Reversal of hyperglycemic preconditioning by angiotensin II: role of calcium transport

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Pastukh, Viktor, Songwei Wu, Craig Ricci, Mahmood Mozaffari, and Stephen Schaffer. Reversal of hyperglycemic preconditioning by angiotensin II: role of calcium transport. Am J Physiol Heart Circ Physiol 288:H1965–H1975, 2005. First published December 16, 2004; doi:10.1152/ajpheart.00855.2004.—Myocardial cell death is an important contributor to the development of diabetic cardiomyopathy. It has been proposed that diabetes-mediated upregulation of the renin-angiotensin system leads to oxidative stress, the trigger for cardiomyocyte death and contractile dysfunction. However, the adverse effect of ANG II on the diabetic heart may extend beyond the development of the cardiomyopathy. ANG II also alters specific modulators of ischemic injury, such as PKC and calcium transport. Therefore, the present study examined the effect of ANG II on hyperglycemic preconditioning, a glucose-mediated condition associated with the elevation of PKC activity and alterations in calcium transport that render the cell resistant to hypoxia. Exposure of the glucose-treated cell to ANG II during the prehypoxic period blocked glucose-mediated cardioprotection. The reversal of hyperglycemic preconditioning was associated with enhanced accumulation of Ca2+ during hypoxia, an effect prevented by inhibition of the Na+/H+ exchanger and the T-type Ca2+ channel. The inhibitors of hypoxia-mediated Ca2+ accumulation also blocked the reversal of hyperglycemic preconditioning by ANG II. Thus ANG II and glucose treatment exert opposite actions on the Na+/H+ exchanger and the T-type Ca2+ channel. Because those transporters are involved in hypoxia-mediated apoptosis, they are logical candidates for the beneficial effects of high glucose and the adverse effects of ANG II on the hypoxic cardiomyocyte.

sodium-hydrogen exchanger; T-type calcium channel; protein kinase C; hyperglycemia; hypoxia; apoptosis; calcium overload

ONE CLINICALLY IMPORTANT COMPLICATION of diabetes mellitus is the development of cardiomyopathy, a condition characterized by contractile dysfunction, diastolic stiffening, altered cardiac autonomic function, impaired myocardial metabolism, and microvascular abnormalities. Although the cardiomyopathy often presents as a preclinical condition, when combined with other complications, such as coronary artery disease, cardiomyopathy hastens the development of congestive heart failure (19). The higher incidence of congestive heart failure among diabetic patients after a myocardial infarction has been attributed to a severe decline in contractile reserve, which results from both cardiomyocyte loss and diabetes-linked excitation-contraction coupling defects of spared cardiomyocytes (2, 12).

Both oxidative stress and impaired calcium transport play key roles in cardiomyocyte loss and impaired excitation-contraction coupling of the diabetic heart. In 1996, Kaul et al. (21) reported that the antioxidant probucol improved contractile function in a rat model of streptozotocin-dependent diabetes. In a related study, Esberg and Ren (6) found that scavengers of nitric oxide, superoxide, and peroxynitrite prevented contractile defects of cardiomyocytes cultured for 24 h in medium containing 25 mM glucose. Because the antioxidants also improved Ca2+ homeostasis, oxidative stress appeared to affect the activity of Ca2+-dependent transporters, such as the Na+/Ca2+ exchanger and sarco(endoplasmic reticulum Ca2+-ATPase (SERCA). However, Fiordaliso et al. (10) implicated the diabetes-mediated upregulation of ANG II in the production of reactive oxygen species, the loss of cardiomyocytes, and the development of cardiomyopathy. In support of their hypothesis, they found that components of the renin-angiotensin system were upregulated in the diabetic heart. Moreover, both the antioxidant Tiron and the AT1 receptor antagonist losartan blocked diabetes-mediated apoptosis and hydroxyl radical production (10, 18).

Upregulation of the renin-angiotensin system may also account for the increased susceptibility of the diabetic heart to ischemia. It is widely recognized that ANG II stimulates the activity of the Na+/H+ exchanger, the Na+/Ca2+ exchanger, and the T-type Ca2+ channel (3, 8, 15), transporters implicated in ischemia-mediated Ca2+ accumulation. Moreover, ANG II promotes the translocation and activation of PKC-δ, an isoform thought by some investigators to adversely affect the ischemic heart (28). Furthermore, several investigators have shown that AT1 receptor blockade and angiotensin-converting enzyme inhibition protect the ischemic heart (17). However, the effects of ANG II on the ischemic myocardium remain controversial. ANG II has also been found to promote the translocation and activation of PKC-ε, an isoform implicated in ischemic preconditioning (14, 23, 34). According to Ford et al. (11), the interaction of ANG II with the AT1 receptor mediates cardioprotection. Moreover, transgenic mice overexpressing human renin and angiotensinogen transgenes show enhanced recovery of contractile function after a hypoxia-reoxygenation insult (37). In view of these controversies and the importance of ANG II in diabetes, further studies on the effect of ANG II on the diabetic cardiomyocyte seem warranted. The present study examined the effect of ANG II on the glucose-treated cardiomyocyte, a model that focuses on the effects of chronic hyperglycemia and mimics many of the changes seen in the diabetic cardiomyocyte (5, 30, 31). Isolated cells were preferred for the present study because interpretation

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of the ANG II effect in the isolated heart would be complicated by the vasoactivity of the peptide.

