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Activation of the sarcoplasmic reticulum Ca^{2+}-ATPase induced by exercise

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Abstract

Prolonged exercise has been shown to cause disruption of intracellular calcium homeostasis in skeletal muscle, which is normally maintained by the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase. We have investigated the response of this enzyme to increased intracellular calcium levels by investigating the functional and physical characteristics of the SR Ca^{2+}-ATPase and membrane lipids following 2 h of treadmill running and throughout a period of post-exercise recovery. The Ca^{2+}-ATPase of SR membranes purified from exercised rats shows increases in enzymatic activity correlating with post-exercise recovery time. Corresponding increases in active Ca^{2+}-ATPase pump units are observed, as measured by the concentration of phosphorylated enzyme intermediate formed from ATP. However, catalytic turnover rates of the Ca^{2+}-ATPase are unchanged. Using spin-label electron paramagnetic resonance to assess both membrane fluidity and associations between individual Ca^{2+}-ATPase polypeptide chains, we find no exercise-induced alterations in membrane dynamics which could explain the observed increases in Ca^{2+}-ATPase activity. Nor do we find evidence for altered membrane purification as a result of exercise. We suggest that the cell responds to the challenge of increased cytosolic calcium levels by increasing the proportion of functional SR Ca^{2+}-ATPase proteins in the membrane for the rapid restoration of calcium homeostasis.

Keywords: Calcium homeostasis; EPR; Exercise; Sarcoplasmic reticulum; ATPase, Ca^{2+}-

1. Introduction

A rise in intracellular calcium concentration is rapidly followed by cellular damage in cardiac or skeletal muscle in such pathological conditions as ischemia or muscular dystrophy [1]. Similar, but transient, damage to skeletal muscle has been reported following strenuous exercise [2]. This damage includes increased sarcolemmal permeability and localized changes in intracellular structures, such as swelling of mitochondria and of the sarcoplasmic reticulum (SR) [2,3]. Although the temporal pattern and extent of changes vary with the exercise protocol employed, these changes are readily reversed during a post-exercise recovery period. Mitochondria and SR appear to rapidly return to normal as early as 30 min after exercise; sarcolemmal integrity often requires several hours further for full recovery [3,4]. In a minor population of muscle fibers (2–5%), exercise-induced focal damage to muscle is irreversible and culminates in cell necrosis [5,6]. Both transient and irreversible muscle damage induced by exercise has been correlated with a dramatic rise in intracellular calcium levels, which has been estimated from mitochondrial calcium concentrations to increase 3-fold in rat muscle following a bout of treadmill running [7,8]. The reversibility of exercise-induced muscle damage, and the correlation of intracellular calcium with sarcolemmal permeability, suggest that calcium homeostasis is readily reattained in muscle cells during the post-exercise recovery period. On the other hand, the inability of a cell to reestablish normal intracellular calcium levels results in its death.

We have used exercise as a means to increase intracellular calcium levels in order to investigate how calcium homeostasis is reattained, specifically focusing on the role of the SR Ca^{2+}-ATPase. This transmembrane protein is

Abbreviations: CK, creatine kinase; E-P, phosphoenzyme; EPR, electron paramagnetic resonance spectroscopy; Mops, 3-(*N*-morpholino)propanesulfonic acid; MSL, maleimide spin label; *S*, order parameter; SASL, stearic acid spin label; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; ST-EPR, saturation transfer electron paramagnetic resonance spectroscopy.

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critically involved in the maintenance of calcium homeostasis by actively sequestering cytosolic calcium into the lumen of the SR against a concentration gradient [9]. Previous work has suggested a pivotal role for SR calcium stores in exercise-induced changes in muscle structure [10].

The present study represents a comprehensive analysis of the function and structure of the SR Ca^{2+}-ATPase and of SR lipids as a means to investigate the mechanism of restoring calcium homeostasis in the cell. We find that in response to high intracellular calcium levels, the Ca^{2+}-ATPase exhibits increased enzymatic activity during post-exercise recovery. These findings may lead to a better understanding of pathological conditions, like muscular dystrophy, which are also associated with elevated intracellular calcium, increased levels of intramuscular enzymes in the blood and similar alterations in muscle architecture as seen with exercise [11,12]. Portions of the information included in this manuscript were previously presented at the American College of Sports Medicine National Meeting [13].

2. Materials and methods

2.1. Exercise protocol

Previously untrained, male Wistar rats (257 ± 10 g) were randomly divided into sedentary control ($n = 6$) or exercise ($n = 12$) groups. The exercise protocol consisted of an initial 15 minute period where the treadmill speed was gradually increased to allow the rats to acclimate, followed by 75 min of continuous level treadmill running at 19 m/min. Several of the rats were unable to maintain the pace of the treadmill after 75 min, and therefore the treadmill speed was then decreased to 16 m/min for the remaining 30 min of the 2 h of treadmill running, allowing all animals to complete the required exercise. A brisk walking pace (10 m/min) was maintained until they were killed for those animals exercising for a total of 2.3 and 2.7 h. The period of walking during the post-exercise recovery period is referred to as 'active recovery'. Thus exercise time includes the 2 h of running plus the additional time involved in 'active recovery' until killing. The intensity of the exercise protocol was approximately 60–65% of the total aerobic capacity for young adult rats [14]. A mild electric shock grid positioned at the rear of the treadmill was used to encourage animals to continue running and walking. Two animals from the exercise group were killed at each time point of 2.0, 2.3, and 2.7 h after starting exercise. Killing occurred within 3 min after removal from the treadmill for all exercise groups. The remaining six rats in the exercise group were killed 48 h post-exercise after exercising for 2.0, 2.3 or 2.7 h (two animals per exercise time). Sedentary control rats were present but maintained in their cage during the exercise session and were killed along with the exercise groups.

2.2. Plasma creatine kinase activity

Following death by CO_2 inhalation, blood was withdrawn from the heart and immediately centrifuged in heparin-treated tubes. Aliquots of plasma were retained on ice (approximately 3–4 h) and then analyzed for total plasma creatine kinase (CK) activity at 37°C by spectrophotometric determination using an Ektachem 700 Analyzer (Kodak). An additional aliquot of plasma was frozen until later determination of CK isoenzyme distribution. Isoenzyme content was determined using a commercially available kit (Paragon-CK, Beckman) which separates CK isoenzymes (MM, MB and BB) in plasma samples by native agarose gel electrophoresis. Quantification of isoenzymes was obtained from measuring fluorescence intensity (Beckman SDS 200 Fluorometer) of NADPH ($\lambda_{ex} = 340$ nm; $\lambda_{em} = 455$ nm) formed in an enzyme-linked assay initiated by the CK present at each isozyme band.

