Exercise prevents diabetes-induced impairment in superficial buffer barrier in porcine coronary smooth muscle

C. A. Witeczak1 and M. Sturek1,2,3

Departments of 1Medical Pharmacology and Physiology 2Internal Medicine, and 3Center for Diabetes and Cardiovascular Health, University of Missouri, Columbia, Missouri 65212

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Witeczak, C. A., and M. Sturek. Exercise prevents diabetes-induced impairment in superficial buffer barrier in porcine coronary smooth muscle. J Appl Physiol 96: 1069–1079, 2004. First published November 21, 2003; 10.1152/japplphysiol.00460.2003.—In healthy coronary smooth muscle cells, the superficial sarcoplasmic reticulum (SR) buffers rise in intracellular Ca\(^{2+}\) levels. In diabetic dyslipidemia, basal Ca\(^{2+}\) levels are increased, yet Ca\(^{2+}\) influx is decreased and SR Ca\(^{2+}\) uptake is increased. Exercise prevents diabetic dyslipidemia-induced increases in basal Ca\(^{2+}\) levels and decreases in Ca\(^{2+}\) influx. We tested the hypothesis that diabetic dyslipidemia impairs Ca\(^{2+}\) extrusion via a decrease in superfical SR and that exercise will prevent these losses. Male Yucatan swine were maintained in four treatment groups: control, hyperlipidemic, diabetic dyslipidemic, and diabetic dyslipidemic plus aerobically exercise trained. Intracellular Ca\(^{2+}\) levels were measured during depolarization-induced Ca\(^{2+}\) influx and caffeine-induced SR Ca\(^{2+}\) release. Na\(^+/\)Ca\(^{2+}\) exchanger and plasmalemmal Ca\(^{2+}\)-ATPase activity were assessed by inhibition with low extracellular Na\(^+\) and 5,6-carboxyfluoresent, respectively. Superficial SR was quantified using the internal membrane dye 3,3′-dihexyloxacarbocyanine iodide (DiOC\(_{6}\)) and novel analysis techniques. We found that, in diabetic dyslipidemia, Ca\(^{2+}\) extrusion was impaired and superficial SR was decreased. Exercise prevented the diabetic dyslipidemia-induced decrease in superficial SR and restored plasmalemmal Ca\(^{2+}\) extrusion. On the basis of these results, we conclude exercise attenuates the diabetic dyslipidemia-induced impairment in intracellular Ca\(^{2+}\) regulation.

sodium-calcium exchanger; plasma membrane calcium-adenosine triphosphatase; sarco(endo)plasmic reticulum calcium-transporting adenosine triphosphatase

DIABETES CURRENTLY AFFLICTS over 16,000,000 Americans (25), creating a total economic burden of over one hundred billion dollars ($100,000,000,000) in medical costs (2). With epidemiological estimates projecting the number of individuals with diabetes to reach almost 22,000,000 by the year 2025 (22), diabetes is rapidly being recognized as a public health threat that is rising to epidemic proportions. Coronary artery disease is increased three- to sixfold in diabetes (45) and represents the leading cause of death in diabetic patients (22). It has been proposed that impaired intracellular Ca\(^{2+}\) regulation underlies the increase in coronary artery disease seen in diabetic dyslipidemia (24).

In healthy coronary smooth muscle cells, intracellular Ca\(^{2+}\) levels are regulated by both the structural localization of the sarcoplasmic reticulum (SR) as well as the functional activity of the intracellular Ca\(^{2+}\) transporters. According to the superficial buffer barrier hypothesis, the superficial SR buffers rises in steady-state intracellular Ca\(^{2+}\) levels because of the sequestration of subplasmalemmal Ca\(^{2+}\) as well as the directed release of SR Ca\(^{2+}\) toward the plasmalemma (6, 7, 21). Thus the superficial SR provides the structural framework for the functional coupling of Ca\(^{2+}\) influx with both SR Ca\(^{2+}\)-uptake and plasmalemmal Ca\(^{2+}\) extrusion mechanisms [i.e., the Na\(^+/\)Ca\(^{2+}\) exchanger (NCX) and the plasmalemmal Ca\(^{2+}\)-ATPase (PMCA)]. In coronary smooth muscle cells from diabetic dyslipidemic swine, basal or steady-state intracellular Ca\(^{2+}\) levels are increased (44), yet Ca\(^{2+}\) influx is decreased (40) and sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) activity is increased (17). These results suggest that plasmalemmal Ca\(^{2+}\) extrusion is impaired in diabetic dyslipidemia.

Disruption of the intracellular cytoskeleton and the endoplasmic reticulum has been shown to impair intracellular Ca\(^{2+}\) regulation (13, 42). In cultured, porcine coronary endothelial cells, retraction of the superficial endoplasmic reticulum with nocodazole uncoupled endoplasmic reticulum Ca\(^{2+}\) release to NCX activity and resulted in a prolonged recovery of intracellular Ca\(^{2+}\) levels after bradykinin stimulation (13). Despite these significant findings on the relationship between impaired intracellular Ca\(^{2+}\) regulation and superficial SR structure, no studies have investigated the relationship between the diabetic dyslipidemia-induced impairment in intracellular Ca\(^{2+}\) regulation and structural alterations in the superficial SR in coronary smooth muscle.

