DENSITY AND DISPOSITION OF Ca^{2+}-ATPase IN SARCOPLASMIC RETICULUM MEMBRANE AS DETERMINED BY SHADOWING TECHNIQUES

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ABSTRACT We have studied the disposition of calcium ATPase in the native sarcoplasmic reticulum (SR) membrane of vertebrate muscles by rotary shadowing of freeze-dried isolated vesicles and of freeze-fractured in situ membranes. The predominant disposition of the ATPase molecules is disorderly, but small oligomers (dimers, tetramers, and occasionally larger aggregates) are seen. In vesicles from white hind legs of rabbits, the density of ATPase over nonjunctional SR is 31–34,000/μm^2. ATPase density is always quite high, but small protein-free lipid patches may be interspersed with it.

INTRODUCTION

In the preceding paper (Ferguson et al., 1985) we have shown that high resolution rotary shadowing allows visualization of single units, presumably heads of individual Ca ATPase, on the cytoplasmic surface of isolated SR vesicles. In this paper we use surface shadowing and freeze-fracture techniques to consider the question of density and disposition of the ATPase in in situ and in isolated, but functional SR, under conditions that do not result in the formation of orderly arrays.

The state of aggregation of the Ca ATPase in the SR membrane has been the subject of numerous investigations. There is no evidence that the Ca ATPase in vertebrate muscle SR forms extensive, naturally occurring, orderly arrays, despite its high density within the membrane. Equatorial x-ray diffraction patterns of stacked SR lamellae do not contain any regular features, and no portions of the x-ray diffraction patterns from living muscle have been assigned to an SR origin, even though the SR membrane has a predominantly longitudinal orientation. The concept of a membrane within which a fluid disorder prevails is currently accepted. Whether the ATPase can and does form small oligomers and whether these are of uniform size are still open questions (see Moller et al., 1982; Ikemoto, 1982*a* for reviews).

One of the arguments for aggregation of the ATPase is the discrepancy between the density of intramembranous particles, seen on the cytoplasmic leaflet of freeze-fractured membranes, and the density of surface projections (heads) visualized either by negative staining or in deep etched shadowed specimens. While the number of surface projections is in fairly good agreement with estimated content of pump molecules in the membrane, the number of intramembranous particles is considerably less. From this it was suggested that the particles must represent ATPase oligomers (Jilka et al., 1975; Scales and Inesi, 1976; Wang et al., 1979). The exact relationship between intramembranous particles and ATPase monomers, however, has been quite difficult to establish. Early calculations indicated a 3–4 ratio between the two (Jilka et al., 1975; Scales and Inesi, 1976; Wang et al., 1979), while more recently a dimeric interpretion of intramembraneous particles was given (Napolitano et al., 1983). Progress in establishing this numerical relationship has been hampered by lack of precise data on the surface disposition of the ATPase in the native membrane. The disorderly arrangement of the protein molecules means that techniques that depend on averaging, such as x-ray diffraction, or on the ability to filter out one of the two superimposed images, as with negatively stained SR vesicles, can provide little useful information on this point.

A related question concerns the surface density of ATPase molecules in the native SR membrane. The currently accepted value of <20,000/μm^2 comes from calculations based on the extent of ATPase phosphorylation (Wang et al., 1979), or from direct counts of the projections on the cytoplasmic surfaces of SR vesicles exposed by deep etching (Scales and Inesi, 1976). The maximum packing density expected for a molecule with the dimensions given by Le Maire et al. (1976), and the average spacing obtained from x-ray diffraction (Worthington and Liu, 1973) also suggest a density of slightly <20,000/μm^2. However, considerations based on the unit cell dimensions from vesicles in which the ATPase has been polymerized would indicate that the density can be as high as 30,000/μm^2 (Ferguson et al., 1985).

In this paper we compare shadowed images of the external surface of isolated SR vesicles and of the freeze-fractured intact SR membranes. Despite the apparent

disorder of the ATPase distribution, we find evidence that demonstrates that the larger intramembranous particles represent tetramers, while others may correspond to a variable number of units. These data, and images obtained using the rotary shadowing technique, indicate that counts of intramembranous particles on fractured membranes are unreliable in estimating ATPase density. Our counts of ATPase density (31–34,000/μm^2) are considerably greater than previous estimates in the literature.

