Effect of endurance exercise on the Ca\(^{2+}\) pumps from transverse tubule and sarcoplasmic reticulum of rabbit skeletal muscle

Viola Becker,¹,⁴ Hugo González-Serratos,² Rocío Álvarez,¹ Michael Bäermann,³ Claudine Irles,⁴ and Alicia Ortega¹

¹Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, México City 04510, México; ²Department of Physiology, School of Medicine, University of Maryland, Baltimore, Maryland 21201; ³Department of Physics E22 (Biophysics), Technical University of Munich, 80333 Garching, Germany; and ⁴Departamento de Bioquímica, Instituto Nacional de Perinatología, México City 11000, México

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Becker, Viola, Hugo González-Serratos, Rocío Álvarez, Michael Bäermann, Claudine Irles, and Alicia Ortega. Effect of endurance exercise on the Ca\(^{2+}\) pumps from transverse tubule and sarcoplasmic reticulum of rabbit skeletal muscle. J Appl Physiol 97: 467–474, 2004. First published April 2, 2004; 10.1152/japplphysiol.00906.2003.—The sarcoplasmic reticulum (SR) Ca\(^{2+}\) pump is the main homeostatic regulatory mechanism in fast skeletal muscle that maintains intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) at the nanomolar level at rest. The transverse tubule (TT) Ca\(^{2+}\) pump is the major homeostatic regulatory mechanism in muscle cells at rest (10) and during mechanical activation (17). The SR has a limited capacity to store Ca\(^{2+}\) at rest (10) and during mechanical activation (17). The SR pump activity was upgraded in association with structural changes to handle the changes in [Ca\(^{2+}\)]\(i\) may increase. TT and SR isolated microsomal pumps of fast skeletal muscle from rabbit at rest. The Ca\(^{2+}\) pump transports cytosolic Ca\(^{2+}\) to the extracellular space. During prolonged muscular activity, [Ca\(^{2+}\)]\(i\) may increase. TT and SR isolated microsomal vesicles were highly purified, and the purity was checked by immunoblotting. The present study shows the effects of endurance exercise on the activities and structures of the TT and SR Ca\(^{2+}\) pumps of fast skeletal muscle from rabbit at rest. The Ca\(^{2+}\) pump activity increased manifolds in TT but did not change in SR. The protein denaturation profiles obtained by differential scanning calorimetry showed 1) a shift in the transition temperature and an increase in the enthalpy of the TT Ca\(^{2+}\) pump and 2) a significant change in the transition temperature of the SR Ca\(^{2+}\) pump Ca\(^{2+}\)-binding domain. We conclude that the TT Ca\(^{2+}\) pump activity was upgraded in association with structural changes to handle the changes in [Ca\(^{2+}\)], and TT lumen Ca\(^{2+}\) concentration that occur during endurance exercise. muscle adaptation; exercise training

ONE OF THE IMPORTANT LINKS in the chain of events that couples excitation with contraction in skeletal muscle during muscle activation is the Ca\(^{2+}\) release from the terminal cisternae of the sarcoplasmic reticulum (SR). This Ca\(^{2+}\) release produces an increase in the free intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) to the micromolar level, which, in turn, produces actomyosin interaction and contraction. Afterward, the reuptake of Ca\(^{2+}\) by the SR Ca\(^{2+}\) pump lowers [Ca\(^{2+}\)]\(i\) to a resting level of 1 \(\times\) 10\(^{-7}\) M, producing relaxation. It has been shown that there is a continuous and significant entry of Ca\(^{2+}\) into skeletal muscle cells at rest (10) and during mechanical activation (17). The SR has a limited capacity to store Ca\(^{2+}\); therefore, once this capacity is reached, [Ca\(^{2+}\)], will increase above the normal resting value of 1 \(\times\) 10\(^{-7}\) M. Consequently, during prolonged muscular activity, a long-term increase in the [Ca\(^{2+}\)], content would seriously impair the function of skeletal muscle and lead to proteolysis and cell damage (13, 14). Therefore, during exercise, resting [Ca\(^{2+}\)], may increase for prolonged periods of time and may lead to cell damage, unless this long-term increase in resting [Ca\(^{2+}\)], is lowered shortly after exercise starts. There are several possible homeostatic membrane mechanisms to buffer the extra Ca\(^{2+}\) that moves into the muscle cells during prolonged activity: Two, calreticulin (1) and calsequestrin (31, 38), are located in the SR intravesicular space; in the SR membrane is the ATP-dependent Ca\(^{2+}\) pump (SERCA). The other Ca\(^{2+}\)-lowering mechanisms are located in the plasma membrane. Although the SR has the capability of removing cytosolic Ca\(^{2+}\) at a high rate (29) and is highly developed in phasic skeletal muscle cells, it has a limited capacity of storing Ca\(^{2+}\) (43). A third mechanism located in the plasma and transverse tubular system (TT) membranes is the Na\(^+/Ca^{2+}\) exchanger driven by the Na\(^+\) electrochemical gradient. When the Na\(^+/Ca^{2+}\) exchanger operates in the forward mode, as it does under normal circumstances, it extrudes cytosolic Ca\(^{2+}\) (5, 21). A fourth mechanism is the calcodulin-stimulated Ca\(^{2+}\) pump located in sarclemma and in the TT membranes (7, 40). This pump has a low activity (7, 26), with the maximum rate of Ca\(^{2+}\) efflux lower than the maximum rate of Ca\(^{2+}\) efflux of the Na\(^+/Ca^{2+}\) exchanger in fast skeletal muscle (12). The two last mechanisms mentioned are located in the TT membranes and, thereby, are able to transport from the cytosol to the lumen of the TT Ca\(^{2+}\) that subsequently moves to the extracellular space. Other Ca\(^{2+}\) regulatory mechanisms that could play an adaptive role during prolonged muscular activity are the TT morphological changes that have been described to occur during chronic low-frequency muscle (through the nerves) stimulation (15, 16).

