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

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Witzak, 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 Ca2+ levels. In diabetic dyslipidemia, basal Ca2+ levels are increased, yet Ca2+ influx is decreased and SR Ca2+ uptake is increased. Exercise prevents diabetic dyslipidemia-induced increases in basal Ca2+ levels and decreases in Ca2+ influx. We tested the hypothesis that diabetic dyslipidemia impairs Ca2+ extrusion via a decrease in superficial 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 Ca2+ levels were measured during depolarization-induced Ca2+ influx and caffeine-induced SR Ca2+ release. Na+/Ca2+ exchanger and plasmalemmal Ca2+-ATPase activity were assessed by inhibition with low extracellular Na+ and 5,6-carboxy-yo-probe, respectively. Superficial SR was quantified using the internal membrane dye 3,3'-dihexyloxacarbocyanine iodide (DiOCl) and novel analysis tech-

iques. We found that, in diabetic dyslipidemia, Ca2+ extrusion was impaired and superficial SR was decreased. Exercise prevented the diabetic dyslipidemia-induced decrease in superficial SR and restored plasmalemmal Ca2+ extrusion. On the basis of these results, we conclude exercise attenuates the diabetic dyslipidemia-induced impairment in intracellular Ca2+ regulation.

In healthy coronary smooth muscle cells, intracellular Ca2+ levels are regulated by both the structural localization of the sarcoplasmic reticulum (SR) as well as the functional activity of the intracellular Ca2+ transporters. According to the super-

ficial buffer barrier hypothesis, the superficial SR buffers rises

in steady-state intracellular Ca2+ levels because of the seques-
tration of subplasmalemmal Ca2+ as well as the directed release of SR Ca2+ toward the plasmalemma (6, 7, 21). Thus the superficial SR provides the structural framework for the functional coupling of Ca2+ influx with both SR Ca2+ uptake and plasmalemmal Ca2+ extrusion mechanisms [i.e., the Na+/Ca2+ exchanger (NCX) and the plasmalemmal Ca2+-ATPase (PMCA)]. In coronary smooth muscle cells from diabetic dyslipidemic swine, basal or steady-state intracellular Ca2+ levels are increased (44), yet Ca2+ influx is decreased (40) and sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA) activity is increased (17). These results suggest that plasmalemmal Ca2+ extrusion is impaired in diabetic dyslipidemia.

Disruption of the intracellular cytoskeleton and the endo-

plasmic reticulum has been shown to impair intracellular Ca2+ regulation (13, 42). In cultured, porcine coronary endothelial cells, retraction of the superficial endoplasmic reticulum with nocodazole uncoupled endoplasmic reticulum Ca2+ release to NCX activity and resulted in a prolonged recovery of intracellu-

lar Ca2+ levels after bradykinin stimulation (13). Despite these significant findings on the relationship between impaired intracellular Ca2+ regulation and superficial SR structure, no studies have investigated the relationship between the diabetic dyslipidemia-induced impairment in intracellular Ca2+ 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 Ca2+ regulation. These include increases in Ca2+ influx (5, 15), increases in SR Ca2+ unloading (i.e., directed release of SR Ca2+ towards the plasmalemma at rest) (34), and, decreases in nuclear Ca2+ signaling (39). Because the SR has a finite capacity to store Ca2+, these results collectively suggest that exercise training enhances plasmalemmal Ca2+ extrusion. Studies conducted by Heaps et al. (15) have demonstrated that the exercise-induced increase in Ca2+ influx in coronary smooth muscle is not compensated by increases in plasmalemmal Ca2+ extrusion via the NCX, suggesting that exercise enhances plasmalemmal Ca2+ 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\(^{2+}\) levels (39). Furthermore, the organ culture model of coronary disease elicits significant changes in cellular morphology and decreased transmural SR distribution, i.e., altered deep SR (18). The clear precedence for changes in SR morphology being associated with altered Ca\(^{2+}\) homeostasis provides a rational basis for hypothesizing that alterations in superficial SR structure will be associated with altered Ca\(^{2+}\) homeostasis, specifically Ca\(^{2+}\) 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\(^{2+}\) 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\(^{2+}\) levels seen in cells from diabetic dyslipidemic swine were due to either 1) an impairment in plasmalemmal Ca\(^{2+}\) extrusion and/or 2) a decrease in the amount of supranuclear SR and that 3) exercise training would prevent the impairment in steady-state intracellular Ca\(^{2+}\) 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 experimental treatment. Arterial samples were collected 60 min after a meal and centrifuged at 1200g for 15 min. The plasma was collected and frozen at −70°C until analyzed. Total cholesterol and triglyceride levels were determined using the Lipid Reagent Kit (Sigma, St. Louis, MO) and standard enzymatic methods (9, 41).

