

Propofol Modulates Na^+–Ca^{2+} Exchange Activity via *Activation of Protein Kinase C in Diabetic Cardiomyocytes*

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Background: **The authors' objective was to identify the role of the Na^+–Ca^{2+} exchanger (NCX) in mediating the contractile dysfunction observed in diabetic cardiomyocytes before and after exposure to propofol.**

Methods: **Freshly isolated ventricular myocytes were obtained from normal and diabetic rat hearts. Intracellular concentration of Ca^{2+} and cell shortening were simultaneously measured in electrically stimulated, ventricular myocytes using fura-2 and video-edge detection, respectively. Postrest potentiation (PRP) and sarcoplasmic reticulum Ca^{2+} load were used to assess propofol-induced changes in the activity of the NCX.**

Results: **Propofol (10 μM) increased PRP in diabetic cardiomyocytes but had no effect on PRP in normal cardiomyocytes. Removal of sodium enhanced and KB-R7943 (reverse mode NCX inhibitor) blocked PRP in both normal and diabetic cardiomyocytes. In the absence of sodium, propofol enhanced PRP in diabetic cardiomyocytes but had no additional effect in normal cardiomyocytes. KB-R7943 completely blocked propofol-induced potentiation of peak intracellular concentration of Ca^{2+} and shortening in both cell types. Propofol increased sarcoplasmic reticulum Ca^{2+} load and prolonged removal of cytosolic Ca^{2+} in diabetic cardiomyocytes, but not in normal cardiomyocytes. Removal of sodium enhanced propofol-induced increases in sarcoplasmic reticulum Ca^{2+} load and further prolonged removal of cytosolic Ca^{2+}, whereas KB-R7943 completely blocked propofol-induced increase in sarcoplasmic reticulum Ca^{2+} load. Protein kinase C inhibition with bisindolylmaleimide I prevented the propofol-induced increase in PRP and prolongation in Ca^{2+} removal.**

Conclusions: **These data suggest that propofol enhances PRP *via* activation of reverse mode NCX, but attenuates Ca^{2+} removal from the cytosol *via* inhibition of forward mode NCX in diabetic cardiomyocytes. The actions of propofol are mediated *via* a protein kinase C–dependent pathway.**

DIABETES-INDUCED cardiac dysfunction is characterized by a decrease in myocardial depression independent of vascular disease. Alterations in Ca^{2+} signaling within the cardiac muscle cells have been a hallmark of cardiomyopathy and heart failure.[1–4] The most significant functional change in the diabetic heart is a slower contraction (especially prolonged relaxation) leading to diastolic dysfunction.[5–7] This contractile dysfunction has the potential to worsen when anesthesia is required for clinical procedures, because induction of anesthesia with inhalational or intravenous anesthetics typically results in a decrease in cardiac depression in patients with and without cardiac disease. Although the cellular and molecular mechanisms responsible for the cardiac dysfunction observed in diabetic patients in the presence or absence of anesthesia are not entirely clear, alterations in either the expression and/or function of key Ca^{2+} regulatory proteins are likely responsible.

Studies examining the effect of propofol on Ca^{2+} handling by the sarcoplasmic reticulum (SR) as an explanation for the reported cardiac dysfunction associated with the use of propofol have yielded conflicting results. Propofol has been shown to prolong Ca^{2+} transients, impair SR Ca^{2+} uptake, and prolong myocardial relaxation in otherwise healthy cardiac muscle, suggesting alterations in the SR Ca^{2+} pump (SERCA2) and/or the sarcolemmal Na^+–Ca^{2+} exchanger (NCX).[8–11] Several studies have concluded that propofol does not influence SR Ca^{2+} handling, indirectly suggesting that propofol may prolong cytosolic Ca^{2+} removal *via* inhibitory effects on the NCX.[9,12] One recent study in cardiac trabeculae indicated that under certain conditions, propofol can increase Ca^{2+} influx *via* activation of the reverse mode of the NCX (Ca^{2+} in, Na^+ out).[13] However, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was not measured in that study,[13] the concentration of propofol was not clinically relevant, and the conditions required for propofol to cause Ca^{2+} influx *via* reverse mode NCX (Ca^{2+} in, Na^+ out) were only observed at slow pacing conditions and low calcium concentrations. No studies have assessed the effect of propofol on the NCX and/or regulation of SR Ca^{2+} load in the diabetic heart.

In this study, we tested the hypothesis that NCX mediates altered Ca^{2+} homeostasis in diabetic cardiomyocytes before and after treatment with propofol. NCX is the main mechanism of Ca^{2+} extrusion from cardiac myocytes. On the basis of thermodynamics, Ca^{2+} influx *via* NCX could occur during the early phase of the action potential. Normally, little Ca^{2+} enters *via* NCX, but Ca^{2+} entry can increase greatly when intracellular Na^+ ($[Na^+]_i$) increases, which can occur as a result of Na^+–K^+ adenosine triphosphatase (ATPase) inhibition or during ischemia and reperfusion.[14,15] Our major findings are that propofol increases the SR Ca^{2+} load by activating the reverse mode of the NCX (Ca^{2+} in, Na^+ out). However, propofol also prolongs cytosolic Ca^{2+} removal by inhibiting forward mode NCX. These effects of propofol in diabetic cardiomyocytes are mediated by protein kinase C (PKC).

Materials and Methods

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

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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. At the time of euthanasia, 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.[16,17] Immediately after euthanasia, 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 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 mM HEPES, 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.[18,19] 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 1 μM fura-2/acetoxy methylester at room temperature for 20 min. Fura-2–loaded ventricular myocytes were placed in a temperature regulated (37°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

Before cessation of electrical stimulation, values representing peak $[Ca^{2+}]_i$ and shortening from at least five contractions were averaged to obtain the mean baseline peak value for each parameter. For analysis of data related to postrest potentiation (PRP) and SR Ca^{2+} load, the change in shortening and/or $[Ca^{2+}]_i$ in response to resuming electrical stimulation or caffeine administration were plotted as the absolute percent change in $[Ca^{2+}]_i$ and/or shortening compared with the value obtained in normal cardiomyocytes, which was set at 100%. For analysis of data related to the time required for cytosolic Ca^{2+} removal ($[Ca^{2+}]_i$ decay), the time required to return to 50% of the baseline value ($t_{1/2}$) from the peak of the caffeine-induced Ca^{2+} transient was calculated before and after treatment with the intervention.

