Enhanced L-type Ca^{2+} channel current density in coronary smooth muscle of exercise-trained pigs is compensated to limit myoplasmic free Ca^{2+} accumulation

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1. We hypothesized that enhanced voltage-gated Ca^{2+} channel current (VGCC) density in coronary smooth muscle cells of exercise-trained miniature Yucatan pigs is compensated by other cellular Ca^{2+} regulatory mechanisms to limit net myoplasmic free Ca^{2+} accumulation.

2. Whole-cell voltage clamp experiments demonstrated enhanced VGCC density in smooth muscle cells freshly dispersed from coronary arteries of exercise-trained *vs.* sedentary animals.

3. In separate experiments using fura-2 microfluorometry, we measured depolarization-induced (80 mM KCl) accumulation of myoplasmic free Ba^{2+} and free Ca^{2+}. Both maximal rate and net accumulation of free Ba^{2+} in response to membrane depolarization were increased in smooth muscle cells isolated from exercise-trained pigs, consistent with an increased VGCC density. Depolarization also produced an enhanced maximal rate of free Ca^{2+} accumulation in cells of exercise-trained pigs; however, net accumulation of free Ca^{2+} was not significantly increased suggesting enhanced Ca^{2+} influx was compensated to limit net free Ca^{2+} accumulation.

4. Inhibition of sarco-endoplasmic reticulum Ca^{2+}-transporting ATPase (SERCA; 10 μM cyclopiazonic acid) and/or sarcolemmal Na^+–Ca^{2+} exchange (low extracellular Na^+) suggested neither mechanism compensated the enhanced VGCC in cells of exercise-trained animals.

5. Local Ca^{2+}-dependent inactivation of VGCC, assessed by buffering myoplasmic Ca^{2+} with EGTA in the pipette and using Ca^{2+} and Ba^{2+} as charge carriers, was not different between cells of sedentary and exercise-trained animals.

6. Our findings indicate that increased VGCC density is compensated by other cellular Ca^{2+} regulatory mechanisms to limit net myoplasmic free Ca^{2+} accumulation in smooth muscle cells of exercise-trained animals. Further, SERCA, Na^+–Ca^{2+} exchange and local Ca^{2+}-dependent inactivation of VGCC do not appear to function as compensatory mechanisms. Additional potential compensatory mechanisms include Ca^{2+} extrusion via plasma membrane Ca^{2+}-ATPase, mitochondrial uptake, myoplasmic Ca^{2+}-binding proteins and other sources of VGCC inactivation.

Exercise training-induced alterations in vasoreactivity of the coronary vasculature have been well documented (Bove & Dewey, 1985; Laughlin, 1985; Laughlin *et al.* 1989; DiCarlo *et al.* 1989; Rogers *et al.* 1991; Oltman *et al.* 1992; Bowles *et al.* 1995; Mombouli *et al.* 1996) and are generally associated with enhanced dilatation and reduced constriction in response to vasoactive agonists (Laughlin & McAllister, 1992; Parker *et al.* 1994). *In vivo*, the coronary circulation of exercise-trained animals exhibits enhanced adenosine-induced vasodilatation (Laughlin, 1985; Laughlin *et al.* 1989; DiCarlo *et al.* 1989) and attenuated α-adrenergic vasoconstriction (Bove & Dewey, 1985). Similarly, isolated coronary rings demonstrate increased relaxation to adenosine (Oltman *et al.* 1992) and diminished vasoconstriction to noradrenaline (norepinephrine) (Oltman *et al.* 1992) and endothelin (Bowles *et al.* 1995). Furthermore, simultaneous measurements of developed tension and myoplasmic free Ca^{2+} in arterial rings isolated from sedentary and exercise-trained pigs indicate that attenuated endothelin-induced contractile responses in

exercise-trained animals are associated with reduced myoplasmic free Ca^{2+} levels (Bowles *et al.* 1995). In apparent contrast to these vascular adaptations with exercise training, Bowles *et al.* (1998) recently reported increased VGCC density in coronary smooth muscle cells isolated from exercise-trained *vs.* sedentary animals. The present study was undertaken to determine whether this enhanced Ca^{2+} current density is compensated in smooth muscle cells from exercise-trained animals to limit net myoplasmic free Ca^{2+} accumulation.

We also evaluated potential cellular mechanisms of Ca^{2+} regulation that may compensate the enhanced VGCC density of smooth muscle cells from exercise-trained animals. Experiments in which SERCA and/or sarcolemmal Na^+–Ca^{2+} exchange were inhibited suggest neither mechanism compensates enhanced VGCC in coronary smooth muscle cells of exercise-trained animals. We also provide evidence that inactivation of VGCC by myoplasmic Ca^{2+} in the immediate vicinity of voltage-gated Ca^{2+} channels (local Ca^{2+}) is not different between sedentary and exercise-trained animals. Taken together, our findings suggest increased VGCC density in smooth muscle cells of exercise-trained animals is compensated by other cellular Ca^{2+} regulatory mechanisms to limit net free Ca^{2+} accumulation; SERCA, Na^+–Ca^{2+} exchange and local Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels do not appear to function as compensatory mechanisms.

METHODS

Exercise training procedures

All animal protocols were in accordance with the *Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training* and approved by the University of Missouri Animal Care and Use Committee. Adult female Yucatan miniature pigs (Charles River, Wilmington, MA, USA) were randomly assigned to either a sedentary or exercise-trained group. Exercise-trained pigs underwent 16 weeks of a progressive treadmill exercise training programme used extensively by our laboratories and described previously (Laughlin, 1985; Laughlin *et al.* 1989; Stehno-Bittel *et al.* 1991; Oltman *et al.* 1992; Bowles *et al.* 1995, 1998). Sedentary pigs were restricted to their pens (2 m × 4 m) for the 16 week duration. Pigs were given positive reinforcement for exercise by being fed after each training bout.

Efficacy of training

Treadmill performance tests were administered before and after completion of the 16 week exercise-training programme or sedentary pen confinement as described previously (Laughlin *et al.* 1989; Bowles *et al.* 1995, 1998). Effectiveness of the exercise-training programme was determined by comparing running time to exhaustion on treadmill performance tests, heart weight to body weight ratio and skeletal muscle oxidative enzyme activity of the exercise-trained *vs.* sedentary animals.

Removal of tissue

Isolation of coronary arteries. Following completion of the 16 week exercise training protocol or sedentary confinement, the animals were anaesthetized using ketamine (35 mg kg^{-1}, I.M.), xylazine (2 mg kg^{-1}, I.M.) and thiopental sodium (10 mg kg^{-1}, I.V.) followed by administration of heparin (1000 u kg^{-1}, I.V.). The hearts were removed, placed in ice-cold Krebs bicarbonate buffer (0–4 °C) and weighed. Hearts were maintained in iced Krebs buffer during isolation of coronary arteries. With the aid of a dissection microscope, segments of the right coronary (RCA) and left circumflex coronary (LCX) arteries were trimmed of fat and connective tissue.