MATERIALS AND METHODS

Cardiomyocyte preparation and incubation conditions. Neonatal cardiomyocytes were prepared as described previously (35). The cells were suspended in minimal essential medium containing 10% newborn calf serum and 0.1 mM 5′-bromo 2′-deoxyuridine and allowed to plate on either glass coverslips or polystyrene-treated petri dishes (Cat. no. 430167, Corning) at a density of ~10 × 10^4 cells/dish (10-cm diameter). They were then placed in serum-free medium containing minimal essential medium supplemented with either no addition (control) or 25 mM glucose (glucose treated). In some experiments, either 1 mM ANG II or varying concentrations of the PKC inhibitor chelerythrine were added to the incubation medium after 48 h. All experiments were initiated after a 3-day incubation at 37°C under a 5% CO2-20% O2 environment. To induce chemical hypoxia, the cells were transferred to medium lacking glucose and ANG II but containing 10 mM deoxyglucose and 3 mM amobarbital. In some experiments, the T-type Ca2+ channel antagonist mibefradil (0.3 μM), the Na+/H+ exchange antagonist cariporide (10 μM), or the combination of mibefradil and cariporide were added to the hypoxic medium.

TdT-mediated dUTP nick-end labeling procedure. The Klenow Frag EL DNA fragmentation detection kit was used to monitor end-labeled DNA fragments of apoptotic nuclei. Cells were assayed for positive TdT-mediated dUTP nick-end labeling (TUNEL) staining before and after 1 h of chemical hypoxia. In each case, the cells were fixed for 15 min in 4% formaldehyde and then resuspended in 80% ethanol for 20 min. After rehydration in PBS, the samples were incubated for 5 min with 20 μg/ml proteinase K in 10 mM Tris (pH 8.0). The slides were then rinsed in PBS. Endogenous peroxidases were inactivated by a 5-min incubation with 3% H2O2. After being rinsed again, the slides were placed in 1× Klenow buffer for 20 min. The Klenow labeling reaction mixture was then added to the samples, and the samples were incubated for 1.5 h at 37°C. The reaction was terminated by a 5-min incubation with buffer containing 0.5 mM EDTA (pH 8.0) followed by a 10-min exposure to blocking buffer. The samples were then treated for 30 min with peroxidase-streptavidin conjugate. After a 15-min incubation with 0.7 mg 3,3-diaminobenzidien and 0.6 mg H2O2-urea, the samples were rinsed and then counterstained with hematoxylin. To fix the color, the glass slides were first immersed in 100% ethanol and then xylene. Four separate fields in the light microscope were examined for dark brown (apoptotic) and purple (normal) nuclei.

Western blot analyses. The cytosolic and particulate content of various PKC isoforms were determined by Western blot analysis. After the cells were scraped from the bottom of the petri dishes, they were suspended in ice-cold Tris buffer (pH 7.4) containing 5 mM EGTA, 2 mM EDTA, 100 mM NaF, 5 mM dithiothreitol, a 1/100 dilution of protease inhibitor cocktail (Calbiochem), and 1% solutions of leupeptin and PMSF. The samples were then centrifuged at 100,000 g for 60 min. The pellet constituted the membrane-particulate fraction, and the particulate-free supernatant fraction was defined as the cytosolic fraction. Each sample containing the particulate fraction was resuspended in homogenizing buffer containing 0.5% Triton X-100 and centrifuged at 100,000 g for 60 min. The resulting detergent-treated supernatant was used in the Western blot analysis. The protein concentration of each sample was determined by the Bradford assay. Cytosolic and membrane proteins were analyzed for PKC isoform content by one-dimensional electrophoresis using 8% SDS-polyacrylamide gels. The samples were then transferred to nitrocellulose membranes, where they were blocked. After incubation with the appropriate antibody, the membranes were washed and then incubated with a secondary antibody, goat anti-rabbit IgG. The Western blots were detected by the enhanced chemiluminescence reaction. All data were analyzed by a computer program, which detected the area under the curve. Gels were also analyzed by Ponceau’s solution to ensure equal protein loading.

Cellular Ca2+ content. Cardiomyocytes were loaded for 20 min with fura-2 using loading buffer (2 ml Krebs buffer with 25 mM HEPES plus 3 mM fura-2 AM and 6 μM Pluronic acid, 37°C). The loading and subsequent rinsing steps took place before the onset of the hypoxic insult. Fura-2 fluorescence at 340 and 380 nm (emission at 510 nm) was measured using an Olympus IX70 inverted microscope at ×400 with a xenon arc lamp photomultiplier system (Photon Technologies; Monmouth Junction, NJ). Data obtained during the course of the hypoxic insult were expressed as the fluorescence ratio (F340/F380), a measure of intracellular Ca2+ concentration ([Ca2+]i). To evaluate the basis for alterations in hypoxia-mediated Ca2+ accumulation, some data were expressed as the difference in fura-2 fluorescence between two experimental conditions (∆F340/380 or ∆F510/380), as described further in RESULTS.

