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Correlation between Ultrastructural and Functional Changes in Sarcoplasmic Reticulum during Chronic Stimulation of Fast Muscle

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Summary. Chronic indirect stimulation of fast twitch rabbit muscle (tibialis anterior and extensor digitorum longus) with a frequency of 10 Hz induced a progressive transformation of the sarcoplasmic reticulum (SR). Ultrastructural changes as studied by electron microscopy of freeze-fractured vesicles consisted in a decrease of intramembranous particles of the concave (*A*) face and an increase of particles in the convex (*B*) face. The asymmetry of the membrane proved to be lowered. Changes in the particle density of the *A* face were mainly confined to the 7–9 nm particles. Electrophoretic analyses revealed a decrease in the 115,000-M_r Ca^{2+} transport ATPase. The reduced density of the 7–9 nm particles correlated well with decreased activities in Ca^{2+}-dependent ATPase as well as with decreases in initial and maximum Ca^{2+} uptake.

Significant differences have been shown to exist between the sarcoplasmic reticulum of fast and slow twitch mammalian skeletal muscle. Morphometric studies revealed a two- to threefold higher volume fraction of sarcoplasmic reticulum in fast than in slow twitch fibers (Tomanek, 1976; van Winkle & Schwartz, 1978). In accordance with this, biochemical studies demonstrated that yield of vesicular protein, rate and capacity of Ca^{2+} transport, Ca^{2+}-dependent ATPase activity, phosphoprotein formation, dephosphorylating activity and concentration of the 115,000-M_r Ca^{2+} pumping protein are considerably lower in sarcoplasmic reticulum from slow twitch muscle (Pette & Heilmann, 1979). Moreover, sarcoplasmic reticulum membranes of fast and slow twitch muscles have been shown to differ in their ultrastructure. According to Bray and Rayns (1976), the number of 7–9 nm particles per unit area which are observed on concave faces of freeze-fractured sarcoplasmic reticulum is significantly lower in slow than in fast twitch muscles. As is suggested by the work of Deamer and Baskin (1969) and others (MacLennan, Seeman, Iles & Yip, 1971; Baskin, 1974; Packer, Mehard, Meissner, Zahler & Fleischer, 1974; Tillack, Boland & Martonosi, 1974; Scales & Inesi, 1976), these 7–9 nm particles represent the structural equivalent of the Ca^{2+} transport ATPase.

The present study was undertaken in order to examine whether biochemical changes as observed after long-term electrical stimulation are paralleled also by ultrastructural alterations of the sarcoplasmic reticulum. The biochemical changes consisted in decreases in Ca^{2+}-uptake, Ca^{2+}-dependent ATPase and the amount of the 115,000-M_r protein (Pette & Heilmann, 1977; Heilmann & Pette, 1979, 1980). The induced transformation of the sarcoplasmic reticulum in fast twitch muscle should result in a reduced density of the 7–9 nm particles if these represent the Ca^{2+} pumping ATPase.

Materials and Methods

Male adult rabbits of the strain "Weisse Wiener" were used. The tibialis anterior and extensor digitorum longus muscles of the left hind leg were subjected to indirect continuous stimulation via electrodes implanted close to the lateral popliteal nerve (Pette, Smith, Staudte & Vrbova, 1973). Stimulation was 24 hr per day (0.15 msec impulses, 2–8 V) at a frequency of 10/sec and lasted 2–16 days. At various times animals were killed and tibialis anterior and extensor digitorum longus muscles from both hind legs were dissected. Muscles were washed in ice-cold sucrose medium (0.25 M sucrose, 5 mM Hepes, pH 7.5), cut into small pieces and minced in a tissue grinder adapted to small samples (Mikrofleischwolf, B. Braun, Melsungen, Germany). The minced tissue was suspended in a twofold volume (w/v) of sucrose medium and homogenized 2 × 30 sec in an Omnimix microattachment (I. Sorvall, Inc., E.I. Du Pont

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de Nemours Co., Newton, Conn.), cooled with ice. The homogenates were diluted to 6% (wt/vol) with sucrose medium and sarcoplasmic reticulum vesicles were isolated by differential centrifugation as described previously (Heilmann, Brdiczka, Nickel & Pette, 1977).