Muscle samples. After removal of the heart, as described above, samples were taken from the long, medial and lateral heads of the triceps brachii muscle for determination of citrate synthase activity as described previously (Stehno-Bittel *et al.* 1991; Oltman *et al.* 1992; Bowles *et al.* 1995, 1998).

Smooth muscle cell dissociation

Segments of RCA and LCX coronary arteries were cut longitudinally and pinned lumen-side up in low-Ca^{2+} physiological buffer containing 294 U ml^{-1} collagenase, 5 U ml^{-1} elastase, 2 mg ml^{-1} bovine serum albumin, 1 mg ml^{-1} soybean trypsin inhibitor, and 0·4 mg ml^{-1} DNase I. Cells were enzymatically dissociated by incubation in a 37 °C shaking water bath for 20 min; this first fraction of dissociated cells, primarily consisting of endothelial cells, was removed. An additional 50 min enzymatic dissociation in fresh enzyme solution generated primarily smooth muscle cells. Dissociated smooth muscle cells from the LCX were then incubated with the acetoxymethyl ester form of the fluorescent Ca^{2+} indicator fura-2 (fura-2 AM; 2·5 μM) at 37 °C for 25 min. Following fura-2 AM incubation, smooth muscle cells were washed in modified Eagle's minimal essential storage medium for 20 min before experiments were initiated. Dissociated smooth muscle cells from the RCA were placed in low-Ca^{2+} physiological mediim for use in voltage clamp experiments.

Myoplasmic free Ca^{2+} measurement

Fura-2-loaded cells were placed in a superfusion chamber and observed using an epifluorescence microscopy system (Nikon, Garden City, NY, USA). Excitation light from a 300 W xenon arc lamp, passed via a liquid light guide, was directed through alternating 340 and 380 nm bandpass filters. Fluorescence emission (510 nm) from user-specified regions of interest (selected smooth muscle cells) was synchronized with the appropriate excitation wavelength and reflected to an integrating CCD monochrome video camera (Cohu, San Diego, CA, USA) with a dichroic mirror. The microscope was equipped with a × 40 oil immersion objective with a numerical aperature of 1·3. Fluorescence images were acquired using InCa dual wavelength Ca^{2+} imaging software, version 2.1 (Intracellular Imaging, Cincinnati, OH, USA). This microfluorometry system provides individual traces of fura-2 fluorescence from multiple smooth muscle cells simultaneously. The fura-2 fluorescence ratio was collected for each cell throughout the experimental protocol. Background fluorescence was determined before the start of the experiment for on-line subtraction during data collection. After the subtraction of background fluorescence, images obtained at 340 and 380 nm were ratioed on a pixel-by-pixel basis. Final data for estimates of myoplasmic free Ca^{2+} are expressed as a fluorescence ratio (F_{340}/F_{380}) because of uncertainties in extrapolating *in vitro* calibrations to *in situ* measurements as described previously (Wagner-Mann *et al.* 1992). Cells were continually superfused (~2·7 ml min^{-1}) under gravity flow. All experiments were conducted at room temperature (22–25 °C) and fluorescence data were sampled every 2 s.

For fura-2 microfluorometry studies, unless otherwise specified, cells were superfused with physiological saline solution (PSS)

containing (mM): 2 $CaCl_2$, 143 NaCl, 1 $MgCl_2$, 5 KCl, 10 Hepes, and 10 glucose, pH 7·4. Cells were depolarized with PSS in which 80 mM KCl replaced equimolar amounts of NaCl. For low Na^+ (5 mM Na^+) protocols, Na^+ was replaced with equimolar amounts of Li^+. For Ba^{2+} protocols, Ca^{2+} was replaced with equimolar amounts of Ba^{2+} and low Na^+ (5 mM Na^+) was used to inhibit Ba^{2+} extrusion via Na^+–Ca^{2+} exchange. Caffeine (5 mM), cyclopiazonic acid (CPA, 10 μM) and nifedipine (3 μM) additions were made directly to appropriate PSS and 80 mM KCl solutions as specified by the experimental protocol.

Whole-cell voltage clamp

In separate experiments, whole-cell Ca^{2+} and Ba^{2+} currents were determined using a standard whole-cell voltage clamp technique as described previously (Bowles *et al.* 1998). Cells were initially superfused with PSS during gigaseal formation. After whole-cell configuration, superfusate was switched to PSS with tetraethylammonium chloride (TEACl) substituted for NaCl and 2 mM Ca^{2+} or 10 mM Ba^{2+} as the charge carrier. Heat-polished glass pipettes (2–5 MΩ) were filled with a solution containing (mM): 120 CsCl, 10 TEACl, 1 $MgCl_2$, 20 Hepes, 2 MgATP, 5 EGTA and 0·5 Tris.GTP. The pipette was connected to the 10 GΩ headstage of a Warner PC-501 patch clamp amplifier and advanced to the cell via a micromanipulator control. Junction potential was offset prior to contact between pipette and cell. After formation of a GΩ seal, pipette capacitance was cancelled and suction applied to achieve whole-cell configuration. Whole-cell currents were filtered through an eight-pole low-pass filter with a cut-off frequency of 400 Hz and digitized at 600 μs intervals. Data acquisition and analysis were accomplished using a Labmaster analog-to-digital converter and microcomputer equipped with AxoBASIC 1.0 software (Axon Instruments, Foster City, CA, USA). Current densities (pA pF^{-1}) were obtained for each cell by normalization of whole-cell current to cell capacitance. Time to half-maximal ($T_{½}$) decay of peak current for whole-cell configuration was determined using both 2 mM Ca^{2+} and 10 mM Ba^{2+} as external charge carriers. We used 10 mM Ba^{2+} to increase current magnitude in these cells. Importantly, Ba^{2+} current inactivation kinetics in smooth muscle cells have been shown previously to not be affected by current magnitude (Nilius *et al.* 1994). Capacity currents were measured for each cell during 10 ms pulses from a holding potential of −80 mV to a test potential of −70 mV. Capacity currents were filtered at a low-pass cut-off frequency of 8·4 kHz and digitized at 25 μs intervals. Leak subtraction was not performed. Cells were continuously perfused under gravity flow. All experiments were conducted at room temperature (22–25 °C).

Statistical analysis

Treadmill test endurance time, heart weight to body weight ratio and citrate synthase activity were evaluated using Student's unpaired t test. Voltage-gated Ca^{2+} channel current–voltage (I–V) relationships were evaluated using analysis of variance and Student's unpaired t test for *post hoc* analyses. Analysis of variance and Student's unpaired t tests were used for comparison of $T_{½}$ decay of peak current. Data for fura-2 experiments were analysed using one-way analysis of variance or split-plot repeated measures analysis of variance, as appropriate. Mean differences were ascertained using Fisher's least significant difference (LSD). Analyses for all experiments were performed on a per cell basis. For all analyses, a P value $\leqslant 0{\cdot}05$ was considered significant. Data are presented as means ± S.E.M., and values in parentheses reflect the number of animals and number of smooth muscle cells.

