Hyperglycemia Inhibits Capacitative Calcium Entry and Hypertrophy in Neonatal Cardiomyocytes

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Hyperglycemia alters cardiac function and often leads to diabetic cardiomyopathy as cardiomyocyte apoptosis causes a hypertrophied heart to deteriorate to dilation and failure. Paradoxically, many short-term animal models of hyperglycemia protect against ischemia-induced damage, including apoptosis, by limiting Ca^{2+} overload. We have determined that, like nonexcitable cells, both neonatal and adult cardiomyocytes respond to depletion of sarcoplasmic/endoplasmic reticulum Ca^{2+} stores with an influx of extracellular Ca^{2+} through channels distinct from voltage-gated Ca^{2+} channels, a process termed capacitative Ca^{2+} entry (CCE). Here, we demonstrate that in neonatal rat cardiomyocytes, hyperglycemia decreased CCE induced by angiotensin II or the Ca^{2+}ATPase inhibitor thapsigargin. Hyperglycemia also significantly blunted Ca^{2+}-dependent hypertrophic responses by ~60%, as well as the Ca^{2+}-sensitive nuclear translocation of a chimeric protein bearing the nuclear localization signal of a nuclear factor of activated T-cells transcription factor. The attenuation of CCE by hyperglycemia was prevented by azaserine, an inhibitor of hexosamine biosynthesis, and partially by inhibitors of oxidative stress. This complements previous work showing that increasing hexosamine metabolites in neonatal cardiomyocytes also inhibited CCE. The inhibition of CCE by hyperglycemia thus provides a likely explanation for the transition to diabetic cardiomyopathy as well as to the protection afforded to injury after ischemia/reperfusion in diabetic models. *Diabetes* 51:3461–3467, 2002

Agonists such as angiotensin II (Ang II), endothelin 1, thrombin, and α-adrenergic agents influence cardiac physiology via signal transduction pathways involving activation of the G_q heterotrimeric G protein complex and phosphoinositol-specific phospholipase C (PLC) (1). In addition to eliciting acute positive inotropic effects (2), this pathway is important over longer time periods to the development of compensatory cardiac hypertrophy (3), as well as to apoptosis and lethal cardiomyopathy (4).

Variations in the magnitude of G_q-mediated stimulation can lead to different outcomes. Transgenic mice expressing in the heart a modest increase in the level of Gαq were characterized by a stable cardiac hypertrophy, whereas those with a higher level of expression developed dilated cardiomyopathy (5) characterized by apoptotic cell death (4). Another important variable may be the balance among the signaling pathways downstream of Gαq. One branch leads to the generation of diacylglycerol and the other to inositol (1,4,5) triphosphate (IP_3). IP_3 is linked to elevated concentrations of cytoplasmic free calcium ($[Ca^{2+}]_i$), and activation of calmodulin (CaM)-regulated enzymes such as Ca^{2+}/CaM-dependent kinase and the protein phosphatase calcineurin. Both of these enzymes have been implicated in cardiac hypertrophy, CaM kinase by activating the transcription factor MEF2 (6) and calcineurin by activating the transcription factor nuclear factor of activated T-cells (NFAT) 3 (7). In addition, calcineurin is able either to suppress (8) or to induce (9) cardiomyocyte apoptosis, depending on the physiological circumstances and its downstream targets (10).

A critical, unresolved issue for our understanding of the $[Ca^{2+}]_i$-dependent pathways in cardiomyocytes is the mechanism that leads from the generation of IP_3 to sustained increases in $[Ca^{2+}]_i$. In a recent study of cultured neonatal rat ventricular myocytes (NRVMs), we (11) established that capacitative calcium entry (CCE) is important to the sustained elevation of $[Ca^{2+}]_i$ seen after exposure to Ang II or phenylephrine (PE). CCE was first described in nonexcitable cells and refers to the influx of Ca^{2+} through plasma membrane calcium channels activated in response to depletion of endoplasmic reticulum (ER) and/or sarcoplasmic reticulum (SR) Ca^{2+} stores (12). It has since been shown to coexist with voltage-dependent calcium entry pathways in smooth (13) and skeletal (14) muscle as well as in NRVMs (11). The channels responsible for CCE in cardiomyocytes are distinct from L-type voltage-gated channels and can be activated both by IP_3-generating agonists and by inhibitors of the SR/ER Ca^{2+} ATPase (SERCA). We (11) also implicated these channels in the hypertrophic response to Ang II seen in NRVMs.

Effects of chronic hyperglycemia on these pathways are likely to be important to the development of diabetic cardiomyopathy, the myocardial dysfunction that characterizes both type 1 and type 2 diabetes independent of coronary artery disease or systemic hypertension (15). The development of diabetic cardiomyopathy in a rat

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ANF, atrial natriuretic factor; Ang II, angiotensin II; AOAC, aminooxyacetic acid; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; CaM, calmodulin; CCE, capacitative Ca^{2+} entry; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; HBSS, Hanks' balanced salt solution; IP_3, inositol (1,4,5) triphosphate; NFAT, nuclear factor of activated T-cells; NRVM, neonatal rat ventricular myocyte; MAP, mitogen-activated protein; PE, phenylephrine; PKC, protein kinase C; PLC, phospholipase C; SERCA, SR/ER Ca^{2+} ATPase; SR, sarcoplasmic reticulum; TTFA, thenoyltrifluoroacetone.

model of type 1 diabetes is dependent on Ang II (16) and can be distinguished from the cardiac hypertrophy induced in nondiabetic models. It is characterized by a high level of cardiomyocyte apoptosis that is dependent on Ang II–initiated signaling pathways (16) and a distinctive remodeling of the heart (17).

