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Propofol Decreases Myofilament Ca^{2+} Sensitivity via *a Protein Kinase C–, Nitric Oxide Synthase–dependent Pathway in Diabetic Cardiomyocytes*

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Background: **The authors' objective was to assess the role of protein kinase C (PKC) and nitric oxide synthase (NOS) in mediating the effects of propofol on diabetic cardiomyocyte contractility, intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), and myofilament Ca^{2+} sensitivity.**

Methods: **Freshly isolated ventricular myocytes were obtained from normal and diabetic rat hearts. $[Ca^{2+}]_i$ and cell shortening were simultaneously measured in electrically stimulated, ventricular myocytes using fura-2 and video-edge detection, respectively. Actomyosin adenosine triphosphatase activity and troponin I (TnI) phosphorylation were assessed in [^{32}P]orthophosphate-labeled myofibrils. Western blot analysis was used to assess expression of PKC and NOS.**

Results: **Propofol (10 μM) decreased peak shortening by 47 ± 6% with little effect on peak $[Ca^{2+}]_i$ (92 ± 5% of control) in diabetic myocytes. Maximal actomyosin adenosine triphosphatase activity was reduced by 43 ± 7% and TnI phosphorylation was greater (32 ± 6%) in diabetic myofibrils compared with normal. Propofol reduced actomyosin adenosine triphosphatase activity by 17 ± 7% and increased TnI phosphorylation in diabetic myofibrils. PKC inhibition prevented the propofol-induced increase in TnI phosphorylation and decrease in shortening. Expression of PKC-α, PKC-δ, PKC-ε, and constitutive NOS were up-regulated and inducible NOS was expressed in diabetic cardiomyocytes. NOS inhibition attenuated the propofol-induced decrease in shortening.**

Conclusion: **Myofilament Ca^{2+} sensitivity and, to a lesser extent, peak $[Ca^{2+}]_i$ are decreased in diabetic cardiomyocytes. Increases in PKC and NOS expression in combination with TnI phosphorylation seem to contribute to the decrease in $[Ca^{2+}]_i$ and myofilament Ca^{2+} sensitivity. Propofol decreases $[Ca^{2+}]_i$ and shortening *via* a PKC-, NOS-dependent pathway.**

DIABETES-INDUCED cardiac dysfunction, commonly called diabetic cardiomyopathy, is characterized by a decrease in myocardial performance independent of vascular disease. It is now widely appreciated that the incidence of death resulting from acute myocardial infarction is significantly greater in people with diabetes. However, the fundamental cellular mechanisms underlying diabetic cardiomyopathy have not been fully elucidated. Furthermore, induction of anesthesia typically results in a decrease in cardiac performance in patients with and without cardiac disease.

Protein kinase C (PKC) is an important signal transduction molecule involved in the regulation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), myofilament Ca^{2+} sensitivity, and contractility of cardiac muscle cells. PKC exists as a family of isoforms, including the conventional PKCs (α, β_1, β_2, γ), novel PKCs (δ, ε, η, θ), and atypical PKCs (λ, ζ).[1] Previous studies have identified that the prominent PKC isoforms expressed in the adult rat heart include PKC-α, PKC-δ, PKC-ε, and PKC-ζ, with a few studies demonstrating the presence of PKC-β_2.[2–4] The roles of the individual isoforms in mediating cellular mechanisms of regulation in the heart are a subject of great debate.

In this study, we assessed the extent to which propofol alters $[Ca^{2+}]_i$, myofilament Ca^{2+} sensitivity, and contractility of cardiomyocytes isolated from diabetic rat hearts. Our major findings are that $[Ca^{2+}]_i$ and myofilament Ca^{2+} sensitivity are reduced in diabetic cardiomyocytes compared with normal cardiomyocytes. Moreover, clinically relevant concentrations of propofol cause a modest reduction in $[Ca^{2+}]_i$ and a marked reduction in myofilament Ca^{2+} sensitivity. The alterations in $[Ca^{2+}]_i$ and myofilament Ca^{2+} sensitivity, in the diabetic cardiomyocyte and following exposure to propofol, are mediated *via* up-regulation and activation of PKC and nitric oxide synthase (NOS).

Materials and Methods

All experimental procedures and protocols were approved by The Cleveland Clinic Foundation Institutional Animal Care and Use Committee (Cleveland, Ohio).

Induction of Experimental Diabetes

Adult, male, Sprague-Dawley rats (6 weeks old) were used for the study. Diabetes was induced by a single intraperitoneal injection of streptozotocin (55 mg/kg). Age-matched controls were injected with the vehicle only (0.1N sodium citrate, pH 4.5). The development of diabetes was assessed by biweekly measurements of urine glucose and ketone using Keto-Diastix (Baxter Scientific, McGaw Park, IL). Animals were maintained with free access to food and water for 12 weeks after streptozotocin administration. When the rats were killed,

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blood glucose levels were assessed using a glucometer (One Touch II; Lifescan, Milpitas, CA).

Ventricular Myocyte Preparation

Freshly isolated adult ventricular myocytes from rat hearts were obtained as previously described.[5,6] Immediately after death, the hearts were rapidly removed and perfused in a retrograde manner at a constant flow rate (8 ml/min) with oxygenated (95% oxygen–5% carbon dioxide) Krebs-Henseleit buffer (37°C) containing the following: 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 37.5 mM $NaHCO_3$, and 16.5 mM dextrose, pH 7.35. After a 5-min equilibration period, the perfusion buffer was changed to a Ca^{2+}-free Krebs-Henseleit buffer containing collagenase type II (309 U/ml). After digestion with collagenase (20 min), the ventricles were minced and shaken in Krebs-Henseleit buffer, and the resulting cellular digest was washed, filtered, and resuspended in phosphate-free HEPES-buffered saline (23°C) containing the following: 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, 11.0 mM dextrose, 25.0 HEPES mM, and 5.0 mM pyruvate, pH 7.35.