MATERIALS AND METHODS

Freeze-fracture

Muscles used for freeze-fracture were: Obliquus inferioris and superioris from the glass fish (*Chanda randa*); sartorius from the frog (*Rana pipiens*); and psoas from the New Zealand white rabbit. The muscles were either pinned in a dissection dish or tied to a stick, immersed in fixative (3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7–7.4), and maintained at room temperature for 2–4 h. The muscles were stored in fixative at 4°C until fractured (up to 2 wk). Following infiltration in 30% glycerol, the muscles were frozen in liquid freon, fractured at −110°C, and shadowed, without etching, at an angle of either 45° (unidirectional) or 25° (rotary).

SR Fractionation

Crude microsomal fractions from rabbit and rat muscle were isolated as described by Herbette et al. (1977); see Ferguson et al. (1985) for details. Some enriched light SR fractions were provided by Dr. D. Pascolini, Department of Chemistry, University of Pennsylvania.

Frog crude microsomal fractions were isolated from saponin skinned muscles as described in Ferguson et al. (1985).

Freeze-drying

Vesicles were freeze-dried and shadowed as described in the preceding paper, except that some rabbit vesicles were washed and frozen in 30% methanol, rather than in 100 mM ammonium acetate.

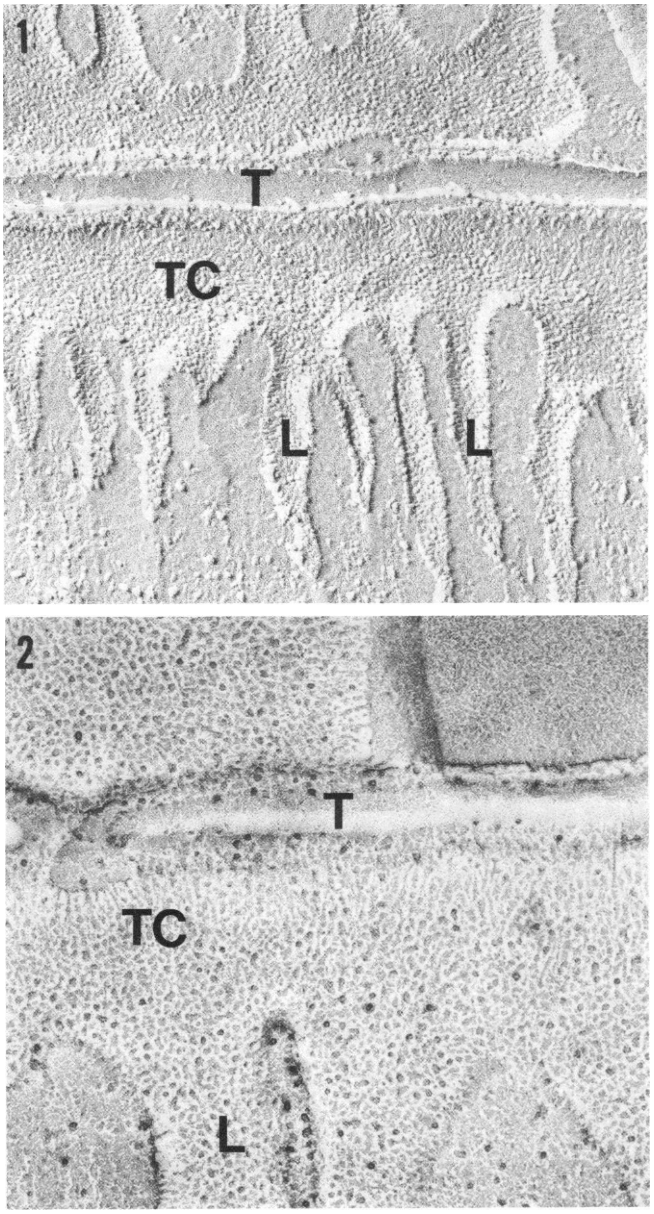
RESULTS

Freeze-fractured Intact SR

Skeletal muscles from three vertebrates, (rabbit, frog, and fish) were fractured. Fish muscle is particularly appropriate for in situ studies because the myofibrils have a flattened shape and large expanses of the SR can be exposed in the same fracture plane. Rabbit and frog were used for comparison to the isolated SR. Since no major differences were found, images from fish muscles are used for most of the illustrations.

Deamer and Baskin (1969) have shown that nonjunctional or free SR membranes facing the myofibrils have asymmetric fracturing properties: virtually all of the particles remain with the cytoplasmic leaflet leaving a smooth luminal leaflet. The content of particles is uniform over the whole free SR surface, regardless of its location in the longitudinal tubules or in the lateral sacs of the triad (Fig. 1).