2.3. Isolation of SR

Native SR vesicles were prepared from rat hindlimb muscles immediately following death as described previously [15]. Briefly, the muscle was homogenized in a Waring blender with a medium containing 0.1 M KCl and 20 mM Mops (pH 7.0) and centrifuged for 20 min at $4000 \times g$. The pellets were homogenized in additional medium and centrifuged ($4000 \times g$) for 30 min. To pellet the mitochondria, the supernatants from the first and second spins were combined, filtered through cheesecloth and centrifuged at $11\,800 \times g$ for 20 min. To eliminate myosin protein from the preparation, the supernatant was centrifuged at $23\,500 \times g$ for 1 h following the addition of solid KCl to the supernatant for a final concentration of 0.6 M KCl. The supernatant was decanted and the pellets were resuspended in a medium containing 0.3 M sucrose and 20 mM Mops (pH 7.0), and centrifuged for 30 min at $100\,000 \times g$. The pelleted SR vesicles were carefully homogenized in a medium containing 0.3 M sucrose and 20 mM Mops (pH 7.0), quick frozen in liquid nitrogen and stored at −70°C. Protein concentration was determined using a modified Biuret assay in which 2% Sterox detergent (Bacharach, Pittsburgh, PA) was included to reduce light scattering. Bovine serum albumin was used as the standard.

2.4. Polyacrylamide gel electrophoresis and Western Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 7.5 or 5% gel with a 3% stacking gel, according to the method of Laemmli [16]. Gels were stained for protein with Coomassie blue. The relative amount of Ca^{2+}-ATPase protein in the SR preparations was determined from densitometric measurements of the band migrating with an

apparent molecular mass of 95 kDa on the 7.5% gel (FB910 Densitometer, Fisher Scientific). The slow and fast isoforms of the Ca^{2+}-ATPase were identified by Western immunoblot analysis using antibodies to the SERCA1 (fast) and SERCA2a (slow) proteins (Affinity Bioreagents) following separation by 5% SDS-PAGE. Densitometry of scanned 5% Coomassie blue stained gels was used to quantify the two isoforms of the Ca^{2+}-ATPase protein.

2.5. Calcium-dependent ATPase activity

Calcium-dependent ATPase activity was measured at 25°C by colorimetric determination of inorganic phosphate released from vesicles made leaky to calcium by the addition of the calcium ionophore A23187 [17]. The reaction medium contained 100 mM KCl, 5 mM $MgCl_2$, 4 μM A23187, 25 mM Mops (pH 7.0), 0.05 mg/ml SR protein, and either 0.1 mM $CaCl_2$ or 1 mM EGTA. The reaction was started by addition of 5 mM ATP and the initial rate of inorganic phosphate release was used to calculate activity. Activity assayed in the presence of EGTA (basal activity) was subtracted from that assayed in the presence of $CaCl_2$ (total ATPase activity) in order to obtain calcium-dependent ATPase activity. Ionophore stimulation was determined to evaluate vesicle integrity by calculating the ratio of calcium-dependent ATPase activities in the presence and absence of A23187.

2.6. Characterization of basal activity

Calcium-independent ATPase (basal) activity corresponds to contamination by ATPase proteins characteristic of other membranes of the muscle cell. Sarcolemmal membrane content was determined by measuring inhibition of basal ATPase activity using 2 mM ouabain. Mitochondrial membrane content was measured from the inhibition of basal ATPase activity by 16 μg/ml oligomycin. Transverse tubule membrane content was determined from the divalent ATPase activity [18]. This assay involved measuring ATPase activity under basal conditions with 1.6 mM $CaCl_2$ and without $MgCl_2$.

2.7. Calcium activation measurements

The calcium dependence of ATPase activity was determined as a function of free calcium using calcium-EGTA buffers. The reaction medium contained 100 mM KCl, 5 mM $MgCl_2$, 4 μM A23187, 25 mM Mops (pH 7.0), 0.1 mM EGTA, 0.05 mg/ml SR protein and sufficient calcium to result in free calcium concentrations ranging from 0.004 to 5 μM calcium ($pCa = 8.4$ to 5.3), as determined using binding constants for calcium in the presence of KCl, $MgCl_2$ and EGTA [19].

2.8. Enzyme-phosphate formation

Steady-state levels of phosphorylated enzyme intermediate formed from [γ-^{32}P]ATP were measured. Phosphorylation was carried out in the following reaction mixture: 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM Mops (pH 7.0), and 0.5 mg/ml protein in the presence and absence of 0.9 mM $CaCl_2$. The reaction was started by addition of 0.1 mM [γ-^{32}P]ATP (Dupont Chemical Co, N. Billerica, MA) and, after 20 s at 0°C, quenched with 7% trichloroacetic acid (w/v) and 4 mM NaH_2PO_4. The quenched protein was washed four times by repeated centrifugations and resuspensions in 3.5% trichloroacetic acid and 2 mM NaH_2PO_4. The final precipitant was dissolved in 2% SDS, 0.1 M NaOH, 2% Na_2CO_3, and 5 mM NaH_2PO_4. ^{32}P, as determined by scintillation counting of the dissolved precipitate, and protein concentration [20] were determined to calculate nanomoles of phosphoenzyme formed per milligram protein. The calcium-dependent phosphoenzyme intermediate was determined from the difference between values derived from measurements in the presence or absence of 0.9 mM $CaCl_2$.

2.9. Spin labeling

Hydrocarbon chain mobility was measured with the fatty acid spin label, an *N*-oxyl-4',4'-dimethyloxazolidine derivative of stearic acid (Aldrich), designated 5- and 16-stearic acid spin label (SASL) using conventional EPR techniques. The spin label was diluted from a stock solution in dimethylformamide into ethanol before adding to SR at a ratio of less than one spin label/200 phospholipids, with the final ethanol concentration less than 1%. To measure rotational motion of the Ca^{2+}-ATPase protein, SR was labeled with a short chain maleimide spin label, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (MSL) (Aldrich) as described previously [21,22].