Measurement of $[Ca^{2+}]_i$ and Shortening

Simultaneous measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and cell shortening was performed, as previously described by our laboratory.[7] Ventricular myocytes exhibiting a rod-shaped appearance with clear striations were chosen for study. Myocytes (0.5×10^6 cells/ml) were incubated in HEPES-buffered saline containing 2 μM fura-2–acetoxy methyl ester at room temperature for 15 min. Fura-2–loaded ventricular myocytes were placed in a temperature-regulated (30°C) chamber (Bioptechs, Inc., Butler, PA) mounted on the stage of an Olympus IX-70 inverted fluorescence microscope (Olympus America, Lake Success, NY). The cells were superfused continuously with HEPES-buffered saline at a flow rate of 2 ml/min and field-stimulated *via* bipolar platinum electrodes at a frequency of 0.3 Hz with a 5-ms pulse using a Grass SD9 stimulator (Grass Instruments, West Warwick, RI).

Fluorescence measurements were performed on individual myocytes using a dual-wavelength spectrofluorometer (Deltascan RFK6002; Photon Technology International, Lawrenceville, NJ) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Because calibration procedures rely on a number of assumptions, the ratio of the light intensities at the two wavelengths was used to measure qualitative changes in $[Ca^{2+}]_i$. Just before data acquisition, background fluorescence was measured and automatically subtracted from the subsequent experimental measurement. The fluorescence sampling frequency was 100 Hz, and data were collected using software from Photon Technology International.

To simultaneously monitor cell shortening, the cells were also illuminated with red light. A dichroic mirror (600-nm cutoff) in the emission path deflected the cell image through a charge-coupled device video camera (Phillips VC 62505T; Marshall Electronics, Culver City, CA) into a video-edge detector (Crescent Electronics, Sandy, UT) with 16 ms resolution. The video-edge detector was calibrated using a stage micrometer so that cell lengths during shortening and relengthening could be measured.

Analysis of $[Ca^{2+}]_i$ and Shortening Data

The following variables were calculated for each individual contraction: resting $[Ca^{2+}]_i$ and cell length; peak $[Ca^{2+}]_i$ and cell length; change in $[Ca^{2+}]_i$ (peak $[Ca^{2+}]_i$ minus resting $[Ca^{2+}]_i$) and twitch amplitude; time to peak for $[Ca^{2+}]_i$ and shortening and time to 50% resting $[Ca^{2+}]_i$ and relengthening. Variables from 10 contractions were averaged to obtain mean values at baseline and in response to the various interventions. Averaging the variables over time minimizes beat-to-beat variation.

Preparation of Cardiac Myofibrils

Cardiomyocytes were washed twice in ice-cold HEPES-buffered saline containing protease and phosphatase inhibitors and pelleted at 400*g* for 3 min, after which an equivalent volume of extraction buffer, 50 mM Tris (pH 7.5) containing Triton X-100 (0.1%), NaF (20 mM), dithiothreitol (0.5 mM), $MgCl_2$ (0.5 mM), EDTA (0.125 mM), antipain (5 μg/ml), leupeptin (10 μg/ml), pepstatin A (5 μg/ml), and paramethylsulfonic acid (43 μg/ml), was added to the suspension. The cells were homogenized and kept on ice for 30 min. The Triton-extracted myofibrils were pelleted at 10,000*g* (5 min, 4°C). The detergent solubilized supernatant was set aside, and the pellet was resuspended in an equivalent volume of extraction buffer and washed twice again. The resultant myofibrillar fraction was resuspended in Ca^{2+}-free extraction buffer and stored at −20°C. Examination of the pellet under the microscope indicated that it was enriched in myofibrils.

Actomyosin ATPase Activity

The Ca^{2+}-stimulated actomyosin adenosine triphosphatase (ATPase) activity of the myofibrillar fraction was measured from the rate of decrease of reduced nicotinamide adenine dinucleotide absorbance (340 nm excitation wavelength) in a reaction coupled to the pyruvate kinase and lactate dehydrogenase reactions, as previously described.[5] Triton X-100 extracted myofibrils were solubilized in a buffer containing the following: 25 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (pH 7.4), 85 mM potassium methanol sulfonic acid, 3 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 0.5 mM dithiothreitol, and 0.5 mM leupeptin. The protein concentration of the extracted myofibrils was determined using the Bradford

protein assay.[8] The reaction mixture consisted of the following: 25 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (pH 7.0), 2.7 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 126 mM potassium methanol sulfonic acid, and varying free $CaCl_2$ concentrations giving free Ca^{2+} concentrations from pCa 9 to 4. The reaction mixture also contained 200 mM phosphoenolpyruvate, 10 mM reduced nicotinamide adenine dinucleotide, 0.5 mg/ml lactate dehydrogenase, 12.5 mg/ml pyruvate kinase, and 1 ml of one of the calcium buffers (pCa 4–9) containing myofibrillar fractions. The reaction was initiated by addition of 2 mM adenosine triphosphate and allowed to continue for up to 10 min (37°C), although the reaction was usually complete within 5 min. Ca^{2+}-stimulated actomyosin ATPase activity was monitored by the formation of adenosine diphosphate, coupled to the oxidation of reduced nicotinamide adenine dinucleotide, and recorded by the change in absorption at 340 nm. The enzyme activity was determined from the rate of adenosine triphosphate hydrolysis and expressed as the percent of maximal actomyosin ATPase activity per milligram of protein.

Labeling of Myofibrils in Intact Ventricular Myocytes with [^{32}P]Orthophosphate