RESULTS

Training status

Effectiveness of the 16 week exercise training programme was demonstrated by significant ($P < 0{\cdot}05$) increases in treadmill endurance time, skeletal muscle oxidative enzyme capacity and an increased heart weight to body weight ratio in exercise-trained animals. Treadmill endurance time increased in exercise-trained (21·6 ± 3·7 *vs.* 31·4 ± 4·2 min), but not sedentary animals (24·0 ± 3·8 *vs.* 24·9 ± 2·9 min), after completion of the 16 week exercise protocol or pen confinement, respectively. Citrate synthase activity was increased in the long heads (15·7 ± 5·3 *vs.* 11·1 ± 1·9 μmol min^{-1} g^{-1}), in the medial heads (19·0 ± 5·3 *vs.* 15·7 ± 2·2 μmol min^{-1} g^{-1}) and in the lateral heads (19·4 ± 6·7 *vs.* 14·2 ± 2·0 μmol min^{-1} g^{-1}) of the triceps brachii muscle in exercise-trained *vs.* sedentary animals, respectively. Heart weight to body weight ratio also increased in exercise-trained *vs.* sedentary pigs (5·3 ± 0·2 *vs.* 4·8 ± 0·1 g kg^{-1}).

Ca^{2+} channel current density

The effect of exercise training on whole-cell Ca^{2+} current was determined using 10 mM external Ba^{2+} as the charge carrier. I–V relationships for sedentary and exercise-trained animals are presented in Fig. 1. Current is plotted as peak inward current measured during a 260 ms step depolarization to the indicated membrane potential (V_m) from a holding potential of −80 mV. Current is normalized to cell membrane capacitance (pA pF^{-1}). Cell capacitance (21 ± 1 *vs.* 22 ± 2 pF) was not different between smooth muscle cells isolated from sedentary ($n = 4$ animals, 8 cells) and exercise-trained ($n = 4$ animals, 11 cells) animals, respectively. These data confirm that exercise training increased peak VGCC density approximately twofold in

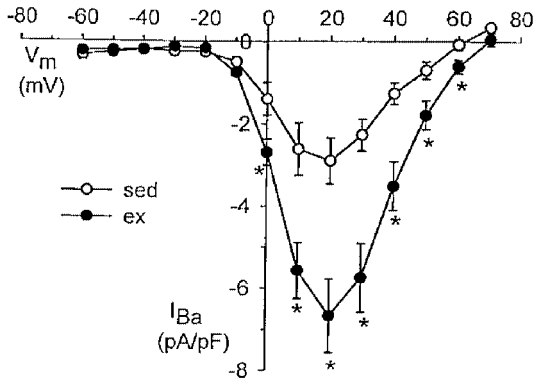

Figure 1. Current–voltage (I–V) relationships for whole-cell VGCCs from coronary smooth muscle of sedentary (sed) and exercise-trained (ex) pigs

Currents were obtained using 10 mM Ba^{2+} as external charge carrier. Current is plotted as peak inward current measured during a 260 ms step depolarization to the membrane potential (V_m) indicated from a holding potential of −80 mV. Current is normalized to cell membrane capacitance (pA pF^{-1}). Data are means ± S.E.M.; * $P < 0{\cdot}05$, ex ($n = 4$ pigs, 11 cells) *vs.* sed ($n = 4$, 8).

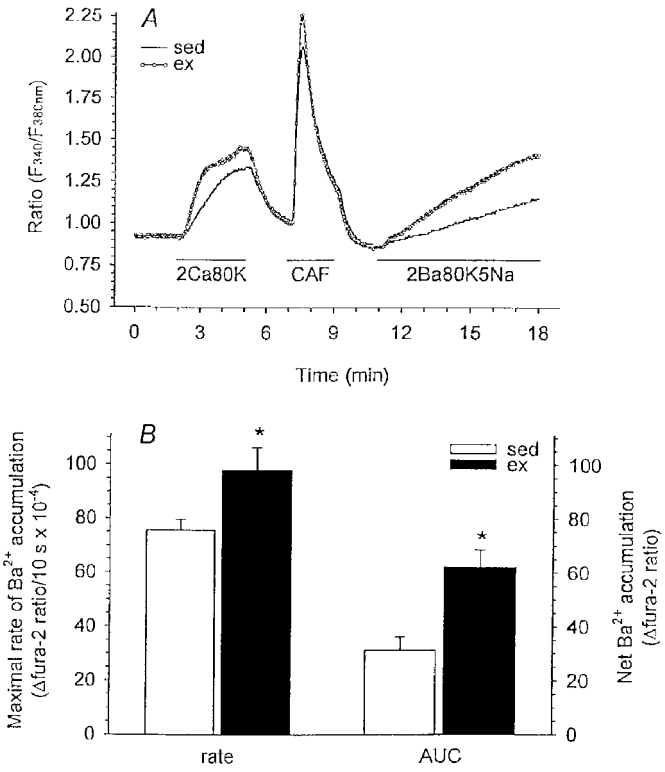

Figure 2. Free Ba^{2+} accumulation during membrane depolarization (80 mM KCl) using fura-2 microfluorometry

A, experimental protocol and representative recordings from single cells of both sedentary and exercise-trained pigs showing change in F_{340}/F_{380} fluorescence ratio. Cells were superfused with PSS unless otherwise specified. Cells were exposed to 80 mM KCl (80K) and caffeine (CAF; 5 mM) in the presence of 2 mM extracellular Ca^{2+} (2Ca) for the durations indicated by the horizontal lines. Cells were then exposed to 80 mM KCl in the presence of 2 mM Ba^{2+} (2Ba; equimolar substitution for Ca^{2+}) and low Na^+ (5Na; 5 mM) for 7 min. *B*, maximal rate (10 s slope) and net (area under the curve, AUC) free Ba^{2+} accumulation for cells from sed ($n = 6$, 71) and ex ($n = 5$, 61) animals. Data are means ± S.E.M.; $*P < 0{\cdot}05$, ex *vs.* sed.

smooth muscle cells isolated from conduit-sized coronary arteries as demonstrated previously (Bowles *et al.* 1998).

Fura-2 measurements of Ba^{2+} influx

We further evaluated alterations in Ba^{2+} influx at the plasma membrane of single smooth muscle cells using the Ca^{2+} indicator, fura-2. Ba^{2+} produces a shift in the fura-2 excitation wavelength spectrum with increasing concentrations similar to that observed with Ca^{2+}, although fura-2 has a higher affinity for Ca^{2+} *vs.* Ba^{2+} (Schilling *et al.* 1989; Kwan & Putney, 1990). Ba^{2+} influx at the plasma membrane of smooth muscle cells is mediated mostly through Ca^{2+} channels (Benham & Tsien, 1987) and the Na^+–Ca^{2+} exchanger (Condrescu *et al.* 1997). However, Ba^{2+} is not sequestered by intracellular organelles (Schilling *et al.* 1989; Kwan & Putney, 1990; Condrescu *et al.* 1997) or transported by ATP-dependent Ca^{2+} pumps (Schilling *et al.* 1989). Therefore, these unique characteristics of Ba^{2+} allow evaluation of unidirectional influx at the plasma membrane under conditions in which corresponding Ca^{2+} fluxes would be difficult to interpret due to multiple pathways for Ca^{2+} removal and sequestration.

