N*-acetylgalactosamine; UDP-GlcNAc, uridine diphosphate-*N*-acetylglucosamine; UDP-HexNAc; uridine diphosphate-*N*-acetylhexosamine.

TABLE 1
Cardiac function in isolated normal and STZ rat hearts

	n	Serum Glc (mmol/l)	RPP (mmHg/min × 10^{-3})	HR (beats/min)	LVDP (mmHg)	+dP/dt (mmHg/min)	−dP/dt (mmHg/min)
Normal	20	6.41 ± 0.22	32.1 ± 0.9	288 ± 5	111 ± 3	4,329 ± 143	2,810 ± 173
STZ	22	26.53 ± 0.62*	21.4 ± 0.9*	273 ± 6	79 ± 4*	3,325 ± 190*	1,469 ± 64*

Data are means ± SE. *$P < 0.05$, normal rats vs. STZ rats by Student's t test. Glc, glucose.

myocytes in the muscular dystrophy model in mice lacking dystrophin (21).

We (22) recently demonstrated that in neonatal rat cardiomyocytes, hyperglycemia decreased CCE induced by PLC activation or the Ca^{2+}ATPase inhibitor thapsigargin. Hyperglycemia also significantly blunted Ca^{2+}-dependent hypertrophic responses. The attenuation of CCE by hyperglycemia was prevented by azaserine, an inhibitor of hexosamine biosynthesis (23). A complementary experiment demonstrated that selectively increasing hexosamine metabolites in neonatal cardiomyocytes with exogenous glucosamine also inhibited CCE (16). The inhibition of CCE by hyperglycemia, mediated by excessive flux through the hexosamine biosynthetic pathway, thus provides a likely explanation for the diminished hypertrophic response seen in this in vitro model.

In light of our recent studies in isolated cardiomyocytes, we hypothesized that the altered contractility in the heart seen after diabetes may in part be due to increased flux through the hexosamine pathway. We found, as have others (11,12), that the positive inotropic response of the whole heart to phenylephrine (PE), a PLC-activating agonist, was blunted after a brief period of diabetes. This decreased inotropic response was attributed to excessive flux through the hexosamine biosynthetic pathway. We also found that an independent inhibitor of CCE decreases the increase in cardiac output in response to these agonists. These treatments had no effect on the responses of the heart to the β-adrenergic agonist isoproterenol. Therefore, we proposed a link between CCE and the increase in flux through L-type channels (3,4) that is likely to be directly responsible for the inotropic response and contribute to altered contractile function in diabetes.

RESEARCH DESIGN AND METHODS

Propanolol, PE, glucosamine, azaserine, streptozotocin (STZ), and angiotensin II (ANG II) were purchased from Sigma. Isoproterenol and SKF96365 were obtained from Calbiochem, and KBR7943 was obtained from TOCRIS.

Induction of experimental diabetes. Animal procedures conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, 1996). Male SD rats (250–320 g) were randomly assigned to either a diabetic or control group. The diabetic group (n = 48) was given a single intravenous injection of STZ (50 mg/kg), dissolved in sodium citrate solution (0.05 mol/l; pH = 4.5), in the lateral tail vein. The control group (n = 71) was given an equivalent volume of citrate solution alone. Control and diabetic rats were fed the same diet, given water, and housed under similar conditions. Blood samples were collected and serum glucose levels were determined with a blood glucose meter (Roche) 2–8 days after STZ administration.

Isolated heart preparations. Rats were anesthetized with intraperitoneal ketamine (100 mg/kg) and decapitated. Their hearts were quickly excised and perfused in a modified Langendorff mode with Krebs-Henseleit bicarbonate buffer equilibrated with 95% O_2/5% CO_2 (38°C, pH 7.4). The composition of the buffer was (in mmol/l): NaCl, 118; KCl, 4.8; Mg_2SO_4, 1.2; $CaCl_2$, 1.25; KH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 5.5 (control group) or 25 (diabetic group).

Physiological measurements. Contractile function was determined using a fluid-filled balloon inserted into the left ventricle through the mitral valve connected to a TXD-310 pressure transducer and analyzed with a heart performance analyzer (HPA-410; Micro-Med). The balloon volume was adjusted to obtain a left ventricular end diastolic pressure (EDP) of 5 mmHg. Coronary flow was adjusted to provide an initial mean coronary perfusion pressure of 75 mmHg. It has been reported that diabetes increases (11), reduces (12), or has no effect on (24) the inotropy elicited by PE. These differing results are at least in part due to variability in the experimental protocol; therefore, in this study, we kept the perfusion flow rate stable rather than the perfusion pressure. In this way, we minimized the vasoactive responses to PE, which themselves can be affected by diabetes (25). Contractile hemodynamics assessed included left ventricular developed pressure (LVDP), EDP, left ventricular maximal dP/dt (+dP/dt), left ventricular minimum dP/dt (−dP/dt), heart rate (HR), and rate-pressure product (RPP; RPP = HR × LVDP).