Exercise training attenuates the progression of coronary artery disease and has been shown to elicit alterations in coronary smooth muscle intracellular Ca\(^{2+}\) regulation. These include increases in Ca\(^{2+}\) influx (5, 15), increases in SR Ca\(^{2+}\) unloading (i.e., directed release of SR Ca\(^{2+}\) towards the plasmalemma at rest) (34), and decreases in nuclear Ca\(^{2+}\) signaling (39). Because the SR has a finite capacity to store Ca\(^{2+}\), these results collectively suggest that exercise training enhances plasmalemmal Ca\(^{2+}\) extrusion. Studies conducted by Heaps et al. (15) have demonstrated that the exercise-induced increase in Ca\(^{2+}\) influx in coronary smooth muscle is not compensated by increases in plasmalemmal Ca\(^{2+}\) extrusion via the NCX, suggesting that exercise enhances plasmalemmal Ca\(^{2+}\) extrusion via the PMCA. However, to date there have been no studies that have directly examined PMCA activity in coronary smooth muscle with exercise training.

Exercise training has been demonstrated to prevent phenotypic changes in smooth muscle in response to organ culturing of coronary artery with the potent mitogen, endothelin-1 (39). These changes included a decrease in total cellular DNA

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content that was observed concurrently with attenuated rises in nuclear Ca\textsuperscript{2+} levels (39). Furthermore, the organ culture model of coronary disease elicits significant changes in cellular morphology and decreased transnuclear SR distribution, i.e., altered deep SR (18). The clear precedence for changes in SR morphology being associated with altered Ca\textsuperscript{2+} homeostasis provides a rational basis for hypothesizing that alterations in superficial SR structure will be associated with altered Ca\textsuperscript{2+} homeostasis, specifically Ca\textsuperscript{2+} extrusion in diabetic dyslipidemia and exercise training.

Presently, to our knowledge, there are no reports demonstrating the protective effects of exercise on diabetic dyslipidemia-induced impairments in coronary smooth muscle intracellular Ca\textsuperscript{2+} regulation. This void of information of cellular mechanisms exists, even though exercise is recommended for the reduction of lipid abnormalities and the prevention of coronary artery disease in individuals with diabetes (14, 27). Thus we tested the hypotheses that the elevated basal intracellular Ca\textsuperscript{2+} levels seen in cells from diabetic dyslipidemic swine were due to either 1) an impairment in plasmalemmal Ca\textsuperscript{2+} extrusion and/or 2) a decrease in the amount of superficial SR and that 3) exercise training would prevent the impairment in steady-state intracellular Ca\textsuperscript{2+} regulation by attenuating and/or preventing these alterations.

MATERIALS AND METHODS

Porcine Model of Cardiovascular Disease and Diabetes

All procedures involving animals were approved by the Animal Care and Use Committee of the University of Missouri and fully complied with the American Veterinary Medical Association Panel on Euthanasia. Male Yucatan swine (aged 8–15 mo) were obtained from the Sinclair Research Center (Columbia, MO) and randomly assigned to one of four treatment groups. These groups included 1) low-fat fed, sedentary (control; n = 8); 2) high-fat, high-cholesterol fed, sedentary (hyperlipidemic; n = 7); 3) alloxan-induced hyperglycemic, high-fat, high-cholesterol fed, sedentary (diabetic dyslipidemic; n = 7); and 4) diabetic dyslipidemic that were aerobically exercise trained (n = 7). The low-fat diet consisted of Purina Minipig chow (Purina Mills, St. Louis, MO). Low-fat-fed animals were maintained at a weight gain of −1.0% gain/wk by 1.050 g (3,182 kcal) of chow ration daily. The high-fat, high-cholesterol diet consisted of 1,000 g (3,957 kcal) Purina Minipig chow supplemented with 14.0 g cholesterol, 119.7 g coconut oil, 16.1 g corn oil, 4.9 g sodium cholate, and 241.8 g of sucrose. High-fat-, high-cholesterol-fed animals were maintained at a 3% weight gain/wk with additional feed as necessary to maintain the expected weight gain as described in detail (4). All animals were fed once daily and given free access to water.

Hyperglycemia was induced by the intravenous administration of 100 mg/kg of the pancreatic β cell toxin, alloxan (9, 16). Diabetic pigs were maintained with plasma glucose levels at 300–400 mg/dl for the duration of the study by using feed and insulin therapy algorithms that maintained a positive energy balance (body weight) and hyperglycemia (4). Exercise training consisted of treadmill running performed 4 days/wk for 20 wk. The training bout consisted of three stages: 1) a gradual 10-min warm-up, 2) 30 min at 65–75% of maximum heart rate (considered moderate intensity), and 3) a 5-min cool-down. Throughout the 20-wk training period, treadmill grade was increased to elicit the target heart rate range. At week 20, the animals were placed in a low-stress sling and attached to a heart rate monitor. After a 10-min acclimation period, 8–10 heart rate values were taken during a 10-min recording phase. These 8–10 values were averaged together to determine the resting heart rate for each animal. The animals did not exercise for 2 days before death to separate the acute effects of exercise from the chronic effects of training.