It is somewhat paradoxical, given these data and the many ways in which diabetes is deleterious to cardiac health (18), that in many animal models short-term hyperglycemia is protective rather than detrimental to ischemia-induced arrhythmias and apoptosis (19), likely because of an inhibition of the $[Ca^{2+}]_i$ overload that characterizes this pathology. Diabetes is also remarkably protective against cardiac damage induced by calcium overload as a result of the calcium paradox (20), in which the heart is exposed to a Ca^{2+}-free extracellular environment and then, upon re-addition of Ca^{2+}, incurs significant damage (21). Ziegelhoffer et al. (20) determined that in contrast to the complete absence of survival seen in control hearts, hearts from rats with streptozotocin-induced diabetes had a survival rate of 83.3%.

Given this background, we sought a better understanding of the influences that hyperglycemia has on signaling downstream of PLC. One candidate for altered response pathways involves the hyperactivation of isoforms of protein kinase C (PKC), which have been implicated in multiple complications associated with diabetes (22). Another is an increase in metabolites within the hexosamine biosynthetic pathway. This metabolic response to hyperglycemia has also been implicated in a spectrum of diabetic complications, including novel transcriptional responses (23–26), insulin resistance (27), and apoptosis in retinal neurons (28). In addition, both hyperglycemia (29,30) and the provision of extracellular glucosamine (11,31), another means of increasing hexosamine metabolites, have been shown to lead to an inhibition of CCE in selected cell types.

Because in NRVMs we recently showed that extracellular glucosamine leads to the rapid inhibition of CCE (11), we asked here whether hyperglycemia also results in an inhibition of CCE in these cells. We present data that confirm this hypothesis and implicate excessive flux through the hexosamine pathway as being responsible for the inhibition. In addition, we show that hyperglycemia compromises Ang II–induced hypertrophic responses in these cells.

RESEARCH DESIGN AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), M199, Hanks' balanced salt solution (HBSS), LipofectAMINE PLUS, and antibiotic/antimycotic solution were obtained from GIBCO. Thapsigargin, Fura 2-AM, and alexa flouro 488 goat anti-rabbit and 568 goat anti-mouse antibodies were purchased from Molecular Probes. Anti-atrial natriuretic factor (ANF) polyclonal antiserum was from Peninsula Laboratories. Anti–α-actinin antibody, Ang II, PE, thenoyltrifluoroacetone (TTFA), azaserine, and aminooxyacetic acid (AOAC) were from Sigma. Calphostin C was from Calbiochem.

Cell cultures. NRVMs were isolated from the hearts of 1-day-old Sprague-Dawley rats by collagenase digestion and cultured as previously described (11). Spontaneously beating or quiescent cells were favored by culturing at dense (400–800 cells/mm^2) or sparse (50–200 cells/mm^2) plating densities, respectively. In some experiments, cells were treated with 5 μmol/l thapsigargin, 1 μmol/l Ang II, or 50 μmol/l PE, in the absence or presence of 5 μmol/l azaserine, 10 μmol/l TTFA, 100 μmol/l AOAC, and 1 μmol/l calphostin C.

Monitoring of $[Ca^{2+}]_i$. NRVMs were plated on glass coverslips and maintained in growth medium (11) with 5.5, 10, 20, or 30 mmol/l glucose or 5.5 mmol/l glucose with 25 mmol/l mannitol for at least 20 h. The cells were loaded with 3 μmol/l Fura 2-AM at 37°C in growth medium for 20 min, washed twice, and incubated in HBSS for 30 min at room temperature to convert the AM ester to Fura-2. Digital images were obtained in room-temperature HBSS using an Olympus 1X70 inverted microscope through a 20× objective lens. Changes in $[Ca^{2+}]_i$ were determined using alternating 340- and 380-nm excitation wavelengths and measuring emission at 510 nm.

Immunocytochemistry. NRVMs were plated at a density of 100–400 cells/mm^2 on eight-well slide chambers (NUNC) and maintained for 48 h in growth media containing either 5.5 or 30 mmol/l glucose in the presence or absence of PE or Ang II. The cells were fixed in ice-cold 100% methanol for 10 min, washed three times in PBS, and blocked in PBS containing 1% BSA for 30 min. Anti–α-actinin antibody and anti-ANF polyclonal antiserum were added at dilutions of 1:400 and 1:150, respectively, in PBS containing 1% BSA and incubated for 1 h at room temperature. Secondary antibodies, Alexa flouro 488 goat anti-rabbit and Alexa flouro 568 goat anti-mouse, were used at a dilution of 1:300 in PBS containing 5% rat serum and incubated for 30 min at room temperature. Percentages of NRVMs positive for ANF were determined by counting the total number of NRVMs in fields based on α-actinin staining and then determining the number with nuclear rings of ANF. At least 100 cells per condition were counted. Cell size was determined by using Universal Image 1 software. Mean cell areas were determined for at least 100 cells in each of the treatment groups for each experiment.