Experimental Protocols

Protocol 1: Effect of Diabetes and Propofol on PRP. A stock solution of propofol was obtained by dissolving the drug in dimethyl sulfoxide. Baseline measurements of $[Ca^{2+}]_i$ and shortening in response to electrical stimulation were collected from individual cardiomyocytes (normal and diabetic) for 20–30 s in the presence or absence of propofol (10 μM). Electrical stimulation was paused (15 s) and then resumed. The PRP achieved with a rest interval of 15 s represents approximately 50% of the maximum PRP achieved at a 60-s rest interval. Dimethyl sulfoxide (0.05% vol/vol) alone had no effect on $[Ca^{2+}]_i$ or shortening. Summarized data for the change in peak $[Ca^{2+}]_i$ and/or shortening in protocols 1–4 are expressed as absolute percent change.

Protocol 2: Effect of Na^+ Removal OR KB-R7943 on PRP. Protocol 1 was repeated, except extracellular NaCl in the HEPES-buffered saline was replaced with 118 mM tetramethylammonium chloride $(CH_3)_4NCl$ during

the quiescent period, which changes the thermodynamic driving force on NCX to favor Ca^{2+} influx. Alternatively, a selective inhibitor of reverse mode NCX (Ca^{2+} in, Na^+ out), KB-R7943 (10 μM), was added to normal HEPES-buffered saline (containing NaCl) for 10 min before initiating electrical stimulation. KB-R7943 has no effect on forward mode NCX (Na^+ in, Ca^{2+} out).[20] Experiments were performed in the presence or absence of propofol (10 μM).

Protocol 3: Effect of Diabetes and Propofol on SR Ca^{2+} Load and $[Ca^{2+}]_i$ Decay. Protocol 1 was repeated in cardiomyocytes, except caffeine (10 mM) was added after cessation of electrical stimulation (15 s) to stimulate SR Ca^{2+} release.

Protocol 4: Effect of Na^+ Removal or KB-R7943 on SR Ca^{2+} Load and $[Ca^{2+}]_i$ Decay. Protocol 2 was repeated, except caffeine was added after cessation of electrical stimulation. Experiments were performed in the presence or absence of propofol (10 μM). In separate experiments, NaCl was again replaced with $(CH_3)_4NCl$, but now Ca^{2+} was omitted and EGTA (1 mM) was added to HEPES-buffered saline (0 Na^+, 0 Ca^{2+}). The composition of this solution effectively blocks both forward and reverse mode NCX (Ca^{2+} in, Na^+ out).[20,21] Alternatively, extracellular NaCl was elevated from 118 to 180 mM in the presence of extracellular Ca^{2+} (1.2 mM) to favor forward mode NCX (Na^+ in, Ca^{2+} out).

Protocol 5: Effect of PKC Inhibition on PRP and SR Ca^{2+} Stores. Protocol 1 was repeated in diabetic cardiomyocytes, except the PKC inhibitor bisindolylmaleimide I (10 μM) was added to normal HEPES-buffered saline for 5 min during the quiescent period. In separate experiments, caffeine (10 mM) was added after cessation of electrical stimulation (15 s) to stimulate SR Ca^{2+} release. Experiments were performed in the presence or absence of propofol (10 μM). Summarized data are presented as absolute change compared with control values before PKC inhibition.

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). Streptozotocin, propofol, bisindolylmaleimide I, and KB-R7943 were purchased from Sigma Chemical Co. (St. Louis, MO). Fura-2 AM was obtained from Texas Fluorescence Labs (Austin, TX).

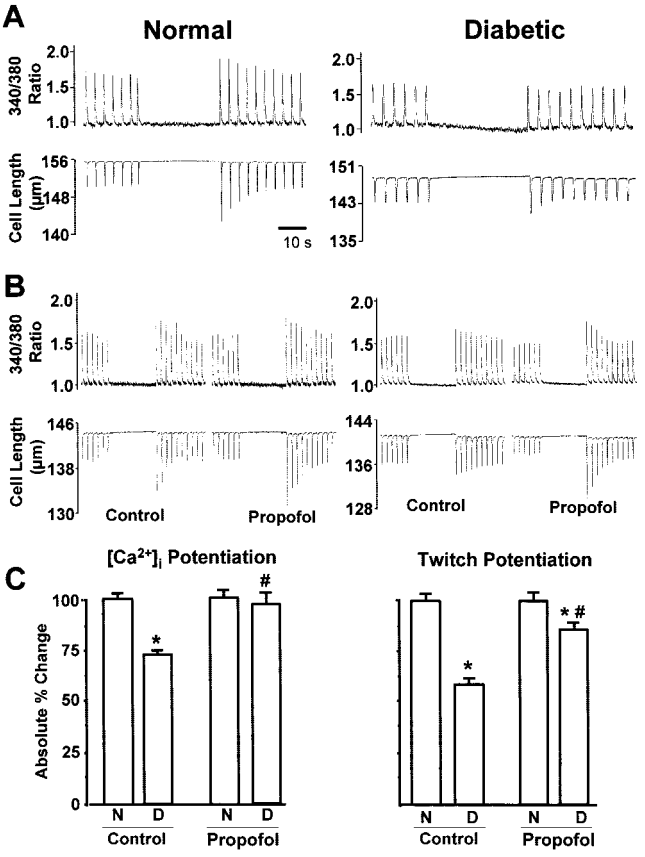

Fig. 1. Representative traces depicting the effect of diabetes (*A*) and propofol (10 μM; *B*) on postrest potentiation of peak $[Ca^{2+}]_i$ and shortening in individual cardiomyocytes. Summarized data are shown in *C*. * $P < 0.05$ *versus* normal. # $P < 0.05$ *versus* control. n = at least 8 cells from 3 different hearts for each group. D = diabetic; N = normal.

Results

Effect of Streptozotocin on Blood Glucose Levels

Diabetic animals (n = 31; 12 weeks diabetic) had blood glucose levels of 447 ± 52 mg/dl (approximately 250 mM) and body weights of 304 ± 21 g at the time of euthanasia. Control animals injected with vehicle only (n = 18) had blood glucose levels of 101 ± 19 mg/dl (approximately 60 mM) and weighed 422 ± 26 g.