Different forms of exercise such as “high-repetition, low-load” (endurance) exercise and “low-repetition, high-load” (strength or resistance) exercise induce specific and distinct structural and functional modifications in muscle fibers. Classical endurance exercise interventions of 6- to 8-wk duration in previously untrained subjects lead to an increase of mitochondria density (28). Endurance exercise influences gene expression in skeletal muscle either by the regulatory genes within a given period of 30 min to some hours or through the structural genes as a consequence of weeks of systematic training (6, 32). In response to strength training of similar duration, fiber volume and muscle mass increase (35). These training responses involve changes in gene expression. The set of expressed mRNAs provides basic instruction for the diversity of trans-

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lated proteins that is finally manifested as a characteristic structural adaptation to exercise training.

In general, endurance-exercised individuals are less predisposed to suffer muscle cell damage after prolonged muscle activity (14). The physiological compensatory response to endurance exercise, known as adaptation, may be among other factors the consequence of a prolonged enhancement of one of the Ca\(^{2+}\) homeostatic regulatory mechanisms. Thereby it will maintain resting [Ca\(^{2+}\)] in or below 1 × 10\(^{-7}\) M. If the SR Ca\(^{2+}\) pump is enhanced during adaptation, more Ca\(^{2+}\) would be transported into the SR; however, the pump’s capacity would be reached and resting [Ca\(^{2+}\)] would be increased. Bezanilla et al. (3) have shown that during repetitive tetanic activation the TT lumen Na\(^{+}\) concentration ([Na\(^{+}\)]\(_{l}\)) decreases, whereas the intracellular Na\(^{+}\) concentration ([Na\(^{+}\)]\(_{i}\)) increases as Na\(^{+}\) moves in during each action potential. These changes in Na\(^{+}\) concentration decrease the Na\(^{+}\)/H\(^{+}\) electrochemical gradient (ΔG\(_{NaH}^{\pm}\)) and, thereby, the Na\(^{+}/Ca^{2+}\) exchange activity, leading to an increase in [Ca\(^{2+}\)]. Consequently, we propose that endurance exercise will induce a major adaptive response of the muscle cell by upgrading the other Ca\(^{2+}\) regulatory mechanism, i.e., the Ca\(^{2+}\) pump located in the TT membranes.

In this study, we focus on the fundamental importance of the ATP-dependent Ca\(^{2+}\) transport in the TT membrane of fast-twitch skeletal muscle composed predominantly of type II muscle fibers. We propose that the activation of the Ca\(^{2+}\) transport from the cytosol to the lumen of the TT, as a result of endurance exercise, occurs to protect the cell from Ca\(^{2+}\) overload and probably delays the state of fatigue.