Fasting arterial blood samples were obtained from anesthetized animals at the time of death. Plasma was assayed for blood glucose levels by using standard methods by the Veterinary Clinical Pathology department at the University of Missouri (Columbia, MO). Plasma was directly assayed for total cholesterol and triglyceride levels by using a standard enzymatic kit (Sigma-Aldrich, St. Louis, MO) (9, 41). Postprandial plasma lipid levels were assessed at week 12 of the intervention treatment. Arterial samples were obtained from fully conscious animals at 2 and 7 h after feed consumption. Plasma was directly assayed for total cholesterol and triglyceride levels using a standard enzymatic kit (Sigma-Aldrich) (9, 41). After 20 wk in a treatment group, animals were anesthetized with isoflurane and the heart surgically removed. The right coronary artery was dissected away from the myocardium and placed in ice-cold Eagle’s minimal essential storage media until ready for the isolation of smooth muscle cells.

Isolation of Coronary Smooth Muscle Cells

The procedure for the enzymatic dispersal of porcine coronary smooth muscle cells from arterial segments has been previously described (16, 17, 34). Briefly, arterial sections were incubated with a collagenase solution containing 294 U/ml collagenase, 5 U/ml elastase, 2 mg/ml bovine serum albumin, 1 mg/ml soybean trypsin inhibitor, and 0.4 mg/ml DNase I. Cells were enzymatically dispersed by incubation in a shaking water bath at 37°C for 60 min. The first fraction, consisting largely of endothelial cells, was removed and discarded. Additional collagenase solution was added, and the cells incubated in the water bath for 30 min. Smooth muscle cells from this fraction were identified morphologically (38) and studied within 6 h of dispersion. These acutely used smooth muscle cells were not cultured in monolayer preparations.

Solutions

The physiological salt solution (PSS) (in mM) 138 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, and 10 glucose, which was titrated to a pH of 7.4 with NaOH. The high-K\textsuperscript{+} solution (80K) contained (in mM) 65 NaCl, 80 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, and 10 glucose, which was titrated to a pH of 7.4 with NaOH. The high-K\textsuperscript{+}, low-Na\textsuperscript{+} solution (80K+5Na) contained (in mM) 80 KCl, 2 CaCl\textsubscript{2}, 63 LiCl, 1 MgCl\textsubscript{2}, 10 HEPES, and 10 glucose, which was titrated to a pH of 7.4 with NaOH. Caffeine (Caf; Sigma-Aldrich) was directly dissolved into the high-K\textsuperscript{+}-containing solutions to a 5 mM final concentration. Stock solutions of thapsigargin (TG: Sigma-Aldrich) and 5,6-carboxyceosine (CBE; Sigma-Aldrich) were prepared in ethanol and dimethyl sulfoxide, respectively. Stock solutions of TG and CBE were diluted in high-K\textsuperscript{+}-containing solutions to final concentrations of 1 and 5 μM, respectively.

Free Intracellular Ca\textsuperscript{2+} Indicator, Fura-2

Basal intracellular free Ca\textsuperscript{2+} levels were assessed using the fluorescent Ca\textsuperscript{2+} indicator, fura-2 (Molecular Probes, Eugene, OR) to compare basal intracellular Ca\textsuperscript{2+} levels with previous studies performed in this laboratory (16, 17, 34, 44). Briefly, freshly dispersed cells were incubated with 2.5 μM of fura-2 for 20 min in a shaking water bath at 37°C. Fura-2 was alternatively excited by 340 and 380 nm light every 0.20 s, and the emitted light was collected at 510 nm. The measurement of intracellular Ca\textsuperscript{2+} levels was made by digital imaging epifluorescence microscopy and assessed as the emission of the fura-2 (340/380 nm) ratio.

Free Intracellular Ca\textsuperscript{2+} Indicator, Fura Red

Changes in steady-state intracellular Ca\textsuperscript{2+} levels were assessed by using the fluorescent Ca\textsuperscript{2+} indicator, fura red (Molecular Probes).
Acutely dispersed cells were incubated with 5.0 μM of fura red for 45 min in a shaking water bath at 37°C. Fura red was excited with 440- and 485-nm light image pairs collected every 10 s with digital imaging epifluorescence microscopy. Images were analyzed offline by using Image Pro Plus (4.0) software. With the use of Image Pro Plus, an area of interest was drawn around the cell edge. This area of interest was then applied to images acquired throughout the experimental protocol, and the mean pixel intensity was determined for each cell throughout the experiment. All cells were depolarized with high K⁺ to induce Ca²⁺ influx. The SR Ca²⁺ store was depleted with 5 mM caffeine. The SERCA, NCX, and PMCA were inhibited by 1 μM TG, 5 mM extracellular Na⁺, and 5 μM CBE, respectively.

**PMCA Inhibitor, CBE**

Specificity for the PMCA. Laser scanning confocal microscopy was used to assess the specificity for the PMCA. Freshly dispersed cells were superfused with 5 μM CBE for 3 min, and the localization of CBE within the cell was observed by using a Noran Oz laser scanning confocal microscope (39, 41). After a thorough wash in PSS for 5 min, the cells were superfused with 2.5 × 10⁻⁷ M of the internal membrane dye, 3,3′-dihexyloxacarbocyanine iodide (DiOCl₂ Molar Probes) for 5 min. Visualization of both the CBE and the DiOCl₂ staining occurred via excitation using the 488-nm line of an argon-krypton laser, and the emission was collected after passage through a 500-nm-long-pass filter. The nucleus was visualized using the fluorescent probe SYTO-64 (Molecular Probes). The SYTO64 (500 nM) was superfused over the cells for 5 min and then excited by using the 568-nm line of the argon-krypton laser. (For simplicity, the labeling of the nucleus with SYTO-64 is not shown.)