Protocol 1: Effect of Diabetes and Propofol on PRP

The overall goal of protocols 1 and 2 was to use PRP as a tool to investigate alterations in reverse mode NCX (Ca^{2+} in, Na^+ out) in diabetic cardiomyocytes, alone and in combination, with propofol. Representative traces from an individual normal and diabetic cardiomyocyte demonstrating the effect of diabetes and propofol on PRP of peak $[Ca^{2+}]_i$ and shortening are shown in figures 1A and B. Summarized data are shown in figure 1C. After a 15-s rest duration, resuming electrical stimulation in normal cardiomyocytes resulted in PRP characterized by an increase in peak $[Ca^{2+}]_i$ and peak shortening that were 130 ± 4 and 236 ± 15%, respectively, of the value obtained before cessation of electrical stimulation. In

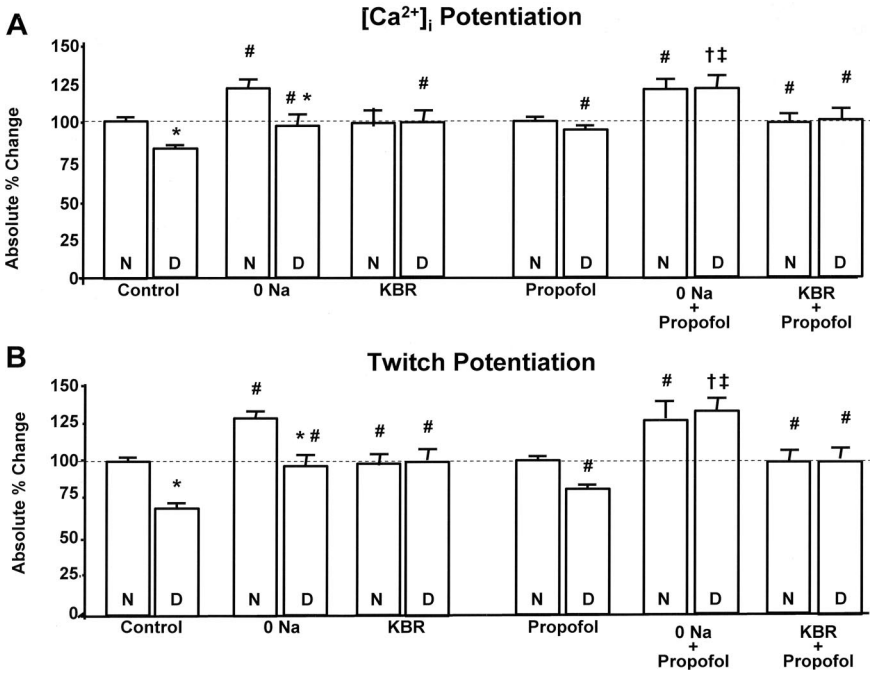

Fig. 2. Summarized data depicting the effect of Na^+ removal (0 Na) or KB-R7943 (KBR; 5 μM) before and after treatment with propofol on postrest potentiation of peak $[Ca^{2+}]_i$ (*A*) and peak shortening (*B*) in normal (N) and diabetic (D) cardiomyocytes. * $P < 0.05$ *versus* normal. # $P < 0.05$ *versus* control. † $P < 0.05$ *versus* propofol. ‡ $P < 0.05$ *versus* 0 Na^+. n = at least 6 cells from 3 different hearts for each group.

diabetic cardiomyocytes, PRP values of peak $[Ca^{2+}]_i$ and peak shortening were 121 ± 6% (71 ± 5% compared with normal cardiomyocytes) and 133 ± 6% (58 ± 9% compared with normal cardiomyocytes), respectively, of the value obtained before cessation of electrical stimulation. In normal cardiomyocytes, propofol (10 μM) exerted no additional effect on peak $[Ca^{2+}]_i$ or shortening after cessation of electrical stimulation compared with untreated controls. In contrast, propofol increased peak $[Ca^{2+}]_i$ and peak shortening by 23 ± 7% (98 ± 4% of value obtained in normal cardiomyocytes) and 49 ± 6% (86 ± 6% of value obtained in normal cardiomyocytes), respectively.

Protocol 2: Effect of Sodium Removal or KB-R7943 on PRP in the Presence or Absence of Propofol

Summarized data depicting the effect of Na^+ removal or inhibition of reverse mode NCX (Ca^{2+} in, Na^+ out) with KB-R7943 on PRP of peak $[Ca^{2+}]_i$ and shortening in the presence or absence of propofol are shown in figure 2. Removal of Na^+ (tetramethylammonium substituted) during the quiescent period enhanced PRP of peak $[Ca^{2+}]_i$ and peak shortening in both normal and diabetic cardiomyocytes. In normal cardiomyocytes, PRP of peak $[Ca^{2+}]_i$ and shortening increased by 24 ± 4 and 29 ± 3%, respectively, whereas PRP of peak $[Ca^{2+}]_i$ and shortening increased by 21 ± 5 and 28 ± 5%, respectively, in diabetic cardiomyocytes. Exposure of normal cardiomyocytes to propofol had no additional effect on peak $[Ca^{2+}]_i$ or shortening in the absence of Na^+ but enhanced peak $[Ca^{2+}]_i$ and shortening in diabetic cardiomyocytes. Selective inhibition of reverse mode NCX (Ca^{2+} in, Na^+ out) with KB-R7923 (10 μM) completely blocked PRP in both normal and diabetic cardiomyocytes. Propofol had no effect on PRP of peak $[Ca^{2+}]_i$ or shortening after inhibition of reverse mode NCX (Ca^{2+} in, Na^+ out) in either normal or diabetic cardiomyocytes.

Protocol 3: Effect of Diabetes and Propofol on SR Ca^{2+} Load and $t_{1/2}$ for $[Ca^{2+}]_i$ Decay

The overall goal of protocols 3 and 4 was to use SR Ca^{2+} load as a tool to further investigate alterations in reverse mode NCX (Ca^{2+} in, Na^+ out), as well as to use $t_{1/2}$ for $[Ca^{2+}]_i$ decay as a tool to explore alterations in forward mode NCX (Na^+ in, Ca^{2+} out) in diabetic cardiomyocytes, alone and in combination with propofol. Representative traces depicting the effect of diabetes and propofol (10 μM) on SR Ca^{2+} load and $t_{1/2}$ for $[Ca^{2+}]_i$ decay are shown in figures 3A and B. Summarized data are shown in figure 3C. Compared with normal cardiomyocytes, SR Ca^{2+} load was reduced by 17 ± 4% in diabetic cardiomyocytes. Moreover, the $t_{1/2}$ for $[Ca^{2+}]_i$ decay was markedly prolonged in diabetic cardiomyocytes (3.5 ± 0.3 s) compared with normal cardiomyocytes (1.7 ± 0.2 s). Addition of propofol had no effect on SR Ca^{2+} load in normal cardiomyocytes. However, propofol enhanced SR Ca^{2+} load in diabetic cardiomyocytes by 14 ± 3% (97 ± 4% of value obtained in normal cardiomyocytes). In addition, propofol further prolonged the $t_{1/2}$ for $[Ca^{2+}]_i$ decay in diabetic cardiomyocytes (5.0 ± 0.3 ms) but had no effect on the $t_{1/2}$ for $[Ca^{2+}]_i$ in normal cardiomyocytes (1.8 ± 0.2 s).