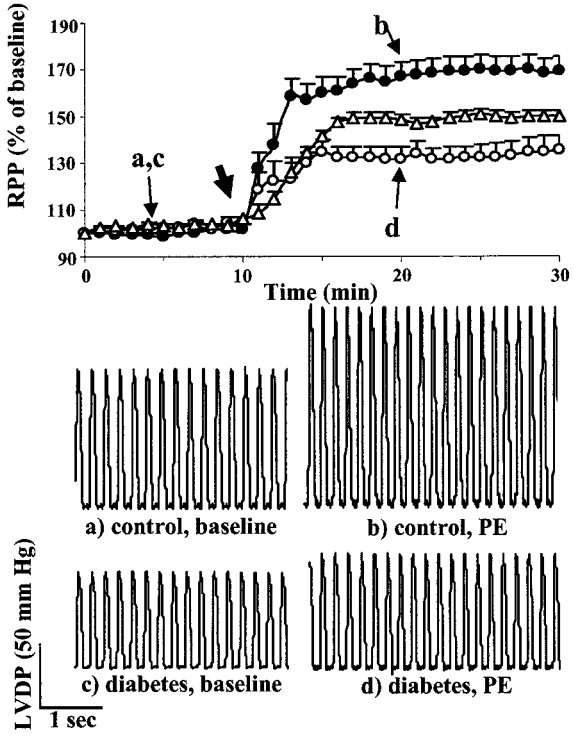

FIG. 1. Changes in contractile function in response to PE in the absence or presence of azaserine in control and diabetic rat hearts. The average changes over time in RPP in response to PE are shown, demonstrating that a short period of diabetes (2–6 days) blunts the inotropic effects caused by PE and that a 45-min pretreatment with azaserine (20 μmol/l), an inhibitor of the hexosamine biosynthetic pathway, partially reverses the diabetes-inhibited inotropy. *a* and *b*: A typical LV pressure trace sampled at the indicated times demonstrating the inotropic effects of 10 μmol/l PE in control rat hearts. *c* and *d*: A typical LV pressure trace demonstrating that the inotropic effects of PE are blunted by a short period of diabetes. Hearts were exposed to PE at the larger arrow. Data are means ± SE. ●, PE in control hearts, n = 8; ○, PE in diabetic hearts, n = 6. Two of the six hearts were harvested 2 days after STZ administration and the remaining four were harvested at 6 days. There were no differences between these groups, and they were combined for the analysis shown here. △, Azaserine + PE in diabetic hearts, n = 6. Two of the six hearts were harvested 2 days after STZ administration, and the remaining four were harvested at 6 days. There were no differences after azaserine treatment between these groups, and they were combined for the analysis shown here. Baseline values are comparable with those given in Table 1.

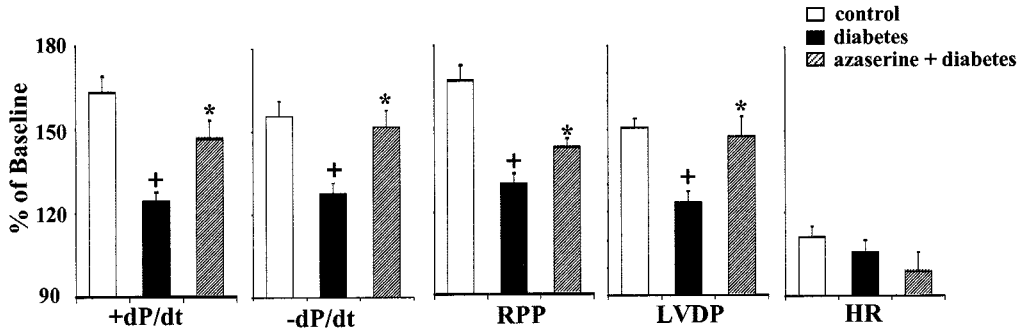

FIG. 2. Positive inotropic effects of PE are inhibited by diabetes, whereas azaserine partially reverses this inhibition. Changes relative to baseline in +dP/dt, −dP/dt, RPP, LVDP, and HR caused by PE (10 μmol/l) in control and diabetic hearts in the presence or absence of a 45-min pretreatment with azaserine (20 μmol/l) are shown. Data are means ± SE. The mean values after the addition of PE are averaged data from 5–15 min after the addition. The baseline values are comparable with those given in Table 1. For all parameters, the increase in the control group after PE was significantly greater than the baseline value ($P < 0.05$). PE in control hearts, $n = 8$; PE in diabetic hearts, $n = 6$; azaserine + PE in diabetic hearts, $n = 6$. $^{+}P < 0.05$ vs. control group; $^{*}P < 0.05$ vs. diabetic group.