Phosphorylation of myofibrils in rat ventricular myocytes by [^{32}P]orthophosphate ($^{32}P_i$) was performed as previously described.[5] Freshly isolated ventricular myocytes were suspended in 12 ml phosphate-free HEPES-buffered saline (5×10^5 cells/ml) and incubated with 250 μCi $^{32}P_i$ for 2 h at room temperature. The cell suspension was gently agitated and maintained under an oxygen hood throughout the duration of the $^{32}P_i$-labeling period. There was no significant loss in myocyte viability (rod-shaped cells) after the 2-h labeling period with $^{32}P_i$. After labeling, the cells were centrifuged (400 rpm) and resuspended in phosphate-free Krebs-Henseleit buffer, divided into 2-ml aliquots and placed in test tubes. To ensure that the cells remained viable and maximally oxygenated throughout the incubation, all tubes were purged with 100% oxygen, then placed on their sides and gently agitated for 10 min at 37°C in a shaking water bath. Myocyte viability remained greater than 75% after the 10-min incubation at 37°C in the presence of propofol and bisindolylmaleimide I. After the incubation period, the reactions were terminated, and interventions were removed by rapidly washing the myocytes in saline (4°C, 5 ml) containing protease inhibitors (5 μ/ml pepstatin A, 10 μg/ml leupeptin, 43 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml antipain, and 5 mM EGTA) and a protein phosphatase inhibitor (0.1 μM sodium orthovanadate) immediately before suspending the pellet in 2 ml ice-cold "inhibiting buffer" (50 mM KH_2PO_4, 70 mM NaF, and 5 mM EDTA), as described by Holroyde *et al.*[9] Myofibrils were extracted on ice for 1 h by adding 1% Triton X-100 plus the protease and phosphatase inhibitors listed above to the inhibiting buffer. Detergent-extracted myofibrils were centrifuged at 5,000*g* (5 min) using a microcentrifuge. The supernatant was discarded. Examination of the pellet under the light microscope indicated that it was enriched in myofibrils.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of $^{32}P_i$-labeled cardiac myofibrils was performed on 12% slab gels, as previously described.[5] The myofibrillar pellet was solubilized and denatured in a sample preparation buffer containing 4% sodium dodecyl sulfate, 0.23 M 2-mercaptoethanol, and 0.2 M Tris(hydroxymethyl)aminoethane HCl (pH 6.5) at 100°C for 5 min. To standardize loading of the gels, protein determinations of the myofibrillar extracts were performed on each of the samples before loading the gel. Protein, 75 μg, protein was applied to each lane, and electrophoresis was performed in the presence of 0.1% sodium dodecyl sulfate at constant current (10 mAmp) overnight (16 h). Gels were dried the following day and subjected to phosphor screen autoradiography for quantitation of labeled proteins. A phosphorImager equipped with a commercial software package (Molecular Dynamics, Piscataway, NJ) was used to quantify the extent of $^{32}P_i$ incorporation into individual protein bands

Immunoblot Analysis of PKC and NOS Isoforms

Immunoblot analysis was performed on cardiomyocyte whole cell lysates as previously described.[5] Protein concentration was assessed using the Bradford method.[8] All samples were adjusted to a protein concentration of 1–2 mg/ml in sample buffer, boiled for 5 min, and then kept at −20°C until use. Equal amounts of protein (50 μg) from each fraction were electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific binding was blocked with Tris-buffered saline solution (0.1%; vol/vol) Tween-20 in 20 mM Tris base, 137 mM NaCl adjusted to pH 7.6 with HCl, containing 3% (wt/vol) bovine serum albumin for 1 h at room temperature. Monoclonal and polyclonal antibodies against PKC-α, PKC-δ, PKC-ε, PKC-ζ, constitutive NOS, and inducible NOS were diluted 1:250–1:1,000 in Tris-buffered saline solution containing 1% bovine serum albumin for immunoblotting (2 h). After washing in Tris-buffered saline solution three times (10 min each), filters were incubated for 1 h at room temperature with horseradish peroxidase–linked secondary antibody (ovine anti-mouse; 1:5,000 dilution in Tris-buffered saline solution containing 1% bovine serum albumin). Filters were again washed and bound antibody detected by the enhanced chemiluminescence method. Protein content was analyzed *via* densitometry using NIH Image (National Institutes of Health, Bethesda, MD).

Experimental Protocols

Protocol 1: Effect of Diabetes on $[Ca^{2+}]_i$ and Shortening. To confirm that 12 weeks of diabetes caused abnormalities in cardiomyocyte function, we examined the extent to which $[Ca^{2+}]_i$ and shortening were altered in diabetic cardiomyocytes compared with normal cardiomyocytes. Cardiomyocytes obtained from normal and diabetic rat hearts were isolated on the same day. Individual cardiomyocytes of similar resting cell length from normal and diabetic hearts were electrically stimulated at 0.5 Hz. Summarized results for peak $[Ca^{2+}]_i$ and shortening are expressed as a percent of control (normal).

Protocol 2: Effect of Propofol on $[Ca^{2+}]_i$ and Shortening. A stock solution of propofol was obtained by dissolving the drug in dimethyl sulfoxide. Baseline measurements were collected from individual diabetic cardiomyocytes for 1.5 min in the absence of any intervention. Cardiomyocytes were exposed to three concentrations of propofol (1, 10, 100 μM) by exchanging the buffer in the dish with new buffer containing propofol at the desired concentration. Data were acquired for 1.5 min after a 5-min equilibration period in the presence of propofol. Summarized results for the concentration–response curves are expressed as a percent of the control value. Dimethyl sulfoxide (0.05% vol/vol) alone has no effect on $[Ca^{2+}]_i$ or shortening. We verified that propofol had no effect on fura-2 fluorescence at the concentrations tested. This was confirmed in separate cell-free experiments using fura-2 (pentapotassium salt) in buffers ranging from pCa 9 to 5 in the presence or absence of propofol (data not shown).

Protocol 3: Effect of Propofol on Myofilament Ca^{2+} Sensitivity and Actomyosin ATPase Activity. Changes in actomyosin ATPase activity were assessed in myofibrils isolated from control and propofol-treated (10 min) cardiomyocytes obtained from normal and diabetic rat hearts. The myocyte suspension was divided into separate aliquots, and each aliquot was treated with or without propofol (10 μM; 10 min) at 37°C with gentle agitation. Propofol had no effect on extracellular pH and did not seem to alter the assay conditions. Actomyosin ATPase activity is expressed as a percent of the maximum rate per milligram of protein.

Protocol 4: Effect of Propofol and PKC Inhibition on Phosphorylation of TnI. Changes in $^{32}P_i$-orthophosphate incorporation into the myofibrillar protein troponin I (TnI) were assessed in myofibrils isolated from control and propofol-treated cardiomyocytes obtained from normal and diabetic hearts. The myocyte suspension was divided into separate aliquots, and each aliquot was treated with propofol (10 μM) alone or pretreatment with the broad-range PKC inhibitor bisindolylmaleimide I (1 μM) for 10 min before addition of propofol at 37°C with gentle agitation. Myofibrillar proteins were isolated and subjected to polyacrylamide gel electrophoresis and phosphor screen autoradiography for quantitation of the incorporated $^{32}P_i$ into contractile proteins. Summarized data are expressed as percent of control (baseline phosphorylation).

Protocol 5: Effect of Diabetes on PKC and NOS Isoform Expression. Immunoblot analysis of PKC-α, PKC-δ, PKC-ε, PKC-ζ, constitutive NOS, and inducible NOS was performed on cardiomyocyte whole cell lysates obtained from normal and diabetic hearts. Rat brain lysate was used as a positive control for the PKC isoforms. Endothelial cell lysate was used as positive controls for constitutive NOS and inducible NOS, respectively.