Infections. A cDNA encoding enhanced green fluorescence protein (EGFP) fused to NFAT4 (32) was provided by Dr. Frank McKeon (Harvard Medical School). Preparation of a truncated form of the chimeric protein (amino acids 1–419 of NFAT4) and the generation of the recombinant adenovirus were carried out as previously described (11). Four hours after plating, NRVMs were infected with EGFP-NFAT4 at a multiplicity of injection of 50 pfu/cell in DMEM for 16–20 h. Fresh media containing 5.5 or 30 mmol/l glucose, or 30 mmol/l glucose plus inhibitors (azaserine, TTFA, AOAC, or calphostin C) was substituted, and cells were incubated for an additional 24 h. The cells were then treated with thapsigargin, PE, or Ang II for 15 min and fixed with 100% methanol. Confocal microscopic images were obtained using a Leica DMIRBE inverted Nomarski/epifluorescence microscope outfitted with Leica TCS NT Laser Confocal optics through a 40× objective lens. A 488-nm argon ion laser was used for excitation, and the emitted fluorescence was collected at 530 ± 30 nm.

Data analysis. All values shown are means ± SE. Comparisons were performed with one-way ANOVA and post hoc (Scheffé's) (StatView; SAS Institute, Cary, NC). Statistically significant differences between groups were defined as $P < 0.05$.

RESULTS

Effects of hyperglycemia on CCE induced by SR Ca^{2+} store depletion. To examine alterations in CCE resulting from hyperglycemia, we incubated NRVMs for at least 20 h in either 5.5 or 30 mmol/l glucose. The cells were then loaded with the Ca^{2+}-sensitive radiometric dye Fura-2, and single-cell digital imaging was used to monitor $[Ca^{2+}]_i$ changes. Thapsigargin, an irreversible SERCA inhibitor (33), was used to deplete Ca^{2+} stores and trigger CCE.

NRVMs can be cultured as either spontaneously beating or quiescent cells, depending on the plating density (34). The left panel in Fig. 1*A* depicts a typical example of a thapsigargin-induced $[Ca^{2+}]_i$ increase for quiescent cells cultured in 5.5 mmol/l glucose growth medium and tested in HBSS containing 5.5 mmol/l glucose and 1.8 mmol/l Ca^{2+}. The addition of thapsigargin produced an immediate rise in $[Ca^{2+}]_i$ and resulted in a stable plateau. Previously, we (11) demonstrated that this increase was dependent on extracellular Ca^{2+} and attenuated by inhibitors of CCE but not L-type voltage-gated Ca^{2+} channels. In contrast, NRVMs cultured for 20 h in 30 mmol/l glucose showed a decreased response to thapsigargin. Because hyperosmolarity can affect both inositol metabolism (35) and $[Ca^{2+}]_i$ (36), NRVMs were also cultured in 5.5 mmol/l glucose with 25 mmol/l mannitol. $[Ca^{2+}]_i$ responses did not differ from those in 5.5 mmol/l glucose alone (Fig. 1*A*). A similar osmolarity control was also carried out in subsequent