Our protocol for assessment of myoplasmic free Ba^{2+} accumulation in response to membrane depolarization with 80 mM KCl and representative recordings from single cells isolated from both sedentary and exercise-trained animals are shown in Fig. 2*A*. Briefly, cells were initially exposed to 80 mM KCl followed by 5 mM caffeine both in the presence of 2 mM extracellular Ca^{2+} for the durations indicated by the horizontal line. Although the SR does not sequester Ba^{2+}, we

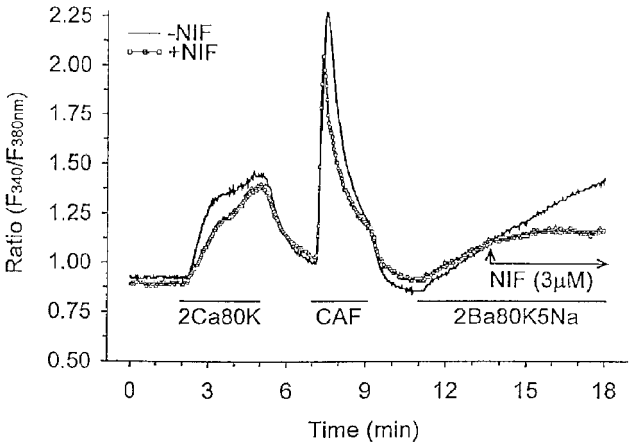

Figure 3. Free Ba^{2+} accumulation during membrane depolarization (80 mM KCl) in the presence of the Ca^{2+} channel blocker, nifedipine

Control (−NIF; continuous line) experimental protocol is the same as presented in Fig. 2*A*. For experiments in the presence of nifedipine (+NIF; ○), a similar protocol was used with the addition of nifedipine (3 μM) between 13·5 and 18 min after the start of the experiment, as indicated by the arrows. Evaluation of the slope between 16 and 18 min indicates that free Ba^{2+} accumulation was abolished in the presence of nifedipine.

applied caffeine in this protocol to maintain consistency with our other fura-2 protocols described below. We then evaluated free Ba^{2+} accumulation in the presence of 80 mM KCl and low extracellular Na^+ (5 mM), a method which has been employed previously to measure unidirectional divalent cation influx in smooth muscle cells (Liu *et al.* 1994). We assessed both the maximal rate and net free Ba^{2+} accumulation (area under the curve; AUC) throughout membrane depolarization (7 min) in sedentary and exercise-trained animals. AUC was obtained by subtracting the baseline fura-2 ratio for each cell (average of 10 data points prior to 80 mM KCl exposure) from each subsequent fura-2 data point throughout the 80 mM KCl exposure (between 11 and 18 min after the start of the experiment). These differences for each data point were then added together to obtain AUC.

Our data demonstrate that exercise training produced a 30% increase in the maximal rate of free Ba^{2+} accumulation and a 98% increase in net free Ba^{2+} accumulation (AUC) compared with sedentary pigs (Fig. 2*B*). Furthermore, the rate of free Ba^{2+} accumulation throughout membrane depolarization (slope of the time period 11–18 min, Fig. 2*B*) was significantly greater in cells isolated from exercise-trained *vs.* sedentary animals. These findings support our whole-cell Ca^{2+} current data indicating that membrane depolarization-induced divalent cation influx through voltage-gated Ca^{2+} channels is enhanced in exercise-trained pigs. In additional experiments we evaluated the effects of the Ca^{2+} channel blocker nifedipine (3 μM) on Ba^{2+} influx as illustrated in Fig. 3. Addition of nifedipine during membrane depolarization completely blocked Ba^{2+} influx as indicated by a levelling off of free Ba^{2+} accumulation (Fig. 3). Slope values representing Ba^{2+} influx for the time period 16–18 min were $8{\cdot}4 \pm 7{\cdot}2$ and $-0{\cdot}8 \pm 2{\cdot}2$ in the absence and presence of nifedipine, respectively. These slope values indicate that in our experiments, Ba^{2+} influx occurred exclusively through dihydropyridine-sensitive Ca^{2+} channels.

Fura-2 measurements of free Ca^{2+} accumulation

Subsequent fura-2 studies evaluating changes in myoplasmic free Ca^{2+} allowed us to assess whole-cell regulation of intracellular free Ca^{2+}. Our protocol for evaluation of free Ca^{2+} accumulation in response to membrane depolarization with 80 mM KCl and representative recordings from single cells isolated from both sedentary and exercise-trained animals are shown in Fig. 4*A*. Briefly, cells were exposed to 80 mM KCl and caffeine in the presence of 2 mM extracellular Ca^{2+} for the durations indicated by the horizontal lines. The use of caffeine depletes the SR adequately to potentiate SERCA activity and allows the examination of the role of Ca^{2+} uptake by SERCA in compensating the enhanced depolarization-induced Ca^{2+} influx observed in cells of exercise-trained animals. We evaluated both the maximal rate and net free Ca^{2+} accumulation (AUC) in response to high KCl-induced membrane depolarization following SR depletion in sedentary and exercise-trained animals. This protocol was the general format used for all subsequent experiments described in this paper. Myoplasmic free Ca^{2+} accumulation in response to high KCl-induced membrane depolarization is dependent on Ca^{2+} influx, Ca^{2+} extrusion at the plasmalemma, and Ca^{2+} sequestration by intracellular organelles. Our findings from these experiments

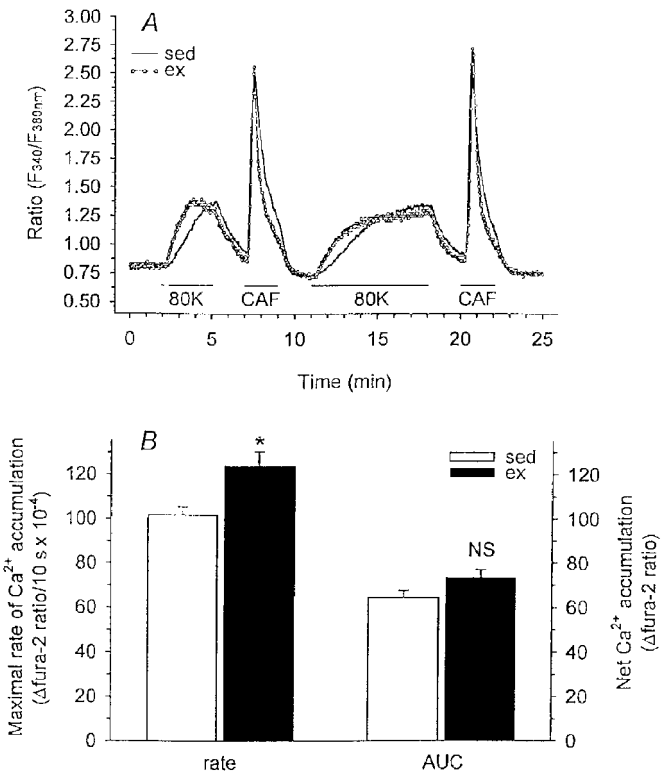

Figure 4. Free Ca^{2+} accumulation during membrane depolarization (80 mM KCl) using fura-2 microfluorometry

A, experimental protocol and representative recordings from single cells isolated from sedentary and exercise-trained animals showing change in F_{340}/F_{380} ratio. Cells were superfused with PSS unless otherwise specified. Cells were exposed to 80 mM KCl and caffeine (5 mM) in the presence of 2 mM extracellular Ca^{2+} for the durations indicated by the horizontal lines. *B*, maximal rate (10 s slope) and net (AUC) free Ca^{2+} accumulation for cells from sed ($n = 7$, 84) and ex ($n = 6$, 76) animals. Data are means ± S.E.M.; * $P < 0{\cdot}05$, ex *vs.* sed.