ATPase activities were determined in a coupled optical assay at 25 °C in 1 ml incubation containing 25 mM Hepes (pH 7.0), 0.1 M KCl, 5 mM $MgCl_2$, 6 mM ATP, 50 µM $CaCl_2$, 1 mM phosphoenolpyruvate, 0.6 mM NADH, 25 µg pyruvate kinase, 25 µg lactate dehydrogenase, 30 µg vesicular protein. For determination of Mg^{2+}-dependent ATPase, 1 mM EGTA was added instead of $CaCl_2$. Ca^{2+}-dependent ATPase activity was calculated as difference between activity measured in the presence of Ca^{2+} and Mg^{2+} and activity in the presence of Mg^{2+} alone. Ca^{2+} uptake was determined in a millipore apparatus (Millipore Corp., Bedford, Mass.) according to the technique of Martonosi and Feretos (1964). The assay mixture contained 37 mM Hepes (pH 7.0), 0.1 M KCl, 6 mM $MgCl_2$, 6 mM ATP, 50 µM $CaCl_2$ containing ^{45}Ca, 5 mM potassium oxalate, 5 mM NaN_3, 5–20 µg/ml vesicular protein. Measurements were performed at 25 °C. Time course of Ca^{2+} uptake was routinely followed up to at least 15 min.

For sodium dodecylsulfate/polyacrylamide gel electrophoresis the procedure of Laemmli (1970) was used. Protein determination was performed according to the procedure of Lowry, Rosebrough, Farr and Randall (1951).

Freeze-Fracture Electronmicroscopy

Pellets of sarcoplasmic reticulum vesicles were supplemented with 30–50% glycerol (Deamer & Baskin, 1969). Small aliquots of the resulting dense supension were frozen in liquid freon. Platinum-carbon replicas were prepared in a Balzers freeze-fracturing apparatus BA 360 M according to Moor and Mühlethaler (1963). The replicas were cleaned with sodium hypochlorite solution and mounted on formvar coated copper grids. All specimens were examined with a Zeiss EM 10 A electron microscope with liquid nitrogen cooling, 30 µm objective aperture and 80 KV accelerating voltage.

Evaluation of Particle Density

In each preparation the particles were counted at least on 50 convex and 50 concave faces which were randomly selected on micrographs at a magnification of 110,000 ×. Particle diameters were determined with a magnifier 10 × equipped with 0.2 mm division scale. Particles with diameter greater than 9 nm, 7 to 9 nm, and smaller than 7 nm were distinguished and called *a, b, c,* respectively. The test circle diameter was 45 nm corresponding to an area of 1590 nm^2. The values for particle density were not corrected for the error due to the curvature of the vesicle membranes (Weibel, Losa & Bolender, 1976).

Results

Figure 1 shows freeze-fracturing faces of vesicles from sarcoplasmic reticulum (SR) preparations of control and stimulated tibialis anterior muscle. The electron micrographs indicate that the number of intramembranous particles is reduced on the concave faces of the SR from stimulated muscle. A detailed analysis of the variations is given for three muscles in Table 1. In the sarcoplasmic reticulum of stimulated muscles, there is a significant decrease in the total number of particles in the concave (*A*) faces and an increase of particles in convex (*B*) faces. As a result, the ratio of intramembranous particles between *A* and *B* faces is reduced by the stimulation. An analysis of the alterations with regard to the size distribution indicates that the changes are confined mainly to the 7–9 nm particle (Table 1). A decrease of this particle population to about 75% of that in the controls is found in the *A* faces of SR vesicles from stimulated muscles. An increase of these particles is seen in the *B* faces.