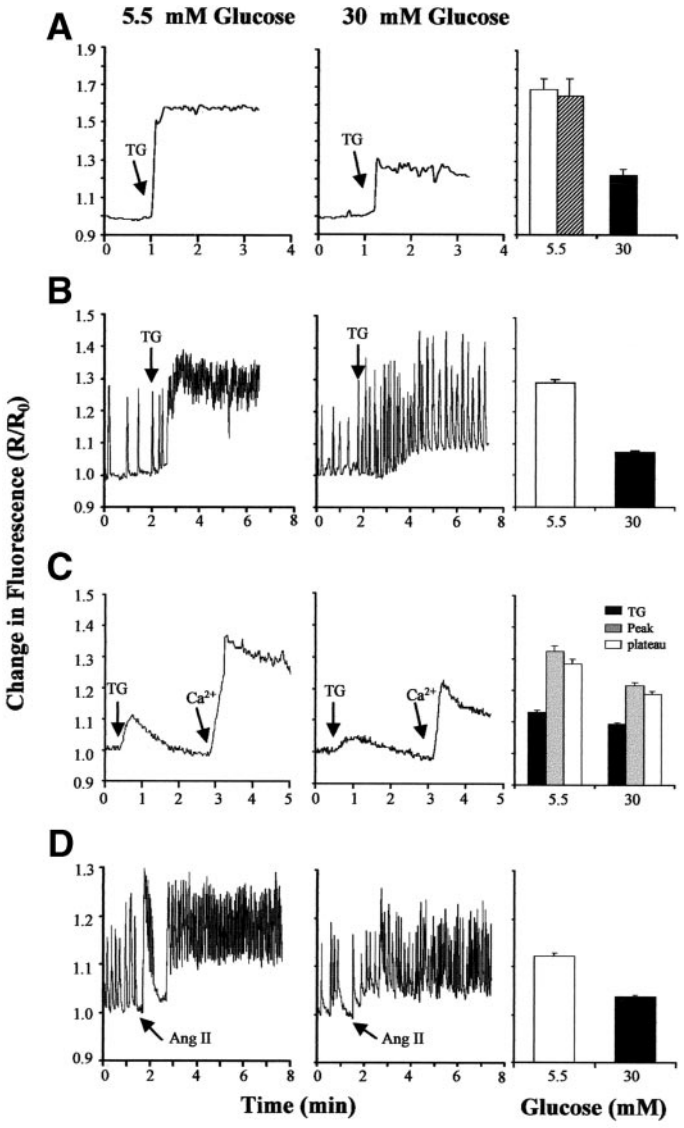

FIG. 1. Hyperglycemia attenuates CCE in NRVMs. Fura-2 responses to thapsigargin (TG) (*A–C*) or Ang II (*D*) of quiescent (*A* and *C*) and beating (*B* and *D*) NRVMs cultured for at least 20 h in 5.5 (left) or 30 mmol/l (center) glucose. Data are expressed as the Fura-2 fluorescence ratio at each time point (R) divided by the initial fluorescence ratio (R_0). The bar graphs depict the mean R/R_0 value for the plateau value in the quiescent cells (*A*) and the "diastolic" Ca^{2+} in the beating cells (*B* and *D*) after CCE was initiated. The mean R/R_0 for the cells cultured in 5.5 mmol/l glucose with 25 mmol/l mannitol (striped bar) was not significantly different from those in 5.5 mmol/l glucose alone (open bar) (*A*). In the bar graph for the Ca^{2+} addback protocol (*C*), the means of the peak fluorescence in the absence of extracellular Ca^{2+}, the peaks after extracellular Ca^{2+} was reintroduced (arrow), and the plateau values are shown. The data are the mean values from at least four experiments ($n \geq 28$ cells). All means for NRVMs in 30 mmol/l glucose were significantly less than those for NRVMs cultured in 5.5 mmol/l glucose.

experiments, and in no case was an effect seen (data not shown).

After the addition of thapsigargin to beating cells cultured in 5.5 mmol/l glucose (Fig. 1*B*), there was a substantial increase in "diastolic" $[Ca^{2+}]_i$ as well as an acceleration of the beat frequency, as seen previously (37). The hyperglycemic group demonstrated a significantly depressed thapsigargin-induced diastolic $[Ca^{2+}]_i$ plateau compared with that observed for the euglycemic group. The increase in beat frequency was not as dramatic in the hyperglycemic group, but the "diastolic to systolic" amplitudes were consistently more robust.

A conventional protocol for the study of CCE utilizes thapsigargin to deplete ER/SR Ca^{2+} stores in the absence of extracellular Ca^{2+}, resulting in a transient increase in $[Ca^{2+}]_i$ that returns to baseline after the stores are thoroughly depleted (12). The subsequent addition of extracellular Ca^{2+} produces a sustained increase in $[Ca^{2+}]_i$ attributable to CCE. With the use of this protocol, there were significant differences in the capacitative Ca^{2+} peaks and plateaus between the euglycemic and hyperglycemic groups (Fig. 1*C*).

CCE induced by IP_3-generating agonists in hyperglycemic conditions was also examined. The left panel of Fig. 1*D* shows a typical response of a spontaneously beating cell in normal glucose to Ang II. Diastolic $[Ca^{2+}]_i$ increased after treatment with Ang II, as did the beat frequency. NRVMs cultured in 30 mmol/l glucose for at least 20 h had a decreased response to Ang II, apparent both in diastolic $[Ca^{2+}]_i$ and in the change in beat frequency.

NRVMs are less sensitive to hypertrophic stimuli in hyperglycemic conditions. A well-established model for the study of cardiac hypertrophy in vitro (38) involves treating NRVMs with Ang II or PE. To examine the effects of hyperglycemia in vitro on cardiac hypertrophy induced by IP_3-generating agonists, we cultured NRVMs in 5.5 or 30 mmol/l glucose in the presence of Ang II or PE for 48 h, and cell size and the presence of the hypertrophic marker ANF were assessed (Fig. 2). Hyperglycemia significantly blunted Ca^{2+}-dependent hypertrophic responses induced by both Ang II and PE, decreasing ANF expression and cell size increases by ~60%.

Hyperglycemia blunts NFAT nuclear translocation in NRVMs in response to thapsigargin or agonists. A chronic increase in $[Ca^{2+}]_i$ is regarded as one of the primary causes of cardiac hypertrophy (39). This leads to the activation of the Ca^{2+}/CaM-dependent protein phosphatase calcineurin, which in turn dephosphorylates members of the NFAT transcription factor family (7). This triggers the translocation of NFATs from cytoplasm to nucleus and results in the initiation of novel transcriptional responses. We recently demonstrated (11) that CCE inhibitors prevented NFAT nuclear translocation induced by IP_3-generating agonists, thus implicating CCE as a primary effector of NFAT-mediated hypertrophy.

To determine the effect of hyperglycemia on NFAT nuclear translocation, we infected NRVMs with a recombinant adenovirus encoding EGFP linked to the best-studied of the NFAT nuclear localization signals, that of NFAT4 (40). They were then cultured in 5.5 or 30 mmol/l glucose for 24 h. Infected NRVMs were exposed to thapsigargin, PE, or Ang II for 15 min, and the localization of the NFAT chimera in the cytoplasm and/or the nucleus was detected using confocal microscopy. Without agonist treatment, fluorescence was restricted to the cytoplasm of NRVMs in both normal and hyperglycemic conditions (Fig. 3*A* and *E*). In the presence of stimuli, the NFAT chimera translocated into the nucleus in 83% of infected NRVMs cultured in 5.5 mmol/l glucose (Fig. 3*B–D*), but this was significantly decreased in cells cultured in 30 mmol/l glucose (Fig. 3*F–H*).