**Delayed onset of PMCA inhibition.** Digital imaging epifluorescence microscopy was used to assess the duration of time needed for CBE to bind the PMCA and inhibit its activity. Freshly dispersed cells loaded with fura red were superfused with PSS for 4 min and then exposed to one of four experimental solutions for 6 min. The composition of the experimental solutions was as follows: S1 = 80K + Ca²⁺ + TG, S2 = 80K + Ca²⁺ + TG + 5Na, S3 = 80K + Ca²⁺ + TG + CBE, and S4 = 80K + Ca²⁺ + TG + 5Na + CBE. The kinetics of the intracellular Ca²⁺ response was determined from the peak of the high-K⁺/caffeine-induced response to 7 min from the start of the experiment and then from 7 to 10 min from the start of the experiment.

**Quantification of Superficial SR by Using DiOCl₂**

The fluorescent dyes utilized for the visualization of the superficial SR and the plasmalemma were DiOCl₂, and 5-(and-6)-carboxyseminalphathorhodofluor (SNARF)-1 acetoxymethyl ester, acetate (SNARF; Molecular Probes), respectively. Freshly dispersed cells were incubated with 2.5 × 10⁻⁷ M DiOCl₂ for 30 min, washed in PSS, and incubated with 5.0 × 10⁻⁸ M SNARF for 30 min. Visualization of the dyes was performed by digital imaging epifluorescence microscopy. The DiOCl₂ and SNARF were excited at 485 and 575 nm, respectively, and the emission was collected through a band-pass filter centered at 528 and 630 nm, respectively. Three images were acquired for each wavelength at z-axis intervals of 0.3 μm. The focal plane of the DiOCl₂ fluorescence was utilized as the midpoint of the cell. On the basis of spatial calibrations previously performed in the laboratory, these specifications allowed us to accurately measure intracellular distances down to ~154 nm. Acquired images were deconvolved by using Image Pro Plus (4.0) software to eliminate out-of-focus fluorescence (18). The digital images were converted into numerical pixel intensity values using the Image Pro Plus Bit-Map Analysis function and then exported into Microsoft Excel spreadsheets. The intensity of the DiOCl₂ fluorescence was normalized to the fluorescence of a 4-μm multispectral bead (Molecular Probes) that was excited at 485 nm. The amount of superficial SR was calculated as the ratio of cell layer (average DiOCl₂ intensity per area) to the bead (average fluorescence per area).

**Statistical Analysis**

The results are presented as the average of every cell ± SE. Animal and cell numbers for groups in each experiment are indicated in the figure legends as n = animal number/cell number. Statistical significance among treatment groups was defined as P < 0.05 and determined by the one-way analysis of variance test. Significance between individual groups was assessed by Student-Newman-Keuls post hoc analysis.

**RESULTS**

**Basic Information on the Methodology**

**PMCA inhibitor, CBE. Specificity for the PMCA.** The specificity of CBE for the PMCA was assessed by laser confocal microscopy. The internal membrane dye, DiOCl₂, was utilized to assess the location of the SR. As shown in Figure 1A, the DiOCl₂ fluorescence is primarily localized centrally within the cell. Figure 1B demonstrates the binding of CBE to both intracellular and plasmalemmal constituents. As shown in Fig. 1, C–E, in which overlaps of both the CBE and DiOCl₂ fluorescence are depicted, CBE does not bind selectively to the PMCA on the plasmalemma but also binds to constituents located on the SR (depicted by the yellow areas). The intense CBE fluorescence in the central portion of the cell denotes binding to the perinuclear SR, as previously described (12, 18, 39, 41).