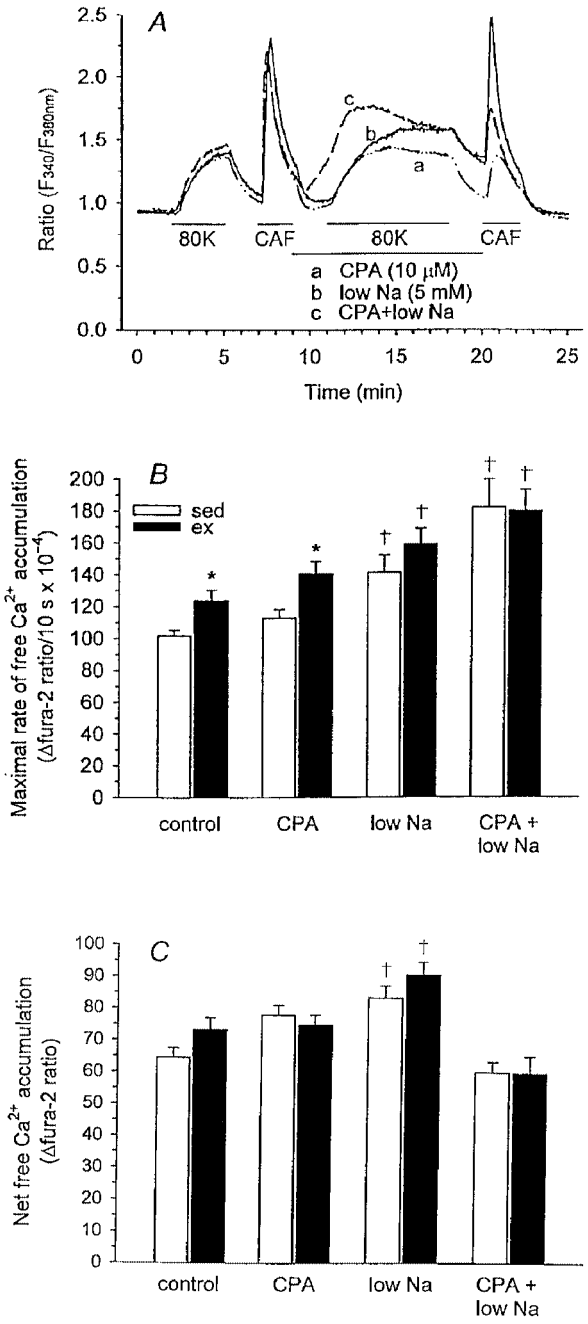

Figure 5. Evaluation of free Ca^{2+} accumulation in the presence of SERCA and/or Na^+–Ca^{2+} exchange inhibition during membrane depolarization (80 mM KCl)

A, experimental protocols are similar to that used in Fig. 4*A* with the addition of CPA and/or low Na^+ for the 11 min period shown by the horizontal line. Representative recordings from single cells of sedentary animals are presented for each protocol showing the change in F_{340}/F_{380} fluorescence ratio. *B*, maximal rate (10 s slope). *C*, net free Ca^{2+} accumulation (AUC) for cells from sedentary and exercise-trained animals for all protocols. n values for control protocol as presented in Fig. 4 legend. CPA protocol: sed, $n = 6, 66$; ex, $n = 6, 62$. NCX protocol: sed, $n = 5, 56$; ex, $n = 5, 45$. Data are means ± S.E.M.: $*P < 0{\cdot}05$, *vs.* respective sed; $\dagger P < 0{\cdot}05$, *vs.* control protocol.

demonstrated that the maximal rate of free Ca^{2+} accumulation in response to membrane depolarization was 22% greater in smooth muscle cells of exercise-trained *vs.* sedentary animals (Fig. 4*B*). However, in contrast to our findings with Ba^{2+}, net myoplasmic free Ca^{2+} accumulation (AUC) throughout the depolarization period was not significantly different between exercise-trained and sedentary animals (Fig. 4*B*). These data suggest that the enhanced maximal rate of free Ca^{2+} accumulation is compensated by additional Ca^{2+} regulatory mechanisms insensitive to Ba^{2+}, thus limiting net myoplasmic free Ca^{2+} accumulation in smooth muscle cells of exercise-trained animals.

To investigate potential mechanisms of Ca^{2+} removal and/or sequestration that may compensate the enhanced Ca^{2+} influx in smooth muscle cells isolated from exercise-trained animals, we systematically evaluated the contribution of SERCA and/or the Na^+–Ca^{2+} exchanger to Ca^{2+} removal from the myoplasm during membrane depolarization. The protocols for these experiments and representative recordings from single cells of sedentary animals are illustrated in Fig. 5*A*. These experimental protocols are similar to that presented in Fig. 4*A* with the addition of CPA and/or low Na^+ for the 11 min period shown by the horizontal line in Fig. 5*A*.

Inhibition of SR Ca^{2+} sequestration

CPA, a reversible inhibitor of SERCA, was used to evaluate the contribution of SR Ca^{2+} sequestration in compensating the enhanced Ca^{2+} influx in exercise-trained animals. Findings from these experiments are presented in Fig. 5*B* and *C* in comparison with the control protocol (in the absence of CPA). CPA had little effect on either maximal rate or net free Ca^{2+} accumulation in smooth muscle cells from both sedentary and exercise-trained animals. The maximal rate of free Ca^{2+} accumulation remained significantly enhanced in smooth muscle cells from exercise-trained animals (Fig. 5*B*) and net free Ca^{2+} accumulation (AUC) was not different between sedentary and exercise-trained pigs (Fig. 5*C*); these results were similar to those observed in the absence of CPA (control protocol; Fig. 5*B* and *C*). Our finding that SERCA inhibition had a negligible influence on net free Ca^{2+} accumulation in either exercise-trained or sedentary smooth muscle cells suggests that the SR does not function to compensate the enhanced Ca^{2+} influx demonstrated in smooth muscle cells from exercise-trained animals. Our findings also indicate that caffeine-releasable SR Ca^{2+} stores in cells of sedentary (6 animals, 66 cells) *vs.* exercise-trained (6 animals, 62 cells) animals were not different after CPA application (Δfura-2 ratio, $0{\cdot}37 \pm 0{\cdot}02$ *vs.* $0{\cdot}36 \pm 0{\cdot}02$, respectively), suggesting similar inhibition of SERCA activity in both groups of pigs. Additionally, caffeine-releasable SR Ca^{2+} stores in the absence of SERCA inhibition in sedentary ($n = 7$ animals, 84 cells) and exercise-trained ($n = 6$ animals, 76 cells) groups (Δfura-2 ratio, $1{\cdot}18 \pm 0{\cdot}06$ *vs.* $1{\cdot}19 \pm 0{\cdot}07$, respectively) were not different, suggesting similar SR Ca^{2+}

store capacity. These data indicate that enhanced SR Ca^{2+} uptake does not compensate increased VGCC in coronary smooth muscle cells isolated from exercise-trained animals to limit net free Ca^{2+} accumulation.