Changes in steady-state intracellular Ca\(^{2+}\) levels were assessed by using the fluorescent Ca\(^{2+}\) 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 1μM 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′-dihexyloxycarbocyanine iodide (DiOC6; Molecular Probes) for 5 min. Visualization of both the CBE and the DiOC6 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 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+ + Caf++ + TG, S2 = 80K+ + Caf++ + TG +5Na, S3 = 80K+ + Caf++ + CBE, and S4 = 80K+ + Caf++ + TG +5Na + CBE. The kinetics of the intracellular Ca2+ response was determined by 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 DiOC6

The fluorescent dyes utilized for the visualization of the superficial SR and the plasmalemma were DiOC6 and 5-(and-6)-carboxyxyami-nophthlorhodolfluor (SNARF)-1 acetoxymethyl ester, acetate (SNARF; Molecular Probes), respectively. Freshly dispersed cells were incubated with 2.5 × 10−7 M DiOC6 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 DiOC6 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. Two images were acquired for each wavelength at z-axis intervals of 0.3 μm. The focal plane of the DiOC6 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 DiOC6 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 DiOC6 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, DiOC6, was utilized to assess the location of the SR. As shown in Figure 1A, the DiOC6 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 DiOC6 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 initiation 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 showed similar kinetic responses (i.e., an increase in intracellular Ca\(^{2+}\)) 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 ~308 nm of the plasmalemma were utilized to assess superficial 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 in vivo 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 Ca\(^{2+}\) levels are elevated in hyperlipidemia and diabetic dyslipidemia. Basal intracellular Ca\(^{2+}\) levels were assessed as the fura-2 emission before any experimental perturbation. As shown in Fig. 4, the basal Ca\(^{2+}\) 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 Ca\(^{2+}\) levels below control. The Ca\(^{2+}\) 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 Ca\(^{2+}\) levels with fura red. The effect(s) of hyperlipidemia, diabetic dyslipidemia, and diabetic dyslipidemia plus exercise training on steady-state intracellular Ca\(^{2+}\) 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 used to assess the relative contribution of the Ca\(^{2+}\) transporters to the maintenance of steady-state intracellular Ca\(^{2+}\) levels. In healthy cells from control swine, steady-state intracellular Ca\(^{2+}\) 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),
or in combination with the NCX (S2), resulted in no significant increase in the steady-state change above the application of 80 mM K+ plus caffeine alone (S0). In cells from hyperlipidemic swine, there was an impaired ability to maintain steady-state intracellular Ca2+ levels with the inhibition of the SERCA and either the NCX and/or the PMCA (S2, S3, and S4) (Fig. 5B). This impairment in the maintenance of intracellular Ca2+ levels above the 80K+Ca2+TG steady-state change (S1) suggested that the PMCA was impaired in the cells from the hyperlipidemic swine. In diabetic dyslipidemia, there was no significant change on exposure to any of the inhibitors (S1, S2, S3, and S4). This striking finding demonstrated that both the NCX and the PMCA were impaired with diabetic dyslipidemia and suggested that impaired plasmalemmal Ca2+ extrusion may contribute to the elevated basal intracellular Ca2+ levels in the diabetic dyslipidemic state (Fig. 5C). Exercise training restored some intracellular Ca2+ 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, the NCX, and the PMCA (S4) resulted in a significant increase in the steady-state change response above S0 (Fig. 5D). Considering that the diabetic dyslipidemic group exhibited a complete loss of intracellular Ca2+ regulation via the SERCA, the NCX, and the PMCA, these results suggested that exercise training attenuated the loss of plasmalemmal Ca2+ 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 DiOC6 fluorescence within ~154 or ~308 nm of the plasmalemma. As shown in Fig. 6A, the ratio of the cell layer (average DiOC6 intensity per area) to the 4-μm multispectral bead (average fluorescence per area) within ~154 nm of the plasmalemma was not significantly altered between the treatment groups (P = 0.067). However, as shown in Fig. 6B, the ratio of the cell layer (average DiOC6 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. Exercise training concurrent with diabetic dyslipidemia prevented this decrease.

**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 Ca2+ regulation and the alteration in superficial SR content in coronary smooth muscle cells from diabetic dyslipidemic swine. Using freshly isolated cells loaded with the Ca2+ indicator fura red, we found more specifically that plasmalemmal Ca2+ extrusion was impaired in diabetic dyslipidemic swine. Exercise training concurrent with diabetic dyslipidemia prevented this decrease.
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^{2+}/H^{+} 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_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^{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^{2+} levels was assessed by direct inhibition with a low-Na^{+}-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^{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^{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^{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^{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^{2+}-transporter inhibitors, suggesting that none of these transporters was functioning to maintain steady-state intracellular Ca^{2+} levels. In other words, all Ca^{2+} efflux mechanisms were already impaired in diabetic dyslipidemia and maximum concentrations of inhibitors did not further decrease Ca^{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^{2+} regulatory mechanisms was compensating to buffer intracellular Ca^{2+} levels to prevent further increases in Ca^{2+} levels. These possible alternative mechanisms include intracellular Ca^{2+} buffering via 1) the nuclear Ca^{2+} store, 2) soluble intracellular Ca^{2+}-binding proteins, and/or 3) the mitochondria.

First, although a number of studies have shown that Ca^{2+} is regulated differentially between the nucleus and the bulk myoplasm due to perinuclear Ca^{2+} uptake (19, 41), several studies have demonstrated a concentration-dependent loss in nuclear Ca^{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^{2+}-binding proteins, including calmodulin. Although diabetic rats showed decreased calmodulin levels (29), the Ca^{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^{2+} regulation under pathophysiological conditions. The mitochondrial Ca^{2+} uniporter has a Michaelis constant for Ca^{2+} that enables substantial Ca^{2+} uptake only when local Ca^{2+} concentrations exceed ~5.0 μM (37). Even though the basal intracellular Ca^{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^{2+} can be reached via a close apposition of the mitochondria to the SR (30, 35).
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 \(\sim 308\) 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 \(\sim 308\) nm of the plasmalemma, which was also prevented by exercise training.

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