2.10. EPR spectroscopy

EPR spectra were obtained with a Varian E-109 spectrometer. Submicrosecond rotational motion of 5- and 16-SASL was detected by conventional EPR (first harmonic absorption in-phase, designated V_1) using 100 kHz field modulation (with peak-to-peak amplitude of 2 G) and a microwave field amplitude of 10 mW. Submillisecond rotational motion of the maleimide spin-label bound to the Ca^{2+}-ATPase was detected by saturation transfer-EPR (ST-EPR) (second harmonic absorption out-of-phase, designated V_2') using 50 kHz field modulation (with a modulation amplitude of 5 G) and a microwave field intensity of 0.25 G. All EPR samples were suspended in 0.3 M sucrose, and 20 mM Mops (pH 7.0).

2.11. Spectral analysis

Spectral characteristics (i.e., lineshapes and peak heights) from conventional EPR spectra using the 5-SASL probe were used to calculate an effective order parameter (S), which depends only on the angular amplitude of the motion of the probe. An order parameter for lipids close to the surface of the lipid bilayer was calculated from 5-SASL spectra using the standard formula relating S to both inner and outer extrema [23,24]:

$$S = \frac{T'_{\parallel} - (T'_{\perp} + C)}{T'_{\parallel} + 2(T'_{\perp} + C)} \times 1.66$$

where $C = 1.4 - 0.053(T'_{\parallel} - T'_{\perp})$. $2T'_{\perp}$ and $2T'_{\parallel}$ are the measured inner and outer extrema resolved in the EPR spectrum in gauss. (These spectral parameters are illustrated in Fig. 6A.)

An effective correlation time (τ_r) for fast isotropic motion of the hydrocarbon chain close to the center of the bilayer was calculated from measurement of peak heights and line widths of the 16-SASL EPR spectrum using the formula [25]:

$$\tau_r = 6.5 \cdot 10^{-10} W_0 \left((h_0/h_{-1})^{1/2} - 1 \right)$$

where W_0 is the line width of the midfield line and h_0 and h_{-1} are the peak-to-peak line heights in the mid- and high-field regions of the spectrum, respectively. (These spectral parameters are illustrated in Fig. 7A.)

ST-EPR spectra are commonly interpreted by either the line shape (ratio of line heights) or the integrated intensity. In the present study, an integrated intensity parameter, the normalized integral of the V'_2 spectrum, was used in order to suppress the interfering signals from weakly immobilized probes that can distort line shapes [26]. The V'_2 integral was calculated for each sample following normalization for spin (label) concentrations, as determined from the double integration of digitized conventional (V_1) EPR spectra from MSL-labeled samples.

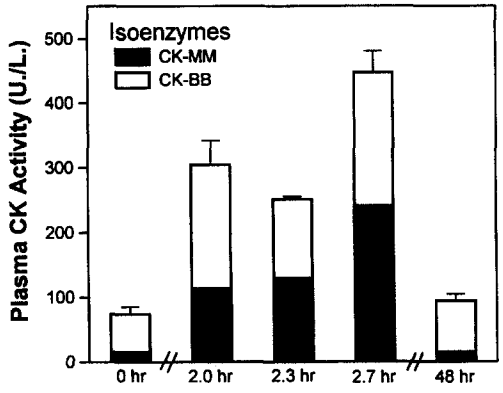
2.12. Statistics

Differences between groups were tested for statistical significance using the non-parametric Mann-Whitney U-test, with the level of significance at $P = 0.05$–0.10. This is the lowest level of significance possible based on our sample size ($n = 2$ for exercise groups compared with $n = 6$ for sedentary controls) [27]. Regression analysis testing for significant trends in enzyme activity with exercise time were determined for the exercise groups (2.0, 2.3 and 2.7 h), with the level of significance at $P < 0.05$. Correlation coefficients were performed comparing Ca^{2+}-ATPase specific activity and the concentration of the enzyme-phosphate intermediate for individual animals, with the level of significance at $P < 0.05$. Data are reported as mean ± S.E. for all groups.

3. Results

3.1. Increased sarcolemmal permeability

The presence of elevated levels of the muscle isoform (CK-MM) of CK in the blood is commonly used as a biochemical indicator of increased permeability of the muscle plasma membrane [2,4,10]. In the rat, CK-MM accounts for less than 10% of the blood isoenzyme profile under resting conditions and has been shown to rise dramatically following exercise [4,10]. We find that following 2 h of treadmill running there is an approximate 10-fold increase in CK-MM activity of rats killed immediately after exercise compared to the sedentary control group (Fig. 1). A 3-fold increase in levels of the CK-BB isoform is also observed. In the sedentary rat, CK-BB is the dominant isozyme (approx. 80–90%) in the blood, originating mainly from brain and liver [4]. Thus, while total CK activity reflects a rise in both isoforms, the greatest change occurs in the muscle specific isoform, CK-MM. Although the relative contribution of CK-MM as well as total CK activity remained elevated up to 2.7 h of exercise, at 48 h after exercise these levels returned to control values, demonstrating the reversibility of exercise-induced changes in skeletal muscle sarcolemmal permeability. The activity levels for total CK and the CK isoenzyme distribution reported here are in agreement with previously published data for male rats following 2 h of treadmill exercise [4,10].



CK-BB	82 ±2%	61 ±6%	50 ±4%	46 ±5%	87 ±4%
CK-MM	18 ±1%	39 ±6%	50 ±4%	54 ±5%	13 ±4%

Fig. 1. Serum creatine kinase (CK) isoenzyme activity (units/liter) determined from sedentary control animals (0 h), rats killed immediately following exercise (2.0 h) or active recovery (2.3 or 2.7 h), and rats killed 48 h post-exercise (48 h). (See Methods for details of exercise protocol.) Bar height and error bars corresponds to CK total activity ± S.E., respectively. The open and solid portions of the bar represent the contribution of CK-BB and CK-MM isoenzyme to the total CK enzyme activity, respectively. The isoenzyme distribution with standard errors as a percentage of total activity is given below the graph. CK-MB was less than 5% of the total CK activities in each case and thus was not included in the distribution.