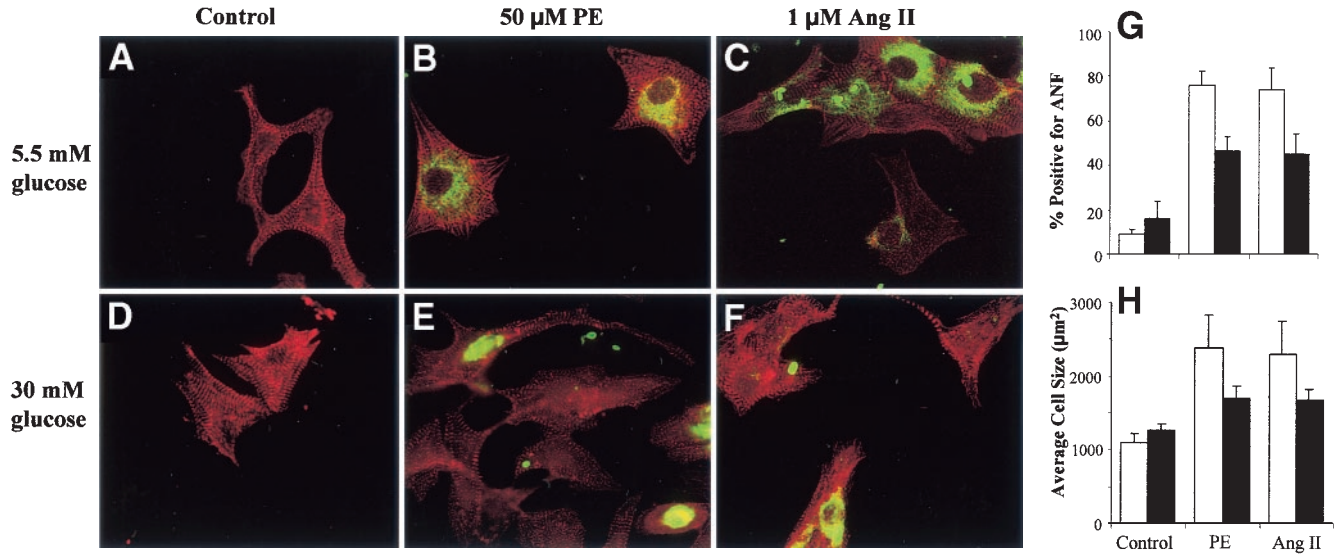

FIG. 2. Hyperglycemia blunts the hypertrophic response in NRVMs. NRVMs were cultured in either 5.5 mmol/l (*A–C*) or 30 mmol/l (*D–F*) glucose in the absence (*A* and *D*) or presence of PE (*B* and *E*) or Ang II (*C* and *F*). After 48 h, the distributions of α-actinin (red) and ANF (green) were assessed. The percentage of cells expressing ANF (*G*) and the average cell size (*H*) are shown (5.5 mmol/l, open bars; 30 mmol/l, filled bars). Mean values are the average of four experiments. For both measurements, the agonist-treated cells were significantly different from controls, and those in 30 mmol/l were significantly less responsive than those in 5 mmol/l.

Inhibition of CCE is seen with less elevated glucose concentrations. To assess better the possible clinical relevance of the inhibition in vitro of CCE seen at 30 mmol/l glucose, we carried out experiments at two less elevated concentrations as well. Figure 4*A* shows that a partial inhibition of CCE was seen at 10 mmol/l (180 mg/dl), whereas at 20 mmol/l (360 mg/dl) the inhibition was as profound as at 30 mmol/l. The extent of the nuclear translocation of the NFAT chimeric indicator in response to Ang II and thapsigargin was also assessed (Fig. 4*B*). A graded inhibition was seen as the glucose concentration was increased from 10 to 30 mmol/l.

The hexosamine biosynthetic pathway is implicated in the hyperglycemia-induced decrease in CCE and NFAT nuclear translocation. Several possible metabolic pathways are known to be associated with hyperglycemia, including the upregulation of selected PKC isoforms (22), increased oxidative stress (41), and increased flux through the hexosamine biosynthetic pathway (27). To determine whether any of these responses might underlie the effects of hyperglycemia on CCE, we examined the effects of various inhibitors on the sustained $[Ca^{2+}]_i$ increases induced by thapsigargin and on the level of NFAT nuclear translocation induced by Ang II or thapsigargin.