**DELAYED ONSET OF PMCA INHIBITION.** The kinetics of the intracellular Ca²⁺ response with application of the inhibitors was assessed to determine the relative rate of inhibitor onset in cells isolated from control, hyperlipidemic, diabetic dyslipidemic, and diabetic dyslipidemic plus exercise-trained swine. For simplicity, typical tracings of the experimental protocols and kinetic measurements are only shown for cells from both the control (Fig. 2, A–D) and diabetic dyslipidemic (Fig. 2, E–H) swine. The proteins inhibited by each of the experimental solutions is shown at the bottom of Fig. 2. As depicted in Fig. 2, A and E, all of the experimental solutions elicited an initial rise in intracellular Ca²⁺ levels due to the depolarization-induced opening of the voltage-gated Ca²⁺ channels and the depletion of the SR Ca²⁺ store by high K⁺ and caffeine, respectively. However, with solutions S3 and S4, this rise in intracellular Ca²⁺ levels was followed by a biphasic response in the fura red ratio. This included an initial decline in intracellular Ca²⁺ levels from the peak response to ~7 min after solution exposure, followed by a subsequent rise in the intracellular Ca²⁺ levels to the end of the experimental protocol. This secondary rise is likely due to the delayed onset of PMCA inhibition by CBE. Evidence to support this hypothesis is shown for control cells in Fig. 2, B–D, in which the rates of the biphasic responses are compared for each of the experimental protocols. As shown in Fig. 2C, in healthy control cells, the initial rate of decline was not significantly different between solutions S1 and S3, or between S2 and S4, suggesting that the CBE was not yet inhibiting the PMCA. However, during the secondary phase of the response, the solutions containing CBE displayed a significant change in the rate of response relative to their non-CBE-containing counterparts (Fig. 2D, S3 and S4). Because previous experiments had already shown that 5 μM CBE would not emit enough light to confound the Ca²⁺ signal, this rise in intracellular Ca²⁺ levels is likely due to the inhibition of PMCA inhibition. The cellular response from the
diabetic dyslipidemic swine was generally the same as those seen in the healthy cells. For example, all cells in both groups showed similar kinetic responses (i.e., an increase in intracellular Ca$^{2+}$ levels) with the application of high K$^+$ and caffeine. Also, significant increases in the rate from 7 to 10 min were seen only in S3 and S4 (Fig. 2, D and H). On the basis of these observations, it is not likely that the diabetic dyslipidemic condition affected the ability of this maximal concentration of CBE to inhibit the PMCA.

Given the slow rate of PMCA inhibition by CBE, kinetic measurements of the decline in the depolarization- and caffeine-induced Ca$^{2+}$ peak would not provide an accurate assessment of the functional capability of the intracellular Ca$^{2+}$ transporters. Thus the effectiveness of the NCX and the PMCA to alter steady state intracellular Ca$^{2+}$ levels was assessed as the change in the fura red ratio from the initial steady-state intracellular Ca$^{2+}$ level to the final steady-state level after experimental perturbation shown in Fig. 2 at 10 min. Steady-state measures were considered of most relevance because a major aim of this study was to determine the Ca$^{2+}$ extrusion mechanism involved in the regulation of basal (steady state) Ca$^{2+}$ levels.

Quantification of superfical SR by using DiOC$_6$. Quantification of the superfical SR was performed using the fluorescent dyes DiOC$_6$ and SNARF and novel analysis techniques. As shown in Fig. 3A, which represents the transmitted light image of a cell isolated from a control animal, only smooth muscle cells were selected for imaging on the basis of their morphological identification as smooth muscle cells. Visible movement in response to the superfusion with PSS was the only other criteria utilized for cell selection to prevent bias in the selection of specific populations of cells on the basis of morphology. Visualization of the dyes was performed by digital imaging epifluorescence microscopy. As stated previously, DiOC$_6$ is a fluorescent, lipophilic dye that stains internal membranes such as the SR and the Golgi apparatus (18). In Fig. 3B, the typical reticular pattern of the DiOC$_6$ staining is shown centrally in the cell with small punctuate “hot spots” denoting superficial SR located more peripherally as shown by the box in Fig. 3D. Figure 3C depicts the typical intracellular staining of SNARF. Although SNARF is a pH indicator, we found it to be a nearly ideal plasmalemmal marker because of its homogeneous distribution in the myoplasm, and its spectral properties yielded no overlap of fluorescence signals with DiOC$_6$. Thus, as shown in Fig. 3D, the use of SNARF as a plasmalemmal marker enabled the identification of the plasmalemmal edge (shown in red) in the DiOC$_6$-stained images. The digital images were converted into numerical pixel intensity values using the Image Pro Plus Bit-Map Analysis function as exported in Microsoft Excel worksheets as shown in Fig. 3E. With the use of the SNARF image to denote the plasmalemmal edge, only the DiOC$_6$ values corresponding to positions within $\sim$308 nm of the plasmalemma were utilized to assess superfical SR content. The intensity of the DiOC$_6$
fluorescence was normalized to the fluorescence of a 4-μm multispectral bead (Fig. 3F, inset) that displayed a linear relationship between fluorescence intensity and exposure time to the 485-nm light (Fig. 3F).

Effects Of Diabetes And Exercise Training

Diabetic dyslipidemia and exercise training. Table 1 summarizes the data for the indicators of diabetic dyslipidemia and exercise training. The percent changes in body weight from week 0 to week 19 were significantly elevated in the hyperlipidemic, diabetic dyslipidemic, and exercise-trained diabetic dyslipidemic swine above control. This was expected because all animals fed the high-fat, high-cholesterol diet were maintained utilizing feed and insulin algorithms to elicit a 3% gain in body weight per week. Fasting plasma glucose levels were elevated ~3.5-fold in both the diabetic dyslipidemic and the diabetic dyslipidemic plus exercise-trained animals, indicative of alloxan-induced hyperglycemia. The glucose levels reported here are ~70 mg/dl greater than our laboratory’s previous reports (16, 17, 41) because the blood was collected under anesthesia. There was no significant difference in the average daily insulin dosage between the diabetic dyslipidemic and exercise-trained swine. Postprandial lipid values for total cholesterol levels were two- to sixfold higher in the hyperlipidemic, diabetic dyslipidemic, and diabetic dyslipidemic plus exercise-trained animals over control. Total triglycerides were only increased in diabetic dyslipidemic and diabetic dyslipidemic plus exercise-trained groups, thus the term “diabetic dyslipidemic,” because hypertriglyceridemia is a hallmark of lipid abnormalities in diabetes (27). In contrast, nondiabetic pigs fed the high-fat and high-cholesterol diet are defined as “hyperlipidemic.” Similarly, fasting cholesterol levels were six- to eightfold higher in the hyperlipidemic, diabetic dyslipidemic, and diabetic dyslipidemic plus exercise-trained swine over control. There was no significant difference in fasting total triglyceride levels. Efficacy of the exercise training protocol was verified by a decrease in the resting heart rate at week 20 from 75 beats/min in the diabetic dyslipidemic animals to 58 beats/min in the exercise-trained diabetic dyslipidemic animals, decreased heart rate during submaximal exercise, and increased physical work capacity (4).