3.2. Characterization of SR preparations

In the SR membranes prepared from the hindlimb skeletal muscles of control (sedentary) and exercised rats, we find no difference in the yield of SR protein (1.6 ± 0.1 mg SR protein/g muscle) or the relative abundance of the Ca^{2+}-ATPase protein. Analysis of SDS-polyacrylamide gels indicate that 52 ± 6% of the protein in these native SR vesicle preparations are Ca^{2+}-ATPase, as assessed by densitometry of the Ca^{2+}-ATPase protein band migrating with an apparent molecular weight of 95 kDa (Fig. 2). Separation and identification by immunoblots of the slow and fast twitch Ca^{2+}-ATPase isoforms on 5% SDS-PAGE indicate that approx. 15% of the total Ca^{2+}-ATPase protein in all samples is the slow twitch isoform (data not shown). Thus despite the observed transient alterations in cellular ultrastructure induced by exercise, the compositions of the isolated SR membranes are not altered [3].

3.3. Enzymatic activity

The specific activity of the SR Ca^{2+}-ATPase, reflected by ionophore stimulated calcium-dependent ATPase activity, progressively increases with exercise time up to 30% after 2.7 h of exercise (including active recovery), and returns to sedentary control levels by 48 h after initiation of exercise (Fig. 3). Indeed, there is a significant trend of increasing specific activity with exercise time, as determined by linear regression analysis for the 2.0, 2.3 and 2.7 h groups ($P = 0.009$ and $r = 0.92$). On the other hand, calcium-independent ATPase activity, accounting for approximately 19% of total ATPase activity in these preparations, is unaltered by exercise (0.49 ± 0.02 μmol/mg per min for all groups). This ATPase activity is attributable to the co-purification of sarcolemmal (< 1%), transverse tubular (3%), and mitochondrial (15%) membranes, as assessed by assays with specific inhibitors to the ATPase proteins associated with these membranes (see Methods for details). The distribution of the activities associated with contaminating membrane proteins is unaffected by exercise, confirming that exercise-induced architectural changes of muscle membranes do not alter the membrane composition of the resulting preparations. Moreover, membrane integrity is not altered by exercise, as evidenced by an identical 3-fold stimulation (3.1 ± 0.1) for all experimental groups by the calcium ionophore A23187.

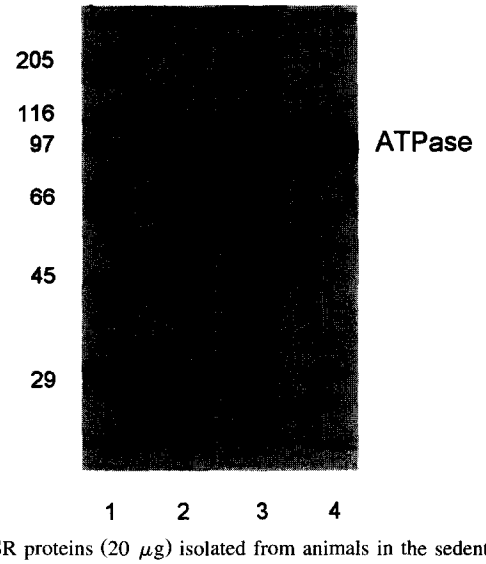


Fig. 2. SR proteins (20 μg) isolated from animals in the sedentary (lane 2), 2.7 h exercise (lane 3) and 48 h post-exercise (lane 4) groups separated on a 7.5% Laemmli denaturing gel and visualized with Coomassie blue stain. ATPase denotes the position of the Ca^{2+}-ATPase band, which comprises 52 ± 6% of total SR protein. Numbers to the left indicate molecular mass in kDa of molecular mass standards (lane 1).

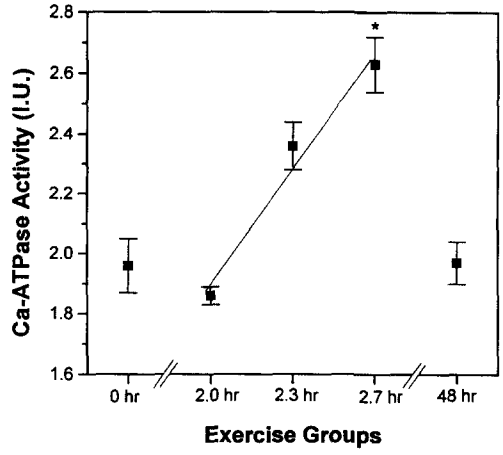


Fig. 3. Ca^{2+}-ATPase activities (in international units, IU) of SR vesicles from sedentary control animals (0 h), rats killed immediately following exercise for 2.0, 2.3, and 2.7 h, and rats killed 48 h post-exercise. Ca^{2+}-ATPase activity was determined as the difference between activity in the presence and absence of calcium. The reaction medium contained 5 mM ATP, 100 mM KCl, 5 mM $MgCl_2$, 4 μM A23187, 25 mM Mops (pH 7.0), 0.05 mg SR protein/ml and either 0.1 mM $CaCl_2$ or 1 mM EGTA. The relationship between increasing enzymatic activity with exercise time is illustrated ($P = 0.009$ and $r = 0.92$ for the 2.0, 2.3 and 2.7 h groups). * Significantly different at $P = 0.05$–0.10, by non-parametric analysis comparing control with the 2.7 h exercise group.

3.4. Calcium concentration-dependent activation

Our assay of Ca^{2+}-ATPase activity utilizes saturating calcium concentrations and therefore may obscure any exercise-induced alterations in enzyme kinetics at more physiological calcium concentrations. Therefore, calcium-dependent ATPase activity was assayed over a range of free calcium concentrations. As demonstrated in Fig. 4, a sigmoidal relationship between calcium concentration and ATPase activity is observed, indicative of positive cooperativity of calcium binding to the Ca^{2+}-ATPase [9]. We find that the extent of cooperativity for these preparations as assessed by the Hill coefficient ($n = 1.51 \pm 0.07$) is unchanged with exercise (Table 1). However, we find a small but statistically significant decrease in the calcium concentration at half maximal activity ($K_{Ca\ (app)}$) for the 2.7 h