Uridine diphosphate-*N*-acetylhexosamine and ATP levels. Frozen hearts were pulverized to a fine powder with mortar and pestle under liquid N_2 and then homogenized in 0.3 mol/l perchloric acid with a PowerGen Homogenizer (Fisher) for 10–20 s. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was neutralized with 2 vol 1:4 trioctylamine:1,1,2-trichloro-trifluoroethane (Freon). The aqueous phase was filtered, and high-performance liquid chromatography was performed with a SAX Partisil 10 anion-exchange column (250 × 4.6 mm) eluted with a gradient of ammonium dihydrogen phosphate from 15 (pH 2.8) to 1 mmol/l (pH 3.7). The concentrations of ATP and uridine diphosphate-*N*-acetylhexosamine (UDP-HexNAc) were determined by ultraviolet detection after calibration with appropriate standards. This method quantifies the sum of uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) and uridine diphosphate-*N*-acetylgalactosamine (UDP-GalNAc) (26).

Experimental protocol. After being stabilized for 30 min, the hearts were exposed to 10 μmol/l PE in the presence of 0.1 μmol/l propanolol (to block β_1-adrenergic receptor activation) (27) or 0.3 μmol/l isoproterenol for 30 min. The concentrations were determined from preliminary experiments to elicit an inotropic response in the heart that persisted for at least 30 min. Inhibitors of CCE, glucosamine (5 mmol/l) or SKF96365 (10 μmol/l), were added 5 min before the agonist and were continued throughout the remainder of the study. These concentrations of glucosamine and SKF96365 were found to be effective in their ability to selectively inhibit SOCs without affecting L-type channels in cardiomyocytes (16,17). In some experiments, azaserine (20 μmol/l), an inhibitor of the hexosamine biosynthetic pathway (23), was included in the perfusate from the beginning of the experiment for 45 min, when the hearts were exposed to 10 μmol/l PE with 0.1 μmol/l propanolol. At the end of the experiment, hearts were freeze-clamped with Wollenberger tongs precooled in liquid N_2. The freeze-clamped tissue was treated as described above to determine the level of UDP-HexNAc.

Data analysis. Data are presented as means ± SE. Comparisons were performed with one-way ANOVA and post hoc Scheffe's test (StatView; SAS Institute). Statistically significant differences between groups were defined as $P < 0.05$ and are indicated in the legends to the figures.

RESULTS

Table 1 shows the baseline heart parameters in the control and diabetic groups after 30 min of equilibration. RPP, LVDP, +dP/dt, and −dP/dt were all significantly depressed in the diabetic group. The mean serum glucose concentrations were 26.5 ± 0.6 and 6.4 ± 0.2 mmol/l in the diabetic and control groups, respectively. In all conditions, RPP decreased slightly during the 90 min of perfusion, with no significant differences in the percent decrease among the groups. There was no significant difference in baseline function between control hearts and hearts perfused with 5 mmol/l glucosamine or 10 μmol/l SKF96365, or between diabetic hearts and diabetic hearts perfused with 20 μmol/l azaserine (data not shown). Thus, at the concentrations used in this study, these inhibitors had no adverse effects on unstimulated heart function.

Diabetes blunts the positive inotropy elicited by phenylephrine. We have previously determined in neonatal cardiomyocytes that hyperglycemia reduces increases in $[Ca^{2+}]_i$ responses to IP_3-generating agonists, such as PE and ANG II (22). Here we tested the effects of short-term diabetes on the positive inotropy induced by PE in the isolated heart.

As seen previously (11,12), a positive inotropic effect attributable to the α-adrenergic pathway was observed in perfused rat hearts in response to PE (Figs. 1 and 2). Figure 1 shows the time course of the mean change in RPP in response to 10 μmol/l PE in the control and diabetic groups. The relative increase in RPP after PE was inhibited ~60% by diabetes. Short-term diabetes also blunted PE-induced increases in +dP/dt, −dP/dt, and LVDP but had no effect on HR (Fig. 2).

Azaserine partially reverses the inhibitory effect induced by diabetes. Brownlee (28) recently reviewed mechanisms likely to be responsible for many diabetic complications, one of which was excessive flux through the hexosamine biosynthetic pathway. We recently demonstrated in neonatal cardiomyocytes that azaserine, a competitive inhibitor of glutamine amidotransferases, including the rate-limiting enzyme in the hexosamine pathway glutamine:fructose-6-phosphate amidotransferase (23), totally prevented the hyperglycemia-induced reduction in calcium responses to IP_3-generating agonists (22). Here we tested whether short-term azaserine could reverse the decreased inotropy caused by diabetes. Diabetic hearts were perfused with azaserine (20 μmol/l) for 45 min before being exposed to 10 μmol/l PE. Longer perfusion periods with azaserine proved impractical because the isolated heart began to deteriorate before completion of the experiment. However, the short-term exposure to azaserine partially reversed the inhibitory effect of diabetes on inotropy elicited by PE (Figs. 1 and 2). Figure 1*B* shows the time course in the change of RPP attributable to PE with or without azaserine in diabetic hearts. The decrease in RPP with diabetes relative to euglycemic controls was >60% reversed after exposure to azaserine. Azaserine also reversed the changes in −dP/dt and LVDP to near control levels and partially restored the diabetes-induced decreases in +dP/dt and RPP (Fig. 2).