Protocol 4: Effect of Na^+ Removal or KB-R7943 on SR Ca^{2+} Load and $t_{1/2}$ for $[Ca^{2+}]_i$ Decay in the Presence or Absence of Propofol

Summarized data depicting the effect of Na^+ removal or inhibition of reverse mode NCX (Ca^{2+} in, Na^+ out)

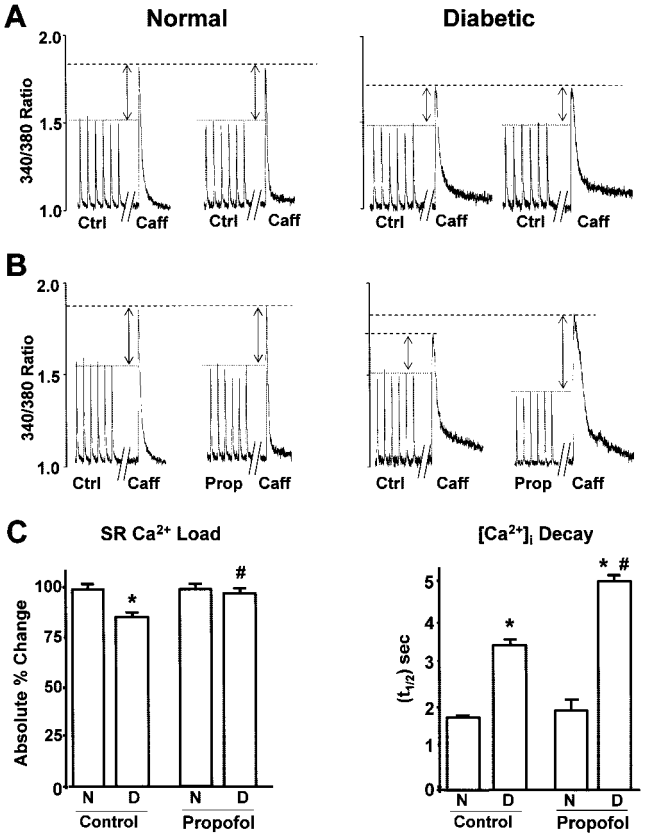

Fig. 3. Representative traces depicting the effect of diabetes (*A*) and propofol (Prop; 10 μM; *B*) on sarcoplasmic reticulum (SR) Ca^{2+} load and $t_{1/2}$ $[Ca^{2+}]_i$ decay after exposure to caffeine (Caff) in normal (N) and diabetic (D) cardiomyocytes. Summarized data are shown in *C*. * *P* < 0.05 *versus* normal. # *P* < 0.05 *versus* control (Ctrl). n = at least 8 cells from 3 different hearts for each group.

with KB-R7943 on SR Ca^{2+} stores in normal and diabetic cardiomyocytes in the presence or absence of propofol are shown in figure 4. Similar to the data depicted in figure 2, Na^+ removal during the quiescent period caused an increase in SR Ca^{2+} load in both normal and diabetic cardiomyocytes. Inhibition of reverse mode NCX (Ca^{2+} in, Na^+ out) with KB-R7943 during the quiescent period reduced SR Ca^{2+} load in both normal and diabetic cardiomyocytes. In the absence of extracellular Na^+, propofol increased SR Ca^{2+} load in diabetic cardiomyocytes by 26 ± 5%, but had no effect in normal cardiomyocytes when compared with the absence of extracellular Na^+ alone. KB-R7943 prevented the propofol-induced increase in SR Ca^{2+} load. The $[Ca^{2+}]_i$ decay was prolonged by Na^+ removal in both normal ($t_{1/2}$ = 3.1 ± 0.4 s) and diabetic cardiomyocytes ($t_{1/2}$ = 3.9 ± 0.4 s). In contrast, KB-R7943 had no effect on the $[Ca^{2+}]_i$ decay in either normal ($t_{1/2}$ = 1.8 ± 0.2 s) or diabetic ($t_{1/2}$ = 3.2 ± 0.3 s) cardiomyocytes. In the absence of Na^+, propofol further prolonged $[Ca^{2+}]_i$ decay in diabetic cardiomyocytes ($t_{1/2}$ = 5.9 ± 0.4 s), whereas the propofol-induced prolongation in $[Ca^{2+}]_i$ decay was not affected by KB-R7943 ($t_{1/2}$ = 5.1 ± 0.4 ms) compared with propofol alone.

Summarized data demonstrating the effect of removing both extracellular Na^+ and Ca^{2+} (0 Na^+, 0 Ca^{2+}) or increasing extracellular Na^+ while Ca^{2+} is held constant on $[Ca^{2+}]_i$ decay in diabetic cardiomyocytes in the presence or absence of propofol are shown in figure 5. In the absence of both Na^+ and Ca^{2+}, $[Ca^{2+}]_i$ decay was markedly prolonged in both normal and diabetic cardiomyocytes, and propofol exerted no additional effect. In contrast, when extracellular Na^+ was elevated, the $[Ca^{2+}]_i$ decay was shortened (faster) in both normal and diabetic

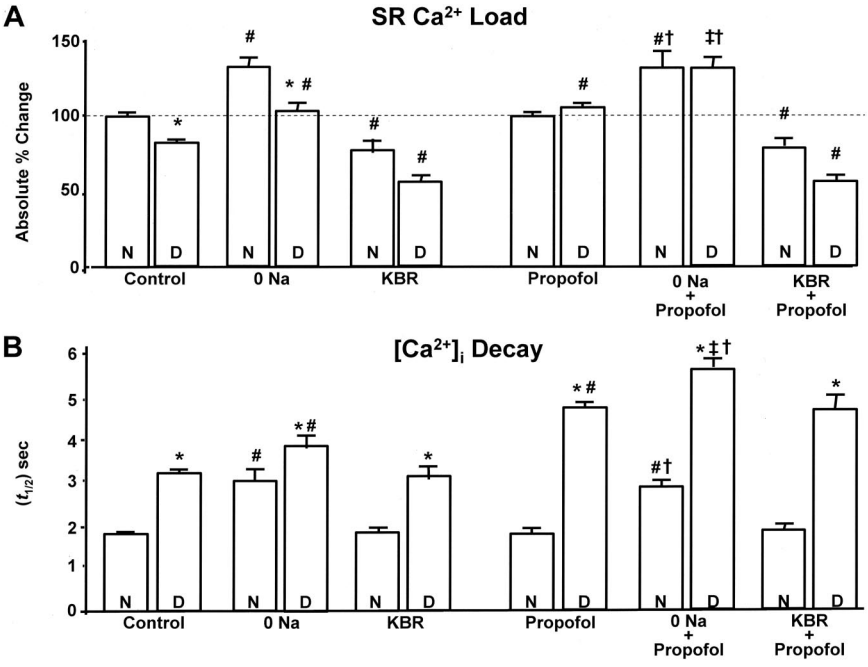

Fig. 4. Summarized data depicting the effect of Na^+ removal (0 Na) or KB-R7943 (KBR; 5 μM) on sarcoplasmic reticulum (SR) Ca^{2+} load (*A*) and $t_{1/2}$ $[Ca^{2+}]_i$ decay (*B*) in normal (N) and diabetic (D) cardiomyocytes. * *P* < 0.05 *versus* normal. # *P* < 0.05 *versus* control. † *P* < 0.05 *versus* propofol. ‡ *P* < 0.05 *versus* 0 Na. n = at least 8 cells from 3 different hearts for each group.