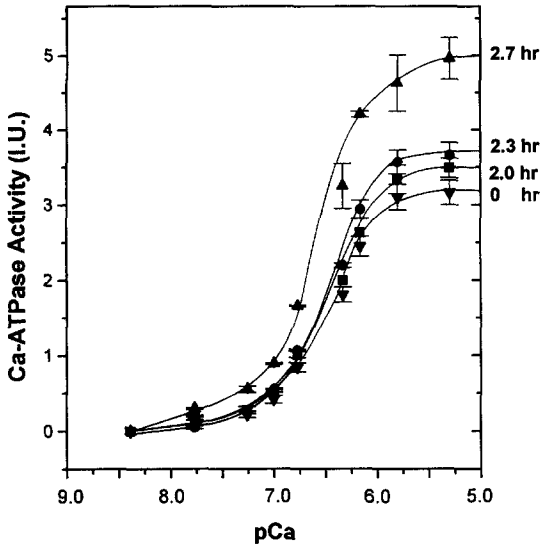


Fig. 4. Calcium-dependent ATPase activity was determined using isolated SR membranes from sedentary control animals (▼) and exercised rats (■ 2.0 h; ● 2.3 h; ▲ 2.7 h). ATPase activity was assayed at 25°C, in a medium of 5 mM ATP, 100 mM KCl, 5 mM $MgCl_2$, 4 μM A23187, 0.1 mM EGTA, 25 mM Mops (pH 7.0), 0.05 mg/ml SR protein and with sufficient calcium to provide the indicated free calcium concentrations ($pCa = 8.4$ to 5.3).

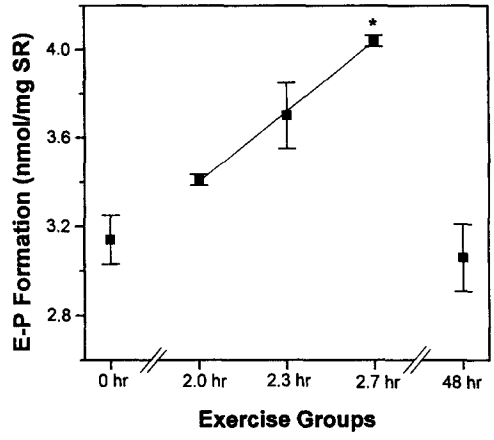


Fig. 5. Steady-state levels of phosphorylated enzyme intermediate (E-P) formed from ATP in SR membranes isolated from sedentary control animals (0 h), rats killed immediately following exercise for 2.0, 2.3, and 2.7 h, and rats killed 48 h post-exercise. Enzyme phosphorylation was carried out at 0°C in a reaction medium of 0.1 mM [γ-^{32}P]ATP, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.9 mM $CaCl_2$, 20 mM Mops (pH 7.0), and 0.5 mg SR protein/ml. The relationship between increasing phosphorylated enzyme formed with exercise time is illustrated ($P = 0.013$ and $r = 0.91$ for the 2.0, 2.3 and 2.7 h groups). * Signifcantly different at $P = 0.05$–0.10, by non-parametric analysis comparing the control with the 2.7 h exercise group.

exercise group relative to the control group; both 2.0 and 2.3 h groups had $K_{Ca\ (app)}$ values identical to those of the sedentary control group.

3.5. Active protein concentration

The observed exercise-induced elevation in maximal enzyme velocity (Table 1 and Fig. 2) could arise from either an increase in the number of enzymatically active proteins or an increased rate of catalytic activity of unchanging numbers of Ca^{2+}-ATPase proteins. In order to differentiate between these two possibilities, we measured the steady-state concentration of the phosphorylated enzyme intermediate formed following reaction with [γ-^{32}P]ATP. Determination of the phosphoenzyme (E-P) concentration is a sensitive measure of the number of functional calcium pump units. We find a significant trend of increasing number of active calcium pumps with increasing exercise time ($P = 0.013$ and $r = 0.91$, as determined by regression analysis for 2.0, 2.3 and 2.7 h groups), up to a 30% increase at 2.7 h of exercise (Fig. 5). By 48 h after exercise, E-P levels return to those comparable with sedentary control animals. Both qualitatively and quantitatively, the pattern of E-P concentration is in agreement with that for specific activity as demonstrated by the significant correlation between these two measures for individual animals ($P = 0.007$ and $r = 0.93$).

Catalytic turnover rates, defined as the maximum number of moles of substrate converted to a product per second per mole of enzyme active site, were determined for the Ca^{2+}-ATPase from the ratio of specific activity to E-P concentration for each experimental group [28]. These rates are the same, i.e., $10.3 \pm 0.8\ s^{-1}$ for all experimental groups. Thus the exercise-induced increases in calcium-dependent ATPase activity are entirely due to increases in the number of active proteins rather than any contribution by changes in catalytic turnover rates.

Table 1
Kinetic characteristics of Ca^{2+}-ATPase activity

	V_{max}(IU)	$K_{Ca\ (app)}$ (μM) [c]	Hill coefficient
Sedentary control [a]	3.2±0.2	0.380±0.067	1.5±0.1
Exercise groups			
2.0 h [b]	3.5±0.3	0.355±0.014	1.7±0.1
2.3 h [b]	3.8±0.2	0.309±0.022	1.4±0.1
2.7 h [b]	5.0±0.3 *	0.263±0.037 *	1.4±0.1

Kinetic characteristics were determined from plots of Ca^{2+}-ATPase activity at free calcium concentrations from 0.004 to 5 μM. V_{max} and $K_{Ca\ (app)}$ were determined from a sigmoid fit to the data plotted as Ca^{2+}-ATPase activity as a function of calcium concentration for each exercise group. The Hill coefficient was determined from Hill plots of the portion of the curve of calcium-dependent activity as a function of calcium concentration which corresponded to 25 to 80% of maximal activity. Data represent mean ± S.E. calculated from duplicate determinations from individual animals for each group.

[a] $n = 6$.

[b] $n = 2$.

[c] Defined as the calcium concentration at half-maximal activity.

* Significantly different at $P = 0.05$–0.10, by non-parametric analysis comparing control with the 2.7 h exercise group.