In unidirectionally shadowed replicas (Fig. 1) the particles on the cytoplasmic leaflet are randomly disposed,



FIGURES 1 AND 2 *Figure 1:* Freeze-fracture of fish muscle, unidirectionally shadowed at 45°. The smooth faced-luminal leaflet of a T tubule (*T*) runs horizontally across the center of the image. Above and below are particle-studded SR membranes, fractured to reveal primarily the cytoplasmic leaflet. Both lateral sacs of the triad (terminal cisternae, *TC*) and longitudinal tubules (*L*) contain a similar random disposition of particles. In this and the following images the fracture plane is parallel to the longitudinal axis of the fiber and thus the jSR membrane, facing the T tubules is not revealed. jSR is the only region whose particle distribution differs from this. ×89,000. *Figure 2:* Fracture similar to above, but rotary shadowed at 25°. The entire surface of the cytoplasmic leaflet of the SR is covered by a continuous carpet of particles of variable sizes and shapes. ×119,000.

and it is difficult to confirm whether or not they cover the entire surface uniformly. Small areas of the membrane seem to have no particles, even though on close examination the surface is not entirely smooth. Rotary shadowing at a lower angle and stereo micrographs (Figs. 2, 3)

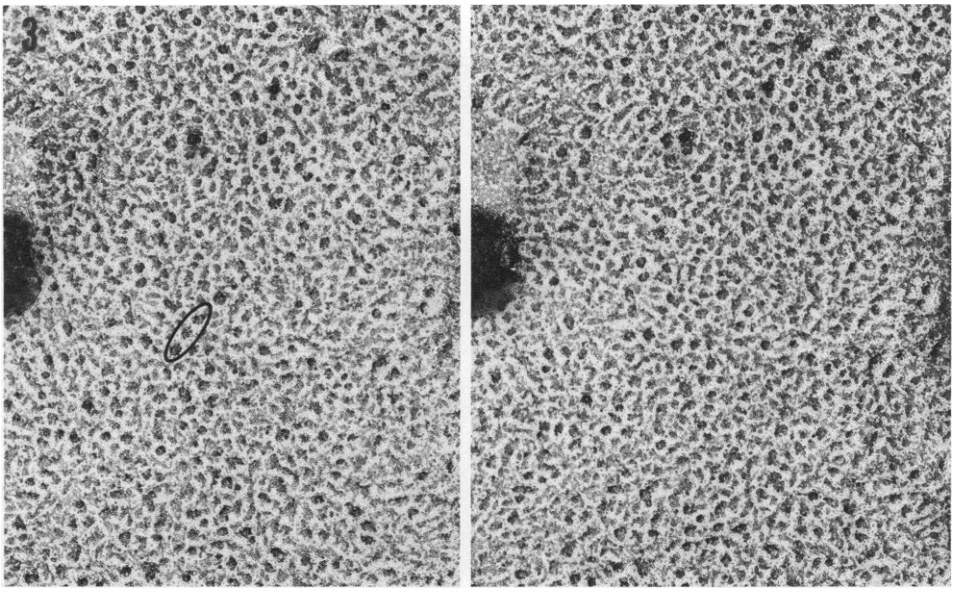


FIGURE 3 Stereo micrograph of a freeze-fracture of fish muscle, rotary shadowed at 25°. Notice that the membrane is far from flat and that the particles vary considerably in height. The taller particles are more heavily decorated by platinum. An aligned set of three large particles is outlined. Tilt ± 10°, ×210,000.

confirm the irregularity of the particle disposition, but add three details not apparent with unidirectional shadowing: (*a*) the great majority of the SR surfaces are covered by a continuous carpet of particles, (*b*) the particles are quite variable in size, shape, and height, (*c*) small sharply delineated lipid patches are occasionally present (Fig. 4). Differences in height of particles are emphasized by variable amounts of platinum deposition, and are quite obvious in stereomicrographs. At the low shadowing angle, taller particles receive substantially more platinum and appear more electron dense (Fig. 3). In between the taller, large-diameter particles there is a dense packing of shorter particles that break closer to the fracture surface and are quite variable in size and shape. Some of these are as large as the taller particles, others are smaller. Clearly, many of the shorter particles would not be revealed by unidirectional shadowing (Fig. 1) as the taller particles lie between them and the platinum source. Thus the appearance of fractured SR membrane is more complex than was revealed from unidirectional shadowing and a simple description of its structure in terms of "8.0-nm particle" does not suffice. Since, as seen in the isolated vesicles, the ATPase is tightly packed and uniformly distributed over the entire surface, these particles must represent ATPase. The particle-free lipid patches are few and small in the muscles examined in this study. They have a fine grain,