**METHODS**

**Animals.** New Zealand male rabbits were exercised on a treadmill. Control animals of the same age and litter were kept in their cages without any particular type of exercise during the 8 wk of the exercise program of the experimental group. Both groups of animals received the same diet and water supply. After the endurance exercise protocol was completed, control and endurance-exercised rabbits were sedated with chloroform and killed by quick cervical dislocation. This work was approved by the Universidad Nacional Autonoma de Mexico review board.

**Endurance exercise protocol.** The protocol consisted of a 4-min warming-up period at a velocity of 11 m/min, followed by a 26-min continuous exercise period divided into four intervals: 6 min at a velocity of 16 m/min, 10 min at a velocity of 27 m/min, 6 min at a velocity 16 m/min, and ending with a 4-min period at 11 m/min to cool down the animals. The total exercise time was 30 min. This exercise session was held once a day for 5 days/wk throughout 8 consecutive weeks. The rabbits were killed as described above 2 days after the last exercise session. The white, predominantly type II fast twitch skeletal muscles from the thigh (vastus lateralis and semitendinosus) were dissected for SR and TT membrane isolation.

**Preparation of membrane vesicles.** Both thighs of each control or trained animal were used for each membrane isolation. TT and SR microsomes were obtained by differential centrifugation as previously described by Rosemblatt et al. (41) and placed on a discontinuous sucrose gradient of 25, 27.5, 30, and 35% wt/vol. The membranes isolated from the sucrose gradient at the 25–27.5% interphase were characterized as TT membranes on the basis of SDS-PAGE, maximum specific dihydropyridine (DHP) binding, and Mg\(^{2+}\)-ATPase activity. The 35% band was placed on a second discontinuous gradient consisting of 27.5, 30, and 35% sucrose. The band obtained at the 30–35% interface was identified as light SR, detected by the maximum ATPase activity stimulated by Ca\(^{2+}\) and based on SDS-PAGE electrophoresis (41). The purity of TT and SR membrane vesicles was assessed as described below and in **RESULTS**. Protein concentration was determined by using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) with BSA as the standard.

**Immunoblotting.** Equal amounts of TT and SR membrane protein, determined by Bradford assay (Pierce), were separated by an 8% SDS-PAGE and transferred to a nitrocellulose Hybrid membrane (Amersham, Little Chalfont, UK). The DHP receptor (DHPR) was targeted using 1 μg/ml sheep anti-DHPR antibody (UBI, Lake Placid, NY) in phosphate-buffered saline solution with 0.5% Tween 20 and 3% nonfat milk and a horeseradish peroxidase-labeled anti-sheep IgG secondary antibody 0.7 μg/ml (Sigma, Steinheim, Germany). Chemiluminescence was detected by using X-Omat Blue (Kodak Eastman, Rochester, NY) according to the manufacturer’s instruction.

An anti-SERCA1 antibody targeted the SERCA1 membrane enzyme. Blotting was performed with the use of 0.004 μg/ml protein mouse anti-SERCA1 monoclonal antibody Ab A52 (46) (kindly supplied by Professor D. MacLennan, University of Toronto, Toronto, ON, Canada) using 0.33 μg/ml of horseradish peroxidase-labeled anti-mouse IgG secondary antibody.

**Ca\(^{2+}\)-uptake.** Ca\(^{2+}\)-transport was measured at room temperature (22–25°C) by the filtration method in a solution containing (in mM) 100 KCl, 5 MgCl\(_2\), 0.1 CaCl\(_2\), 1 μM Ca\(^{2+}\)-EGTA, 20 Tris-malate (pH 7.0), and 4 ATP with 0.05 mg of protein/ml. The reaction was stopped with 0.5 ml of ice-cold quenching solution containing (in mM) 5 MgCl\(_2\), 4 EGTA, and 20 Tris-malate (pH 7.0). Membrane vesicles were filtered through 0.45-μm Millipore filters, washed, dried, and then counted by scintillation.

**High-affinity 
\[^{110}\text{H}\]PN20–110 binding.** Specific binding of DHP was determined by the filtration method using 
\[^{110}\text{H}\]PN20–110 as previously described by Hamilton et al. (24). TT membrane aliquots of 7.5 μg/ml were incubated for 3 h in the dark at room temperature in a solution containing 50 mM MOPS (pH 7.4) and 
\[^{110}\text{H}\]PN20–110 (86 Ci/mmol). Nonspecific binding was determined in the presence of 0.001 nifedipine. Samples were filtered and washed with 25 ml of ice-cold deionized water by using 0.45-μm Millipore filters. Filters were dried and radiation was counted with a scintillation counter. Specific binding was calculated as the difference of total and nonspecific binding.

**Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase activities.** Total ATPase activity and specific Ca\(^{2+}\)-ATPase activity were measured by colorimetric determination of Pi, using malachite green. Aliquots of 0.005 mg/ml of protein were incubated in a solution containing (in mM) 100 KCl, 5 MgCl\(_2\), 5 Na\(_3\)N, 20 Tris-malate, and 0.33 ATP, pH 7.0 (30). The reaction was stopped with a solution containing 0.045% malachite green hydrochloride, 4.2% ammonium molybdate in 4 N HCl, 0.8 ml Trion X-100 for each 100 ml of solutions, and 0.25 ml of 34% sodium citrate; absorbance was read at 660 nm. The Ca\(^{2+}\)/Mg\(^{2+}\)-ATPase activity was measured in the presence of 1 mM EGTA, and the total ATPase activity was measured in the presence of 0.1 mM CaCl\(_2\). The Ca\(^{2+}\)/Mg\(^{2+}\)-ATPase activity was calculated as the difference between the total and the Ca\(^{2+}\)/Mg\(^{2+}\)-ATPase activity.

**Differential scanning calorimetry.** Important information concerning the structure of membrane proteins can be obtained by determining the changes in the specific heat of denaturation (C\(_p\)) as a continuous function of increasing temperature at a constant rate by using a differential scanning microcalorimeter MicroCal VP-DSC. The transition temperatures (T\(_{m} \)) of the TT and SR membrane proteins were obtained from the resulting endothermic transition. Both membrane systems were suspended in a buffer containing (in mM) 100 KCl and 20 Tris-malate (pH 7.0). Differential scanning calorimetry (DSC) data were analyzed by the method of Cahn vs. temperature were obtained. The membrane sample and reference solution were degassed under vacuum for 5 min before loading the DSC cells (0.6 ml). When equilibrium at 10°C was reached, the temperature was increased to 100°C. The scan rate for all
scans was 1°C/min. To assess for protein denaturation reversibility, the temperature was cooled back to 10°C and a rescan up to 100°C was obtained. The baseline was corrected by subtracting the rescan measurement from the scan, which showed in all cases no evidence of reversibility. The T_m was defined as the temperature at which half of the protein is denatured. DSC profiles were deconvoluted, and the best theoretical fit was calculated by assuming there was irreversible denaturation as previously described for SR (33) and for TT membranes (40).

All values are expressed as means ± SE. All figures are represented as normalized values taking 1 as the maximum activity of the control experiments.

RESULTS

Assessment of the purity of isolated TT and SR microsomal vesicles. Isolated TT membranes from control and adapted muscles were highly purified as described in METHODS. Specific ryamodine binding was not detected in any of the isolated TT membranes, indicating that the membranes were not contaminated with SR terminal cisternae vesicles. The specific DHP binding, determined to test the enrichment of TT, was 9.3 ± 2.7 and 11.2 ± 4.3 pmol/mg protein, respectively, from muscles of control (n = 3) and endurance-exercised rabbits (n = 3).

To further assess the purity of the isolated vesicles of TT and SR, specific immunoblotting was performed as described in METHODS by using antibody protein binding. Figure 1 shows immunoblotting results against DHPR (Fig. 1A) and against SERCA1 (Fig. 1B) in membrane fractions obtained by differential centrifugation. The membrane fraction obtained at the interface 25/27.5%, contains a protein at 170 kDa, which reacts with the anti-DHPR antibody and corresponds to the α-subunit of the DHPR. In the fraction obtained at the 30/35% interface, which has been shown to correspond to SR, where the highest SERCA activity is observed, the anti-DHPR antibody did not detect DHPR. This membrane fraction was further used to isolate light SR as described in METHODS. Figure 1B shows the Western blot analysis in the 25/27.5% and 30/35% membrane fractions against SERCA1. The membrane fraction obtained at the interface 30/35% contains a protein around 115 kDa that reacts with the anti-SERCA1 antibody, which therefore we are led to conclude corresponds to SERCA1. However, in the fraction obtained at the interface 25/27.5% (TT membrane), there was no activity. On the basis of the above, the fraction isolated at the interface 25/27.5%, which was previously characterized as TT membranes (41), was used for the present study.