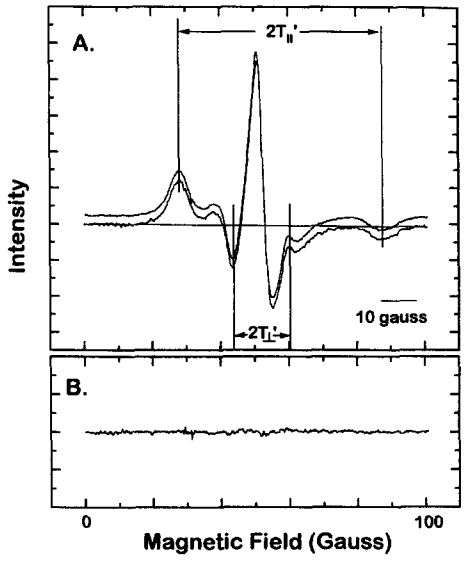


Fig. 6. (A) Conventional EPR spectra representing 5-SASL incorporated into SR membranes isolated from a sedentary rat (below) and an animal exercised for 2.7 h (above) where Ca^{2+}-ATPase activity was substantially increased relative to control activity. (Spectra are offset in order to facilitate visualization of line shapes.) Spectra were obtained at 4°C and contained approximately 35–40 mg SR protein/ml in 0.3 M sucrose and 20 mM Mops (pH 7.0). Spectra were recorded with a 100 gauss scan range. Order parameters (S) were calculated from outer ($2T_{\parallel}'$) and inner ($2T_{\perp}'$) hyperfine splitting constants, as described in Materials and Methods. (B) Residual difference remaining following subtraction of normalized spectra from the sedentary and the exercised animal.

Table 2
Apparent order parameters and correlation times for 5-SASL and 16-SASL

	Order parameter (S)	Correlation time (ns)
Sedentary control [a]	0.761 ± 0.003	2.39 ± 0.06
Exercise groups		
2.0 h [b]	0.757 ± 0	2.33 ± 0.01
2.3 h [b]	0.760 ± 0.004	2.34 ± 0.02
2.7 h [b]	0.766 ± 0.006	2.31 ± 0.03

Hydrocarbon chain motion near the surface and center of the bilayer was calculated from conventional EPR spectra using 5- and 16-SASL probes, respectively. Data represent mean ± S.E. calculated from single determinations from individual animals for each group.
[a] $n = 6$.
[b] $n = 2$.

3.6. Membrane structure analysis

Exercise-induced changes in the physical structure of the SR membrane may be responsible for the observed increases in enzymatic activity. Support for this suggestion arises from in vitro experiments which have correlated structural changes of lipid or protein membrane components with changes in Ca^{2+}-ATPase function [29]. Therefore, we have used spin-label EPR to directly measure the rotational motion of the Ca^{2+}-ATPase and of surrounding lipids. Rotational motion of the Ca^{2+}-ATPase is sensitive to both lipid viscosity and the volume of the rotating protein unit [30]. Thus, parallel measurements of protein rotational motion and lipid viscosity allow unambiguous interpretation of changes in protein motion.

We have measured membrane fluidity at two depths in the bilayer with two stearic acid spin labels with the nitroxide spin moiety positioned at the 5 and 16 position along the fatty acyl chain (5- and 16-SASL). Thus, 5-SASL probes lipid hydrocarbon chain mobility near the bilayer surface, while 16-SASL probes mobility near the bilayer center. Spectra of 5-SASL probes incorporated into SR membranes isolated from sedentary and exercised animals were analyzed from inner and outer hyperfine splittings to determine effective order parameters (S) (Fig. 6A). As illustrated in Fig. 6A and Table 2, neither spectral lineshape nor calculated order parameters differed between groups for average membrane order near the bilayer surface. Likewise, comparison of 16-SASL spectral shapes (Fig. 7A), and the correlation times (Table 2) calculated from line height ratios for average hydrocarbon motion close to the center of the bilayer, demonstrate no difference between sedentary control and exercise groups.

Spectra of lipid hydrocarbon chain mobility in SR (i.e., Fig. 6A, Fig. 7A) contain contributions from two motional lipid populations, one consisting of lipids which are freely mobile in the bilayer (bulk lipids); the other consisting of a less mobile population of lipids intimately associated with the Ca^{2+}-ATPase protein (annular or boundary lipids) [31].

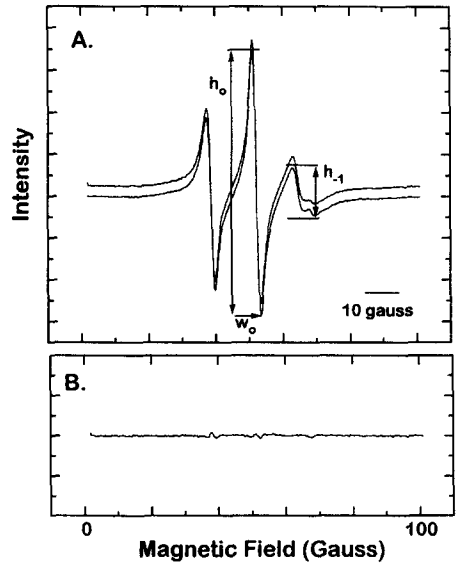


Fig. 7. (A) Conventional EPR spectra representative of 16-SASL incorporated into SR membranes isolated from a sedentary rat (below) and an animal exercised for 2.7 h (above) where Ca^{2+}-ATPase activity was substantially increased relative to control activity. (Spectra are offset in order to facilitate visualization of lineshapes.) Spectra were obtained at 4°C and contained approximately 35–40 mg SR protein/ml in 0.3 M sucrose and 20 mM Mops (pH 7.0). Spectra were recorded with a 100 G scan range. Correlation time (T_r) was calculated from peak heights and widths as described in Materials and Methods. (B) Residual difference remaining following subtraction of normalized spectra from the sedentary and the exercised animal.

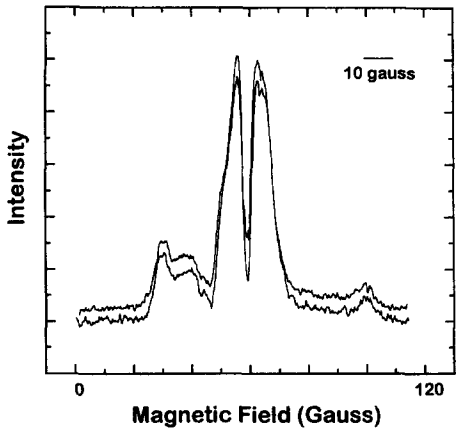


Fig. 8. ST-EPR spectra of maleimide spin-labeled Ca^{2+}-ATPase measured at 4°C in SR membranes isolated from a sedentary rat (below) and an animal exercised for 2.7 h (above) where Ca^{2+}-ATPase activity was substantially increased relative to control activity. (Spectra are offset in order to facilitate visualization of lineshapes.) All spectral samples contained 10 mg SR protein/ml in 0.3 M sucrose and 20 mM Mops (pH 7.0). Spectra were recorded with a 200 G scan range.