Table 2 summarizes results obtained in a second experimental series from nine animals. The analyses of ultrastructural changes were limited to the 7–9 nm particles in the *A* faces. A significant decrease of these particles in the *A* faces is evident for all muscles stimulated more than 2 days. The maximum change con-

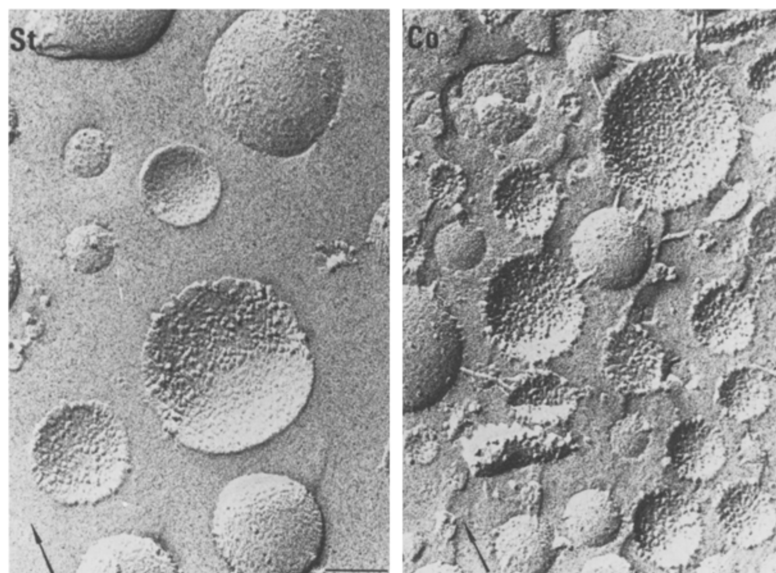

Fig. 1. Electron micrographs of freeze-fractured sarcoplasmic reticulum vesicles from a 6-day continuously stimulated (*St*) and contralateral unstimulated tibialis anterior muscle (*Co*). Magnification 110,000 × shown here at reduction to 78%. Bar length 0.1 µm. The arrows indicate the direction of platinum shadowing

Table 1. Particle density on freeze-fracture faces of sarcoplasmic reticulum from unstimulated control (co) and stimulated (st) tibialis anterior muscles[a]

Length of stimulation	Muscle	Convex face ($N=100$)				Concave face ($N=100$)			
		Particles per μm^2 surface area (M ± SE)							
d		*a*	*b*	*c*	*a+b+c*	*a*	*b*	*c*	*a+b+c*
5	co	13 ± 9	220 ± 44	465 ± 69	698 ± 81	157 ± 38	4346 ± 192	1415 ± 98	5919 ± 180
	st	63 ± 24	403 ± 72	667 ± 79	1132 ± 112	226 ± 47	3818 ± 159	1308 ± 115	5353 ± 179
12	co	13 ± 9	277 ± 60	1157 ± 117	1447 ± 132	189 ± 40	5152 ± 177	1604 ± 102	6944 ± 161
	st	50 ± 21	755 ± 106	1447 ± 130	2252 ± 143	233 ± 42	4007 ± 189	1554 ± 111	5793 ± 169
11	co	44 ± 16	359 ± 55	711 ± 79	1113 ± 99	214 ± 36	4648 ± 121	1617 ± 89	6479 ± 145
	st	67 ± 20	475 ± 79	981 ± 89	1524 ± 129	253 ± 50	3404 ± 248	1438 ± 97	5094 ± 281

[a] Three types of particles were distinguished according to their diameter: *a*) greater than 9 nm, *b*) 7–9 nm, *c*) smaller than 7 nm in diameter, respectively. M and SE are mean and standard error of the particle density (particles/μm^2). *N* gives the number of the evaluated fracture faces.

Table 2. Density (M ± SE) of intramembranous 7–9 nm particles on concave (*A*) fracture faces of stimulated and contralateral unstimulated (control) tibialis anterior muscles

Length of stimulation	Number of vesicles counted	Density of 7–9 nm particles on concave fracture faces		
		control	stimulated	stimulated/control ratio
d	*n*	particles/μm^2 ± SE		
2	50	4994 ± 144	5133 ± 210	1.028
3	50	4680 ± 159	4340 ± 146	0.927
5	100	4346 ± 192	3818 ± 159	0.878
6	50	2981 ± 158	1686 ± 229	0.565
8	50	5120 ± 185	3497 ± 243	0.683
11	100	4648 ± 121	3404 ± 248	0.732
12	100	5152 ± 177	4007 ± 189	0.778
13	50	3573 ± 169	2101 ± 229	0.588
16	50	5422 ± 183	4139 ± 268	0.763