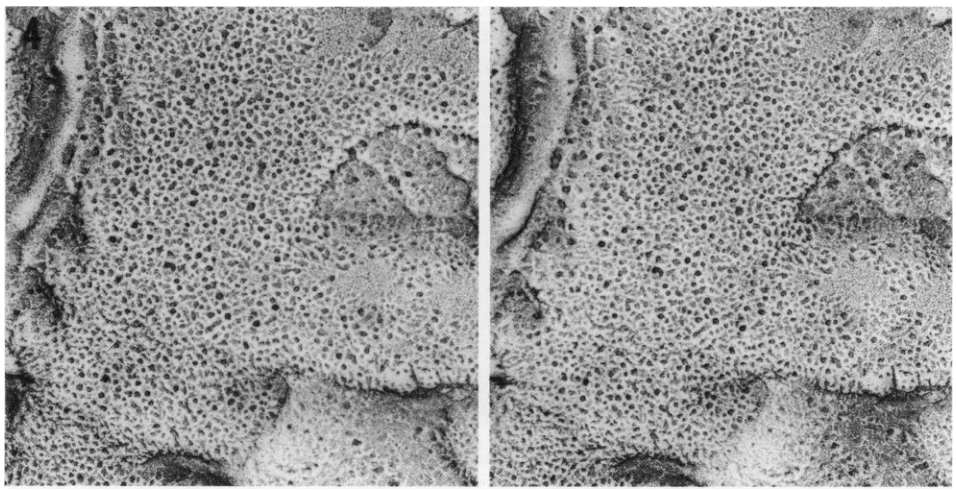


FIGURE 4 Stereo micrograph from the same muscle as above, but at a lower magnification. In the two longitudinal SR tubules (*L*) there are four patches of lipids. Lipid patches have a fine grain and are at a lower level than the areas of the membrane which contain ATPase. Tilt ± 10°, ×100,000.

presumably the basic grain of the platinum shadow, and are at a level lower than the surrounding particle-covered areas (Fig. 4).

The diameter of an intramembranous particle is difficult to determine accurately because (*a*) it depends on the amount of shadow deposited, and (*b*) the variable shapes of the particles make measurements difficult. To avoid these problems we used the center-to-center distance between selected particles in close apposition (e.g., see circled particles in Fig. 3) to determine the surface area of the domain occupied by them. The particles chosen were the most prominent both in diameter and height, i.e., they were all large particles. The average center-to-center distance between 28 particles was 13.3 ± 1.1 nm (mean ±1 SD), and from this we calculate that on the average a large particle occupies a surface area of 138.9 ± 23.7 nm^2.

Particles do not have orderly dispositions but occasionally several large particles occur in rows (Fig. 3). The significance of this will be discussed below.

Freeze-dried Isolated SR Vesicles

When spread on mica surfaces, most SR vesicles flatten out (Fig. 5). We used those with flat upper surfaces for this study. Regardless of origin (rabbit, rat, guinea pig, frog) all vesicle surfaces (except those obviously belonging to junctional SR, mitochondria, and perhaps sarcolemma or T-tubules) are covered by a similar complete, fine-grained, random arrangement of dots. On the basis of the one-to-one relationship between surface dots and ATPase monomers established in the preceding paper (Ferguson et al., 1985), we identify each dot as the head of an ATPase molecule.

A few vesicles, primarily from the frog (Fig. 6), have a mosaic appearance, due to the presence of round patches with a finer grain which are recessed. We interpret these as patches of lipids, where the true membrane surface is visible. One vesicle from a sample of vanadate-treated SR had a similar lipid patch, except that in this case its outlines were not round (Fig. 7). Obvious lipid patches like these are quite rare in the frog. In rabbit, guinea pig, and rat SR we found only very small lipid patches. This tendency for the ATPase to aggregate at high density, while leaving excess lipids, if available, to form separate patches was already clear in published images of reconstituted SR (Wang et al., 1979). It is also a characteristic of another membrane ion pump: the Na,K ATPase (Deguchi et al., 1977).