Current-voltage relationship. Conventional whole cell voltage-clamp configurations were used as described previously (39). Briefly, to measure transmembrane currents in single rat cardiomyocytes by the standard gigaseal patch-clamp technique, whole cell recordings were performed. Recording pipettes were made of hemo capillaries (Warner Instruments; Hamden, CT) pulled by a two-stage puller (PC-10, Narishige; Tokyo, Japan) and heat polished with a microforge (model MF-200, World Precision Instruments; Sarasota, FL) before use. Pipette resistance was in the range of 2–5 MΩ when filled with intracellular solution. All experiments were performed at room temperature (22–25°C). An EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht; Pfalz, Germany) was used to acquire data with Pulse/PulseFit software (HEKA) and filtered at 2.9 kHz. Voltage-dependent currents were corrected for linear leak and residual capacitance using an on-line Pin subtraction paradigm; the definition of the so-called Pin leakage correction protocol is as follows: in a voltage range, where voltage-dependent channels are not active, a scaled-down version of the pulse protocol is applied n times, and the resulting current is averaged, scaled, and subtracted from that elicited by the main test pulse. The extracellular (bath) solution contained (in mmol/l) 130 NaCl, 2 CaCl2, 110 tetraethylammonium chloride, 10 CsCl, and 10 HEPES 10 (pH 7.4, adjusted with tetraethanolammonium hydroxide). The intracellular (pipette) solution contained (in mmol/l) 130 N-methyl-d-glucamine, 10 EGTA, 5 BAPTA, 10 HEPES, 6 MgCl2, 4 CaCl2, and 2 Mg-ATP (pH 7.2, adjusted with methane sulfonic acid). All solutions were adjusted to 290 to 300 mosM with sucrose.

Statistical analysis. The statistical significance of the data was determined using the Student’s t-test for comparison within groups and ANOVA combined with Tukey’s post hoc test for comparison between groups. Values of P < 0.05 were considered statistically significant.

RESULTS

Figure 1A shows that chemical hypoxia triggers a time-dependent increase in apoptosis among isolated cardiomyocytes in culture. Before hypoxia, 11.9 ± 1.3% of control and 14.2 ± 1.6% of glucose-treated cardiomyocytes were apoptotic, where control and glucose treatment refer to cells incubated for 3 days with medium containing 5 and 25 mM glucose, respectively. Two hours of chemical hypoxia carried out in the absence of glucose increased the percentage of apoptotic cells in the control and glucose-treated groups to 54.5 ± 5.1% and 33.6 ± 5.5%, respectively. Taking into consideration the number of apoptotic cells found in culture before chemical hypoxia, the 2-h hypoxic insult caused 42.6% of the control cells and 19.4% of the glucose-treated cells to undergo apoptosis. The difference between the two values (23.2%) represents the degree of hyperglycemic preconditioning.
ing, a term used to describe protection arising from prehypoxic (not hypoxic) exposure of cardiomyocytes to medium containing 25 mM glucose. As seen in Fig. 1B, the maximum degree of glucose protection (23.2 ± 1.1%) was achieved after 2 h of chemical hypoxia. However, the addition of ANG II (1.0 nM) to the prehypoxic incubation medium completely blocked the cardioprotective effect of high glucose; note that ANG II was also excluded from the hypoxic medium. This adverse effect of ANG II appears to be largely unrelated to its proapoptotic activity, because ANG II-mediated apoptosis disappears after prolonged periods of hypoxia. While the addition of ANG II to the prehypoxic incubation medium increased the number of apoptotic cells in culture by 8.5% before hypoxia, after 120 min of hypoxia, the extent of hypoxia-induced death was virtually identical in control cells incubated with medium containing ANG II (54.8 ± 6.3%) or those that had been exposed to ANG II during the prehypoxic period (54.5 ± 5.1%).

PKC activation is a key step in ischemic preconditioning (14, 23, 34); therefore, the effect of hyperglycemia on the particulate and cytosolic content of various PKC isoforms was

Fig. 1. Effect of ANG II on hyperglycemic preconditioning. Isolated neonatal cardiomyocytes were incubated for 3 days with medium containing either 5 (control) or 25 mM glucose (glucose). Some of the cells were treated during the last 24 h with medium containing 1 nM ANG II. The cells were then exposed for 45–150 min to medium lacking glucose and ANG II but containing 10 mM deoxyglucose and 3 mM amobarbital. The presence of nuclear end-labeled DNA fragments in these cells after various periods of chemical hypoxia was detected using the TdT-mediated dUTP nick-end labeling (TUNEL) technique. Data are expressed as the percentage of cells undergoing positive brown TUNEL staining, with 15–20 cells examined per field and 4–5 fields examined per preparation. A: time course of hypoxia-mediated increase in TUNEL staining of glucose-treated and control cells. Values shown represent means ± SE of 4–5 different cell preparations. *Significant difference between control and glucose-treated cells (P < 0.05).