Table 3. Maximum Ca^{2+} uptake by sarcoplasmic reticulum of stimulated and contralateral unstimulated (control) tibialis anterior muscles[a]

Length of stimulation *d*	Maximum Ca^{2+} uptake		
	control	stimulated	
	μmol × mg protein^{-1}		% of control
3	4.6	3.75	81.5
6	5.3	2.26	42.6
8	4.8	3.16	65.8
11	4.2	3.1	73.8
12	4.75	3.0	63.1
13	5.66	2.9	51.2
16	7.2	3.3	45.8

[a] Time course of Ca^{2+} uptake was followed up to 15 min. Maximum Ca^{2+} uptake was usually reached after 5-min incubations.

Table 4. Initial Ca^{2+} uptake by sarcoplasmic reticulum of stimulated and contralateral unstimulated (control) tibialis anterior muscles in the presence of potassium oxalate

Length of stimulation *d*	Initial Ca^{2+} uptake		
	control	stimulated	
	μmol × mg protein^{-1}		% of control
3	1.5	1.4	93.3
6	2.16	0.91	42.1
8	2.0	0.66	52.0
11	2.4	1.6	66.6
12	2.3	1.0	66.1
13	2.5	1.1	44.0
16	3.4	2.08	61.1

sisted in a decrease to about 57% of the control in a 6-day stimulated animal. A similar decrease was found in a 13-day stimulated animal. Although changes appear to be more pronounced after prolonged stimulation periods, no clear time dependence can be derived from the data in Table 2. A possible explanation of these variations in time dependence is that animals might not have been stimulated at strictly identical conditions. This may occur because of differences in the position of electrodes. Position of the electrodes and their distance to the nerve may be altered by movements of the animal. It is evident that such variations in experimental conditions create greater scattering of the induced changes in short than in long term stimulations.

Results of measurements on Ca^{2+} uptake by isolated SR vesicles both from control and stimulated tibialis anterior and extensor digitorum longus are presented in Tables 3 and 4. A decrease in Ca^{2+} uptake by SR of stimulated muscles is observed for both maximum (Table 3) and initial (Table 4) uptake.

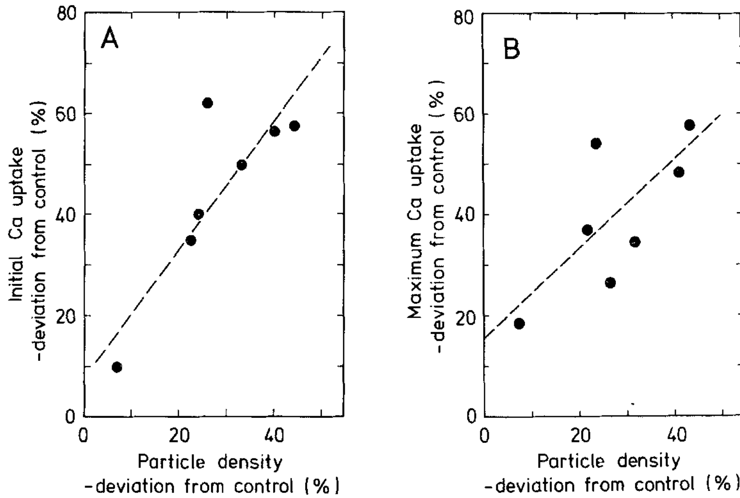

Fig. 2. Correlation between particle density and initial (*A*) and maximum Ca^{2+} uptake (*B*) of sarcoplasmic reticulum from stimulated tibialis anterior muscles. Data have been plotted as percentage deviations from the respective values of sarcoplasmic reticulum from contralateral unstimulated muscles which were taken as controls. Data on the abscissa refer exclusively to particles with 7–9 nm diameter. Straight line was obtained by least-squares linear regression