Azaserine, an inhibitor of the rate-limiting enzyme (glutamine: fructose-6-phosphate amidotransferase) in the hexosamine biosynthetic pathway (27), completely restored the $[Ca^{2+}]_i$ increase induced by thapsigargin of cells cultured in 30 mmol/l glucose (Fig. 5). Partial restoration was seen with inhibitors of superoxide production (26),

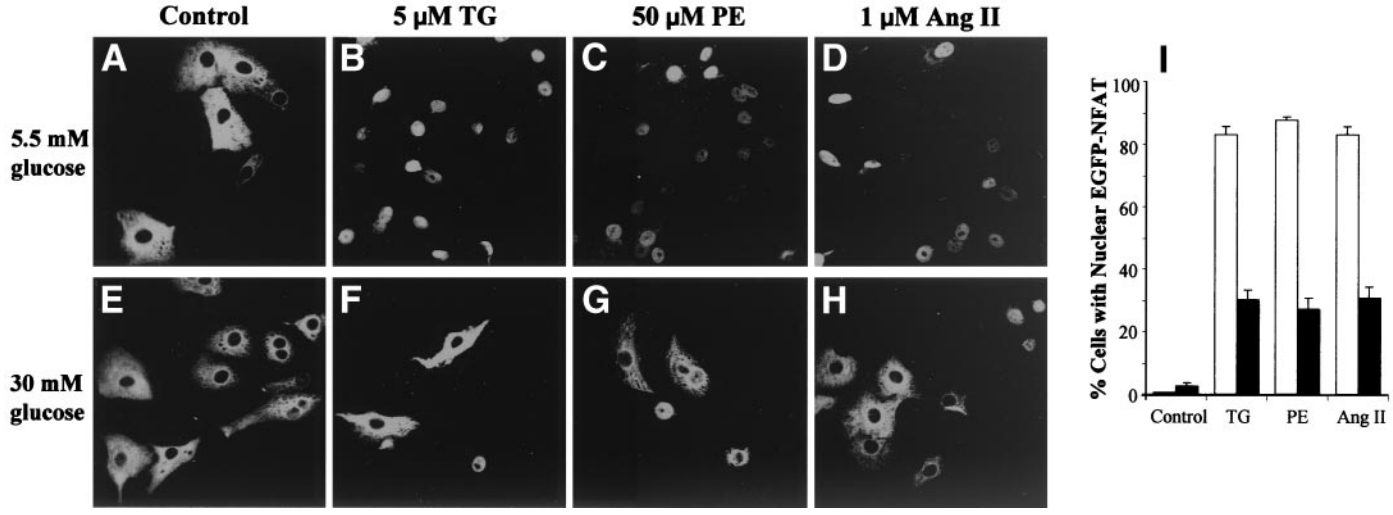

FIG. 3. Hyperglycemia inhibits EGFP-NFAT nuclear translocation induced by thapsigargin or agonists. NRVMs were infected with an adenovirus expressing EGFP-NFAT and cultured in either 5.5 (*A–D*) or 30 mmol/l (*E–H*) glucose in the absence (*A* and *E*) or presence of thapsigargin (*B* and *F*), PE (*C* and *G*), or Ang II (*D* and *H*). The percentage of cells cultured in 5.5 (open bars) and 30 mmol/l (filled bars) glucose in which the chimeric protein was localized to the nucleus is shown (*I*). Mean values are the average of four experiments. In all cases the agonist-treated cells were significantly different from controls, and those in 30 mmol/l were significantly less responsive than those in 5 mmol/l.

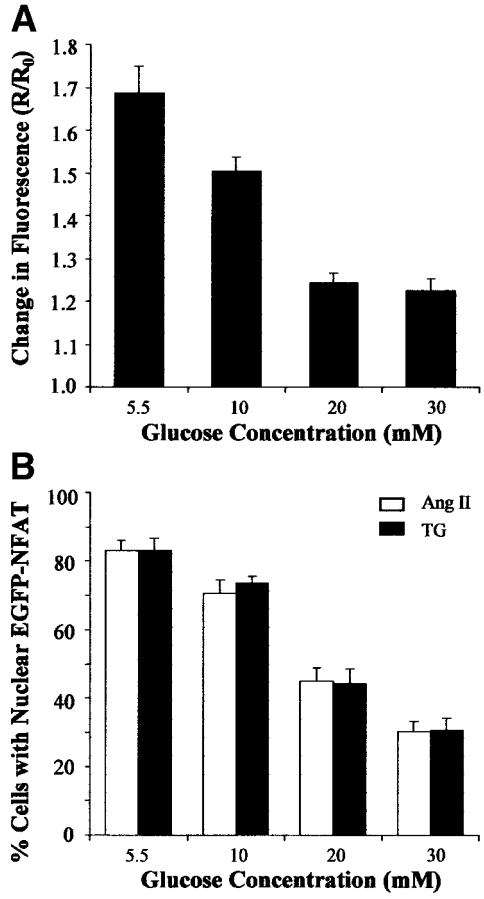

FIG. 4. Inhibition of CCE and EGFP-NFAT nuclear translocation is seen with less elevated glucose concentrations. *A*: The mean R/R_0 value ($n \geq 12$ cells) for the plateau value of the Fura-2 signal after thapsigargin treatment of quiescent NRVMs cultured with 5.5, 10, 20, or 30 mmol/l glucose is shown. *B*: The percentage of cells cultured in 5.5, 10, 20, or 30 mmol/l glucose in which EGFP-NFAT was localized to the nucleus in response to either Ang II (open bars) or thapsigargin (filled bars) is summarized. Mean values are the averages of three independent experiments. The changes in fluorescence and percentage of cells with nuclear EGFP-NFAT at 10, 20, and 30 mmol/l glucose were all significantly different from those at 5.5 mmol/l glucose.