Protocol 6: Effect OF PKC and NOS Inhibition on Propofol-induced Decreases in $[Ca^{2+}]_i$ and Shortening. Individual cardiomyocytes obtained from diabetic hearts were continuously perfused with or without bisindolylmaleimide I (1 μM) or the NOS inhibitor nitro-L-arginine methyl ester (100 μM) before exposure to propofol (10 μM). Summarized results are expressed as a percent of the control value.

Statistical Analysis

All experimental protocols were repeated in myocytes obtained from at least five different hearts. Results obtained from each heart were averaged so that all hearts were weighted equally. Within group comparisons were made using one-way analysis of variance with repeated measures and the Bonferroni *post hoc* test. Comparisons between groups were made using two-way analysis of variance. Differences were considered statistically significant at $P < 0.05$. All results are expressed as mean ± SD.

Materials

Collagenase was obtained from Worthington Biochemical Corp. (Lakewood, NJ). Polyclonal antibodies for PKC-δ and PKC-ζ and a monoclonal antibody against PKC-α were purchased from Upstate Biotechnology (Charlottesville, VA). A monoclonal antibody for PKC-ε was obtained from Becton Dickinson Biosciences (San Diego, CA). Streptozotocin, propofol, bisindolylmaleimide I, and nitro-L-argine methyl ester were purchased from Sigma Chemical Co. (St. Louis, MO). The mammalian protein extraction reagent was purchased from Pierce (Rockford, IL). Fura-2/AM was obtained from Texas Fluorescence Labs (Austin, TX).

Results

Effect of Streptozotocin on Blood Glucose Levels

Diabetic animals (n = 27; 12 weeks diabetic) had blood glucose levels of 438 ± 44 mg/dl and body weights of 311 ± 27 g at the time of euthanasia. Control animals injected with vehicle only (n = 23) had blood glucose levels of 89 ± 12 mg/dl and weighed 437 ± 32 g.

Effect of Diabetes on $[Ca^{2+}]_i$ and Shortening

A representative trace depicting the effect of 12 weeks of diabetes on peak $[Ca^{2+}]_i$ and shortening of individual

cardiomyocytes is shown in figure 1A. Compared with cardiomyocytes isolated from normal rats, shortening of diabetic cardiomyocytes was reduced by 28 ± 5% (P = 0.006), whereas peak $[Ca^{2+}]_i$ was attenuated by 17 ± 6% (P = 0.013). To more clearly illustrate changes in the timing variables, the data in figure 1A were normalized to peak height (fig. 1B). Time to peak shortening was prolonged from 161 ± 17 to 258 ± 21 ms (P = 0.004), whereas time to peak $[Ca^{2+}]_i$ was unaltered (111 ± 15 *vs.* 116 ± 13 ms; not significant, P = 0.179). Time to 50% relengthening from peak shortening was prolonged from 115 ± 14 to 201 ± 16 ms (P = 0.005). Similarly, time to 50% baseline $[Ca^{2+}]_i$ from peak $[Ca^{2+}]_i$ was prolonged from 170 ± 6 to 240 ± 13 ms (P = 0.001). Summarized data depicting the effect of diabetes on peak $[Ca^{2+}]_i$ and shortening are summarized in figure 1C.

Effect of Propofol on $[Ca^{2+}]_i$ and Shortening

A representative trace depicting the dose-dependent effect of propofol on peak $[Ca^{2+}]_i$ and shortening in a diabetic cardiomyocyte are shown in figure 2A. Addition of propofol (10 μM) resulted in a depression in peak shortening of 47 ± 12% (P = 0.001), with little effect on peak $[Ca^{2+}]_i$ (95 ± 6% of control; not significant). In contrast, 10 μM propofol had no effect on peak shortening (97 ± 4% of control; not significant) or peak $[Ca^{2+}]_i$ (98 ± 5% of control; not significant) in normal myocytes. Time to peak $[Ca^{2+}]_i$ was unaltered by propofol (118 ± 9 ms, normal *vs.* 121 ± 14 ms, diabetic), nor was time to peak shortening (170 ± 14 ms, normal *vs.* 270 ± 15 ms, diabetic). Propofol prolonged time to 50% $[Ca^{2+}]_i$ (288 ± 11 ms; P = 0.006) and relengthening (249 ± 14 ms; P = 0.003) in diabetic cardiomyocytes. The summarized data for the effect of propofol on $[Ca^{2+}]_i$ and shortening in diabetic and normal cardiomyocytes are shown in figure 2B. In normal cardiomyocytes, propofol only attenuated peak $[Ca^{2+}]_i$ and shortening at the highest concentration tested. Moreover, the inhibitory effect of propofol on shortening was greater in diabetic compared with normal cardiomyocytes at all concentrations tested (fig. 2).

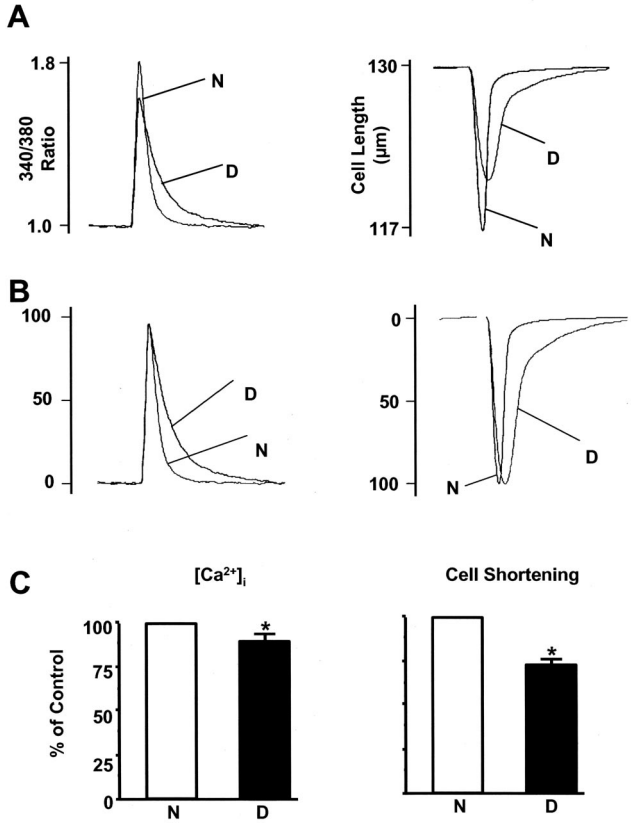

Fig. 1. (*A*) Overlay of typical traces depicting differences in intracellular free Ca^{2+} concentration (340/380 Ratio) and shortening in individual normal (N) and diabetic (D) cardiomyocytes. (*B*) Same as *A*, but normalized to peak height to illustrate changes in timing. (*C*) Summarized data. Values represent mean ± SD in this and all subsequent figures. * P < 0.05 *versus* N. n = 24 cells from six hearts for N; n = 35 cells from eight hearts for D. $[Ca^{2+}]_i$ = intracellular free Ca^{2+} concentration.