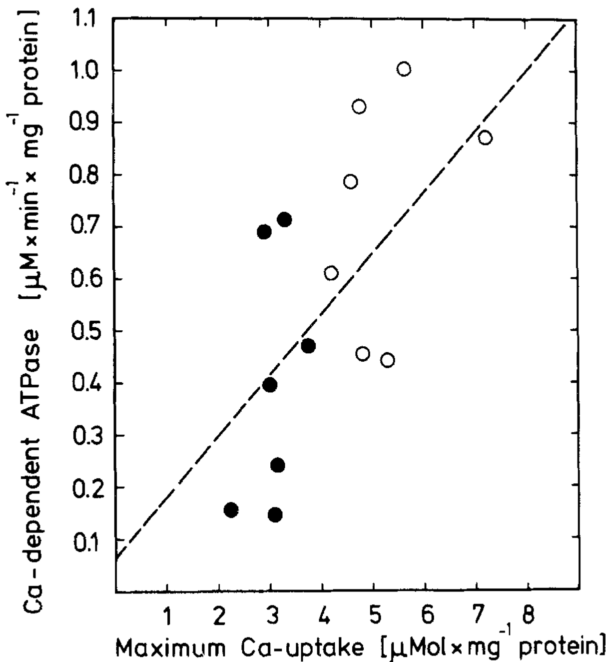

Fig. 3. Correlation between maximum Ca^{2+} uptake and Ca^{2+}-dependent ATPase activities of sarcoplasmic reticulum from control (○) and stimulated (●) tibialis anterior muscles

As observed for the ultrastructural changes, no strict relation exists between duration of stimulation and extent of reduction in Ca^{2+} uptake. It is evident, however, that a correlation exists between the extent of ultrastructural and biochemical alterations. This correlation is illustrated by Fig. 2 in which initial (Fig. 2*A*) and maximum (Fig. 2*B*) Ca^{2+} uptake have been plotted against densities of 7–9 nm particles in the concave faces. In order to illustrate more clearly the changes induced by stimulation, both Ca^{2+} uptake and particle density have been expressed as per-

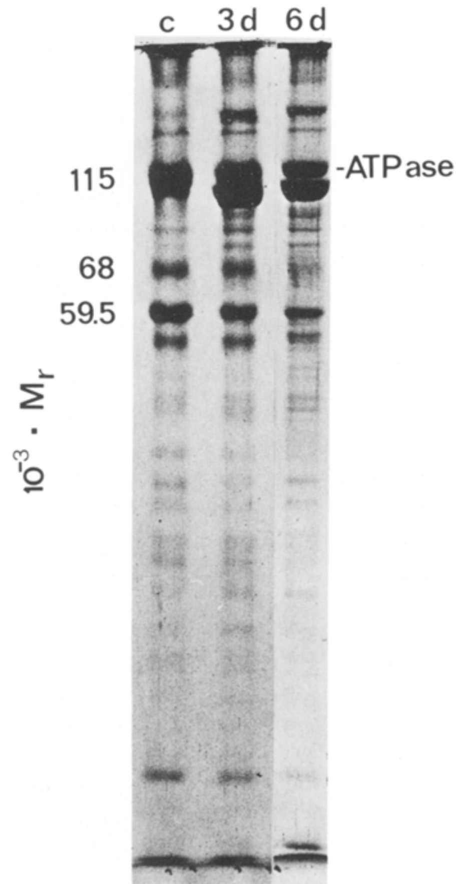

Fig. 4. Polyacrylamide gel electrophoreses in the presence of sodium dodecylsulfate of sarcoplasmic reticulum from unstimulated (*c*), 3- and 6-day stimulated tibialis anterior muscles. Electrophoresis was performed according to Laemmli (1970) in 12.5% separating gels loaded with 10 μg vesicular protein