Basal intracellular Ca2+ levels are elevated in hyperlipidemia and diabetic dyslipidemia. Basal intracellular Ca2+ levels were assessed as the fura-2 emission before any experimental perturbation. As shown in Fig. 4, the basal Ca2+ levels were significantly increased in cells from both the hyperlipidemic and diabetic dyslipidemic swine above control. In this study, exercise training concurrent with diabetic dyslipidemia did not significantly lower the basal intracellular Ca2+ levels below control. The Ca2+ level intermediate between control and diabetic dyslipidemic, however, indicated that exercise did not prevent, but attenuated, the effects of diabetic dyslipidemia.

Assessment of steady-state intracellular Ca2+ levels with fura red. The effect(s) of hyperlipidemia, diabetic dyslipidemia, and diabetic dyslipidemia plus exercise training on steady-state intracellular Ca2+ regulation was assessed by using the indicator fura red. The steady-state change in the fura red ratio from the preexperimental solution start point to the end of the response was utilized to assess the relative contribution of the Ca2+ transporters to the maintenance of steady-state intracellular Ca2+ levels. In healthy cells from control swine, steady-state intracellular Ca2+ levels were not elevated until both the SERCA and the PMCA were simultaneously inhibited (S3 and S4) (Fig. 5A). Direct inhibition of SERCA alone (S1),
Intracellular Ca\(^{2+}\) in swine, there was an impaired ability to maintain steady-state levels in the cells from diabetic dyslipidemic plus exercise swine (DDX; 0.4 ± 0.1) compared with control (C; 0.5 ± 0.2) (Fig. 5B). This impairment in the maintenance of intracellular Ca\(^{2+}\) may contribute to the elevated basal intracellular Ca\(^{2+}\) and suggested that impaired plasmalemmal Ca\(^{2+}\) extrusion may contribute to the elevated basal intracellular Ca\(^{2+}\) in diabetic dyslipidemia. Basal intracellular Ca\(^{2+}\) levels were significantly increased (\(P < 0.05\)) in diabetic dyslipidemic (S2, S3, and S4) (Fig. 5C). Exercise training restored some intracellular Ca\(^{2+}\) regulatory activity to the cells from the exercise-trained, diabetic dyslipidemic swine. In cells isolated from diabetic dyslipidemic plus exercise-trained swine, only the simultaneous inhibition of the SERCA and the PMCA (S4) resulted in a significant increase in the steady-state change response above SO (Fig. 5D). Considering that the diabetic dyslipidemic group exhibited a complete loss of intracellular Ca\(^{2+}\) regulation via the SERCA, the NCX, and the PMCA, these results suggested that exercise training attenuated the loss of plasmalemmal Ca\(^{2+}\) extrusion.

**Exercise prevented the diabetic dyslipidemia-induced decrease in superficial SR.** The amount of superficial SR was quantified to assess the effect of hyperlipidemia, diabetic dyslipidemia, and exercise training concurrent with diabetic dyslipidemia on the structure of the superficial buffer barrier. The superficial SR was defined as the amount of DiOC\(_6\) fluorescence within ~154 or ~308 nm of the plasmalemmal. As shown in Fig. 6A, the ratio of the cell layer (average DiOC\(_6\) intensity per area) to the 4-μm multispectral bead (average fluorescence per area) within ~154 nm of the plasmalemmal was not significantly altered between the treatment groups (\(P = 0.067\)). However, as shown in Fig. 6B, the ratio of the cell layer (average DiOC\(_6\) intensity per area) to the 4-μm multispectral bead (average fluorescence per area) within ~308 nm of the plasmalemma was significantly (\(P < 0.05\)) decreased in cells from the hyperlipidemic and diabetic dyslipidemic swine.

**DISCUSSION**

The major findings from these studies support the overall hypotheses that exercise training would attenuate and/or prevent the diabetic dyslipidemia-induced impairment in intracellular Ca\(^{2+}\) regulation and the alteration in superficial SR content in coronary smooth muscle cells from diabetic dyslipidemic swine. Using freshly isolated cells loaded with the Ca\(^{2+}\) indicator fura red, we found more specifically that plasmalemmal Ca\(^{2+}\) extrusion was impaired in diabetic dyslipidemic swine, consistent with the diminished ability to maintain intracellular Ca\(^{2+}\) levels with the inhibition of the SERCA and the PMCA, these results suggested that exercise training attenuated the loss of plasmalemmal Ca\(^{2+}\) extrusion.
Yucatan swine and that exercise attenuated this impairment. These novel findings on the effects of exercise training are a significant extension of studies on Ca\textsuperscript{2+}/H\textsuperscript{1001} regulation in vascular smooth muscle of human uterine artery (10), in vitro studies mimicking diabetes (33, 43), and coronary artery studies from sedentary animals (16, 41). Also, using freshly isolated cells loaded with the internal membrane dye, DiOC\textsubscript{6}, we found that the amount of superficial SR within ~308 nm of the plasmalemma is decreased with diabetic dyslipidemia and that exercise prevented this decrease. This novel finding suggested that the diabetic dyslipidemia-induced impairment in coronary smooth muscle intracellular Ca\textsuperscript{2+} regulation may be linked to changes in the localization of the superficial SR.