Treatment of native SR vesicles with uranyl acetate produces only a minor difference in appearance: the molecular heads form small aggregates of variable size, separated by thin, irregular, interconnected, shadow-free strips (Fig. 8). In vesicles not treated with uranyl acetate (Fig. 9) the grouping is less obvious, but one can still discern groups of projections and intervening spaces.

ATPase molecules in native SR vesicles are clearly not aggregated into large polymers or chains comparable to those seen following vanadate-induced polymerization, or in native isolated scallop SR. The few vesicles with some degree of order shown in the preceding paper (Ferguson et al., 1985) are rare and difficult to find. However, the particle arrangement is not totally random. Images of orderly arrays from vanadate-treated vesicles (Fig. 10, inset) provide appearances and ranges of intermolecular distances within dimers and aggregates of higher order (tetramers, hexamers, etc). Using these as a guide, group-

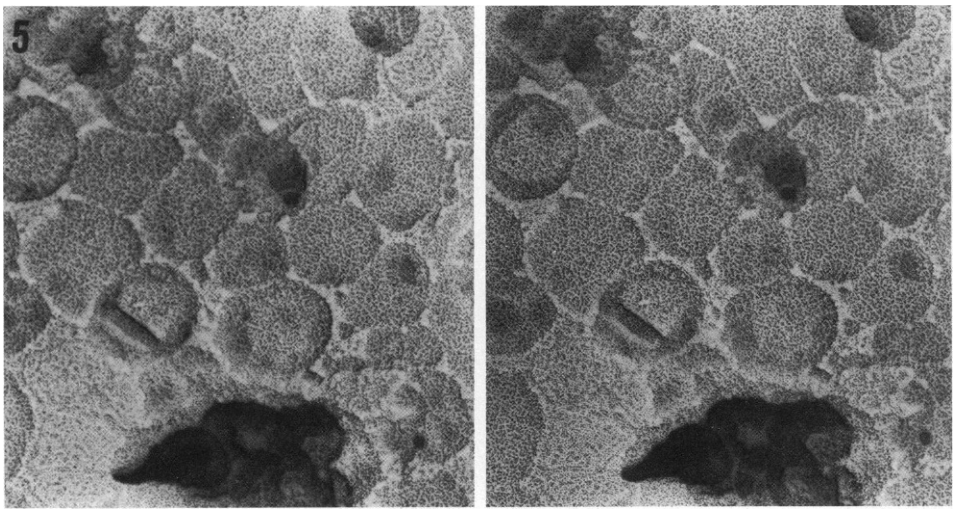
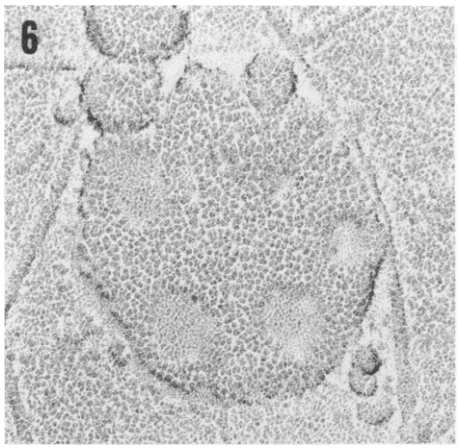


FIGURE 5 Stereo micrograph of freeze-dried SR vesicles from rabbit skeletal muscle illustrating the manner in which the vesicles flatten out on the mica surface. Depth effect is emphasized by steep tilt. This particular suspension was quite concentrated so the vesicles are crowded together and often overlap. Note that all vesicles are covered by a very fine-grained random pattern of dots. Flat-surfaced vesicles from this and similar images were used for counting. In this and all subsequent images of isolated SR shadow was applied at an angle of 25°. Final wash solutions are indicated in parentheses. (Methanol). Tilt ± 10°, ×100,000.



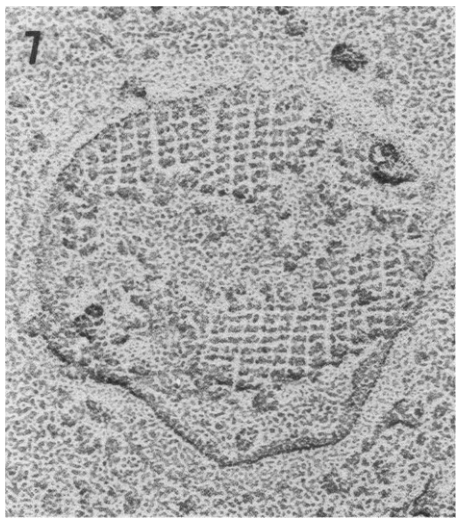


FIGURE 6 SR vesicle isolated from frog skeletal muscle. Lipid patches are clearly visible as depressed, fine grained regions of the surface. The remainder of the surface is covered by ATPase heads. Vesicles showing this much free lipid are rare in frog and even less frequent in rabbit SR fractions. Filaments are actin. (Ammonium acetate). ×185,000.