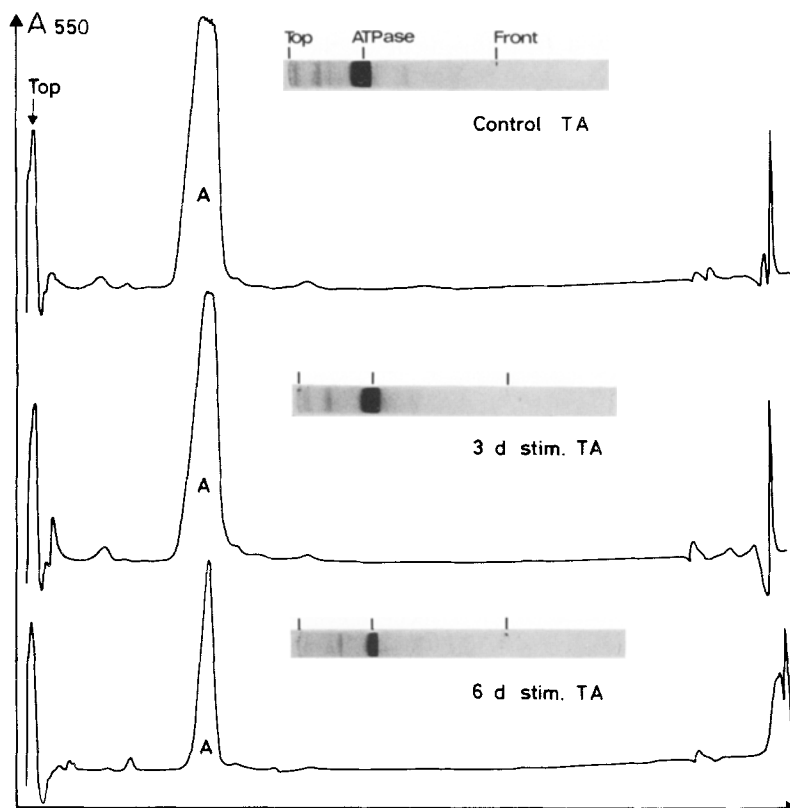

Fig. 5. Polyacrylamide gel electrophoreses in the presence of sodium dodecylsulfate of pre-extracted sarcoplasmic reticulum from unstimulated (control), 3- and 6-day stimulated tibialis anterior muscles (*TA*). Pre-extraction of sarcoplasmic reticulum was performed similar to Meissner, Connor and Fleischer (1973). Equal amounts of vesicular protein (1 mg/ml) were incubated for 10 min at 0 °C in a medium containing 0.3 mg deoxycholate/ml, 0.3 M sucrose, 0.6 M KCl, 2 mM $MgCl_2$, 2 mM mercaptoethanol, 35 mM Hepes (pH 7.5). After centrifugation at $100{,}000 \times g$ for 40 min the pellets were washed and lysed for electrophoresis according to Laemmli (1970). Gels were loaded with exactly identical volumes of the lysed residues. After staining with Coomassie blue, densitometry was performed at 550 nm. The 115,000-M_r Ca^{2+} transport ATPase is marked as *A*

centage deviations from the respective controls, as determined in SR from the contralateral unstimulated muscles. Since the activity of Ca^{2+}-dependent ATPase was also decreased in the stimulated muscles, a correlation (Fig. 3) was also found between maximum Ca^{2+} uptake and ATPase activity in control and stimulated muscles. The decrease in 7–9 nm particle density is thus paralleled by a reduction both in Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity. This latter activity has been ascribed to a peptide of 105,000–115,000-M_r (MacLennan, 1970). Figure 4 shows electrophoretically separated peptide patterns of SR from unstimulated and 3- and 6-day stimulated tibialis anterior muscles. The band of the 115,000-M_r Ca^{2+} pumping protein is prominent in each of these electrophoreses. Due to the presence of other peptides, especially those of similar molecular weight, it is difficult, to decide whether the amount of the 115,000-M_r peptide is decreased. In order to study changes in the concentration of this peptide, a pre-extraction of extrinsic membrane proteins by deoxycholate (0.3 mg/mg vesicle protein) was performed before electrophoresis. Figure 5 shows the electrophoreses of the preextracted samples. Since equal amounts of SR vesicle of protein were subjected to the extraction, the electrophoretic patterns of the residuals may be compared at least semiquantitatively. As may be seen both from the reproductions of the electrophoreses and the densitometric tracings, there is a pronounced decrease in the amount of the 115,000-M_r Ca^{2+} pumping protein after 3 and 6 days of stimulation.