ATPase activity and ATP-dependent Ca^{2+} transport. The TT ATP-dependent Ca^{2+} transport was notably affected in muscles from control animals. Fig. 2A shows that, at the end of the 30-min reaction, the normalized TT ATP-dependent Ca^{2+} transport from endurance-exercised rabbits compared with muscles from control animals. The Ca^{2+} transport at 30 min increased from 58.6 ± 3.8 to 161.20 ± 11.0 nmol/mg protein (n = 4). Figure 2B shows the normalized SR Ca^{2+} transport, which, up to 15 min, is practically the same for preparations from control and endurance-exercised animals. At the end of 30 min, the reaction from endurance-exercised preparations decreased by 13% compared with controls. The specific activities measured 30 min after the beginning of the

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Fig. 1. Immunolabeling of dihydropyridine receptor (DHPR) and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA1) in different fractions of muscle microsomes after a sucrose gradient separation. Membrane fractions obtained after sucrose-density gradient centrifugation, collected at the interfaces 25/27.5% and 30/35% were separated on SDS-PAGE 8% (wt/vol) and silver stained (lanes 1 and 2). Molecular mass markers are indicated for the SDS-PAGE in lanes 1 and 4.

Proteins were then transferred onto nitrocellulose. The corresponding Western blots stained with antibodies to the DHPR are shown in lanes 5 and 6 for membrane fraction obtained at the interfaces 25/27.5% and 30/35%, respectively. Arrows in lane 7 indicate the molecular mass markers of individual proteins in the immunoblot. TT, transverse tubule; SR, sarcoplasmic reticulum; STD, protein standard; IB, immunoblot. B: equal amounts of membrane protein (5 μg per lane) of the membrane fraction at the 25/27.5% and 30/35% interface were separated on SDS-PAGE 8% (wt/vol) and silver stained (lanes 1 and 2). Molecular weight markers are indicated for the SDS-PAGE in lanes 1 and 4. Proteins were then transferred onto nitrocellulose. The corresponding Western blots stained with antibodies to SERCA1 in lanes 5 and 6 for membrane fractions 25/27.5 and 30/35%, respectively. Arrows in lane 7 indicate the molecular mass markers of individual proteins in the immunoblot.
reaction were 300 ± 2 \,(n = 4) \,and \,260 ± 3 \,nmol/mg \,protein for control and endurance-exercised muscles, respectively.

The TT Ca\(^{2+}\) pump hydrolytic activity, measured 30 min after the reaction started, increased from 0.7 ± 0.027 \,μmol/mg \,in TT vesicles obtained from untrained animals to 1.3 ± 0.28 \,μmol/mg \,(n = 4) \,in TT vesicles obtained from endurance-exercised rabbits. This corresponds to a 1.8-fold increase (Fig. 3A). The Mg\(^{2+}\)-ATPase activity, which is the most abundant protein and ATPase activity in TT membranes, increased from 2.9 ± 0.4 to 9.3 ± 1.4 \,μmol/mg \,protein in endurance-exercised animals. This corresponds to a 3.2-fold increase with respect to the control (Fig. 3B). No important changes in Ca\(^{2+}\) pump activity were observed in SR as a consequence of adaptation to exercise (Fig. 3C). The SR Ca\(^{2+}\) pump hydrolytic activity was 13.5 ± 0.8 and 14.2 ± 3 \,μmol/mg \,protein measured 30 min after the reaction started, for control and adapted animals, respectively.

**Differential scanning calorimetry.** To investigate whether endurance exercise induced conformational changes in TT and SR membrane proteins, we studied by DSC the denaturation profile of membranes from control and endurance-exercised rabbit muscles. In our studies, isolated TT and SR membranes showed important changes in the protein denaturation profile after adaptation to exercise. In all experiments, denaturation was completely irreversible after the temperature reached 100°C. The Tₘ and enthalpy (heat of transition) of Ca\(^{2+}\) pumps indicate that the native structure of the proteins had changed.

The thermal denaturation profiles of isolated TT membranes obtained from a heating rate of 1°C/min are shown in Fig. 4A for membranes obtained from control and in Fig. 4B for membranes obtained from endurance-exercised rabbits.