FIGURE 7 Vesicle from a vanadate-treated, rabbit SR sample. Note the long rows of polymerized ATPase. This was one of the few vesicles that had a large, easily distinguishable lipid patch on its surface. (Uranyl acetate). ×230,000.

ings of molecules suggestive of dimers, tetramers, and occasionally hexamers are easily found (Figs. 8–11). Putative dimers are best identified where the shadow fills the gap between the two ATPases (Fig. 10).

It is likely that some small polymers escape identification. However, it is also clear that numerous molecules cannot be classified into groupings, and thus belong to randomly disposed dimers and/or isolated monomers.

Particle Density Counts

Vesicles from rabbit muscle having a fine grain shadow (Figs. 5, 9, and 10) were selected for counting the surface density of the Ca ATPase. Information from the shadowed images of polymerized vesicles was used as a guide in identifying heads. For example, two shadowed dots at a distance much less than 6 nm were considered to be part of the same molecule. In the vesicles used for counting, however, the question of identity rarely arose. Stereo pairs were used to select areas for counting that were flat (Fig. 5). The two authors independently counted the same areas from 56 different vesicles, each containing 49–244 ATPases. The two independent counts gave means and standard deviations of 31,000 ± 2,000 and 34,000 ± 4,000 molecules/ μm^2. The 10% difference in the two counts can be assigned to slight uncertainty in locating the position of a small fraction of the molecules.

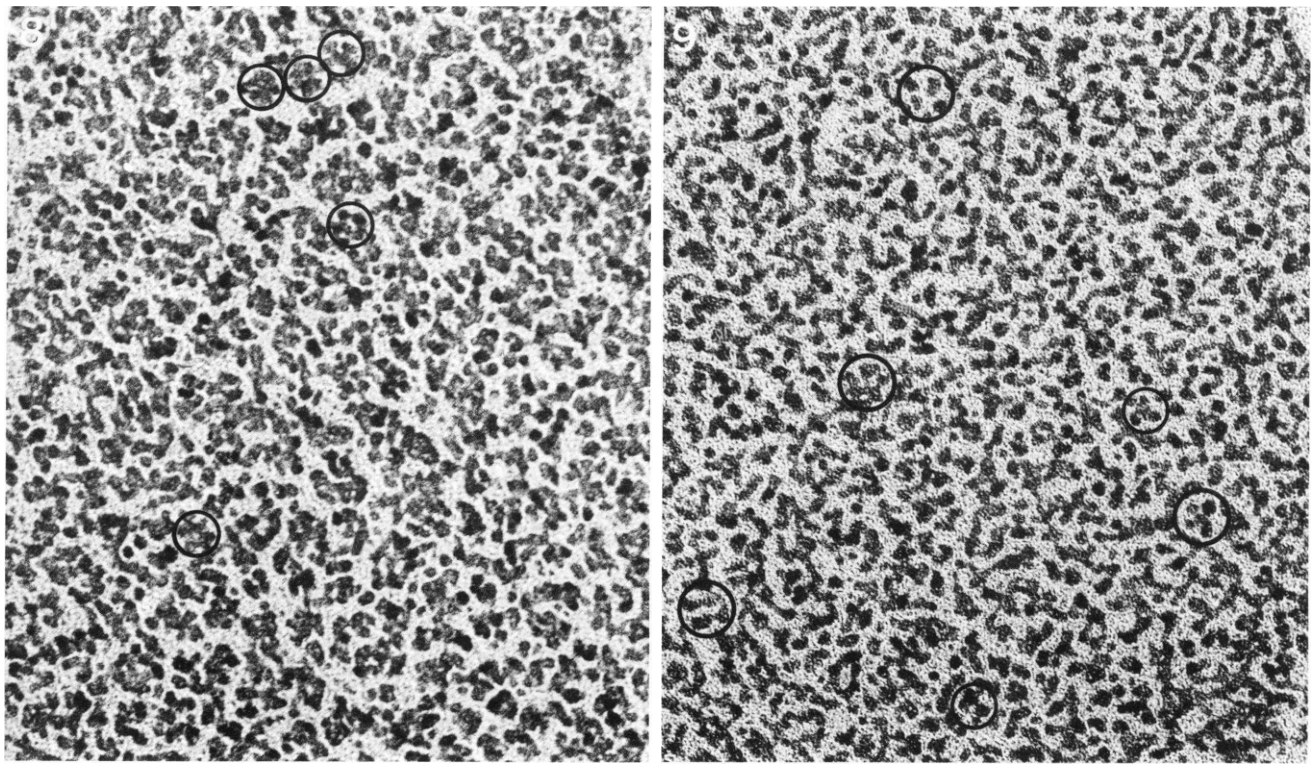
Comparison of Intramembranous Freeze-fracture Particles and Surface Projections