Therefore, we digitally subtracted the spectrum of each exercise animal from that of control animals as a means to detect any residual spectral shapes indicative of changes in the distribution of bulk or boundary lipids. Spectral subtraction of all 5- and 16-SASL spectra of each exercise animal from each sedentary control spectra do not result in a lineshape characteristic of either rapid or slow motion, but rather small and randomly distributed residuals (Fig. 6B, Fig. 7B). Thus, we find no evidence for alterations in the distribution or mobility of either bulk or boundary lipids as a result of exercise.

Protein rotational mobility was analyzed using the V_2' integral from ST-EPR spectra, an intensity parameter that eliminates errors due to the line-shape distortion caused by weakly immobilized probes. Since we observed no exercise-induced change in fluidity, any observed changes in protein rotational mobility are likely to be indicative of an altered protein association state. Fig. 8 is an example of the ST-EPR (V_2') spectra from a sedentary control and an animal exercised for 2.7 h, whose Ca^{2+}-ATPase activity was significantly elevated over the control values. Comparison of mean V_2' integrals (Table 3) for all samples demonstrate that there are no differences in protein mobility as a result of exercise.

Table 3
Integrated intensities of the V_2' saturation transfer EPR spectra from MSL-SR

	$\int V_2'$
Sedentary control [a]	3.4±0.3
Exercise groups	
2.0 h [b]	4.4±0.2
2.3 h [b]	4.2±0.1
2.7 h [b]	3.8±0.3

The V_2' integral was calculated for each sample following normalization for spin (label) concentrations, as determined from the double integration of digitized conventional (V_1) EPR spectra from MSL-labeled samples. Data represent mean±S.E. calculated from single determinations from individual animals for each group.
[a] $n=6$.
[b] $n=2$.

4. Discussion

4.1. Summary of results

In this study, we have investigated how the SR Ca^{2+}-ATPase is affected by the disruption in intracellular calcium homeostasis, i.e., increased calcium levels, which occurs during prolonged exercise. We have correlated biochemical measurements of Ca^{2+}-ATPase function with spin-label EPR used as a sensitive measure of SR membrane dynamics. Based on measurements of SR yield, gel densitometry of the Ca^{2+}-ATPase protein (Fig. 2), and marker enzyme activities of other muscle membranes, we find that all SR preparations, whether isolated from exercised or sedentary animals, had identical membrane compositions. Concomitant with exercise-induced increases in sarcolemmal permeability as assayed by plasma CK levels (Fig. 1), a condition which has been associated with increased concentrations of intracellular calcium, there is a positive correlation between time of active recovery after strenuous exercise and increases in Ca^{2+}-ATPase activity in isolated SR membranes (Figs. 2 and 3; Table 1) [8]. These increases in activity are due to corresponding increases in the number of active protein pumps as measured by steady-state formation of the phosphorylated enzyme rather than any alterations in catalytic turnover (Fig. 4). Moreover, based on the results from EPR analysis of membrane fluidity (Figs. 5 and 6; Table 2) and protein rotational motion (Fig. 7; Table 3), we can rule out possible activating mechanisms that involve increased bulk or boundary lipid fluidity or decreased size of the rotating protein unit.

4.2. Relationship to other exercise studies

A number of other studies have reported alterations in SR function following exercise. Direct comparison of our results with other investigations is difficult because of differences in the tissue selected for study, in the exercise protocol employed (mode, duration and intensity), as well as the differences in SR Ca^{2+}-ATPase preparations and biochemical measures of protein function. A few aspects of our experimental design and methodology warrant discussion. Previous studies with specific fiber types have demonstrated exercise-induced alterations specific to either fast or slow twitch muscle, suggesting that tissue selection may be an important consideration [32–35]. However, we have chosen to use all muscles of the rat hindlimb in order

to provide sufficient SR protein for parallel biochemical and biophysical measurements for individual animals. The fiber type composition of these muscles is mixed (76% fast-twitch glycolytic, 19% fast-twitch oxidative glycolytic, and 5% slow-twitch); quantitation of Ca^{2+}-ATPase isoforms indicates 85% fast twitch and 15% slow twitch Ca^{2+}-ATPase, thus precluding identification of any changes specific to fiber type or isoform in our study [36].

Another significant variable influencing research findings of SR function includes the intensity of the exercise employed. In comparison with our finding of increased SR Ca^{2+}-ATPase function following strenuous exercise and through a period of 'active recovery', a number of other investigators have reported diminished SR protein function correlating with muscle fatigue immediately following exhaustive exercise or low-frequency stimulation of muscle fibers [32,34,37–40]. We have used a strenuous exercise protocol of defined length which all rats were able to complete, indicating that exhaustion was not achieved. In addition, the focus of our investigation has been to determine how the SR Ca^{2+}-ATPase responds to an extended period of increased calcium transients associated with muscle contraction, i.e., exercise. This was accomplished by sampling animals both immediately after and throughout an 'active recovery' period following exercise, during which time animals walked until being killed. The observed increased protein function during this active recovery period after exercise may be an important mechanism for rapidly restoring calcium homeostasis in the cell. These results are in agreement with other studies which examined SR Ca^{2+}-ATPase function following 30 min of recovery after exercise, where the functional parameters investigated had either returned to normal or had increased above control values, demonstrating the ready reversibility or overcompensation of protein function [32,37,38].