We observed three major transitions. These transitions are the same as those previously described by Ortega and Lepock (40), who attributed transition A to the Ca\(^{2+}\) pump and transition B to the Mg\(^{2+}\)-ATPase. Transition C was found to correlate with the denaturation of the DHP receptor components (data not shown). The Tₘ and enthalpy computed from the DSC profiles shown in Fig. 3B indicate important changes in all three transitions (A, B, and C), in membranes isolated from muscles of endurance-exercised animals. However, the...
of transition associated with component C
denaturation (Cₚ) vs. temperature

A pattern of light SR used in these experiments reveals an
increase in thermal stability and enthalpy as the result of the denaturation of the TT
membranes; and a considerable change in thermal stability and enthalpy of the SR Ca²⁺
pump caused by changes in the Ca²⁺-binding domain, although the function of the SR Ca²⁺
pump was not modified.

most dramatic changes are observed in transitions A and C. Transition A yields a 2.5-fold increment in enthalpy from 367 ± 45 to 919 ± 102 kJ/mol (n = 3) and a shift in Tₘ from 3°C from 49 ± 0.4 to 52 ± 0.1°C (n = 3), indicating that an important conformational change had occurred in the Ca²⁺
pump. Transition B shows a shift in the Tₘ from 56 ± 1 to 60 ± 1°C (n = 3) and a decrease in enthalpy of 60%, from 2,570 ± 112 (n = 3) to 1,030 ± 98 kJ/mol (n = 3). The heat of transition associated with component C decreased ~50%; it appears as a single, broad component with the same Tₘ at 65°C in both conditions.

The denaturation profile of isolated light SR shows mainly the denaturation of the Ca²⁺ pump. The protein electrophoretic pattern of light SR used in these experiments reveals an 80–95% composition of Ca²⁺ pump. Lepock et al. (33) demonstrated that, in the presence of 1 mM CaCl₂, two different transitions, A and B, appear in the DSC. Transition A corresponds to the denaturation of the nucleotide binding domain, and transition B corresponds to the denaturation of the transmembrane Ca²⁺-binding domain. Figure 5 shows the DSC profile of SR membranes isolated from muscles of control (A) and endurance-exercised (B) rabbits. A conformational change was detected in the Ca²⁺-binding domain (transition B), mainly as a shift in the Tₘ from 63 to 59 ± 0.2°C (n = 3). The transition associated with the nucleotide-binding domain (transition A) has the same Tₘ at 51°C in both conditions.

**DISCUSSION**

Muscle is a very adaptable tissue with an intrinsic ability to change its mass and phenotype in response to activity. Adaptation to different muscle work regimes is brought up by changes in fiber type and cross-sectional area. Endurance exercise or intermittent daily muscle nerve stimulation leads to adaptive phasic skeletal muscle responses consisting in a reduction in the number of type IIB fibers as they get transformed into IIA fibers (36). It has been widely documented that both mass and phenotype are markedly altered by strength training within a short period of time (35). Prolonged exercise has been associated with quantitative as well as qualitative changes in gene expression for contractile proteins (18, 19) and compensatory biochemical changes (27). Other types of adaptive response studied in the context of endurance exercise or chronic electrical stimulation experiments are changes in Ca²⁺ regulatory proteins. Such adaptive changes have been observed in the SR Ca²⁺ pump (23), parvalbumin (23, 31), and calsequestrin (31, 38), the major SR Ca²⁺ binding protein (37).

The results described in this paper show that the TT and SR Ca²⁺ pumps of animals that have been subjected to 8 wk of endurance exercise exhibit the following characteristics compared with nontrained animals: 1) a considerable increase in Ca²⁺ uptake and Ca²⁺ pump hydrolytic activity in vesicles of TT membranes; 2) an important increase in Mg²⁺-ATPase activity in TT; 3) no considerable differences in Ca²⁺ transport and Ca²⁺ pump hydrolytic activity in light SR vesicles compared with controls; 4) a considerable change in thermal stability and enthalpy as the result of the denaturation of the TT Ca²⁺ pump and Mg²⁺-ATPase in the adapted muscles; and 5) a modification in thermal stability and enthalpy of the SR Ca²⁺ pump caused by changes in the Ca²⁺-binding domain, although the function of the SR Ca²⁺ pump was not modified.
Our results lead us to conclude that one of the most important adaptive changes in endurance exercise involves the upgrading of the TT Ca\(^{2+}\) pump. To our knowledge, this is the first study that shows the effect of endurance exercise on the TT ATP-dependent Ca\(^{2+}\) uptake and the TT Ca\(^{2+}\) pump hydrolytic activity. These two processes result in a 2.8- and 1.8-fold increased activity, respectively. Specific binding of \(^{3}H\)RyR and \(^{3}H\)DHPR and immunoblot studies show the enriched concentration of DHPR in the fraction associated with TT and the absence of SERCA1 antibody immunolabeling, indicating the purity of the fraction used in the present study.