Fig. 2. Effect of glucose treatment and ANG II exposure on the distribution of various PKC isoforms. Neonatal rat cardiomyocytes were incubated for 3 days with medium containing 5 (control) or 25 mM glucose. Some of the cells were exposed to medium containing 1 nM ANG II during the last 24 h. On the third day, cells were harvested and separated into cytosolic and membrane fractions by centrifugation. PKC isoform contents of the two fractions were measured by Western blot analysis, with bands detected by the enhanced chemiluminescence reaction after exposure to goat anti-rabbit IgG. A: representative Western blots of PKC isoforms (PKC-α, PKC-β2, PKC-δ, PKC-ε, and PKC-ζ) from cytosolic and membrane fractions. B: relative levels of PKC-α, PKC-β2, PKC-δ, PKC-ε, and PKC-ζ in control and glucose-treated cells. Values shown represent means ± SE of 4–5 preparations. *Significant difference between control and glucose-treated cells (P < 0.05).

C: relative levels of PKC-α, PKC-β2, PKC-δ, PKC-ε, and PKC-ζ in control and glucose-treated cells exposed to ANG II. Values of 1 were assigned to bands corresponding to control PKC-α, control PKC-β2, control PKC-δ, control PKC-ε, and control PKC-ζ. Data shown are means ± SE of 5–6 different preparations. *Significant differences between control and glucose-treated cells exposed to ANG II (P < 0.05).
examined. Figure 2 shows that membrane levels of PKC-β2, PKC-δ, PKC-ε, and PKC-ζ were elevated in cardiomyocytes incubated for 3 days with medium containing elevated glucose (25 mM). Despite fairly substantial increases in the particulate content of some PKC isoforms, such as PKC-β2 and PKC-δ, only small increases in the cytosolic content of these and other PKC isoforms were observed (Fig. 2, A and B). PKC-α was unique, as both the cytosolic and particulate content were reduced in the glucose-treated cells. These data revealed that of the five PKC isoforms examined, only PKC-β2 and PKC-δ preferentially associated with the particulate fraction in response to chronic hyperglycemia.

It has been documented that ANG II promotes the translocation of PKC-δ and PKC-ε from the cytosol to the particulate fraction, where they are activated (25). In accordance with previous studies, it was found that ANG II increased the particulate levels of PKC-δ in both glucose-treated and control cells (Fig. 2, A and C). Exposure of control cells to ANG II led to a 60.2 ± 9.9% increase in particulate PKC-δ while reducing cytosolic PKC-δ content by 34.3 ± 7.7%. By comparison, glucose-treated cells exposed to ANG II exhibited a 96 ± 18% increase in particulate PKC-δ content compared with a 56 ± 11% increase arising from glucose treatment in the absence of ANG II. There was also a net decrease in the cytosolic content of PKC-δ after ANG II exposure (Fig. 2, A and C). Although glucose treatment caused a nearly equal elevation in the cytosolic (20 ± 9%) and particulate (24 ± 8%) content of PKC-δ, ANG II mediated a typical, but muted, translocation reaction (Fig. 2, A and C). In the control cell, ANG II exposure led to a 49.2 ± 8.9% increase in particulate PKC-ε while slightly decreasing cytosolic PKC-ε. By comparison, exposure of the glucose-treated cells to ANG II led to a small increase in the particulate content of PKC-ε (from 24 ± 8% to 39 ± 15%) while decreasing the cytosolic PKC-ε content from 20 ± 9% to 11 ± 7%. These data reveal that ANG II promotes the translocation of both PKC-δ and PKC-ε from the cytosol to the particulate fraction, with both glucose and ANG II having a greater effect on PKC-δ than on PKC-ε. The other PKC isoform that preferentially associates with the particulate fraction in response to glucose treatment is PKC-β2. After a 3-day incubation with medium containing 25 mM glucose, the particulate content of PKC-β2 increased over twofold, whereas the cytosolic content of the isoform was unaffected. ANG II diminished the glucose response, reducing glucose-mediated elevations in particulate PKC-β2 from 130% to 57%. Thus, although ANG II modulated the distribution of several PKC isoforms, it was effective in only partially reversing the effects of hyperglycemia on one PKC isoform, namely, PKC-β2.