Inhibition of Ca^{2+} extrusion via Na^+–Ca^{2+} exchange

We also evaluated the potential contribution of Na^+–Ca^{2+} exchange activity to the extrusion of Ca^{2+} from the cell. During depolarization with 80 mM KCl, the membrane potential of smooth muscle cells typically increases to approximately −15 mV (Ito *et al.* 1980) causing reversal of the Na^+–Ca^{2+} exchanger and, therefore, Ca^{2+} influx at the exchanger (Sturek *et al.* 1992). During steady-state depolarization, low extracellular Na^+ would have little further influence on the reversal of the exchanger. However, during the initial development of membrane depolarization following exposure to 80 mM KCl, the Na^+–Ca^{2+} exchanger may contribute to alterations in Ca^{2+} handling of coronary smooth muscle cells of sedentary *vs.* exercise-trained animals. Therefore, this low Na^+ protocol was used to evaluate a potential role for the Na^+–Ca^{2+} exchanger as a compensatory mechanism for enhanced Ca^{2+} influx during the early stages of depolarization in smooth muscle cells of exercise-trained pigs. Our data from these experiments are presented in Fig. 5*B* and *C* and indicate that decreased extracellular Na^+ produced significant but similar increases in maximal rate and net free Ca^{2+} accumulation (AUC) of cells from both sedentary and exercise-trained animals. These increases in maximal rate and net free Ca^{2+} accumulation may result from impaired Ca^{2+} extrusion or enhanced Ca^{2+} entry at the exchanger. However, similar increases in sedentary and exercise-trained groups indicate that Na^+–Ca^{2+} exchange activity does not compensate the enhanced Ca^{2+} influx demonstrated in cells from exercise-trained animals to limit net free Ca^{2+} accumulation. Furthermore, our data also demonstrate that inhibition of Na^+–Ca^{2+} exchange abolished the significant difference observed in the maximal rate of free Ca^{2+} accumulation between cells of sedentary and exercise-trained animals in the control protocol. Conversely, if Na^+–Ca^{2+} exchange activity compensated the enhanced VGCC observed in smooth muscle cells of exercise-trained pigs, inhibition of Na^+–Ca^{2+} exchange would potentiate the difference between cells of sedentary and exercise-trained animals.

Simultaneous inhibition of Ca^{2+} removal via SR and Na^+–Ca^{2+} exchange

Previous studies have demonstrated that inhibition of one cellular mechanism of Ca^{2+} removal may prompt an additional regulatory mechanism to compensate to some extent for its absence (Bers & Bridge, 1989). For this reason, we simultaneously inhibited SERCA and the sarcolemmal Na^+–Ca^{2+} exchanger to unequivocally evaluate the role of either pathway of Ca^{2+} removal as a potential compensatory mechanism for increased Ca^{2+} current density in cells of exercise-trained animals. Our data from these experiments are presented in Fig. 5*B* and *C* and indicate that simultaneous inhibition of both the SERCA and Na^+–Ca^{2+} exchanger produced significant but similar increases in maximal rate of free Ca^{2+} accumulation in cells from both sedentary and exercise-trained animals. Furthermore, net free Ca^{2+} accumulation (AUC) was similar between cells of sedentary and exercise-trained animals following inhibition of both mechanisms of Ca^{2+} removal. These findings further suggest that SERCA and Na^+–Ca^{2+} exchange do not compensate the enhanced Ca^{2+} influx demonstrated in cells from exercise-trained animals. Interestingly, simultaneous inhibition of SERCA and Na^+–Ca^{2+} exchange produced a gradual increase in myoplasmic free Ca^{2+} prior to membrane depolarization (between 9 and 11 min after the start of the experiment, Fig. 5*A*, representative trace c). The subsequently diminished depolarization-induced net free Ca^{2+} accumulation (AUC) compared with control data illustrated in Fig. 5*C* suggests that these cells may possess a regulatory mechanism to limit maximal myoplasmic free Ca^{2+} concentrations and, perhaps, thereby protect the cell from Ca^{2+} concentrations which may compromise cell viability.

Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels

We also evaluated inactivation of voltage-gated Ca^{2+} channel current for both Ca^{2+} and Ba^{2+} in the presence of EGTA (5 mM). EGTA generally buffers Ca^{2+} or Ba^{2+} at a distance of approximately 100 nm from voltage-gated Ca^{2+} channels (Gutnick *et al.* 1989); thus, local Ca^{2+} gradients near the channel that are not buffered by EGTA can influence inactivation of the channel. In contrast, more 'distant' Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels is greatly attenuated in the presence of EGTA. Furthermore, Ba^{2+} does not directly inactivate voltage-gated Ca^{2+} channels (Ohya *et al.* 1988); therefore, using Ba^{2+} as charge carrier in the presence of high EGTA results in little to no local Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels.

The voltage clamp template for these experiments is illustrated in Fig. 6*A*. The holding potential was −80 mV and the cells were depolarized to the test potential that elicited peak current for Ca^{2+} or Ba^{2+}; generally +10 mV for both charge carriers. Representative current traces from a single cell for both Ca^{2+} and Ba^{2+} are presented in Fig. 6*A*. The time for the peak current to decay to half-maximal amplitude ($T_{½}$ decay) was used to assess Ca^{2+} current inactivation. As illustrated in Fig. 6*B*, $T_{½}$ decay of peak current was significantly shorter in the presence of Ca^{2+} *vs.* Ba^{2+}, suggesting that local Ca^{2+} gradients did play a role in Ca^{2+} channel inactivation. However, $T_{½}$ decay of peak current was not significantly different between cells of sedentary and exercise-trained animals in the presence of either Ca^{2+} or Ba^{2+} as charge carrier. These findings indicate that local Ca^{2+}-dependent inactivation of the voltage-gated Ca^{2+} channel did not compensate for the enhanced VGCC observed in exercise-trained animals. Furthermore, our findings that $T_{½}$ decay of peak current was not significantly different between cells of sedentary and exercise-trained animals in the presence of Ba^{2+} as charge carrier suggest

that the voltage-dependent inactivation of voltage-gated Ca^{2+} channels in cells of sedentary and exercise-trained pigs was not different.