TTFA, an inhibitor of electron transport complex II, and AOAC, an inhibitor of the malate-aspartate shuttle, whereas no improvement was seen with a PKC inhibitor previously shown to be effective in cardiomyocytes, calphostin C (42). In addition, the level of NFAT nuclear translocation in response to Ang II or thapsigargin doubled in the presence of azaserine. TTFA was again partially effective, whereas calphostin C had no effect (Fig. 6).

DISCUSSION

Diabetic cardiomyopathy is characterized by hypertrophy, but rather than being stable and compensatory, it often deteriorates into a loss of cardiac mass as a result of apoptotic cell death, dilation, and failure (15). In rats made diabetic with streptozotocin, this progression is dependent on Ang II (16). The signal transduction pathways that link Ang II and other IP_3-generating stimuli to both cardiac hypertrophy and cardiomyocyte apoptosis are complex but becoming more completely understood (3).

Diabetes and chronic hyperglycemia might be expected to influence Gαq-mediated signaling pathways in at least two ways. First, diabetic complications have been linked to elevated levels of diacylglycerol as a result of increased de novo synthesis (22). Because nine isoforms of PKC are activated to varying extents by diacylglycerol (41), such an increase could clearly affect the balance among the various signal transduction pathways. Recent data suggest that stimulation of PKC ϵ is linked to the activation of the ERK kinases and hypertrophy, whereas activation of PKC δ leads to activation of p38 and apoptosis (43). Second, we demonstrate here that hyperglycemia decreases agonist-initiated increases in $[Ca^{2+}]_i$ as a result of an inhibition of CCE. This inhibition has a clear effect on the CaM/calcineurin-dependent nuclear localization of NFAT family members. Because calcineurin is critical to the hypertrophic response (7) as well as to both proapoptotic (8) and antiapoptotic (9) signals, disruption of normal $[Ca^{2+}]_i$ signaling in response to agonists such as Ang II might be expected to alter cellular responses and thereby contribute to the progression to diabetic cardiomyopathy. Such a duality in calcineurin's influence on apoptosis is not peculiar to cardiomyocytes, as it also has been described in myeloid leukemic cells (44).

Insight into the effects that a disruption in the balance among signaling pathways might have on cardiac outcome is provided by a recent study of signal transduction pathways in human hearts characterized either by hypertrophy or by advanced heart failure (45). In hypertrophied hearts, the dominant activated signaling pathway centered

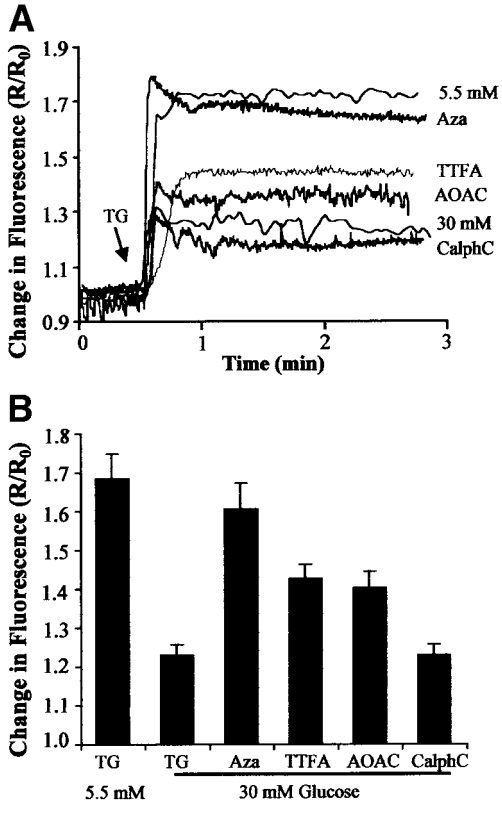

FIG. 5. Azaserine reverses the inhibitory effect of hyperglycemia on CCE induced by thapsigargin. *A*: Characteristic Fura-2 responses of quiescent NRVMs to thapsigargin (TG) cultured in 5.5 or 30 mmol/l glucose, or 30 mmol/l glucose in the presence of azaserine (Aza), TTFA, AOAC, or calphostin C (CalphC). *B*: The mean R/R_0 value ($n \geq 14$ cells) for the plateau value after thapsigargin treatment in the absence or presence of inhibitors is shown. Aza, TTFA, and AOAC all are significantly greater than the 30-mmol/l control.