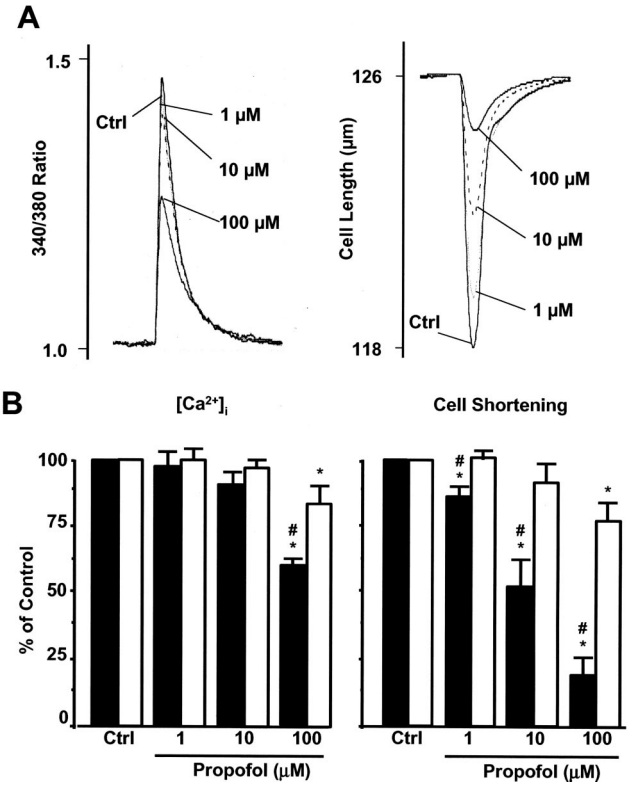

Fig. 2. (*A*) Overlay of typical traces depicting the dose-dependent effects of propofol (1, 10, 100 μM) on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and shortening in an individual diabetic cardiomyocyte. (*B*) Summarized data for the effects of propofol on $[Ca^{2+}]_i$ and shortening in diabetic (*filled bars*) and normal (*open bars*) cardiomyocytes. * P < 0.05 *versus* control (Ctrl). # P < 0.05 *versus* normal at the same concentration of propofol. n = 20 cells from five hearts (diabetic); n = 12 cells from four hearts (normal).

Effect of Propofol on Myofilament Ca^{2+} Sensitivity and Actomyosin ATPase Activity

We assessed the effects of propofol (10 μM) on myofibrillar actomyosin ATPase activity in myofibrils isolated

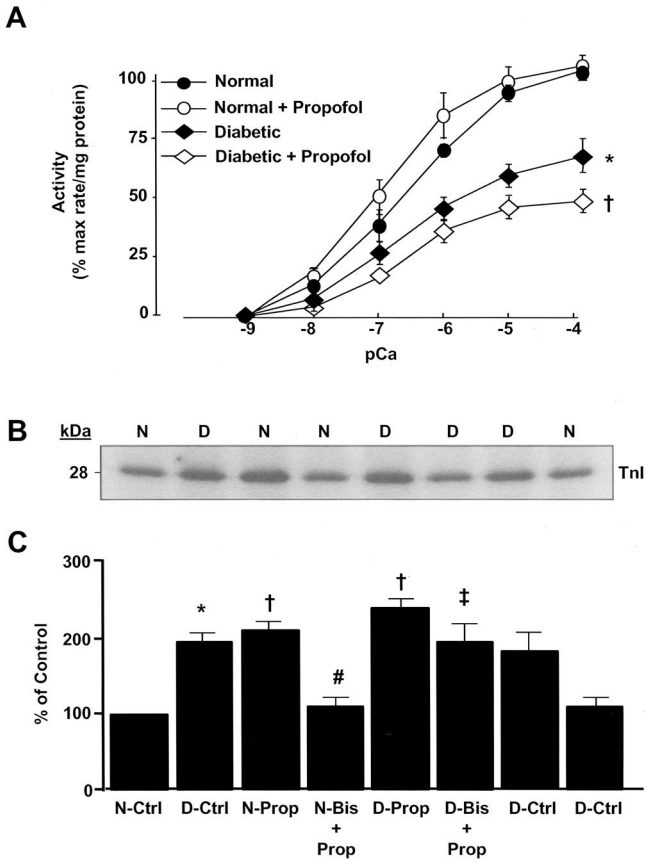

Fig. 3. (*A*) Summarized data depicting the effect of propofol on actomyosin adenosine triphosphatase activity in myofibrils isolated from normal and diabetic cardiomyocytes. * $P < 0.05$ *versus* normal. † $P < 0.05$ *versus* diabetic. n = myofibrils from seven hearts in each group. (*B*) Autoradiograph depicting the effect of propofol (Prop) alone (10 μM) or after pretreatment with bisindolylmaleimide (Bis; 1 μM) on troponin I (TnI) phosphorylation in normal (N) and diabetic (D) cardiomyocytes. (*C*) Summarized data for *B*. * $P < 0.05$ *versus* control (Ctrl) N. † $P < 0.05$ *versus* Ctrl diabetic. # $P < 0.05$ *versus* N Prop. ‡ $P < 0.05$ *versus* D Prop. n = myocytes obtained from six different hearts in each group.