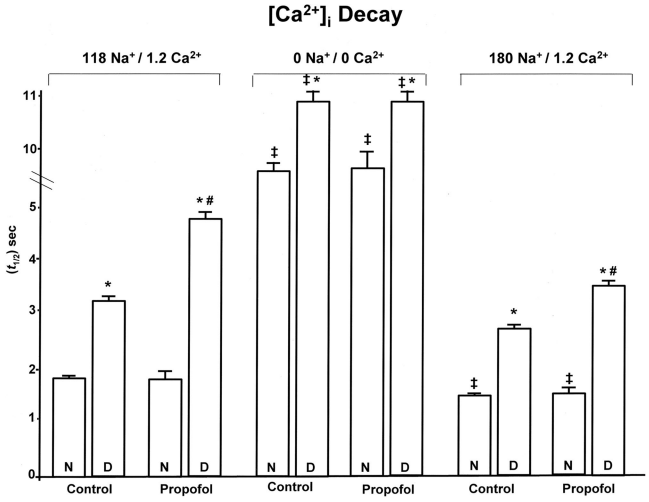

Fig. 5. Summarized data depicting the effect of Na^+ and Ca^{2+} removal (0 Na^+/0 Ca^{2+}) or elevated Na^+ (180 Na^+/1.2 Ca^{2+}) on $[Ca^{2+}]_i$ decay after exposure to caffeine in normal (N) and diabetic (D) cardiomyocytes. * $P < 0.05$ *versus* normal. # $P < 0.05$ *versus* control. ‡ break point in y axis. n = at least 9 cells from 3 different hearts for each group.

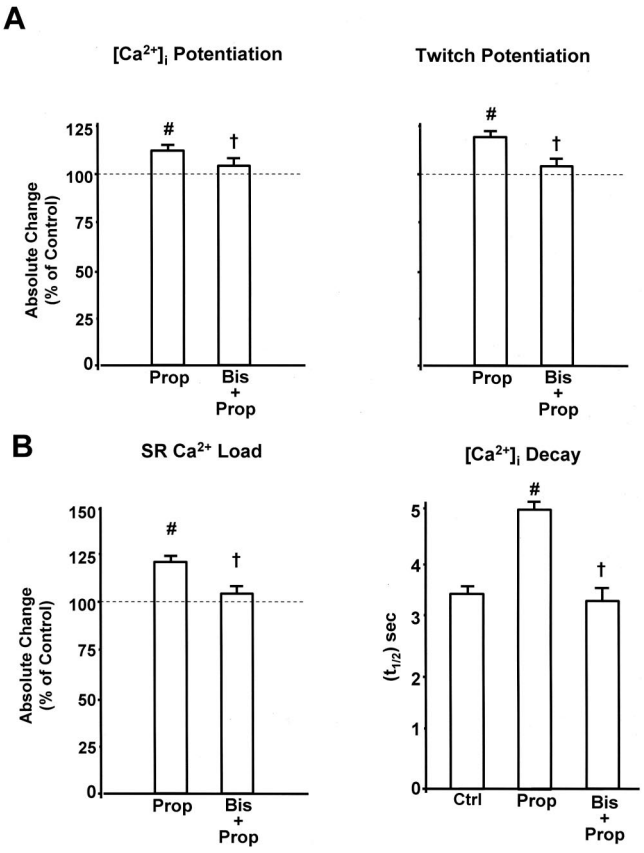

Fig. 6. Summarized data depicting the effect of protein kinase C inhibition with bisindolylmaleimide 1 (Bis; 5 μM) on propofol (Prop)–induced increase in postrest potentiation of peak $[Ca^{2+}]_i$ and shortening (*A*) or sarcoplasmic reticulum (SR) Ca^{2+} load and $[Ca^{2+}]_i$ decay (*B*). # $P < 0.05$ *versus* control (Ctrl). † $P < 0.05$ *versus* propofol. n = at least 10 cells from 3 different hearts for each group.

control cells compared with 118 mM Na^+ controls. Similarly, the $[Ca^{2+}]_i$ decay was significantly faster in propofol-treated cardiomyocytes.

Protocol 5: Effect of PKC Inhibition on PRP, SR Ca^{2+} Load, and $t_{1/2}$ $[Ca^{2+}]_i$ Decay in the Presence or Absence of Propofol

The overall goal of protocol 5 was to investigate the role of PKC as a mediator of alterations in reverse mode NCX (Ca^{2+} in, Na^+ out), SR Ca^{2+} load, and forward mode NCX in diabetic cardiomyocytes, alone and in combination with propofol. Summarized data depicting the effect of PKC inhibition on PRP of peak $[Ca^{2+}]_i$ and peak shortening as well as SR Ca^{2+} load and $t_{1/2}$ $[Ca^{2+}]_i$ decay in diabetic cardiomyocytes are shown in figures 6A and B, respectively. Addition of the PKC inhibitor bisindolylmaleimide I (10 μM) during the quiescent period (10 min) prevented the propofol-induced increase in PRP of peak $[Ca^{2+}]_i$ and shortening. Moreover, bisindolylmaleimide I also prevented the propofol-induced prolongation in SR Ca^{2+} load and $t_{1/2}$ $[Ca^{2+}]_i$ decay. In the absence of propofol, bisindolylmaleimide I alone slightly restored PRP of peak $[Ca^{2+}]_i$ and peak shortening as well as SR Ca^{2+} load and $[Ca^{2+}]_i$ decay (data not shown).

Discussion

This is the first study to assess the extent to which propofol alters the bimodal actions of the NCX on removal of cytosolic Ca^{2+} (forward mode NCX, Na^+ in, Ca^{2+} out) and SR Ca^{2+} load (reverse mode NCX, Ca^{2+} in, Na^+ out) in the setting of diabetes-induced cardiac dysfunction using freshly isolated ventricular myocytes. Previous studies suggest that altered Ca^{2+} homeostasis is the primary abnormality contributing to cardiomyocyte dysfunction in diabetic hearts. Our key findings are that altered Ca^{2+} homeostasis involving the bimodal actions of the NCX is a contributor to diabetic cardiomyocyte dysfunction. Moreover, propofol modulates the bimodal actions of the NCX in diabetic cardiomyocytes *via* activation of PKC. These findings are summarized in figure 7.