Since the external surfaces of isolated vesicles bear (with few exceptions) a complete coverage of ATPase molecules, the intramembranous particles on the cytoplasmic leaflet must all represent fractures of the ATPase. The size of intramembranous particles and of the ATPase heads is compared in Figs. 12 and 13. In Fig. 12 several possible tetramers on the exposed surface of a vesicle are circled, and in Fig. 13 circles of the same diameter surround large intramembranous particles. It is quite clear that a large intramembranous particle occupies a surface area on the P face of the fractured membrane equivalent to that occupied by a tetramer on the cytoplasmic free surface of the vesicle. The same conclusion is derived from the following considerations: the surface areas of unit cells in the polymerized SR, calculated from the published data (Buhle et al., 1983, 1984; Castellani and Hardwicke, 1983; Taylor et al., 1984) range from 56.5 to 66 nm^2. Since the unit cells contain dimers, the surface area covered by a tetramer must be 113 to 132 nm^2. The average surface area of a large particle, calculated from the average diameter given above, is 138 nm^2, very close to that of a tetramer. Since smaller particles are quite variable in diameter, their relationship to ATPase molecules is more difficult to establish. From the variable height and appearance of the particles we suggest that where the molecules have a random arrangement, the fracture may be guided only in part by groupings of the protein molecules.

DISCUSSION

The three main conclusions that we derive from these studies are: (*a*) the disposition of ATPase in the native SR membrane from vertebrates is primarily disorderly, but small aggregates of the molecules are found; (*b*) the density of ATPase is extremely high, and (*c*) the larger intramembranous particles seen in freeze-fractured SR represent tetramers.

Our conclusion that in SR membranes in situ the ATPase does not form large polymers is critically dependent on the assumption that the fracturing process and



FIGURES 8 and 9 SR vesicles from rat and rabbit treated with (Fig. 8) and without (Fig. 9) uranyl acetate. In both cases the disposition of dots is random, but in Fig. 8 there is a marked tendency to form small groups separated by larger spaces (see also Fig. 11). Groups of four (tetramers) are circled in both images. ×580,000.

glutaraldehyde fixation are not primarily responsible for the disorder seen in the replicas from freeze-fracture of in vivo membranes. This seems likely on the basis of the following observations: (*a*) The long rows that are characteristic of extensively polymerized ATPase in vanadate-treated preparations are clearly visible in nonfixed, fractured vesicles (Peracchia et al., 1983, 1984; Scales and Highsmith, 1984). Thus distortion during fracturing does not alter the order seen in extensively polymerized membranes. (*b*) Glutaraldehyde fixation does not destroy the orderly arrays seen in vanadate-treated vesicles. (*c*) Freeze-fracture of rapid frozen intact muscle does not reveal any regular arrangement of particles (unpublished observations on fractures kindly donated by Dr. J. E. Heuser, Department of Physiology, Washington University and Dr. T. S. Reese, National Institutes of Health, National Institute of Neurological and Communicative Disorders and Stroke). Thus disposition of intramembranous particles is not regular in muscles not exposed to glutaraldehyde. However, we have shown in the preceding manuscript that disposition of ATPase in polymerized SR is quite sensitive to freezing and thus further careful consideration should be given to this question. (*d*) Isolated vesicles not exposed to fixatives show a random arrangement of particles in freeze-fracture (our unpublished observations).