DISCUSSION

In the present study, we document the novel finding that increased Ca^{2+} influx through voltage-gated Ca^{2+} channels observed in smooth muscle cells from exercise-trained animals is compensated such that net free Ca^{2+} accumulation in sedentary *vs.* exercise-trained animals was not significantly different. Inhibition of SERCA and/or sarcolemmal Na^+–Ca^{2+} exchange did not support a role for either mechanism in compensating the enhanced VGCC in exercise-trained animals. Also, Ca^{2+}-dependent inactivation of VGCC by local Ca^{2+} gradients was not different between sedentary and exercise-trained animals and therefore did not appear to compensate the initial increase in Ca^{2+} influx. The present study also confirmed previous findings using whole-cell voltage clamp experiments that exercise training does indeed increase peak VGCC density in coronary smooth muscle cells.

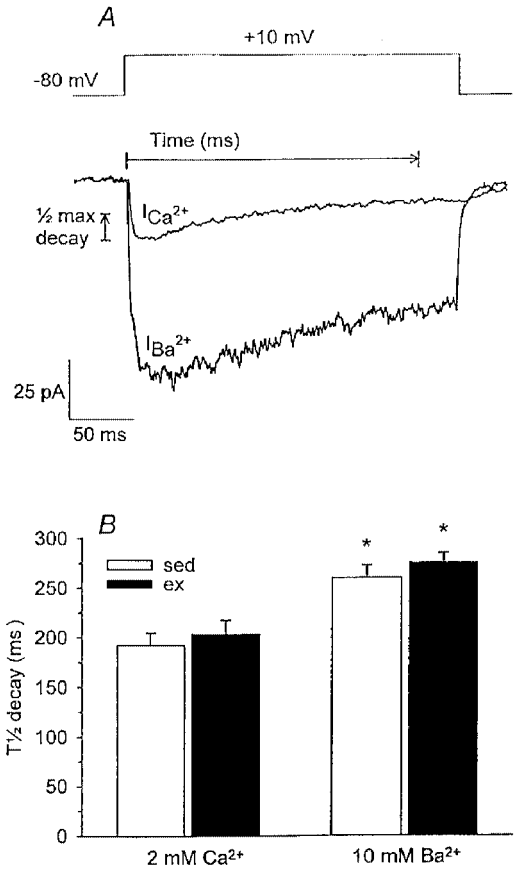

Figure 6. Evaluation of local Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels during membrane depolarization

A, voltage clamp template and representative current traces from a single cell using both Ca^{2+} and Ba^{2+} as charge carriers were elicited by 260 ms step depolarizations to +10 mV from a holding potential of −80 mV. Measures for calculation of $T_{½}$ decay of peak Ca^{2+} current are illustrated. *B*, $T_{½}$ decay of peak current was significantly shorter in the presence of Ca^{2+} *vs.* Ba^{2+} for cells of both sed ($n = 8$, 36) and ex ($n = 8$, 29) animals. However, $T_{½}$ decay was not significantly different between cells of sed and ex animals in the presence of Ca^{2+} or Ba^{2+}. Data are means ± S.E.M.; $*P < 0{\cdot}05$ *vs.* respective Ca^{2+} current.

Previous studies have shown that chronic exercise training attenuates contractile responses of coronary arterial rings to vasoactive agents (Oltman *et al.* 1992; Bowles *et al.* 1995), which are associated with reduced myoplasmic free Ca^{2+} levels (Bowles *et al.* 1995). Paradoxically, in the same experiments, the rate of endothelin-stimulated divalent cation influx was significantly greater in arterial rings from exercise-trained *vs.* sedentary animals (Bowles *et al.* 1995). More recent findings of enhanced VGCC density in coronary smooth muscle cells from exercise-trained animals (Bowles *et al.* 1998) prompted our hypothesis that enhanced Ca^{2+} influx via voltage-gated Ca^{2+} channels is compensated to limit net free Ca^{2+} accumulation in smooth muscle cells of exercise-trained animals.

Evaluation of Ba^{2+} accumulation in the presence of low extracellular Na^+ has been used previously as a measure of unidirectional divalent cation influx in smooth muscle cells (Liu *et al.* 1994). Ba^{2+} is transported via voltage-gated Ca^{2+} channels (Benham & Tsien, 1987) and the Na^+–Ca^{2+} exchanger (Condrescu *et al.* 1997) but is a poor substrate for ATP-dependent Ca^{2+} pumps (Schilling *et al.* 1989). Furthermore, our results in the presence of nifedipine indicate that accumulation of intracellular Ba^{2+} during high KCl-induced membrane depolarization was exclusively dependent on influx through dihydropyridine-sensitive L-type Ca^{2+} channels. In contrast, Ca^{2+} can be removed from the myoplasm by a variety of cellular mechanisms and thus accumulation of myoplasmic free Ca^{2+} is dependent upon the balance of influx and efflux/sequestration pathways. Therefore, increased net free cation accumulation (AUC) in the presence of Ba^{2+}, but not Ca^{2+}, suggests compensation for exercise training-enhanced Ca^{2+} influx via the increased activity of additional Ca^{2+} uptake and/or extrusion mechanisms. Our experiments also demonstrated a faster rate of rise in free Ca^{2+} *vs.* Ba^{2+} in response to membrane depolarization (Fig. 4 *vs.* 2). Although Ba^{2+} current is typically larger than Ca^{2+} current through VGCC, the faster rate of rise in free Ca^{2+} *vs.* Ba^{2+} appears to reflect the increased binding affinity of fura-2 for Ca^{2+} as documented previously (Schilling *et al.* 1989; Kwan & Putney, 1990).

We conducted experiments to evaluate the relative role of potential cellular mechanisms of Ca^{2+} extrusion and/or sequestration that may function to compensate enhanced VGCC density and thus limit net free Ca^{2+} accumulation in smooth muscle cells from exercise-trained animals. While maximal rate of free Ca^{2+} accumulation remained significantly elevated in cells from exercise-trained *vs.* sedentary animals, SERCA inhibition had a negligible influence on maximal rate and net free Ca^{2+} accumulation in

cells from both groups of animals (Fig. 5*B* and *C*). These data suggest that SR Ca^{2+} uptake made minimal contribution to Ca^{2+} removal during membrane depolarization in sedentary and exercise-trained animals, and provide support for another Ca^{2+} regulatory mechanism as the primary mechanism for the compensation of enhanced VGCC density.