Acute treatment with glucosamine partially blunts positive inotropy elicited by phenylephrine. Because

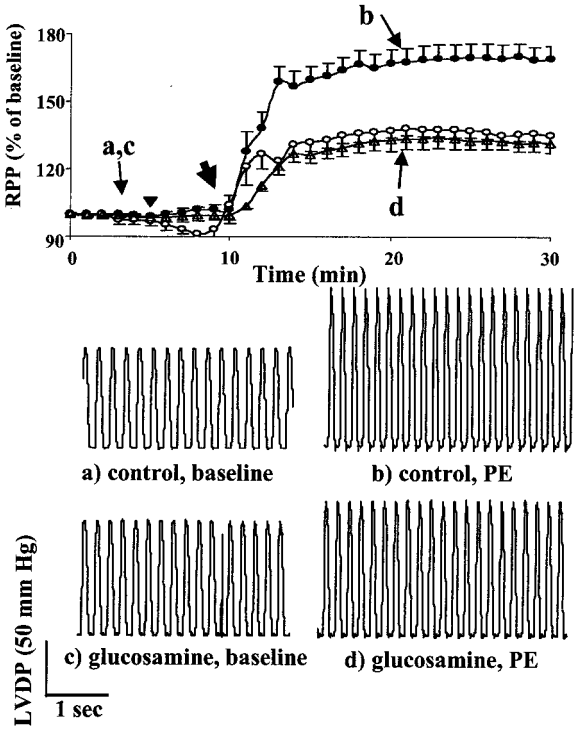

FIG. 3. Changes in contractile function in response to PE in the absence or presence of inhibitors of CCE. The average changes over time in RPP in response to PE, demonstrating the effects of the CCE inhibitors SKF96365 (10 μmol/l) and glucosamine (5 mmol/l) added 5 min before PE and continued until the end of the experiment are shown. *a* and *b*: A typical LV pressure trace at the indicated times demonstrating the inotropic effects of 10 μmol/l PE in control rat hearts. These data are the same as those shown in Fig. 1 and are included to facilitate comparison. *c* and *d*: A typical LV pressure trace demonstrating that the inotropic effects of PE are blunted by 5 mmol/l glucosamine. Hearts were exposed to glucosamine or SKF96365 at the arrowhead and to PE at the larger arrow. Data are means ± SE. ●, PE in control hearts, $n = 8$; ○, PE in control hearts pretreated with glucosamine, $n = 8$; △, PE in control hearts pretreated with SKF96365, $n = 8$. Baseline values are comparable with those given in Table 1

we were able to partially reverse the effects of diabetes on inotropic responses with short-term azaserine treatment, we attempted to induce the inhibition with short-term exposure of hearts from control nondiabetic rats to hyperglycemic buffers. A 45-min preincubation with 25 mmol/l glucose, either with or without added insulin, resulted in only a modest decrease in inotropic responses (RPP = 92% of control; $n = 6$; $P > 0.05$). This lack of effect with elevated glucose, however, served to control for the possibility that an increase in osmolality alone was responsible for the decreases seen with diabetic hearts. A more effective and selective method of increasing metabolites in the hexosamine biosynthetic pathway is to expose cells to exogenous glucosamine (23). Glucosamine enters cells via glucose transporters and is phosphorylated to form glucosamine-6-phosphate. It then enters directly into hexosamine biosynthesis (23). A 5-min exposure to 5 mmol/l glucosamine was sufficient to inhibit the $[Ca^{2+}]_i$ responses to IP_3-generating agonists in cardiomyocytes (16). Therefore, we exposed normal isolated hearts to 5 mmol/l glucosamine and continued the exposure in the presence of PE.

Figure 3 shows the time course in the change of RPP in response to PE in the absence and presence of glucosamine. The increase in RPP with 10 μmol/l PE was inhibited by ~50% after a 5-min preincubation with glucosamine. Elevation of left ventricular maximal pressure attributable to PE was also significantly blunted (Fig. 3*D*). The inhibitory effect was also seen in +dP/dt, −dP/dt, LVDP, and HR (Fig. 4). Another IP_3-generating agonist, ANG II, was also tested. Although the positive inotropy induced by ANG II was smaller than that seen with PE (RPP = 111% of control; $n = 5$; $P < 0.05$ vs. control), glucosamine totally inhibited the response (data not shown; RPP after exposure to ANG II = 96% of pre-ANG II value; $n = 6$; $P < 0.05$).