Effect of Diabetes and Propofol on PRP

The cellular mechanisms contributing to PRP are not entirely clear but likely result from Ca^{2+} entry during rest *via* reverse mode NCX causing an increase in the SR Ca^{2+} load.[21,22] In the current study, PRP was attenuated in diabetic cardiomyocytes when compared with that observed in normal cardiomyocytes. Both diminished function and expression of the cardiac NCX have been observed in diabetic cardiomyocytes.[23,24] Therefore, it is likely that diminished function of NCX in the diabetic cardiomyocyte is responsible for the diminished PRP observed in the current study. Diabetic cardiomyocytes have been shown to have a higher baseline $[Na^+]_i$ than normal cardiomyocytes,[25] and $[Na]_i$ drives the NCX in the reverse

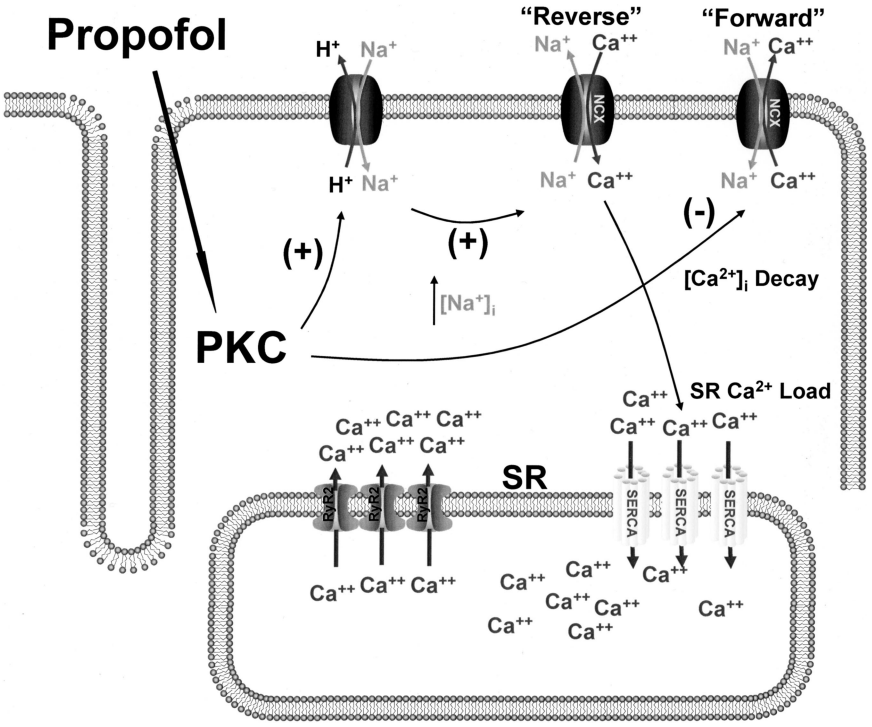

Fig. 7. Schematic diagram depicting the proposed signaling pathway for propofol-induced modulation of Na^+–Ca^{2+} exchange (NCX) in diabetic cardiomyocytes. Elevated levels of intracellular Na^+ ($[Na^+]_i$) *via* reduced Na^+–K^+ adenosine triphosphatase activity leading to a more positive membrane potential results in a reversal potential that thermodynamically slightly favors Ca^{2+} efflux *via* forward mode NCX. This effectively limits sarcoplasmic reticulum (SR) Ca^{2+} loading at rest. However, the NCX is dysfunctional in diabetic cardiomyocytes and therefore results in prolonged Ca^{2+} removal. Propofol further exacerbates the situation *via* activation of protein kinase C (PKC), which then turns on the Na^+–H^+ exchanger or further reduces Na^+–K^+ adenosine triphosphatase activity to further elevate $[Na^+]_i$. This result shifts the reversal potential to favor Ca^{2+} influx at rest and effectively increases the SR Ca^{2+} load. At the same time that Ca^{2+} influx is favored *via* reverse mode, PKC may also inhibit Ca^{2+} efflux *via* the forward mode NCX, resulting in an even greater prolongation in Ca^{2+} removal. RyR2 = ryanodine receptor; SERCA = sarcoplasmic reticulum Ca^{2+} pump.

mode. This should facilitate an increase in SR Ca^{2+} load and enhance PRP, but this was not observed in the current study. The lack of an increase in SR Ca^{2+} load may be due to reports of diminished Na^+–K^+ ATPase activity in diabetic cardiomyocytes.[26] Diminished activity would result in a more positive membrane potential such that the reversal potential of NCX would not favor Ca^{2+} influx *via* reverse mode (Na^+ out, Ca^{2+} in). It is questionable whether diminished Na^+–K^+ ATPase activity itself, independent of changes in NCX activity, could contribute to the alterations in Ca^{2+} handling observed in this model. The reasons for this are that an increase in $[Na]_i$ can inhibit Na^+–H^+ exchange *via* a Na^+-activated G protein resulting in an accumulation of protons (H^+) in the cytoplasm, but acidosis increases both diastolic and peak $[Ca^{2+}]_i$,[27] which should increase SR Ca^{2+} load. In contrast, increased $[Na]_i$ can also activate K^+ channels resulting in a shortening of action potential duration that would limit Ca^{2+} influx and SR Ca^{2+} load consistent with our findings; however, action potential prolongation is typically observed in diabetic cardiac myocytes[28] primarily *via* decreases in transient outward and steady state K^+ current density.[29] If Na^+ levels are truly higher in our diabetic cardiomyocytes, our current findings could still be explained by reports of decreased expression and function of the cardiac ryanodine receptor[30] and/or decreased SR Ca^{2+} uptake by the SR Ca^{2+} ATPase.[2,31,32] Either of these could contribute to the decreased $[Ca^{2+}]_i$ and shortening observed in diabetic cardiomyocytes. We also cannot discount the potential of reports indicating increased expression of nonphosphorylated forms of phospholamban in diabetic cardiomyocytes, which would attenuate SR Ca^{2+} loading and diminish PRP of peak $[Ca^{2+}]_i$ and shortening.[2,31,33]