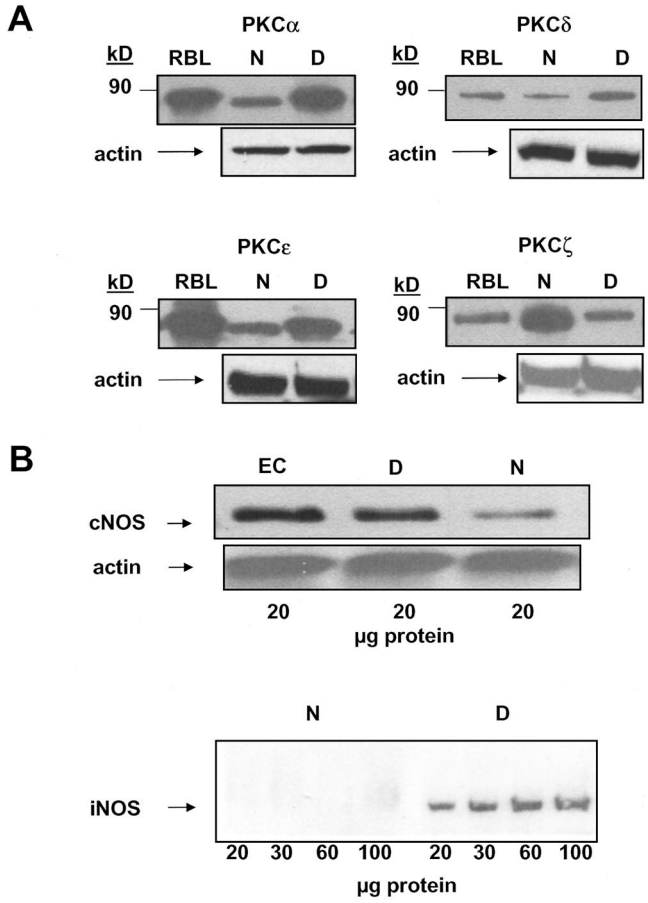

Fig. 4. (*A*) Representative immunoblot analysis of protein kinase C (PKC) isoforms including PKC-α, PKC-δ, PKC-ε, and PKC-ζ in normal (N) and diabetic (D) cardiomyocytes. Actin was used as a control for protein loading. Positive control = rat brain lysate (RBL). (*B*) Immunoblot analysis of constitutive nitric oxide synthase (cNOS) and inducible NOS (iNOS) in normal (N) and diabetic (D) cardiomyocytes.

from normal and diabetic cardiomyocytes. As previously demonstrated,[6] propofol caused a leftward shift in the actomyosin ATPase activation curve with no significant effect on maximal actomyosin ATPase activity (Vmax) in myofibrils isolated from normal cardiomyocytes (fig. 3A). Compared with myofibrils isolated from normal cardiomyocytes, Vmax was reduced by 35 ± 8% ($P = 0.007$) from a control value of 184 ± 8 nmol · min^{-1} · mg^{-1} in diabetic myofibrils. Moreover, propofol decreased the EC_{50} (pCa) value (*i.e.*, increased the Ca^{2+} requirement) from 6.2 ± 0.2 to 5.6 ± 0.1 ($P = 0.011$).

Effect of Propofol and PKC Inhibition on $^{32}P_i$ Incorporation into TnI

A representative autoradiogram depicting the phosphorylation state of TnI in normal and diabetic cardiomyocytes is shown in figure 3B. We also assessed whether propofol further modifies TnI phosphorylation and the extent to which PKC is involved. Compared with normal myofibrils (lanes 1, 8), baseline incorporation of $^{32}P_i$ into TnI was increased by 76 ± 22% ($P = 0.004$) in myofibrils from diabetic cardiomyocytes (lanes 2, 7). Addition of propofol (10 μM) further increased incorporation of $^{32}P_i$ into TnI in both normal (lane 3) and diabetic myofibrils (lane 5). The propofol-induced increase in $^{32}P_i$ incorporation was reduced in both normal (lane 4) and diabetic (lane 6) myofibrils when the cardiomyocytes were pretreated with the PKC inhibitor bisindolylmaleimide I. Summarized data for the effects of diabetes and propofol with and without PKC inhibition on $^{32}P_i$ incorporation into TnI are shown in figure 3C.

Effect of Diabetes on Expression of PKC and NOS Isoforms

Immunoblot analyses of PKC and NOS isoform expression in normal and diabetic cardiomyocytes are shown in figures 4A and B, respectively. At 12 weeks of diabetes, expression of PKC-α, PKC-δ, and PKC-ε were up-regulated, whereas expression of PKC-ζ was down-regulated

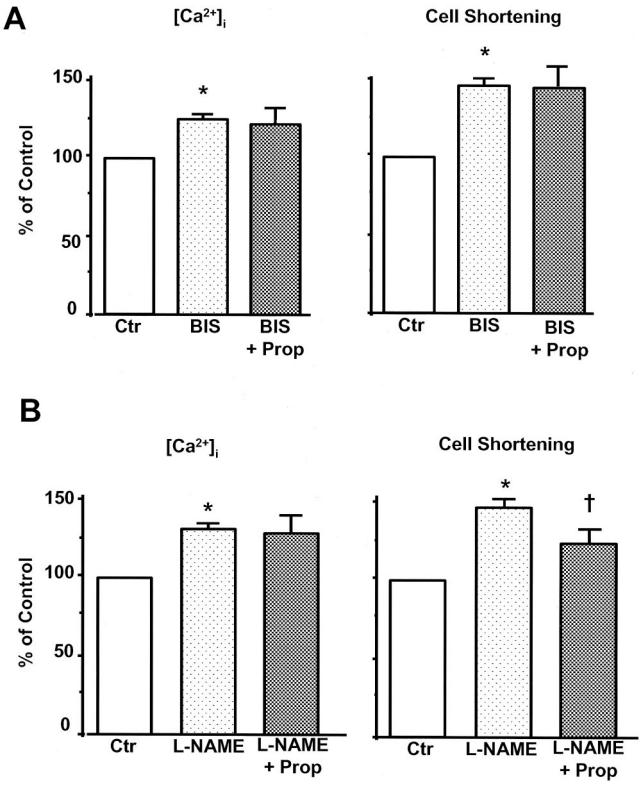

Fig. 5. (*A*) Summarized data for the effects of bisindolylmaleimide I (Bis; 1 μM) alone and bisindolylmaleimide I plus propofol (10 μM) on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and shortening in diabetic cardiomyocytes. (*B*) Summarized data for the effects of nitro-L-arginine methyl ester (L-NAME, 100 μM) alone and L-NAME plus propofol on $[Ca^{2+}]_i$ and shortening in diabetic cardiomyocytes. * $P < 0.05$ *versus* control (Ctr). † $P < 0.05$ *versus* L-NAME alone. n = 20 myocytes from five hearts.