Positive inotropy elicited by PE is partially blunted by an independent inhibitor of CCE. Glucosamine is an effective inhibitor of CCE in several cell types, including cardiomyocytes (16,17). However, although increases in $[Ca^{2+}]_i$ have been clearly linked to the positive inotropy induced by IP_3-generating agonists (3,4,15,16), it has not been determined if CCE might contribute to this response. Therefore, another CCE inhibitor, 10 μmol/l SKF96365 (18), was used to pretreat a series of hearts for 5 min before exposure to PE. Figure 3 also shows the time course in the change of RPP in response to PE in the absence and presence of SKF96365. The increase in RPP after PE was inhibited by ~55% by SKF96365. SKF96365 also blunted PE-induced increases in LVDP, −dP/dt, and +dP/dt (Fig. 4).

As one indicator of whether the inhibitory effects of glucosamine on inotropy were attributable to its effects on CCE, a series of experiments was performed in which isolated

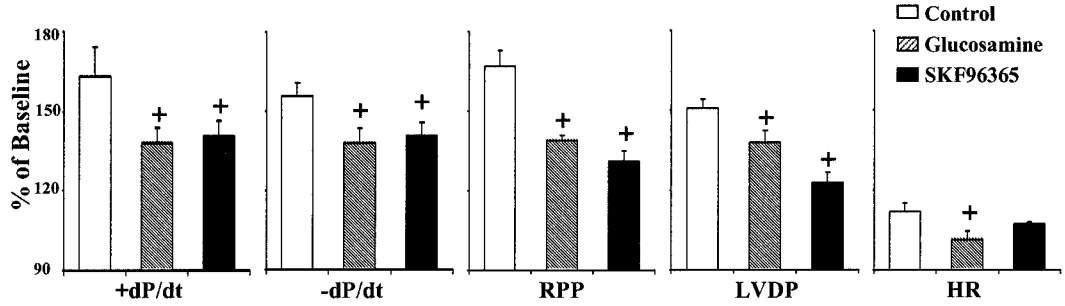

FIG. 4. Positive inotropic effects of PE are inhibited by glucosamine and SKF96365. Five parameters are shown. Changes relative to baseline in +dP/dt, −dP/dt, RPP, LVDP, and HR caused by PE (10 μmol/l) in control hearts in the presence or absence of a 5-min pretreatment with 5 mmol/l glucosamine or 10 μmol/l SKF96365 are seen. Data are means ± SE. The mean values after the addition of PE are averaged data from 5–15 min after the addition. Baseline values are comparable with those given in Table 1. For all parameters, the increase after PE was significantly greater than the baseline value ($P < 0.05$). +Significant decreases ($P < 0.05$) of the inhibitor group vs. PE alone. PE, $n = 8$; PE + SKF96365, $n = 5$; PE + $GlcNH_2$, $n = 5$.

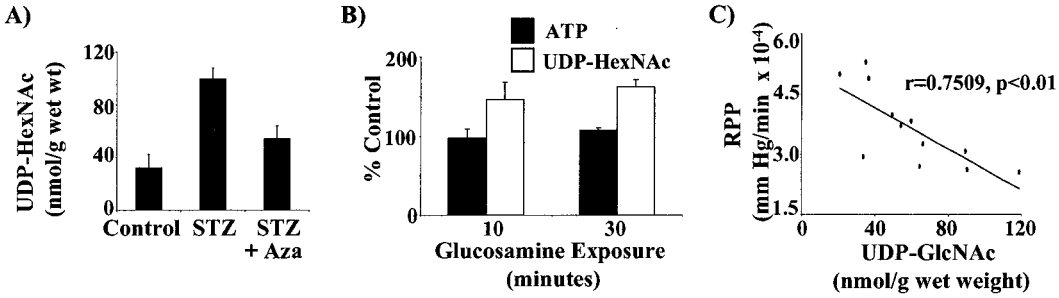

FIG. 5. Elevated levels of UDP-HexNAc correlate inversely with inotropic responses. *A*: Levels of UDP-HexNAc in hearts from rats in control, 7-day diabetic, and diabetic plus 45-min perfusion with 20 μmol/l azaserine (Aza) groups. Data are means ± SE from at least three experiments. *B*: Levels of UDP-HexNAc and ATP in hearts 10 and 30 min after perfusion with 5 mmol/l glucosamine. The 100% values for UDP-HexNAc are 32 (10 min) and 34 (30 min) nmol/g wet wt and for ATP are 3.34 (10 min) and 3.27 (30 min) μmol/g wet wt. *C*: Correlation between UDP-HexNAc levels from the four groups noted above and RPP 10 min after exposure to PE (10 μmol/l).

hearts were treated with both 5 mmol/l glucosamine and 10 μmol/l SKF96365. The initial inhibition in LVDP, −dP/dt, and +dP/dt was no greater than that seen with either agent alone ($n = 6$; data not shown), although with time the two inhibitors together caused a deterioration in heart function in both the presence and absence of PE that prevented clear interpretation.