The relative contribution of the NCX and the PMCA to the maintenance of steady-state intracellular Ca\textsuperscript{2+} levels was assessed by direct inhibition with a low-Na\textsuperscript{+}-concentration perfusate and the fluorescein derivative, CBE, respectively. Although the SERCA was directly inhibited with TG, the activity...
In this study, exercise training attenuated, but did not prevent, the diabetic dyslipidemia-induced increase in basal intracellular Ca\textsuperscript{2+} levels as we found in a preliminary study (44). Although results demonstrating the efficacy of the exercise training protocol (i.e., decreased resting heart rates) were similar to those in a previous study (40), there were no significant alterations in the fasting plasma lipid profile with exercise. Thus the lack of a significant decrease in the basal intracellular Ca\textsuperscript{2+} levels in the cells from the diabetic dyslipidemic plus exercise-trained swine, together with the lack of a significant change in fasting plasma cholesterol and triglyceride values, suggested that the additional weight gain in this study hindered at least some of the beneficial effects of the exercise training bout. The absence of exercise training-induced attenuation of plasma lipids and glucose is very intriguing in that it strongly argues for either a more direct action of exercise on the vasculature or changes in other systemic (humoral factors) that might elicit the restoration in Ca\textsuperscript{2+} extrusion and superficial SR distribution. Such direct vascular actions of exercise may be analogous to vascular actions of pharmacological agents, such as statins, which prevent coronary atherosclerosis and Ca\textsuperscript{2+} signaling with no change in plasma cholesterol or glucose (23, 41).

The cells from the diabetic dyslipidemic swine displayed a lack of response to any and/or all of the Ca\textsuperscript{2+}-transporter inhibitors, suggesting that none of these transporters was functioning to maintain steady-state intracellular Ca\textsuperscript{2+} levels. In other words, all Ca\textsuperscript{2+} efflux mechanisms were already impaired in diabetic dyslipidemia and maximum concentrations of inhibitors did not further decrease Ca\textsuperscript{2+} efflux. This lack of a response was consistent with other studies performed in coronary smooth muscle from diabetic dyslipidemic swine (26) and suggested that at least one of several alternative intracellular Ca\textsuperscript{2+} regulatory mechanisms was compensating to buffer intracellular Ca\textsuperscript{2+} levels to prevent further increases in Ca\textsuperscript{2+} levels. These possible alternative mechanisms include intracellular Ca\textsuperscript{2+} buffering via 1) the nuclear Ca\textsuperscript{2+} store, 2) soluble intracellular Ca\textsuperscript{2+}-binding proteins, and/or 3) the mitochondria. First, although a number of studies have shown that Ca\textsuperscript{2+} is regulated differentially between the nucleus and the bulk myoplasm due to perinuclear Ca\textsuperscript{2+} uptake (19, 41), several studies have demonstrated a concentration-dependent loss in nuclear Ca\textsuperscript{2+} reloading in response to the SERCA inhibitor, thapsigargin (1, 20). Thus, even if SERCA-like pumps are found on the outer nuclear envelope, the use of TG in our experimental protocols would have inhibited this uptake. Second, the myoplasm of a coronary smooth muscle cell is rich with a number of soluble Ca\textsuperscript{2+}-binding proteins, including calmodulin. Although diabetic rats showed decreased calmodulin levels (29), the Ca\textsuperscript{2+}-buffering capacity of calmodulin was not studied, nor have any studies assessed this possible perturbation in coronary smooth muscle cells. Third, the mitochondria have long been thought to contribute to intracellular Ca\textsuperscript{2+} regulation under pathophysiological conditions. The mitochondrial Ca\textsuperscript{2+} uniporter has a Michaelis constant for Ca\textsuperscript{2+} that enables substantial Ca\textsuperscript{2+} uptake only when local Ca\textsuperscript{2+} concentrations exceed ~5.0 \textmu M (37). Even though the basal intracellular Ca\textsuperscript{2+} levels did not reach this concentration in the cells from the diabetic dyslipidemic swine, a number of studies have demonstrated that locally high levels of Ca\textsuperscript{2+} can be reached via a close apposition of the mitochondria to the SR (30, 35).