The translocation of PKC-ε is considered a key step in ischemic preconditioning and other types of cardioprotection (4, 14, 23, 29, 34). However, its role in hyperglycemic preconditioning remains unclear. One concern is that despite the elevation in particulate levels of PKC-ε, glucose treatment did not promote the translocation of the isoform. Moreover, ANG II caused some translocation of PKC-ε while reversing hyperglycemic preconditioning. Therefore, the potential role of cardioprotective PKC isoforms in glucose-mediated cardioprotection was further examined by evaluating the effectiveness of the PKC inhibitor chelerythrine in blocking hyperglycemic preconditioning. As expected, chelerythrine triggered a concentration-dependent increase in apoptosis of normoxic cells, with the degree of chelerythrine-mediated apoptosis being identical in control and glucose-treated cardiomyocytes during normoxia but not hypoxia. Figure 3 shows the effect of chelerythrine on hypoxia-mediated apoptosis in control and glucose-treated cells after hypoxic data were corrected for the effect of chelerythrine during normoxia. A similar pattern was observed using another PKC inhibitor, calphostin C. In the absence of chelerythrine, 90 min of hypoxia caused ∼29% of the control cells and 11% of the glucose-treated cells to undergo apoptosis, with the difference between the two groups representing the degree of hyperglycemic preconditioning. Chelerythrine had no effect on hypoxia-mediated apoptosis in the control cells. However, it caused a concentration-dependent decrease in the degree of hyperglycemic preconditioning. The Ki for chelerythrine-mediated inhibition of hyperglycemic preconditioning was ∼1.0 μM, whereas the Ki for PKC inhibition is reportedly 0.7–0.8 μM (16), suggesting a role for PKC in hyperglycemic preconditioning.

One apparent paradox of the observed reversal of hyperglycemic preconditioning by ANG II is that the signaling pathway of ANG II requires the activation of PKC, whereas chelerythrine blocks hyperglycemic preconditioning. This could occur if ANG II promoted the translocation of PKC-ε and PKC-δ while activating transporters involved in Ca2+ overload, such as the Na+/H+ exchanger, the Na+/Ca2+ exchanger, and the T-type Ca2+ channel (3, 8, 15). In support of this calcium transport hypothesis, it was found that glucose treatment significantly reduced hypoxia-mediated Ca2+ accumulation, whereas pretreatment with ANG II had the opposite effect (Fig. 4). More importantly, the two effects were nearly additive, with ANG II reversing the beneficial effect of glucose treatment on hypoxia-mediated Ca2+ accumulation (Fig. 4).

Figure 5 shows that two PKC inhibitors, chelerythrine and calphostin C, reduce hypoxia-mediated Ca2+ accumulation in cells treated with both 25 mM glucose and ANG II. A similar reduction in [Ca2+]i was observed with KB-R7943, an inhib-

Fig. 3. Effect of chelerythrine on hyperglycemic preconditioning. Neonatal rat cardiomyocytes were incubated for 3 days with medium containing either 5 (control) or 25 mM glucose. After 48 h, cells from each group were exposed to 0, 0.2, 1.0, 3.5, or 10 μM chelerythrine for 24 h. Cells were then subjected to either 1 h of normoxia or chemical hypoxia, the latter with medium containing 10 mM deoxyglucose and 3 mM amobarbital but no glucose or chelerythrine. The extent of apoptosis was assessed from the number of cells exhibiting positive TUNEL staining. The data were corrected for the effects of chelerythrine during normoxia. Values shown represent means ± SE of 4 preparations.
itor of the Na+/Ca2+ exchanger, a transporter that is regulated by PKC (3). Interestingly, KB-R7943 was nearly as effective as hyperglycemic preconditioning in preventing hypoxia-mediated Ca2+ overload. To further examine the role of the Na+/H+ and Na+/Ca2+ exchangers in hyperglycemic preconditioning, control and hyperglycemic-preconditioned cells were subjected to chemical hypoxia using medium supplemented with either no addition or 10 μM cariporide, a Na+/H+ exchange inhibitor. Figure 6, A and B, shows that glucose-treated cells exhibited less Ca2+ accumulation during hypoxia than control cells. The difference between the fura-2 fluorescence ratios of control and glucose-treated cells, which we designate as ΔF340/380, was used to glean information on the mechanism underlying the hyperglycemia-mediated decline in Ca2+ accumulation during hypoxia. As seen in Fig. 6C, ΔF340/380...
was 0.9 in the absence of cariporide but was reduced to 0.5 in cells treated with cariporide. Because $\Delta F_{340/380}$ is a direct measure of the glucose-mediated attenuation in hypoxia-mediated Ca$^{2+}$ accumulation, the reduction in $\Delta F_{340/380}$ indicates that the Na$^{+}$/H$^+$ exchanger contributes to the improvement in Ca$^{2+}$ homeostasis of the glucose-treated cell.

The T-type Ca$^{2+}$ channel is also a potential source of Ca$^{2+}$ influx; therefore, the effect of glucose treatment on T-type Ca$^{2+}$ channel function was examined. Significantly, a concentration of mibefradil commonly used to specifically block the T-type Ca$^{2+}$ channel (7) was found to be effective in reducing hypoxia-mediated Ca$^{2+}$ accumulation. However, mibefradil is considered a weak inhibitor of the Na$^{+}$/Ca$^{2+}$ exchanger (9). Therefore, to mitigate the concern that some or all of the mibefradil-mediated improvement in hypoxia-mediated Ca$^{2+}$ uptake was caused by reduced flux through the Na$^{+}$/H$^+$ and Na$^{+}$/Ca$^{2+}$ exchangers, the effect of mibefradil was reexamined in control cells treated with the Na$^{+}$/H$^+$ exchange inhibitor cariporide. Although cariporide diminished the response to mibefradil, a significant difference in hypoxia-mediated Ca$^{2+}$ accumulation was noted between the mibefradil plus cariporide group and the cariporide group (Fig. 7). Thus, in the control cell, the T-type Ca$^{2+}$ channel contributes to hypoxia-mediated Ca$^{2+}$ accumulation.