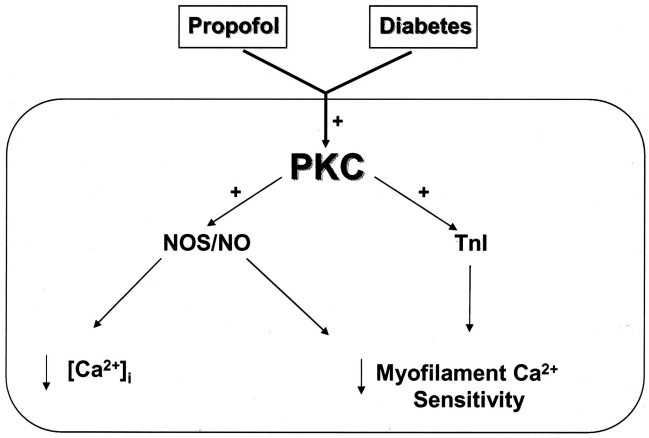

Fig. 6. Schematic diagram illustrating the putative signaling pathways and cellular mechanisms by which diabetes and propofol alter cardiomyocyte intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and myofilament Ca^{2+} sensitivity. NO = nitric oxide; NOS = nitric oxide synthase; PKC = protein kinase C; TnI = troponin I.

(fig. 4A). Similarly, expression of constitutive NOS was up-regulated in diabetic cardiomyocytes compared with normal cardiomyocytes (fig. 4B). Moreover, inducible NOS was absent in normal cardiomyocytes but expressed in diabetic cardiomyocytes.

Effect of PKC or NOS Inhibition on Propofol-induced Decreases in $[Ca^{2+}]_i$ and Shortening

The functional significance of the increased expression of PKC and NOS isoforms on $[Ca^{2+}]_i$ and shortening in diabetic cardiomyocytes is demonstrated in figures 5A and B, respectively. PKC inhibition with bisindolylmaleimide I alone increased $[Ca^{2+}]_i$ and shortening and prevented the propofol-induced decrease in shortening. NOS inhibition with nitro-L-arginine methyl ester alone increased $[Ca^{2+}]_i$ and markedly increased shortening. Moreover, pretreatment with nitro-L-arginine methyl ester only partially attenuated the propofol-induced decrease in shortening.

Discussion

This is the first study to assess the effects of propofol on cardiomyocyte $[Ca^{2+}]_i$, myofilament Ca^{2+} sensitivity, and contractility in the setting of diabetic cardiomyopathy. Previous studies suggest that altered Ca^{2+} homeostasis is the primary abnormality contributing to cardiomyocyte dysfunction in diabetic hearts.[10–12] Our key findings are a confirmation of altered Ca^{2+} homeostasis as a contributor to diabetic cardiomyocyte dysfunction. However, a decrease in myofilament Ca^{2+} sensitivity and Vmax of the actomyosin ATPase also seem to be important factors. Moreover, propofol further exacerbates the dysfunction *via* an activation of PKC and NOS, which are up-regulated in diabetic cardiomyocytes, resulting in an even greater decrease in myofilament Ca^{2+} sensitivity, actomyosin ATPase activity, and Ca^{2+} homeostasis. These findings are summarized in figure 6.

Effect of Diabetes on $[Ca^{2+}]_i$ and Shortening

Alterations in Ca^{2+} signaling within the cardiac muscle cell have been a hallmark of cardiomyopathy and heart failure. Although several recent studies[10,12–14] have used isolated cardiomyocytes as a model system to identify the cellular and subcellular derangements in diabetic cardiomyopathy, the precise mechanisms are still largely unknown and/or controversial. Decreases in peak $[Ca^{2+}]_i$ and peak shortening have been demonstrated in some studies using cardiomyocytes from diabetic rats,[10,15,16] but not in others.[13,17] Moreover, a prolongation in the increase and the decrease of $[Ca^{2+}]_i$ and shortening has been demonstrated in cardiomyocytes,[13,16] as previously observed in papillary muscle.[11] Our current findings confirm a decrease in peak $[Ca^{2+}]_i$ and shortening as well as a prolongation in the increase and decrease of the contraction. We also confirm a prolongation of the decay of the Ca^{2+} transient. These data are consistent with previous studies that suggest that alterations in multiple cellular mechanisms regulating $[Ca^{2+}]_i$ including the L-type Ca^{2+} channel, Na^+–

Ca^{2+} exchange, sarcoplasmic reticulum Ca^{2+} ATPase, and ryanodine receptor are altered in diabetic cardiomyocytes.[10,12,14,15]

Effect of Propofol on $[Ca^{2+}]_i$ and Shortening

There are no previous studies examining the effect of propofol on $[Ca^{2+}]_i$ or shortening in diabetic cardiomyocytes. We previously demonstrated that propofol decreased $[Ca^{2+}]_i$ and shortening only at supraclinical concentrations in normal cardiomyocytes.[7] In the current study of diabetic cardiomyocytes, propofol had no effect on $[Ca^{2+}]_i$ at clinically relevant concentrations (1, 10 μM), whereas it attenuated shortening at both concentrations. These data suggest that propofol decreases myofilament Ca^{2+} sensitivity and/or the Vmax of the actomyosin ATPase in diabetic cardiomyocytes. Therefore, we directly assessed the extent to which propofol alters myofilament Ca^{2+} sensitivity in myofibrils isolated from propofol-treated cardiomyocytes.