Because both the forward and reverse modes of the plasma membrane Na^+/Ca^{2+} exchanger have been reported to be stimulated by PE (29), we sought to determine if KBR7943, a selective inhibitor of Ca^{2+} influx attributable to the reverse mode of the exchanger (30), could influence the inotropic responses to PE. We found that 5 μmol/l KBR7943 did not blunt the positive inotropy attributable to PE (RPP = 101% of control; $n = 4$; $P > 0.05$).

Diabetes and glucosamine increase UDP-HexNAc levels without decreasing ATP, whereas azaserine partially reverses the increase in UDP-HexNAc induced by diabetes. To determine if the alterations in inotropic responses described above correlated with altered flux in the hexosamine biosynthetic pathway, we monitored concentrations of the end products of the pathway, UDP-GlcNAc and UDP-GalNAc. We found that 4 days after the onset of diabetes, threefold increases in UDP-HexNAc (UDP-GlcNAc and UDP-GalNAc) levels were seen relative to control hearts (Fig. 5*A*). When hearts from the STZ-administered animals were perfused with 20 μmol/l azaserine for 45 min, the same 45-min perfusion period used in the physiological experiments before PE administration, a significant reduction in the elevated levels of UDP-HexNAc induced by diabetes was observed. We also treated hearts for 10 or 30 min with 5 mmol/l glucosamine to span the interval over which inhibition by this sugar of the inotropic response to PE was evident (Figs. 3 and 4). At the 10-min time point, the UDP-HexNAc level had already increased by 46%, whereas at 30 min it was up by 62% (Fig. 5*B*). ATP levels were also monitored 10 and 30 min after glucosamine administration. At neither time was there a significant decrease relative to control hearts (Fig. 5*B*).

The relation in individual hearts between UDP-HexNAc levels assessed 30 min after administration of PE and measures of cardiac performance from all four of these experimental conditions (control, STZ-treated, STZ and azaserine, 5 mmol/l glucosamine) was also examined. When mean ratios between baseline and final values were compared for the groups, highly significant inverse correlations were observed ($r = 0.91$ for RPP, $r = 0.98$ for dP/dt). In addition, absolute values for assessments of cardiac performance 10 min after exposure to PE were plotted for individual hearts from the four groups. Figure 5*C* shows this relation for RPP. After PE treatment, an inverse and significant ($r = 0.75$, $P < 0.05$) correlation was observed.

CCE inhibitors do not blunt the positive inotropy elicited by isoproterenol. Isoproterenol, acting primarily through β-adrenergic receptors, has a positive inotropic effect on the heart because of the activation of cAMP-dependent protein kinase A and phosphorylation of several protein kinase A substrates (2). To test whether CCE inhibitors blunt all means of inducing positive inotropy, we perfused the hearts with 0.3 μmol/l isoproterenol in the absence or presence of 10 μmol/l SKF96365 or 5 mmol/l glucosamine. Isoproterenol at the concentration used resulted in a significant positive inotropic effect, similar in magnitude to that seen with PE (Fig. 6). However, in contrast to the changes seen with PE, the increases in LVDP, RPP, +dP/dt, −dP/dt, and HR were not affected by SKF96365 or glucosamine (Fig. 7). These results suggest that the signaling pathways activated by isoproterenol are distinct from those activated by PE.

DISCUSSION

Our data implicate excessive flux through the hexosamine biosynthetic pathway as being responsible for the compromise in inotropic responses to PE seen in hearts taken from rats with experimentally induced diabetes. These findings thus demonstrate that the compromise with hyperglycemia in response to PLC-activating agonists previously reported in isolated cardiomyocytes (22) is also relevant to the intact heart. Furthermore, the effectiveness in blunting the inotropic responses of inhibitors of CCE, a pathway previously demonstrated in isolated cardiomyocytes (16,17), suggests that CCE is important to this physiological aspect of the intact heart. Neither glucosamine nor SKF96365 affected the normal function of the heart, demonstrating that acute inhibition of CCE does not interfere with Ca^{2+} fluxes involved in normal excitation-contraction coupling. Rather, these inhibitors significantly blunted the positive inotropic effect of PE without affecting the increase in function stimulated by the β-adrenergic stimulus isoproterenol.