Discussion

A faces of freeze-fractured SR vesicles from unstimulated fast-twitch muscle displayed an average density of 6447 ± 513 particles per μm^2 (combined *a*, *b*, *c* particles, Table 1*A*). This value is in good agreement with data from Packer et al. (1974) and Scales and Inesi (1976) who found 5625 and 5730 particles per μm^2, respectively, on *A* faces of SR vesicles from predominantly fast twitch rabbit skeletal muscle. In accordance with Packer et al. (1974) we found a large scattering in particle size with 7–9 nm particles in greatest number. The particle density in the *B* faces was much less as compared to the *A* faces with a ratio *A*:*B* of 6:1 (*a*, *b*, *c* particles, Table 1) and 16:1 if only the 7–9 nm particles were compared. A similar asymmetry of 8.6:1 was reported by Packer et al. (1974) for SR of rabbit fast twitch muscle. Rayns, Devine and Sutherland (1975) determined a ratio of 6.0:1 for SR of guinea pig fast twitch muscle. It follows from the data in Table 1 that the asymmetric

distribution of intramembranous particles between *A* and *B* faces may be altered under the influence of stimulation. Significantly higher particle densities were found on *B* faces of SR vesicles from stimulated muscles as compared to those from unstimulated muscles. The ratio *A*:*B* of 6:1 (*a, b, c* particles, Table 1) in unstimulated muscles was changed to 3.3:1 in stimulated muscles. A similar shift of the ratio *A*:*B* was observed if only type *b* particles (7–9 nm) were considered. These findings are in agreement with the data of Bray and Rayns (1976) who found a 2.4-fold higher density of intramembranous particles on *B* faces of SR in avian slow twitch as compared to fast twitch muscle.

Phospholipids are a prerequisite for the insertion and orientation of the Ca^{2+} transport ATPase within the membrane (MacLennan et al., 1971; Packer et al., 1974; Green, 1975). Moreover, it has been shown by Packer et al. (1974) that SR which was dissolved and reassembled to form membrane vesicles, completely abolished the asymmetric distribution of intramembranous particles. Following this concept, the reduced asymmetry of the vesicular membrane from stimulated muscle may result from an altered protein/lipid ratio and a rearrangement of the phospholipid pattern. SR of slow twitch muscle has been shown by freeze-fracturing *in situ* (Bray & Rayns, 1976), to contain considerably less particles per μm^2 *A* face than SR of fast twitch muscle. A ratio (fast/slow) of 1.68 has been reported (Bray & Rayns, 1976). A similar ratio of 1.7 (control/stimulated) was found for the density of 7–9 nm particles on the *A* faces of SR in the 6-day stimulated animal muscle.

The decrease in density of 7–9 nm particles on *A* faces is accompanied by a decrease in the concentration of the Ca^{2+} transport ATPase (Fig. 5), and a reduction of the activities of initial and maximum Ca^{2+} uptake (Tables 3 and 4) and Ca^{2+}-dependent ATPase (Fig. 3). The present analyses reveal a correlation between these biochemical and ultrastructural changes, and therefore support the concept that the intramembranous particles represent the structural equivalent of the Ca^{2+} transport ATPase molecule or an oligomer of it within the membrane. Studies during ontogenesis of muscle in various mammalian species demonstrated a similar correlation between functional and ultrastructural properties of SR (Baskin, 1974; Tillack et al., 1974; Sarzala, Pilarska, Zubrzycka & Michalak, 1975). An increase in the density of intramembranous particles of concave fracturing faces was accompanied by increases in ATPase activity, Ca^{2+} transport, phosphoprotein formation and in phospholipid content (Baskin, 1974; Tillack et al., 1974). As has been communicated recently (Pette & Heilmann, 1977; Heilmann & Pette, 1979; 1980), the transformation of the SR is illustrated also by a rearrangement of the peptide pattern with appearance of an intrinsic 30,000-M_r membrane peptide and an altered pattern of phosphorylatable peptides.

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