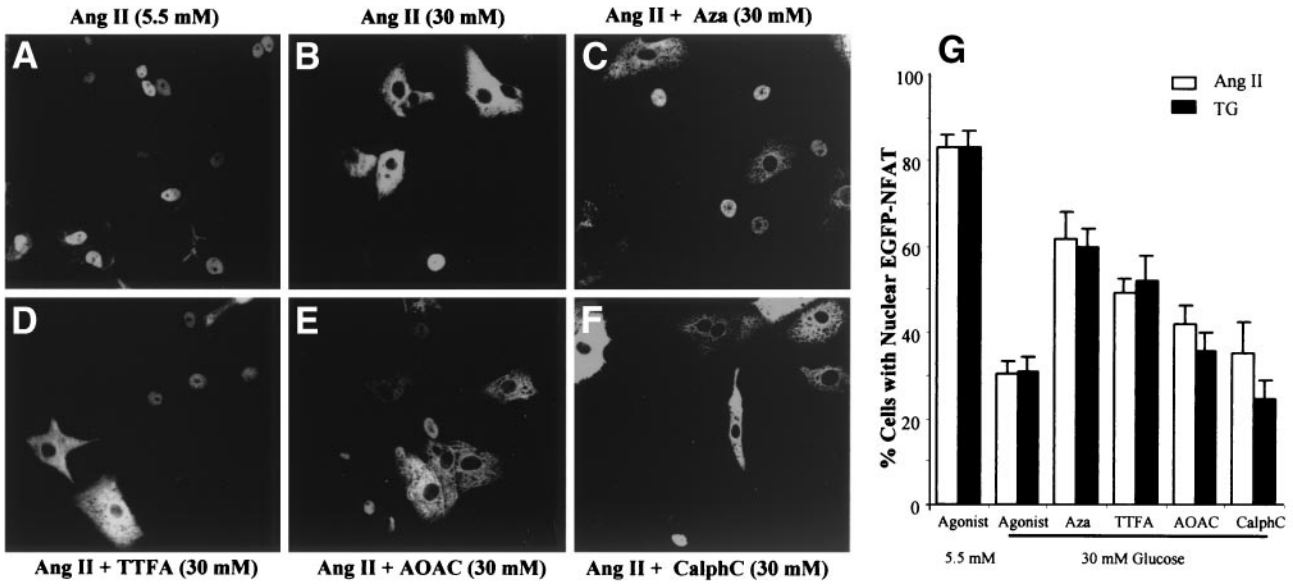

FIG. 6. Azaserine doubles EGFP-NFAT nuclear translocation induced by thapsigargin or Ang II in hyperglycemic NRVMs. NRVMs were infected with a recombinant adenovirus expressing EGFP-NFAT and cultured in either 5.5 (*A*) or 30 mmol/l (*B–H*) glucose and Ang II in the absence (*A* and *B*) or presence of azaserine (*C*), TTFA (*D*), AOAC (*E*), or calphostin C (*F*). The percentage of cells in which the chimeric protein was localized to the nucleus in response to either Ang II (open bars) or thapsigargin (filled bars) is summarized (*G*). Mean values are the average of three experiments. Azaserine and TTFA resulted in translocation significantly greater than that in the 30-mmol/l control.

on calcineurin. Little activation of the ERK, JNK, or p38 mitogen-activated protein (MAP) kinase pathways or the Akt pathway was seen. In failing hearts, calcineurin was still activated but less so, but in addition, all three MAP kinase pathways and Akt were activated (45). If in diabetes a shift away from calcineurin's dominance is seen as a result of inhibition of CCE, then a tendency to progress to failure might be predicted.

Brownlee (41) recently put forward a unifying hypothesis for the molecular mechanisms responsible for many diabetic complications. He suggested that increased oxidative stress as a result of hyperglycemia inhibits glyceraldehyde-3-phosphate dehydrogenase, the key enzyme controlling entry into the glycolytic cascade and an enzyme particularly vulnerable to the effects of oxidants. This diverts glucose from energy metabolism to other pathways implicated in diabetic complications, including the hexosamine biosynthetic pathway. In studies in endothelial cells, a complete recovery from hyperglycemia-induced changes was achieved either by inhibiting hexosamine biosynthesis directly with azaserine or by inhibiting mitochondrial superoxide production (26). These data substantiate the links among increased oxidative stress, the inhibition of glyceraldehyde-3-phosphate dehydrogenase, and the diversion of fructose-6-phosphate from glycolysis to hexosamine metabolism. Here, the direct inhibition of hexosamine biosynthesis with azaserine completely reversed hyperglycemia's inhibition of CCE, whereas the inhibitors of superoxide production were partially effective.

Previous work investigating the ability of IP_3-generating agonists to increase $[Ca^{2+}]_i$ in cardiomyocytes has implicated increased flux through the Na^+/Ca^{2+} exchanger operating in the reverse mode (46). However, our recent data show that inhibitors of the exchanger are not effective in preventing the influx seen in CCE, whereas exogenous glucosamine has no effect on calcium influx via the exchanger. Patch clamp analyses have also now characterized the store-operated channels responsible for CCE (Hunton et al., unpublished data).

Previous studies linking hyperglycemia to a cardioprotective effect against calcium overload and hypoxia-induced cell death have also focused on a reduction in influx of calcium via the Na^+/Ca^{2+} exchanger (19). However, Woodcock et al. (47) determined that Ca^{2+} influx via the exchanger in the absence of IP_3 accumulation is not sufficient to cause reperfusion-induced arrhythmias. They suggested that increases in $[Ca^{2+}]_i$ as a result of the high levels of IP_3 are critical contributors. These increases in $[Ca^{2+}]_i$ could be due solely to changes at the SR. Alternatively, our data suggest that IP_3-mediated store depletion and the subsequent increase in $[Ca^{2+}]_i$ that results from activation of CCE is a primary contributor to the development of these reperfusion-induced arrhythmias. Importantly, as is the case with the calcium paradox (20), hyperglycemia in many animal models affords protection to the development of such arrhythmias (19). The data presented here suggest that excessive flux through the hexosamine biosynthesis pathway and the resulting inhibition of CCE could be critical.

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