In the presence of 80 mM KCl, the plasma membrane is depolarized to values more positive than the reversal potential for the Na^+–Ca^{2+} exchanger and therefore the exchanger contributes to Ca^{2+} influx rather than efflux (Sturek *et al.* 1992). However, we were concerned that during the early stages of depolarization when changes in membrane potential are more dynamic and have not yet attained reversal potential or steady state, Na^+–Ca^{2+} exchange may contribute to Ca^{2+} removal from the intracellular space. Therefore, we applied low extracellular Na^+ superfusate for 2 min prior to high KCl-induced membrane depolarization. Our data indicate that inhibition of Na^+–Ca^{2+} exchange activity produced significant, but similar, increases in maximal rate and net free Ca^{2+} accumulation in cells from both sedentary and exercise-trained animals. These findings indicate that Na^+–Ca^{2+} exchange activity alters Ca^{2+} influx in smooth muscle cells from sedentary and exercise-trained animals to a similar extent and thus provides evidence against Na^+–Ca^{2+} exchange activity as the compensating mechanism for increased VGCC density in cells of exercise-trained pigs. The increase in maximal rate and net free Ca^{2+} accumulation (AUC) in the presence of Na^+–Ca^{2+} exchange inhibition was probably the result of enhanced Ca^{2+} influx via the exchanger during 80 mM KCl-induced membrane depolarization. Preliminary experiments indicate a further reduction of extracellular Na^+ to 5 mM in the presence of 80 mM KCl-induced depolarization has no further influence on the membrane potential of smooth muscle cells (M. Sturek, unpublished observations). These findings suggest that the increases in maximal rate and net free Ca^{2+} accumulation in the presence of low Na^+ are the result of Ca^{2+} influx via Na^+–Ca^{2+} exchange and are not a response to greater membrane depolarization. Subsequent experiments in which SERCA and Na^+–Ca^{2+} exchange were inhibited simultaneously unequivocally demonstrate that neither of these Ca^{2+} regulatory mechanisms compensates the increased VGCC density to limit net free Ca^{2+} accumulation in cells of exercise-trained pigs.

Ca^{2+} removal from smooth muscle myoplasm occurs primarily via Na^+–Ca^{2+} exchange, plasma membrane Ca^{2+}-ATPase (PMCA) and SERCA (Nazer & van Breemen, 1998), while the role of mitochondria in Ca^{2+} regulation remains controversial (Drummond & Tuft, 1999; Ganitkevich, 1999). Previous studies have documented that following stimulated Ca^{2+} influx, PMCA-mediated extrusion accounts for approximately 50% of intracellular Ca^{2+} removal (Furukawa *et al.* 1988; Nazer & van Breemen, 1998) with SERCA and Na^+–Ca^{2+} exchange equally accounting for the remaining 50%, in the presence of experimentally induced collapse of the mitochondrial potential gradient (Nazer & van Breemen, 1998). In the present study, Ca^{2+} removal during 80 mM KCl-induced membrane depolarization would be primarily dependent on PMCA, SERCA and, potentially, mitochondrial uptake since the membrane potential would be more positive than the reversal potential for Na^+–Ca^{2+} exchange (Ito *et al.* 1980). Therefore, in the light of our findings that Na^+–Ca^{2+} exchange and SERCA contribute to Ca^{2+} removal to a similar extent in sedentary and exercise-trained pigs, enhanced PMCA activity or mitochondrial uptake potentially compensates the enhanced Ca^{2+} influx in exercise-trained animals.

Previous studies have suggested that increased spontaneous release of Ca^{2+} from the SR towards the plasma membrane in smooth muscle cells isolated from exercise-trained miniature pigs may contribute to enhanced subsarcolemmal Ca^{2+} gradients (Stehno-Bittel *et al.* 1991; Stehno-Bittel & Sturek, 1992). We hypothesized that enhanced Ca^{2+} concentrations in the vicinity of voltage-gated Ca^{2+} channels (increased local Ca^{2+}) of exercise-trained animals may function to rapidly inactivate VGCC and thereby attenuate net free Ca^{2+} accumulation. To test this hypothesis, we evaluated Ca^{2+} current inactivation in the presence of high EGTA to eliminate more distant Ca^{2+}-dependent inactivation. Distant Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels may involve activation of an intracellular mediator, such as a Ca^{2+}-dependent phosphatase, which might dephosphorylate the Ca^{2+} channel, rendering it inactive (Chad, 1988). Furthermore, because Ba^{2+} does not directly inactivate voltage-gated Ca^{2+} channels (Ohya *et al.* 1988), the more rapid $T_{½}$ decay in the presence of Ca^{2+} *vs.* Ba^{2+} indicates that local Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels does exist in these cells. However, our findings suggest that local Ca^{2+}-dependent inactivation of the voltage-gated Ca^{2+} channel is similar in sedentary and exercise-trained animals.

Our data indicate that the enhanced rate of free Ca^{2+} accumulation in smooth muscle cells from exercise-trained animals may be compensated by increased Ca^{2+} removal ultimately producing increased movement of Ca^{2+} (both influx and efflux) across the plasma membrane. The resultant increase in Ca^{2+} movement across the plasma membrane without any increase in net free Ca^{2+} accumulation (AUC) may increase the Ca^{2+} concentration in a restricted subdomain of the plasma membrane as originally proposed by Rasmussen *et al.* (1989). This change in subsarcolemmal Ca^{2+} could potentially regulate the activities of numerous membrane-associated Ca^{2+}-sensitive proteins and thus, in turn, strongly influence cellular Ca^{2+} efflux and influx mechanisms. For example, increased subsarcolemmal Ca^{2+} concentrations in smooth muscle cells from our exercise-trained pigs might stimulate Ca^{2+}-dependent K^+ channel current and subsequently hyperpolarize the cell, thereby limiting subsequent Ca^{2+} influx through voltage-gated Ca^{2+} channels (Guia *et al.* 1999). Increased subsarcolemmal Ca^{2+} concentrations could also

directly activate mechanisms of Ca^{2+} extrusion (i.e. PMCA) or potentially provide a direct inhibition at L-type Ca^{2+} channels to limit Ca^{2+} influx. Additional studies have endorsed the presence of a subsarcolemmal Ca^{2+} regulatory process in smooth muscle cells, dissociated from global Ca^{2+} regulation, which may alter cellular contractile states (Stehno-Bittel *et al.* 1991; Stehno-Bittel & Sturek, 1992; Sturek *et al.* 1992; Ganitkevich & Isenberg, 1996; Guia *et al.* 1999). The presence of an increased Ca^{2+} concentration in a restricted subdomain of the plasma membrane might provide a mechanistic link between the cellular adaptation of increased Ca^{2+} influx and the general functional adaptation of enhanced vasodilatation and reduced vasoconstriction in the coronary circulation of exercise-trained animals.

In conclusion, we report the novel finding that the increased Ca^{2+} influx observed in smooth muscle cells from exercise-trained animals is compensated by other cellular Ca^{2+} regulatory mechanisms, and thus net myoplasmic free Ca^{2+} accumulation in cells of sedentary *vs.* exercise-trained animals is not significantly different. We also verify previous findings that exercise training produces adaptations in coronary smooth muscle cells that increase VGCC density. Our evaluations of cellular mechanisms that may function to compensate enhanced VGCC density in exercise-trained pigs indicate that neither Na^+–Ca^{2+} exchange nor SERCA plays a compensatory role in Ca^{2+} removal. Furthermore, local Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels does not appear to compensate enhanced VGCC. Our findings suggest that increased Ca^{2+} extrusion via PMCA, Ca^{2+} sequestration by mitochondria, increased Ca^{2+}-binding protein number/affinity, or inactivation of voltage-gated Ca^{2+} channels by means other than local Ca^{2+}-dependent inactivation, are potential compensatory mechanisms for the enhanced rate of free Ca^{2+} accumulation in smooth muscle cells from exercise-trained animals.

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