4.3. Interpretation of results

Based on our observations that increasing concentrations of phosphoenzyme correlate with exercise time without change in the catalytic turnover number, we suggest that exercise results in an increased active Ca^{2+}-ATPase proteins in the SR membrane. There are several possible mechanisms which could cause an increase in active Ca^{2+}-ATPase proteins. First, previous physical studies of SR Ca^{2+}-ATPase have demonstrated that changes in SR membrane dynamics, through an alteration in protein rotational mobility or bilayer fluidity, can influence enzymatic activity. For example, selective crosslinking of Ca^{2+}-ATPase proteins demonstrates that protein rotational mobility is directly proportional to enzyme activity [41]. Thus, increases in active Ca^{2+}-ATPase proteins could be explained in terms of disaggregation of large, inactive protein complexes that would initiate activation of the Ca^{2+}-ATPase. Similarly, an increase in Ca^{2+}-ATPase activity and protein rotational mobility can result from specific increases in the fluidity of SR lipids, as previously reported upon induction by addition of diethyl ether or increased temperature [42]. Another relevant means that alters membrane order is from free-radical-induced addition of a hydroperoxide group to a polyunsaturated lipid acyl chain [43]. The production of free radicals has been reported in contracting muscle, and thus this mechanism might provide a potential explanation for our observations [44–47]. Nevertheless, in this study we find no evidence for exercise-induced changes in either protein associations or membrane fluidity and thus we can rule out such mechanisms involving changes in membrane dynamics as an explanation for activation of the Ca^{2+}-ATPase. However, we cannot rule out altered interactions of specific lipids with the Ca^{2+}-ATPase that may be important for function but which have no effect on the physical properties of annular lipids. Similarly, exercise-induced refolding of misfolded but non-aggregated Ca^{2+}-ATPase protein cannot be ruled out as a mechanism for the observed increased active sites.

Other mechanisms, whereby an inactive population of Ca^{2+}-ATPase proteins may become activated with exercise, include changes in either the active site or an allosteric site that would relieve a pre-existing inhibition. One such mechanism may include activation of the slow twitch isoform (SERCA2) of the Ca^{2+}-ATPase following its phosphorylation by calcium/calmodulin-dependent protein kinase (Ca-CaM kinase), which has been reported to result in a 2-fold increase in enzymatic activity of this isoform in slow twitch skeletal muscle fibers without altering calcium afffinity [48]. Considering that approximately 15% of the total Ca^{2+}-ATPase protein consists of the slow twitch isoform, this mechanism may provide a feasible explanation for the observed 30% increase in enzymatic activity reported. Increased calcium transients associated with exercise may act as a physiological trigger to activate the calcium/calmodulin protein kinase pathway. However, it is not known whether this activation mechanism involves increased catalytic turnover or increased numbers of active sites.

A similar mechanism is suggested by the observed Ca-CaM kinase-mediated phosphorylation of phospholamban that activates the SERCA2 isoform in the heart [49]. However, it has been reported that phosphorylation of phospholamban in SR from canine slow twitch fibers is not accompanied by activation of the Ca^{2+}-ATPase and therefore it is unlikely that this mechanism is responsible for the 30% increase in enzymatic activity that we observe [50].

Finally, the augmentation of protein turnover, i.e., changes in the relative rates of Ca^{2+}-ATPase degradation and synthesis, may explain our results. Either removal of damaged, dysfunctional molecules or the addition of newly synthesized molecules (or both) into the pool of proteins would result in a greater percentage of fully functional proteins. Protein turnover is an integral part of the adapt-

ability and remodeling that muscle exhibits with changes in contractile activity [51,52]. With exercise, two processes of remodeling and repair may be involved. The first involves the complete necrosis and replacement of damaged muscle fibers. Histological analysis of rat muscle following a bout of treadmill running has found that approximately 2–5% of the muscle is involved, with the peak of necrosis observed 48 to 72 h post-exercise [5,6]. It is unlikely that this process significantly contributes to our observations due to both the different timeframe of cell necrosis and the small percentage of the fibers involved.

A second type of remodeling and repair which plays a primary role in the maintenance of the terminally differentiated myocyte is the replacement of individual proteins within the cell. The alterations in Ca^{2+}-ATPase function that we observe are more likely due to this type of constitutive process occurring within the majority of muscle cells which do not experience complete replacement, but rather entail the exercise-stimulated degradation and replacement of a select population of proteins which are damaged.

Exercise has been shown to stimulate the turnover of individual proteins by increasing degradation of damaged cell components via an exercise-induced increase in activity of endogenous proteolytic enzymes [53–56] and through an increase in protein synthesis rates. Regulation of protein synthesis can occur at several different levels, from the gene to the final protein product, and investigations of several metabolic proteins provide evidence that a single bout of running or swimming can increase protein expression up to 10-fold [57–60]. Rapid increases in enzymatic activity of the proteins hexokinase II and lipoprotein lipase have been observed after exercise. For example, within less than 30 min of running, a 2-fold increase in mRNA levels of hexokinase II has been observed, along with a 50% increase in its specific activity during the post-exercise recovery period [60]. In the case of lipoprotein lipase, a nearly 200% increase in enzyme activity has been reported immediately after exercise [59]. Thus, despite the relatively slow turnover of the Ca^{2+}-ATPase, there is precedence for rapid exercise-induced changes in turnover of proteins and their specific activities occurring on the scale of the changes that we observe for the Ca^{2+}-ATPase [61].

Whereas gene transcription or mRNA levels of the Ca^{2+}-ATPase have not been specifically examined after a single exercise bout, several studies have demonstrated that the concentration of SR Ca^{2+}-ATPase protein in skeletal and cardiac muscle is sensitive to changes in contractile activity [62–65]. In addition, in tissue culture studies, exposure of muscle cells to high intracellular calcium resulted in an increase in steady-state levels of Ca^{2+}-ATPase proteins [66]. Thus, an exercise-induced rise in intracellular calcium may be responsible for the observed transient changes in both sarcolemmal permeability and some of the changes in muscle structure (i.e., swollen mitochondria and sarcoplasmic reticulum) and in addition, may activate a signal pathway which stimulates changes in protein synthesis and degradation, especially in those proteins directly involved in calcium regulation [3,4,7,10]. Thus, this type of mechanism may partially account for the remarkable plasticity exhibited by skeletal muscle to changes in contractile activity.

4.4. Conclusions

In this study, we have used exercise as a way to perturb intracellular calcium homeostasis in the skeletal muscle cell. Our results indicate that the SR rapidly responds to the challenge of increased intracellular calcium by increasing the proportion of functional Ca^{2+}-ATPase proteins in the membrane. Additional studies to identify the mechanism of this process will further our understanding of cellular calcium regulation and may offer therapeutic approaches to pathologies involving a loss of calcium homeostasis.

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