Propofol had no effect on PRP of peak $[Ca^{2+}]_i$ and shortening in normal cardiomyocytes but enhanced these values in diabetic cardiomyocytes. One potential explanation for our current findings may be related to our previous findings that propofol activates the Na^+–H^+ exchanger in normal cardiomyocytes.[17] It is possible that a propofol-induced increase in $[Na^+]_i$ (*via* Na^+–H^+ exchange) could serve to further facilitate and/or result in favoring reverse mode NCX in diabetic cardiomyocytes by increasing $[Na^+]_i$. Assuming a resting $[Na]_i$ level of 12–14 mM and a $[Ca^{2+}]_i$ level of 100 nM in normal rat cardiomyocytes, the predicted NCX reversal potential would be −67 to −79 mV.[20] If $[Na^+]_i$ increased by just 3 mM, and it is already elevated in diabetic cardiomyocytes,[25] perhaps by a decrease in Na^+–K^+ ATPase activity,[26] the reversal potential for NCX would become −85 to −95 mV, negative to the resting membrane potential, and strongly favor net Ca^{2+} influx at rest.[20] The lack of effect of propofol on PRP in normal cardiomyocytes, despite a propofol-induced activation of Na^+–H^+ exchange,[34] suggests that other factors in diabetic cardiomyocytes, such as decreased Na^+–K^+ ATPase activity,[26] abnormal K^+ channel activity,[29] prolonged action potential duration,[28] and/or decreased membrane potential,[35] may contribute to the propofol-induced alterations in Ca^{2+} handling observed in diabetic cardiomyocytes. Although we previously demonstrated that propofol-induced activation of Na^+–H^+ exchange causes an increase in myofilament Ca^{2+} sensitivity in normal cardiomyocytes,[17] we recently demonstrated that propofol decreases peak $[Ca^{2+}]_i$, prolongs $t_{1/2}$ $[Ca^{2+}]_i$ decay,

and decreases myofilament Ca^{2+} sensitivity in diabetic cardiomyocytes.[36] Moreover, it should be noted that the increase in peak $[Ca^{2+}]_i$ release by propofol in diabetic cardiomyocytes is much larger than the percent increase in fractional shortening, again implying a propofol-induced decrease in myofilament Ca^{2+} sensitivity, despite enhanced fractional Ca^{2+} release.

Effect of Na^+ Removal or KB-R7943 on PRP in the Presence or Absence of Propofol

The bimodal action of the NCX is primarily regulated by the concentration gradients of Na^+ and Ca^{2+} across the sarcolemma. Therefore, removal of Na^+ from the perfusion buffer facilitates reverse mode NCX (Ca^{2+} in, Na^+ out). KB-R7943 is a novel agent that preferentially blocks reverse mode NCX and has no effect on forward mode NCX.[20,37] In the current study, removal of Na^+ completely restored, and KB-R7943 completely blocked, PRP of peak $[Ca^{2+}]_i$ and shortening in both normal and diabetic cardiomyocytes. This indicates that the reduced PRP in diabetic cardiomyocytes is entirely due to a defective NCX. In addition, these data support findings that PRP in both cell types depends on activation of reverse mode NCX (Ca^{2+} in, Na^+ out).[21,22,38]

In diabetic cardiomyocytes, removal of Na^+ further enhanced the propofol-induced increase in PRP, whereas blockade of reverse mode NCX with KB-R7943 blocked propofol's effect on PRP. These data further indicate that the actions of propofol on PRP are mediated by a propofol-induced activation of the reverse mode NCX (Ca^{2+} in, Na^+ out). A recent report demonstrated that propofol enhances PRP in rat cardiac trabeculae by increasing Ca^{2+} influx *via* the reverse mode NCX (Ca^{2+} in, Na^+ out).[13]

Effect of Diabetes and Propofol on SR Ca^{2+} Load and $t_{1/2}$ for $[Ca^{2+}]_i$ Decay

Caffeine-induced SR Ca^{2+} release was used to assess the effect of diabetes and propofol on SR Ca^{2+} load driven by reverse mode NCX (Ca^{2+} in, Na^+ out) and $t_{1/2}$ for $[Ca^{2+}]_i$ decay driven by forward mode NCX (Na^+ in, Ca^{2+} out) in cardiomyocytes. Although it is well documented that the SR Ca^{2+} ATPase contributes approximately 90% to the decline of twitch $[Ca^{2+}]_i$ in rat cardiomyocytes, the SR only releases approximately 50% of the stored Ca^{2+} during a twitch.[39] In the current study, peak twitch $[Ca^{2+}]_i$ and SR Ca^{2+} release in response to caffeine were reduced in diabetic cardiomyocytes compared with normal cardiomyocytes. These data imply a reduced SR Ca^{2+} load in diabetic cardiomyocytes as previously suggested, although not directly tested, in one recent study.[30] Moreover, the $t_{1/2}$ $[Ca^{2+}]_i$ decay was significantly prolonged in diabetic cardiomyocytes compared with normal cardiomyocytes, implying a defect in removal of Ca^{2+} by forward mode NCX (Na^+ in, Ca^{2+} out), and not SERCA2, because caffeine-induced activation of the SR Ca^{2+} release channels is so strong in promoting SR Ca^{2+} release that it prevents Ca^{2+} accumulation by the SR.[39–41] Therefore, removal of Ca^{2+} is dependent on other transport systems (*e.g.*, NCX, mitochondria, sarcolemmal Ca^{2+} ATPase). These various transport systems and their relative roles in Ca^{2+} removal may be altered in diabetic cardiomyocytes and could contribute to the "fast" and "slow" decay in Ca^{2+} removal observed in diabetic cardiomyocytes after caffeine exposure, which is not observed in normal cardiomyocytes. Our data are consistent with studies demonstrating alterations in the expression and/or function of these key Ca^{2+} regulatory proteins in diabetic hearts,[2,31] as well as functional studies demonstrating prolonged relaxation in cardiomyocytes and papillary muscles.[4,6,31,36,42,43]

In diabetic cardiomyocytes, propofol increased peak $[Ca^{2+}]_i$ achieved in response to caffeine after a rest that resembled levels observed in normal cardiomyocytes. We believe this is due to a propofol-induced increase in reverse mode NCX (Ca^{2+} in, Na^+ out) leading to an increase in the SR Ca^{2+} load. More striking is the marked prolongation in the $t_{1/2}$ $[Ca^{2+}]_i$ decay by propofol. These data further suggest a defect in the forward mode NCX (Na^+ in, Ca^{2+} out) rather than a defective SERCA2, as mentioned above. Our data are consistent with other studies demonstrating that propofol prolongs Ca^{2+} transients and impairs myocardial relaxation,[8,9,18,44] suggesting that propofol exerts inhibitory effects on SERCA2 and/or forward mode NCX (Na^+ in, Ca^{2+} out). There are numerous conflicting reports regarding the effects of propofol on SR Ca^{2+} uptake by SERCA2,[9,10,12,18,44] and only one report of propofol exerting effects on the cardiac NCX, in particular, activation of reverse mode NCX in trabecular muscle.[13] There are no reports of propofol exerting effects on forward mode NCX (Na^+ in, Ca^{2+} out). Therefore, we further examined the role of NCX as the target for propofol and the mediator of prolonged $t_{1/2}$ $[Ca^{2+}]_i$ decay in diabetic cardiomyocytes.