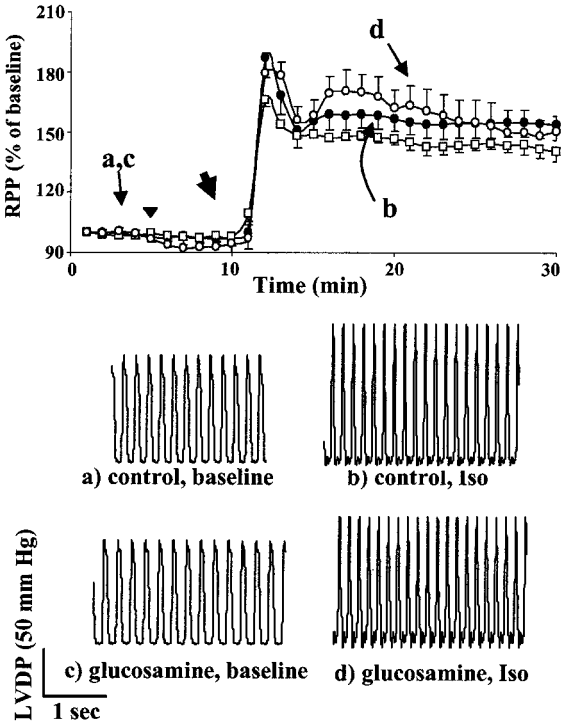

FIG. 6. Changes in contractile function in response to isoproterenol in the absence or presence of CCE inhibitors. The average changes over time in RPP in response to 0.3 μmol/l isoproterenol in the absence or presence of the CCE inhibitors SKF96365 (10 μmol/l) and glucosamine (5 mmol/l) added 5 min before isoproterenol and continued until the end of the experiment are shown. *a* and *b*: A typical LV pressure trace at the indicated times demonstrating the inotropic effects of 0.3 μmol/l isoproterenol in control rat hearts. *c* and *d*: A typical LV pressure trace demonstrating that the inotropic effects of PE are not significantly blunted by 5 mmol/l glucosamine. Hearts were exposed to glucosamine or SKF96365 at the arrowhead and to isoproterenol at the larger arrow. Data are means ± SE. ●, Isoproterenol in control hearts, $n = 5$; ○, isoproterenol in control hearts pretreated with glucosamine, $n = 4$; □, isoproterenol in control hearts pretreated with SKF96365, $n = 8$. Baseline values are comparable with those given in Table 1. The mean value is averaged data from 5–15 min after the addition of isoproterenol. For all parameters, the increase after isoproterenol was significantly greater than the baseline value ($P < 0.05$). There was no significant difference between the inhibitor groups and the isoproterenol group

The prolonged hyperglycemia that characterizes diabetes is clearly detrimental to cardiac health and outcomes (1). However, some of the underlying cellular responses may actually have evolved because they give rise to selective advantages in the context of the short-term hyperglycemia that characterizes the organismal response to stresses such as hemorrhage or sepsis (31). Because Ca^{2+} overload is central to stress-induced cell death (32), an inhibition of Ca^{2+} entry into pathways such as CCE could indeed be advantageous. Following this line of reasoning, we suggest that the dampened response to PE observed here may have been an unintended consequence of an adaptive response to stress-induced hyperglycemia. Other diabetic complications may have similar roots. For example, transforming growth factor-β attenuates myocardial ischemia-reperfusion injury in the short term (33), but over extended time periods it contributes to diabetic complications (34).

This observation, if substantiated, may help explain a confusing series of findings. In animal models of diabetes, short-term hyperglycemia is protective rather than detrimental to ischemia-induced cardiac damage, including the development of arrhythmias and apoptosis (35), likely because of an inhibition of the overload in Ca^{2+} that characterizes this pathology (36). Diabetes is also remarkably protective against cardiac damage induced by calcium overload because of the calcium paradox (37), in which the heart is exposed to a Ca^{2+}-free extracellular environment and then, upon re-addition of Ca^{2+}, suffers significant damage (38). In the former case, Woodcock et al. (6) established that damage can be greatly lessened by inhibiting PLC, with high levels of IP_3 being central to the Ca^{2+} overload. In the case of the calcium paradox, SR Ca^{2+} store depletion is one consequence of the removal of extracellular Ca^{2+} (39,40). The resulting inhibition of CCE in either condition could be at least one step in which diabetes offers protection. In fact, our unpublished data support these interpretations. It is possible, therefore, that in addition to contributing to positive inotropy, CCE plays an important role in regulating Ca^{2+} entry into the cell under conditions of Ca^{2+} overload and thus represents a potential target for therapeutic intervention.

The inhibition of CCE with diabetes may also contribute to some of the cardiac complications that characterize the disease by altering the balance among agonist-induced signaling pathways. For example, failing hearts are characterized by a lower level of activation of calcineurin (41), a protein phosphatase dependent on sustained elevation of $[Ca^{2+}]_i$ (42) and CCE (22), relative to hearts with compen-