The adaptive modifications previously described by other researchers indicate, in general, that sustained contractile activity during prolonged time periods leads to modifications in the cytosolic Ca\(^{2+}\) regulatory proteins. Chronic electrical stimulation of fast-twitch rabbit muscle nerves using a frequency pattern typical of slow-twitch muscles induces progressive transformations of structural, functional, and molecular characteristics of the SR. These experiments indicated that after 2 days of stimulation the SR Ca\(^{2+}\) pump SERCA1 isoform, which is mainly expressed in fast-twitch type II fibers, is converted to the SERCA2 isoform, mainly expressed in slow-twitch type I fibers (8, 23, 25, 34, 36). Under these conditions, the ATPase hydrolytic activity and the corresponding Ca\(^{2+}\) transport associated with the ATPase activity show a decrease (22). Mabuchi et al. (36) have shown that long-term intermittent stimulation (10 Hz, 8 h/day for 7 wk) of the fast-twitch tibialis cranialis muscle results in a complete transformation of type IIB fibers to type IIA fibers with a decrease in the SR Ca\(^{2+}\) transport activity. These adaptive changes are consistent with the characteristics that correspond to slower [Ca\(^{2+}\)] buffering systems. Therefore, these fibers would not be very efficient in handling a continuous long-term Ca\(^{2+}\) entry that may take place during many days of exercise. All of these studies underline the importance of the stimulation protocol parameters in determining under what condition a fast-to-slow type could take place as a consequence of endurance exercise. Also, single bouts of intense exercise may lead to a modified Ca\(^{2+}\) handling, similar to the alterations found by repetitive prolonged mechanical activation leading to fatigue. Under these conditions of stimulation, relaxation slows down to a point that a sustained contracture can arise, suggesting that a low-level increase in cytosolic [Ca\(^{2+}\)] is maintained for a long period of time (20). This extra cytosolic Ca\(^{2+}\) may be the consequence of a decrease in the rate of Ca\(^{2+}\) transport by the SR, because it occurs in animals that exercise without having gone through previous endurance exercise (2, 22, 44). Thus the intracellular Ca\(^{2+}\) buffering mechanisms become overwhelmed, and the extra Ca\(^{2+}\) is extruded through the sarcolemmal and TT Ca\(^{2+}\) pumps to the extracellular space, thereby increasing the TT lumen [Ca\(^{2+}\)] (4). However, under normal conditions and before adaptive changes take place, also the TT Ca\(^{2+}\) pumps have a limited capacity and they cannot extrude the cytosolic Ca\(^{2+}\) that builds up during prolonged exercise.

We found that after 8 wk of endurance exercise, the Ca\(^{2+}\) pump from light SR had experienced a conformational change in the transmembrane Ca\(^{2+}\)-binding domain as indicated by a change in the thermal stability of transition B in the DSC experiments. Ortega and Lepock (40) and Sineistra et al. (42) have previously associated a conformational change of the SR transmembrane Ca\(^{2+}\)-binding domain of the Ca\(^{2+}\) pump with a decrease in the activity of Ca\(^{2+}\) transport. However, we did not find substantial differences in Ca\(^{2+}\) transport and pump hydrolytic activity associated to the conformational change. This is in agreement with Green et al. (22), who found that SR Ca\(^{2+}\)-ATPase activity, which reflects SR Ca\(^{2+}\) uptake, decreases by 31% after 60 min of exercise before training, but that, after 12 wk of exercise training the ATPase activity was unchanged. Wilson et al. (44) studied the rate of Ca\(^{2+}\) uptake and SR Ca\(^{2+}\) pump hydrolytic activity in SR vesicles isolated from the middle gluteal muscle of the horse, before the last exercise session after 12 wk of training. However, these authors found that there was a 17% and a 62% increase in the rate of Ca\(^{2+}\) uptake and SR Ca\(^{2+}\) pump hydrolytic activity, respectively. They also observed 31 and 76% improvements in the postexercise decreased rate of Ca\(^{2+}\) uptake and Ca\(^{2+}\) pump hydrolytic activity. The difference between the results of Wilson et al. and our results could be attributed to the use of different animal species and muscles or to contamination by TT membranes in the isolated SR vesicles. Wilson and collaborators did not discriminate between having isolated pure light SR and TT membranes as separated or attached vesicles. It is not uncommon that during the isolation process SR vesicles remain attached to TT vesicles, especially if a microsomal fraction is used (41).