A similar set of experiments was performed to examine T-type Ca$^{2+}$ channel function in glucose-treated cells. After 1 h of hypoxia, the glucose-treated cell exhibited a fura-2 fluorescence ratio of 1.03 compared with a value of 1.93 in the control cell, yielding a difference ($\Delta F_{340/380}$) of 0.9 (Fig. 6, A–C). Inhibition of the Na$^{+}$/H$^+$ exchanger dramatically lowered fura-2 fluorescence of the hypoxic cell, reducing $\Delta F_{340/380}$ to 0.5 (Fig. 8, A–C). The addition of mibefradil to the hypoxic medium caused a further decrease in $\Delta F_{340/380}$ to 0.18 (Fig. 8C). Because cariporide eliminated the contribution of the Na$^{+}$/H$^+$ exchanger dramatically low-

![Fig. 7. Contribution of T-type Ca$^{2+}$ current to hypoxia-mediated Ca$^{2+}$ accumulation. Neonatal rat cardiomyocytes were loaded with fura-2, and fluorescence was monitored at 340 and 380 nm. After a steady-state fura-2 fluorescence was obtained for cells incubated under normoxic conditions, the cells were subjected to chemical hypoxia with medium containing no additions (control), cariporide (10 μM), or the combination of cariporide and the T-type Ca$^{2+}$ channel antagonist mibefradil (0.3 μM). Shown is the time course of the hypoxia-induced rise in $[Ca^{2+}]$, of representative cells.](image-url)
Na\textsuperscript{+}/H\textsuperscript{+} and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers toward hypoxia-mediated Ca\textsuperscript{2+} accumulation, the reduction in \(\Delta F_{340/380}\) by mibebradil implies that the T-type Ca\textsuperscript{2+} channel contributes to the beneficial effects of glucose on [Ca\textsuperscript{2+}]i.

Current-voltage curves provided further support for T-type Ca\textsuperscript{2+} channel involvement in the regulation of Ca\textsuperscript{2+} homeostasis by glucose. As seen in Fig. 9, glucose-treated cells exhibited significantly less T-type Ca\textsuperscript{2+} current than control cells, whereas L-type Ca\textsuperscript{2+} current was unaffected by glucose treatment.

Because ANG II activates Ca\textsuperscript{2+} transporters that are down-regulated in the hyperglycemic-preconditioned cell (3, 8, 15), it seemed logical that exposure of the cardiomyocyte during the prehypoxic period to medium containing 1.0 nM ANG II rendered the cell sensitive to hypoxia-mediated elevations in [Ca\textsuperscript{2+}]i (Fig. 10). After 1 h of hypoxia, the difference in the fura-2 fluorescence ratio between control and ANG II-treated cells (designated \(\Delta F_{340/380}\)) was 2.68. Exposure of both the control and ANG II-treated cells to cariporide (10 \(\mu\)M) during the hypoxic period reduced \(\Delta F_{340/380}\) to 0.66, implying that enhanced flux through the Na\textsuperscript{+}/H\textsuperscript{+} and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers contributed to the elevation in hypoxia-mediated Ca\textsuperscript{2+} accumulation by ANG II. Interestingly, the T-type Ca\textsuperscript{2+} channel antagonist mibebradil (0.3 \(\mu\)M) was as effective as cariporide in inhibiting the ANG II effect (data not shown). More importantly, the combination of mibebradil and cariporide nearly abolished the ANG II effect, as evidenced by the decrease in \(\Delta F_{340/380}\) to 0.14. Because cariporide blocks Ca\textsuperscript{2+} influx via the Na\textsuperscript{+}/H\textsuperscript{+}-Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger combination, the mibebradil-mediated reduction in \(\Delta F_{340/380}\) indicates the involvement of T-type Ca\textsuperscript{2+} current in the elevation of hypoxia-mediated Ca\textsuperscript{2+} influx by ANG II.

The effect of ANG II on the glucose-treated cell reflects the opposing actions of glucose treatment and ANG II on hypoxia-mediated Ca\textsuperscript{2+} accumulation. Therefore, it is not surprising that the potentiation of hypoxia-mediated Ca\textsuperscript{2+} accumulation by ANG II was inhibited by both cariporide and mibebradil (Fig. 11). Because glucose treatment and ANG II have opposing actions on flux through the individual transporters involved in hypoxia-mediated Ca\textsuperscript{2+} accumulation, it follows that the effects of cariporide and mibebradil on [Ca\textsuperscript{2+}]i are significantly diminished in the glucose plus ANG II group compared with the ANG II group (Figs. 10 vs. 11).

The ability of mibebradil and cariporide to reverse the adverse effects of ANG II on glucose-mediated cardioprotection was then examined. As seen in Fig. 12, both the T-type Ca\textsuperscript{2+} channel inhibitor and the Na\textsuperscript{+}/H\textsuperscript{+} exchanger inhibitor diminished hypoxia-induced apoptosis in glucose-treated cells exposed to ANG II. However, neither drug affected hypoxia-induced apoptosis in the absence of ANG II.