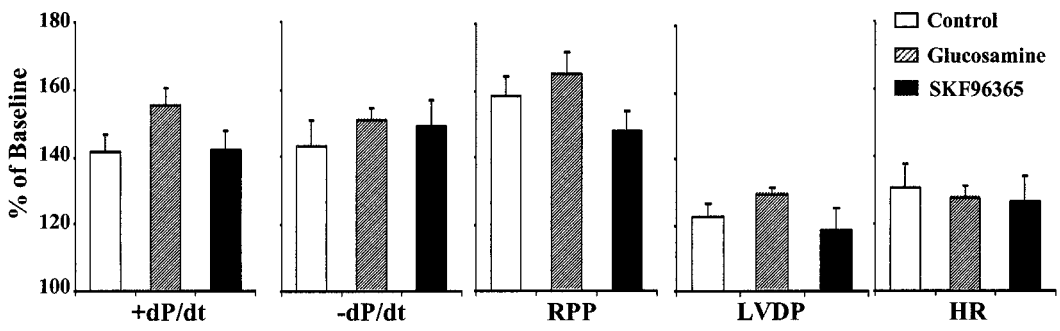

FIG. 7. CCE inhibitors do not block the positive inotropic effect of isoproterenol. Changes relative to baseline in +dP/dt, −dP/dt, RPP, LVDP, and HR caused by isoproterenol (0.3 μmol/l) in control hearts in the presence or absence of a 5-min pretreatment with 5 mmol/l glucosamine or 10 μmol/l SKF96365. Data are means ± SE. The mean values after the addition of isoproterenol are averaged data from 5–15 min after the addition. The baseline values are comparable with those given in Table 1. For all parameters, the increase after isoproterenol was significantly greater than the baseline value ($P < 0.05$). There were no significant differences for either of the inhibitor groups relative to isoproterenol alone. Isoproterenol, $n = 5$; isoproterenol + $GlcNH_2$, $n = 4$; isoproterenol + SKF96365, $n = 4$.

satory hypertrophy. The inhibition of CCE with diabetes could contribute to the oft-observed progression to failure by muting calcineurin-mediated signaling.

As seen by others (43), we have shown here that diabetes leads to significant increases in the level of UDP-GlcNAc, an end product of hexosamine biosynthesis. It is interesting that a 45-min perfusion with azaserine was sufficient to cause a reduction in the elevated levels, likely because of ongoing utilization coupled with a reduction in de novo synthesis (23). Changes in UDP-GlcNAc levels were not significant in 45-min perfusions of control hearts with 25 mmol/l glucose. However, UDP-GlcNAc levels were increased 10 min after glucosamine perfusion was initiated. In a previous study with tissue culture cells (44), glucosamine treatment led to a decrease in ATP levels. In this study, however, the cells were concomitantly deprived of glucose. Here, glucose was maintained at 5 mmol/l and ATP levels were not depressed. Furthermore, glucosamine treatment leads to elevation of hexosamine metabolites in addition to UDP-GlcNAc, such as glucosamine-6-phosphate (45). An elevation of these intermediaries is not seen in diabetes (45), however, thus focusing attention on the metabolite elevated in both conditions, UDP-GlcNAc.

One possibility as to how an increase in UDP-GlcNAc could be transduced into an inhibition of CCE lies in a glycosylation reaction that is now well-characterized but highly atypical (46). The reaction is unique in that it is catalyzed by an O-*N*-acetylglucosamine (GlcNAc) transferase that is found in the cytoplasm and transfers GlcNAc from UDP-GlcNAc to serine or threonine residues of cytoplasmic and nuclear proteins in O-linkage. Like phosphorylation, this modification is reversible, and under at least certain conditions, the number of proteins with O-GlcNAc residues within the cell is comparable with the number of phosphorylated proteins (46). The capacity to transform an increase in UDP-GlcNAc into complex cellular responses comes about because the O-GlcNAc transferase appears to recognize distinct proteins when the concentration of UDP-GlcNAc increases (47,48). Levels of glycosylation increase on certain proteins, and new proteins become modified. This response is seen in perfused hearts at 10 min of perfusion with 5 mmol/l glucosamine (J. Liv, R.B.M., unpublished data). The presence of the sugar itself could no doubt affect protein function, but, in addition, the sites that become glycosylated are often also sites that can be phosphorylated (46). Thus, this one modification can influence numerous signal transduction pathways. The pathway leading to CCE could be among those affected.

Previous investigations of the effects of IP_3-generating agonists on isolated hearts have yielded differing results. α-Adrenergic agonists have produced sustained positive inotropic responses due to activation of α_{1a} receptors in most species, although a brief negative response, mediated by α_{1b} receptors (49), is sometimes seen. The dog, however, appears to be devoid of α_{1a} receptors (50) and the mouse has a compromise in the signal transduction pathway leading to IP_3 generation (51). Positive inotropy with PE has been previously reported in the rat (49,51).

Although diabetes, glucosamine, and SKF96365 all substantially blunted the inotropic response to PE, the response was not completely abolished. PE acts on both β- and α-adrenoreceptors; 0.1 μmol/l propanolol was added to block the β-adrenoreceptor effects of PE. However, because there was still some stimulation of function in the presence of CCE inhibitors, this blockade may not have been complete. Alternatively, inotropic stimulation via α-adrenoreceptors may only be partially mediated via CCE, and release of Ca^{2+} from SR stores may be sufficient for a partial response.

In conclusion, our data implicate the hexosamine biosynthetic pathway as being responsible for the inhibition in inotropic responses to PE seen with diabetes. In addition, the effects of selective inhibitors of CCE on these responses implicate CCE as being an important step in the mechanism linking PLC-activating agonists to positive inotropy.

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