Our results show a small change in the SR ATP-dependent Ca\(^{2+}\) transport and no change in ATPase hydrolytic activity measured at rest after endurance exercise. Thus the underlying conformational change described in this paper may well be associated with a change in the activity of the SR Ca\(^{2+}\) pump that we have observed. The mechanism by which a modified conformation of the SR Ca\(^{2+}\)-ATPase does not reveal a change in activity is yet unknown and is under investigation in our laboratory.

Another homeostatic mechanism that could extrude the extra cytosolic Ca\(^{2+}\) that might be accumulated during prolonged periods of muscle activity is the Na\(^{+}\)/Ca\(^{2+}\) exchanger located in the plasma membrane (12) when operating in the forward mode (5, 21). The exchanger is driven by the \(\Delta\mu_{\text{Na}}\). However, at the normal [Na\(^+\)]\(_{\text{r}}\) of 120 mM, [Na\(^+\)]\(_{\text{r}}\) is reduced to \(-60\) mM during a 60-Hz, 2- to 3-s tetanus (3). The other 60 mM move into the cytosol, increasing [Na\(^+\)]\(_{\text{i}}\). The [Na\(^+\)]\(_{\text{r}}\) depletion and [Na\(^+\)]\(_{\text{i}}\) increase cause a substantial decrease in \(\Delta\mu_{\text{Na}}\). The decreases in \(\Delta\mu_{\text{Na}}\) and in resting membrane potential that occur during high-frequency activation (3) lead to low activity of the exchanger, resulting in an increased cytosolic [Ca\(^{2+}\)]. The SR may take up the extra Ca\(^{2+}\) not extruded by the Na\(^{+}\)/Ca\(^{2+}\) exchanger. However, the frequency of stimulation does not allow for a complete Ca\(^{2+}\) removal by the SR as the rate of Ca\(^{2+}\) uptake decreases (43). These combined factors will lead to an increase in cytosolic [Ca\(^{2+}\)] (21) that will alter excitation-contraction coupling, contractility, and, subsequently, viability of the muscle cells. Thus another mechanism must be upgraded to reduce the increased long-term cytosolic [Ca\(^{2+}\)]. The results from our DSC measurements show that the changes in TT Ca\(^{2+}\)-ATPase hydrolytic and transport activities are accompanied by conformational changes of the TT membrane proteins. Thereby we propose that endurance exercise upgrades the TT Ca\(^{2+}\) pump. This modification leads to an increased TT ATP-dependent Ca\(^{2+}\) transport and thus to an increased transfer of Ca\(^{2+}\) out of the cell into the TT lumen. The changes described
in the present study cannot be attributed to the morphological changes of the TT system that have been observed during and after chronic low-frequency muscle electric stimulation (15, 16). Chronic low-frequency electric stimulation decreases the TT system density. If this had happened during the long-term exercise, it would have led to a decrease in the density of the TT Ca\(^{2+}\) pump and thereby to a decrease, not an increase, in total Ca\(^{2+}\) pump activity.

In conclusion, the experiments described in this paper support the idea that two of the most important changes resulting from endurance exercise are the upgrading of the TT Ca\(^{2+}\) pump and the Mg\(^{2+}\)-ATPase activity. The upgrading of the activity of the Ca\(^{2+}\) pump may become one of the homeostatic compensatory mechanisms to handle the changes in [Ca\(^{2+}\)], that take place during endurance exercise. Otherwise, during prolonged muscular activity, i.e., endurance exercise, resting [Ca\(^{2+}\)] may increase long term. This long-term increase in the cytosolic Ca\(^{2+}\) content would seriously impair the function of skeletal muscle. Ca\(^{2+}\) is an activator of the protease calpain, which has been found in skeletal muscle (11). Calpains produce proteolysis (13, 45) and also decapitation of dystrophin (9). Therefore, an excessive amount of Ca\(^{2+}\) will cause an overproduction of calpains, which may lead through the combination of the above effects to severe muscle damage. Unless this long-term increase in resting [Ca\(^{2+}\)] is lowered by the upgraded TT Ca\(^{2+}\) pump shortly after training starts, it would lead to proteolysis and cell damage (13, 14, 45). The precise nature of the conformational changes described in this paper remains unknown. We are presently investigating the nature of these conformational changes.

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