**DISCUSSION**

In the present study, the effect of ANG II on hyperglycemic preconditioning was examined using isolated cardiomyocytes...
exposed to medium containing high concentrations of glucose. According to Davidoff and co-workers (5, 31), isolated cardiomyocytes incubated with medium containing 25 mM glucose are converted into a diabetes-like phenotype, characterized by impaired excitation-contraction coupling with reduced SERCA function. These defects appear to be related in part to ANG II, because AT1 receptor blockade prevents glucose-induced cardiac dysfunction (30). Because NADPH oxidase inhibition also blocks hyperglycemia-mediated contractile dysfunction, it has been proposed that AT1 receptor-mediated NADPH oxidase activation and the generation of reactive oxygen species are involved in hyperglycemia-induced cardiomyocyte dysfunction (30). There is also evidence that ANG II might cause glucose-mediated alterations in troponin phosphorylation and myofilament Ca\(^{2+}\) sensitivity (25). The present finding that ANG II adversely affects the response of the glucose-treated cardiomyocyte to chemical hypoxia adds to the long list of adverse effects mediated by ANG II.

The present study supports a role for both PKC and improved Ca\(^{2+}\) handling in hyperglycemic preconditioning. PKC-\(\beta_2\) is a potential candidate for the dependence of hyperglycemic preconditioning on PKC. Not only are the levels of PKC-\(\beta_2\) elevated in the diabetic heart (13, 24), but PKC-\(\beta_2\) has been implicated in the development of the diabetic cardiomyopathy.
antagonist mibefradil (0.3 M). PKC-ε fraction contributes to ischemic preconditioning and other upregulation of PKC-ε/H9252 system and enhanced calcium influx. In light of these actions, cemic preconditioning: the activation of the renin-angiotensin pression mediates two events that adversely impact hypergly-

There is substantial evidence that the translocation of one of the PKC isoforms, PKC-ε, from the cytosol to the particulate fraction contributes to ischemic preconditioning and other forms of cardioprotection (4, 14, 23, 29, 34). Not only does PKC-ε translocate to the membrane in response to ischemic preconditioning (29), but downregulation of PKC-ε through gene disruption or use of a selective PKC-ε antagonist blocks ischemic/hypoxic preconditioning (14, 34). Moreover, overexpression of PKC-ε protects the ischemic heart while leaving H+ generation unchanged (4). However, a role for PKC-ε in hyperglycemic preconditioning is less clear. Levels of PKC-ε in the particulate fraction of the glucose-treated cardiomyocyte were elevated relative to those of the control cell, but the 24% increase in particulate PKC-ε content occurred without the translocation of the isoform from the cytosol to the membrane fraction. Moreover, ANG II modestly promoted the translocation of PKC-ε from the cytosol to the particulate fraction in the glucose-treated cell despite reversing hyperglycemic preconditioning.

Cell signaling initiated by ANG II is dependent on the activation of PKC (32). Therefore, the substantial translocation of PKC-δ from the cytosol to the particulate fraction is likely an important event. Indeed, both glucose treatment and ANG II increase particulate levels of PKC-δ; however, the increase in particulate PKC-δ by ANG II is associated with a decrease in cytosolic levels of the isoform, whereas glucose treatment elevates both the particulate and cytosolic content of PKC-δ. Thus ANG II promotes the translocation of PKC-δ from the cytosol to the particulate fraction, whereas glucose treatment primarily enhances the expression of PKC-δ, causing only a modest shift in the localization of PKC-δ to the particulate fraction. The significance of elevated particulate PKC-δ content on the hypoxic cardiomyocyte remains controversial. According to Zhao and Kukreja (41) and Miyawaki et al. (26), PKC-δ exerts cardioprotective activity, whereas Murriel and Mochly-Rosen (28) feel that PKC-δ promotes cell damage. Nonetheless, the study by Murriel and Mochly-Rosen (28) using state-of-the-art techniques provides compelling evidence linking PKC-δ to cardiac injury. Assuming PKC-δ worsens ischemic injury, as reported by Murriel and Mochly-Rosen (28), it is possible that glucose-mediated elevations in PKC-δ levels act to minimize the extent of hyperglycemic preconditioning. Moreover, it would be attractive to suggest that a downstream effector of PKC-δ might be involved in the reversal of hyperglycemic preconditioning by ANG II.