Effect of Sodium Removal or KB-R7943 on SR Ca^{2+} Load in the Presence or Absence of Propofol

Under normal conditions, NCX is responsible for approximately 10% of the Ca^{2+} removal from the cytosol, and SERCA2 is responsible for the remainder.[45] We suspected it was unlikely that prolongation in $t_{1/2}$ $[Ca^{2+}]_i$ decay involved SERCA2, because this would be inconsistent with our findings of enhanced PRP by propofol, which would require an active SERCA2 to increase Ca^{2+} load of the SR. Our findings that Na^+ removal enhanced, and KB-R7943 reduced, fractional Ca^{2+} release in response to caffeine were predictable based on data depicted in figure 2 that have previously been discussed. Interestingly, we found that under conditions where both forward and reverse mode NCX (Ca^{2+} in, Na^+ out) were blocked (0 Na^+ and 0 Ca^{2+}), propofol no longer

prolonged the $t_{1/2}$ $[Ca^{2+}]_i$ decay and restored values to control levels. These data provided strong evidence that propofol was inhibiting forward mode NCX (Na^+ in, Ca^{2+} out) and not exerting effects on SERCA2 in diabetic cardiomyocytes. These novel data may help to explain the controversy regarding propofol's effects on cytosolic Ca^{2+} removal, SR Ca^{2+} handling, and impairment of myocardial relaxation in cardiac muscle. We next explored a possible cellular mechanism to explain the propofol-induced effects on NCX.

Effect of PKC Inhibition on PRP and SR Ca^{2+} Stores in the Presence or Absence of Propofol

We recently demonstrated that several PKC isoforms are up-regulated in diabetic cardiomyocytes[36] and that propofol causes PKC-dependent phosphorylation of contractile proteins.[16] Our current data indicating that inhibition of PKC prevents the propofol induced increase in PRP of peak $[Ca^{2+}]_i$ and shortening as well as increased SR Ca^{2+} load and $t_{1/2}$ $[Ca^{2+}]_i$ decay suggest that a PKC-dependent mechanism is involved. Not only could propofol-induced, PKC-dependent activation of Na^+–H^+ exchange be involved in facilitating reverse mode NCX (Ca^{2+} in, Na^+ out), as discussed above, but it may also be responsible for inhibiting forward mode NCX (Na^+ in, Ca^{2+} out). Alternatively, PKC-dependent phosphorylation of the NCX may also work in parallel with changes in Na^+ and H^+ to regulate/modulate the NCX in diabetic cardiomyocytes. This would be consistent with a report indicating that reduced NCX activity resulting from diabetes may be related to changes in PKC activity but is not related to altered expression of the transporter.[24]

Limitations

As with all *in vitro* studies, there are always some limitations to the model system and its extrapolation to the clinical situation. It should be noted that this model system represents type 1 diabetes, and that the lack of insulin may induce changes in cardiomyocytes different from those seen in insulin-resistance and a high or normal level of insulin as occurs in type 2 diabetes. However, a recent report indicates that cardiomyocytes bathed in high glucose exhibit all the same changes in excitation–contraction coupling that are observed *in vivo* as well as in chemically induced models of type 1 diabetes. Moreover, patients with type 2 diabetes also exhibit a prolongation in action potential duration, and intracellular Ca^{2+} clearing and mechanical relaxation are slowed. It is also well established that species differences can contribute to difficulties in extrapolating *in vitro* data to the *in vivo* situation, and in this model system, rat cardiomyocytes are know to have an abnormally high $[Na^+]_i$, which means that the contribution of NCX in clearing intracellular Ca^{2+} is far greater than that observed in other species. In addition, this *in vitro* study only deals with intrinsic myocardial function, whereas changes in cardiac function after propofol administration *in vivo* also depend on a variety of other factors, including venous return, afterload, and neurohumoral compensatory mechanisms. However, we believe that the strengths of this model system far outweigh the limitations.

Clinical Significance

It is well established that the response to propofol is widely variable among patients given the same dose. Its binding to serum proteins exceeds 98%,[46] so small changes in protein concentrations can be amplified in the unbound fraction of the drug and in its effect. It is likely that that part of the variability in the response among patients is because of differences in protein levels among individuals, and particularly among those with pathologies such as liver disease. The clinical relevance of *in vitro* studies using propofol is often questioned because the concentrations of propofol that cause changes in cell, tissue, or organ function are typically outside of our estimations of what we perceive as a clinically relevant concentration. However, estimations of the clinically relevant plasma concentrations of propofol *in vivo* as well as relating these clinical plasma concentrations to free aqueous drug concentrations in *vitro* are difficult for several reasons. First, the rate of exchange between propofol-containing liposomes, the aqueous phase, serum proteins, and cellular constituents is not precisely known, which could significantly affect plasma concentrations *in vivo*. In addition, protein binding *in vivo* is unlikely to be instantaneous, so free drug concentrations with a bolus injection would probably be higher than the steady state value. Peak plasma levels after a bolus injection have been estimated at 50 μM, and stable levels of approximately 10–25 μM during maintenance infusion.[47] Additional factors such as speed of injection, volume of distribution, and pH are all factors that can affect plasma concentrations of anesthetics. Given the difficulty and uncertainty in estimating the *in vivo* concentrations and the likelihood that these estimations may be different in pathologic conditions (hemodilution, liver disease, diabetes), we believe that the concentration of propofol used in this study is likely to be similar to that encountered in clinical practice.

A hallmark of cardiac dysfunction in diabetic patients is the depressed contractility accompanied by prolonged relaxation. Although our study identifies that propofol enhances PRP in diabetic cardiomyocytes, the clinical significance of this finding is uncertain because propofol also causes a decrease in myofilament Ca^{2+} sensitivity resulting in an overall negative inotropic effect. However, a more important clinically related finding is that propofol exacerbates the already prolonged relengthening of the cardiomyocyte causing a negative lusitropic effect and suggests that propofol may increase the risk for additional diastolic dysfunction in diabetic patients. Moreover, this negative lusitropic effect can influence cardiac inotropy because diastolic function significantly

influences systolic cardiac function, left ventricular filling, and coronary blood flow. Although difficulties arise when attempting to extrapolate results from *in vitro* studies to the *in vivo* situation, we believe that the results from this study support observations in the clinical setting. In light of our findings, we propose that caution should be used when administering propofol in patients with limited inotropic reserve and/or diastolic dysfunction. Further studies are required to assess the role of PKC-dependent modulation of NCX by propofol in the diabetic heart.

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