Fig. 6. Exercise prevented the diabetic dyslipidemia-induced decrease in superficial sarcoplasmic reticulum. The superficial SR was defined as the SR within ~154 nm (A) or ~308 nm (B) of the plasmalemma. The amount of superficial SR was quantified as the ratio of the DiOC\textsubscript{6} fluorescence (within ~154 or 308 nm of the plasmalemma) per area of these cell layers to the fluorescence of the multispectral bead per bead area (ratio of cell to bead). A: ratio of cell to bead within ~154 nm approached, but did not reach, statistical difference between groups (P = 0.067). B: ratio of cell to bead was significantly decreased (**P < 0.01) within ~308 nm in cells from the H (0.53 ± 0.03, n = 8/46) and DD swine (0.51 ± 0.03, n = 5/38) below C (0.65 ± 0.05, n = 5/29). In DDX swine (0.67 ± 0.04, n = 6/59), the decrease in the ratio of the cell to bead was prevented.

of the SERCA was not isolated in these experiments. The reason for this omission in the experimental protocols was due to the ability of CBE to bind to constituents on the SR (i.e., SERCA) (Fig. 1, C and E). A number of previous studies had utilized CBE as a specific and direct inhibitor of the PMCA while minimizing (3, 8) or neglecting (31) its possible effects on the SERCA. Using scanning laser confocal microscopy, we have clearly demonstrated that CBE is not solely localized to the PMCA in porcine coronary smooth muscle cells.

The activity of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was also not examined in these experiments, even though studies have demonstrated a diabetes-induced impairment in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in vascular smooth muscle (28, 32). This was because both of the diabetic swine groups were given daily injections of exogenous insulin (see Table 1) to maintain targeted weight gain and plasma glucose levels throughout the 20-wk study. Administration of insulin in doses that do not fully normalize blood glucose prevents the diabetes-induced reduction in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity (28, 32). Although our study design involving insulin treatment is likely to have prevented impairment in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, we cannot conclude definitively without having directly measured Na\textsuperscript{+}-K\textsuperscript{+}-ATPase or the use of ouabain in our Ca\textsuperscript{2+} regulation studies.

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Our data favor mitochondria as the alternative intracellular Ca\(^{2+}\)-buffering mechanism, but largely by exclusion of the other mechanisms. Future studies should involve direct measurement of mitochondrial Ca\(^{2+}\) levels.

In cells from hyperlipidemic and diabetic dyslipidemic swine, there was a significant decrease in the amount of superficial SR as defined as the average DiOC\(_6\) fluorescence within ~308 nm of the plasmalemma (Fig. 6). These data suggested that the impaired Ca\(^{2+}\) extrusion in chronic hyperlipidemia and diabetic dyslipidemia could be due to a decrease in the amount of superficial SR. Another provocative implication of this finding is that the functional superficial buffer barrier might extend beyond the rare junctional SR located ~50 nm from the plasmalemma (11). Precedence for functional effects of acute changes in superficial buffer barrier was shown by the loss of subplasmalemmal Ca\(^{2+}\) localization by pharmacologically induced retraction of endoplasmic reticulum deep (several micrometers) into vascular endothelial cells (13). Alternatively, the diabetic dyslipidemia-induced impairment in Ca\(^{2+}\) extrusion and the rise in basal intracellular Ca\(^{2+}\) levels could initiate the morphological changes that are characteristic of dedifferentiated coronary smooth muscle cells in coronary artery disease models (18).

All of our previous studies had addressed the functional alteration of the superficial buffer barrier, i.e., the effects on Ca\(^{2+}\) regulation. This is the first study to directly determine whether the structure of the superficial buffer barrier is altered, which in turn influences function of the superficial buffer barrier. Van Breemen’s (36) seminal findings that laid the groundwork for the superficial buffer barrier hypothesis 25 years ago included this structural component of the SR, but until the present study there was no direct evidence for alteration of the structure of superficial SR associated with functional alterations in superficial buffer barrier regulation of Ca\(^{2+}\). In other words, van Breemen proposed that the exquisite positioning of the SR in close proximity to the sarcolemma (superficial SR) facilitated the superficial buffer barrier function. Our significant finding of retraction of the superficial SR is a key feature of the mechanism for functional changes in the superficial buffer barrier. Our work on the structure of the superficial buffer barrier has filled a major gap in the superficial buffer barrier hypothesis. Further mechanistic studies involving biochemical steps in the retraction of the superficial SR in diabetic dyslipidemia and prevention by exercise is very likely to involve cytoskeletal protein regulation and so forth. On the basis of the important findings in this manuscript, future studies of these biochemical regulators of superficial SR retraction are warranted.

In this study, exercise training prevented the diabetic dyslipidemia-induced changes in superficial SR content. This finding is a significant extension of previous studies performed in our laboratory that demonstrated an exercise-induced prevention of morphological changes in organ-cultured, coronary smooth muscle cells treated with endothelin-1 (39). However, on the basis of these findings, it is presently unclear whether exercise primarily acts to attenuate changes in intracellular structure, which then modulate intracellular Ca\(^{2+}\) regulation. Time course studies showing that alterations in superficial SR precede (result in) impaired Ca\(^{2+}\) homeostasis would provide more compelling evidence for a cause-and-effect relationship, in addition to the association noted in this study.

In conclusion, we have definitively shown that aerobic exercise training attenuated the impairment of plasmalemmal Ca\(^{2+}\) extrusion from coronary smooth muscle in diabetic dyslipidemic swine. Furthermore, this functional impairment was associated with a decrease in the amount of superficial SR within ~308 nm of the plasmalemma, which was also prevented by exercise training.

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