One of the unique and extremely important cardioprotective mechanisms in the diabetic heart is the improvement in Ca2+ homeostasis. Tani and Neely (36) made the initial discovery that diabetic hearts were resistant to ischemia-mediated Ca2+ accumulation. This improvement was subsequently traced in part to a diabetes-linked reduction in Na+/H+ exchanger activity (22). In a related study, Schaffer et al. (35) found that the glucose-treated cardiomyocyte was also resistant to hypoxia-induced injury, an effect associated with an improvement in Na+ and Ca2+ handling. The present study provides additional lines of evidence supporting the notion that hyperglycemic preconditioning depends on transporters involved in hypoxia-mediated Ca2+ accumulation. First, glucose treatment renders the cell resistant to hypoxia-mediated cell death while lowering [Ca2+]. Second, glucose treatment reduces the activity of two transporters, the Na+/H+ exchanger and the T-type Ca2+ channel, which contribute to hypoxia-mediated Ca2+ accumulation. Third, inhibitors of the Na+/H+ exchanger and the T-type Ca2+ channel protect the control cell against hypoxia-mediated Ca2+ accumulation and cell death. Fourth, ANG II reverses hyperglycemic preconditioning while elevating [Ca2+]. Finally, inhibitors of the Na+/H+ exchanger and the T-type Ca2+ channel prevent reversal of hyperglycemic preconditioning by ANG II.

Although the combined actions of the Na+/H+ and Na+/ Ca2+ exchangers have been touted as a major source of hypoxia-mediated Ca2+ accumulation (20), a role for the T-type Ca2+ channel in Ca2+ overload has received little attention. Yet, in the present study, the T-type Ca2+ channel antagonist mibefradil was found to be a potent inhibitor of hypoxia-mediated Ca2+ accumulation. Some of mibefradil’s effect have been attributed to its weak inhibition of the Na+/ Ca2+ exchanger; nonetheless, we found that the T-type Ca2+ channel antagonist kurtoxin reduces hypoxia-mediated Ca2+ accumulation without affecting the activity of the Na+/Ca2+ exchanger (data not shown). Moreover, mibefradil significantly reduces hypoxia-mediated Ca2+ accumulation in cells exposed to cariporide. Therefore, in rat neonatal cardiomyocytes, the T-type Ca2+ channel is an important contributor to hypoxia-mediated Ca2+ accumulation.
Glucose-treated cells show a reduction in hypoxia-mediated Ca$^{2+}$ accumulation that is only partially related to reduced Na$^+$/Ca$^{2+}$ exchanger activity. In the present study, the difference in the fura-2 fluorescence ratio between control and hyperglycemic-preconditioned cells ($\Delta F_{340/380}^{340/380}$) was about 0.9. In the presence of cariporide, which blocks hypoxygenated Ca$^{2+}$ influx via the Na$^+$/H$^+$-Na$^+$/Ca$^{2+}$ exchanger combination, mibefradil reduced $\Delta F_{340/380}^{340/380}$ from 0.5 to 0.18. These data show that glucose-mediated reductions in T-type Ca$^{2+}$ current also contribute to the reduction in [Ca$^{2+}$]$_i$ of the hypoxic cell.

In contrast to glucose treatment, exposure of the control cell to ANG II during the prehypoxic period promotes hypoxygenated Ca$^{2+}$ accumulation. On the basis of $\Delta F_{340/380}^{340/380}$, the fluorescence difference between the control and the ANG II-treated cell, ANG II elevates [Ca$^{2+}$]$_i$ of the hypoxic cell by enhancing Ca$^{2+}$ influx via the Na$^+$/H$^+$-Na$^+$/Ca$^{2+}$ exchanger combination and the T-type Ca$^{2+}$ channel.

ANG II also elevates hypoxygenated Ca$^{2+}$ accumulation by the glucose-treated cell. Indeed, the ANG II-mediated increase in $F_{340/380}$ of the control and glucose-treated cells is comparable, indicating that glucose treatment has no effect on ANG II signaling. Therefore, it is not surprising that exposure of the glucose-treated cell to ANG II prevents hyperglycemic preconditioning. Several lines of evidence support the notion that Ca$^{2+}$ influx via the two exchangers and the T-type Ca$^{2+}$ channel play crucial roles in the reversal of hyperglycemic preconditioning by ANG II. First, ANG II reverses hyperglycemic preconditioning while activating transporters that enhance hypoxygenated-elevated elevations in [Ca$^{2+}$]$_i$ (Figs. 1, 10, and 11). Second, glucose treatment promotes cardioprotection while reducing hypoxygenated-Ca$^{2+}$ influx via the exchanges and the T-type Ca$^{2+}$ channel (Figs. 1, 6, and 8). Third, the T-type Ca$^{2+}$ channel antagonist mibefradil and the Na$^+$/H$^+$ exchange inhibitor cariporide reduce hypoxygenated-Ca$^{2+}$ accumulation in glucose-treated cells exposed to ANG II (Figs. 6 and 8). Finally, mibefradil and cariporide partially block the increase in hypoxygenated-apoptosis arising from ANG II exposure (Fig. 12).

The present study may have pathological significance. Recently, Mozafari and Schaffer (27) proposed that ANG II was a likely regulator of infarct size in the diabetic preconditioned heart. This hypothesis was based on the finding that elevations in afterload reversed prediabetic preconditioning. Because a major consequence of elevated afterload is cell stretching, which in turn causes the release of ANG II from the heart (33), the investigators proposed that ANG II was a logical candidate for the adverse effects of elevated afterload, particularly because ANG II activates Ca$^{2+}$ transporters involved in hypoxygenated-Ca$^{2+}$ overload.

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