Effect of Propofol on Myofilament Ca^{2+} Sensitivity and Actomyosin ATPase Activity

There have been no consistent findings regarding the sensitivity of the myofilaments to Ca^{2+} in diabetic cardiomyocytes. The sensitivity of isolated myofibrils to $[Ca^{2+}]_i$ has been reported to be unchanged[18,19] or increased[20,21] during diabetes. In contrast, a decreased Ca^{2+} sensitivity of the myofibrillar ATPase has been reported in several studies.[22–24] Diminished Ca^{2+} sensitivity and troponin T-band shifts have been observed in skinned cardiac trabeculae obtained from diabetic rats,[22] as well as in skinned cardiac myocytes.[25] In our study, we found that both myofilament Ca^{2+} sensitivity (shift in EC_{50} for Ca^{2+}) and the Vmax for the actomyosin ATPase were decreased in myofibrils isolated from diabetic cardiomyocytes. PKC-dependent phosphorylation of TnI is known to decrease myofilament Ca^{2+} sensitivity and Vmax of the actomyosin ATPase.[26–28] We observed a significant increase in TnI phosphorylation in myofibrils isolated from diabetic cardiomyocytes compared with those obtained from normal cardiomyocytes. This finding is consistent with one previous study[29] and suggests that TnI phosphorylation may be responsible for the altered sensitivity and activity of the contractile apparatus. In contrast to our earlier findings of a propofol-induced increase in myofilament Ca^{2+} sensitivity with no change in Vmax of the ATPase in myofibrils obtained from normal hearts[6] (which were confirmed again in this study), propofol caused a decrease in both myofilament Ca^{2+} sensitivity and the Vmax of the actomyosin ATPase in diabetic myofibrils. Moreover, we observed a propofol-induced increase in TnI phosphorylation in diabetic cardiomyocytes, as previously observed in normal cardiomyocytes,[5] which could be prevented by the PKC inhibitor bisindolylmaleimide I. Collectively, these data suggest that PKC isoform expression and activation may be up-regulated in diabetic cardiomyocytes resulting in decreased myofilament Ca^{2+} sensitivity and Vmax of the actomyosin ATPase. Moreover, propofol stimulates a PKC-dependent increase in TnI phosphorylation, further contributing to altered sensitivity and activity of the ATPase. However, alterations in additional pathways involved in regulation of myofilament Ca^{2+} sensitivity could be involved, including NOS signaling pathways and nitric oxide production or alterations in intracellular pH.

Effect of Diabetes on Expression of PKC and NOS Isoforms

The extent to which PKC and NOS isoform expression is altered in diabetic myocardium has not been extensively examined. A few recent studies have specifically examined alterations in PKC isoform expression in diabetic cardiomyocytes, and the results have been inconsistent.[30,31] In one study, PKC-α and PKC-δ expression were up-regulated in diabetic heart homogenates, whereas PKC-ε and PKC-ζ expression were decreased.[31] In yet another study, PKC-α, PKC-β, PKC-ε, and PKC-ζ isoforms were all increased in diabetic hearts.[30] However, the cellular origin of the isoforms (cardiomyocytes, endothelial cells, fibroblasts, and others) was not determined in either study.[30,31] In our study, we found that expression of PKC-α, PKC-δ, and PKC-ε were up-regulated, whereas PKC-ζ was down-regulated in diabetic cardiomyocytes. All four of the isoforms are capable of phosphorylating TnI, which could explain the altered sensitivity and activity of the actomyosin ATPase.[32] Moreover, we recently demonstrated that propofol activates PKC and causes translocation of PKC-α, PKC-δ, PKC-ε, and PKC-ζ to distinct intracellular locations, as well as causing PKC-dependent production of nitric oxide in normal cardiomyocytes.[33] These data indicate that propofol stimulates PKC-dependent activation of constitutive NOS in normal cardiomyocytes. In diabetic cardiomyocytes, we observed an up-regulation of constitutive NOS and *de novo* expression of inducible NOS, which is consistent with previous reports.[34,35] Activation of NOS and production of nitric oxide has been shown to decrease myofilament Ca^{2+} sensitivity and reduce cardiomyocyte contractility.[36–38] In addition, a nitric oxide–mediated increase in the phosphorylation of TnI has been reported, resulting in decreased cardiomyocyte contraction.[37] Taking these data together, we believe that a propofol-induced activation of PKC and NOS contribute to the altered sensitivity and activity of the actomyosin ATPase and also contributes to the abnormal Ca^{2+} handling in diabetic cardiomyocytes. Therefore, we next investigated the functional significance of PKC and NOS up-regulation on cardiomyocyte $[Ca^{2+}]_i$ and contractility.

Effect of PKC or NOS Inhibition on Propofol-induced Decreases in $[Ca^{2+}]_i$ and Shortening

Only one recent report examining the role of PKC inhibition on diabetic cardiomyocyte contractility has been reported,[39] and a single report evaluating the effect of NOS inhibition on ventricular performance in diabetic rat hearts has been described.[34] In these studies, PKC inhibition improved diabetic cardiomyocyte contractility, and NOS inhibition with nitro-L-arginine methyl ester improved ventricular performance in isolated rat hearts. In our study, we observed that PKC inhibition or NOS inhibition improved diabetic cardiomyocyte peak $[Ca^{2+}]_i$ and markedly improved peak shortening. These data are consistent with the observed up-regulation of PKC and NOS isoform expression, and suggest that increased activity of PKC and NOS contribute primarily to the decreased Ca^{2+} sensitivity but also play a role in regulating $[Ca^{2+}]_i$ in diabetic cardiomyocytes. Moreover, PKC inhibition completely prevented, whereas NOS inhibition only partially prevented, the propofol-induced decrease in peak shortening. Peak $[Ca^{2+}]_i$ was unaltered by propofol (10 μM) in the absence of PKC or NOS inhibition, and therefore, no changes in peak $[Ca^{2+}]_i$ were observed in the presence of PKC or NOS inhibition. These data suggest that NOS activation and nitric oxide production are only partially responsible for the propofol-induced decrease in myofilament Ca^{2+} sensitivity, and other PKC-dependent mechanisms are involved.

Summary and Conclusions

Diabetes causes decreases in peak $[Ca^{2+}]_i$, myofilament Ca^{2+} sensitivity, and actomyosin ATPase activity resulting in depressed contractility of isolated cardiomyocytes. Alterations in multiple mechanisms responsible for Ca^{2+} handling as well as mechanisms regulating myofilament responsiveness likely contribute to additional abnormalities observed during the decline of the Ca^{2+} transient and relengthening of the cardiomyocyte. An increase in PKC and NOS expression are key factors contributing to the altered contractility observed in the diabetic cardiomyocyte. This may be explained by an increase in TnI phosphorylation. Propofol has little effect on peak $[Ca^{2+}]_i$ at clinically relevant concentrations but further exacerbates the depressed contractility *via* a PKC-, NOS-dependent decrease in myofilament Ca^{2+} sensitivity and Vmax of the actomyosin ATPase. Further studies are required to determine the roles of individual PKC and NOS isoforms in mediating the contractile dysfunction observed in the diabetic heart, as well as their roles in mediating the propofol-induced myocardial depression.

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