There is no question that a standard preparation of isolated, functional SR, of the type commonly used to probe ATPase structure and function, does not contain large polymers. In this respect our observations agree very well with those from x-ray diffraction (Herbette et al., 1977). One can be less certain about the aggregation of the ATPase into small groupings. In the isolated vesicles we detect small aggregates that, on the basis of the relative positions and the number of units, we describe as dimers, tetramers, and larger oligomers. However, the shadowed images do not reveal whether the orientation of the units in each group is the same as that in the dimeric rows, i.e., with units facing each other. Even though small aggregates are seen, we still cannot accurately determine how many actually exist, since many may be sufficiently distorted to escape detection. Freeze-fracture images might solve this problem. It is possible that the fracture produces a tall, large particle wherever it meets a tight grouping of four molecules (a tetramer), and it fractures in a random way, producing particles of variable height and size, wherever it meets molecules having random orientations, i.e., monomers or nonoriented dimers. If this is the case, then tetramers are not very numerous Fig. 3, for example, contains 33 large particles, giving an approximate density of 1,794/μm^2. Large particles are sometimes in rows or groups and this may correspond to the occasional groups of

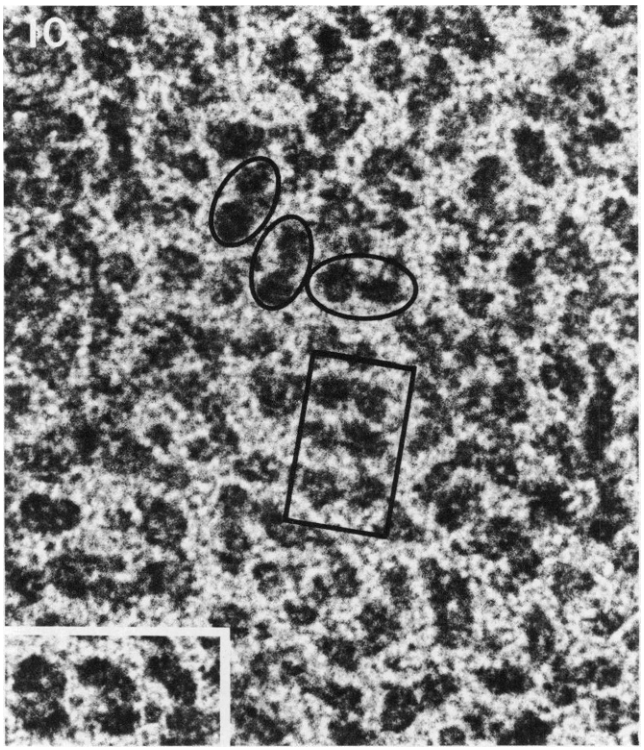


FIGURE 10 Detail of a rabbit muscle SR vesicle. Three clearly recognizable dimers, randomly oriented in relation to each other, and an aligned group of three dimers are circled (compare with Fig. 11). Putative dimers are far more numerous than those circled. (Methanol). *Inset:* detail of a short segment of a row from vanadate-polymerized ATPase showing three adjacent, aligned sets of dimers. See Ferguson et al. (1985) for further images. (Uranyl acetate.) ×1,400,000.

tetramers that we have found on the free surface. The rarity of such groupings is consistent with the lack of extensive polymerization in native SR from vertebrates.

The observation that ATPase molecules form small groupings is in keeping with the natural tendency of the ATPase to aggregate in the presence of lipids (Le Maire et al., 1976; Dean and Tanford, 1977; Moller et al., 1980; see also Ikemoto, 1982*b* and Moller et al., 1982 for reviews), but provides no definitive answer as to the size of the minimum functional unit of the Ca pump. Recent irradiation inactivation experiments have been interpreted to indicate that the minimum functional unit is a dimer (Chamberlain et al., 1983; Hymel et al., 1984). However, the calculations on which this conclusion is based are still subject to some uncertainty. Stoichiometry of phosphorylation (or catalytic) sites and enzyme units in SR vesicles have for many years given results accounting for roughly half of the total protein, and these also are consistent with a dimeric requirement for function. In the same vein, Ikemoto (1982) states that: "extensive interactions of subunits are involved in the reaction steps in which the Ca-induced conformational changes take place." On the other hand, technical refinements and more careful experiments have recently given a stoichiometric ratio of approximately one, indicating that each chain can be independently phosphorylated (Barrabin et al., 1984), and thus opening the possibility that a single polypeptide may act as the catalytic subunit.

Our counts of 31–34,000/μm^2 for the total denisty of ATPase are considerably higher than previous estimates of 12–20,000/μm^2 (Scales and Inesi, 1976; Wang et al., 1979). We have paid considerable attention to the identification of the molecules, by first studying polymerized ATPase (Ferguson et al., 1985) and have obtained particle

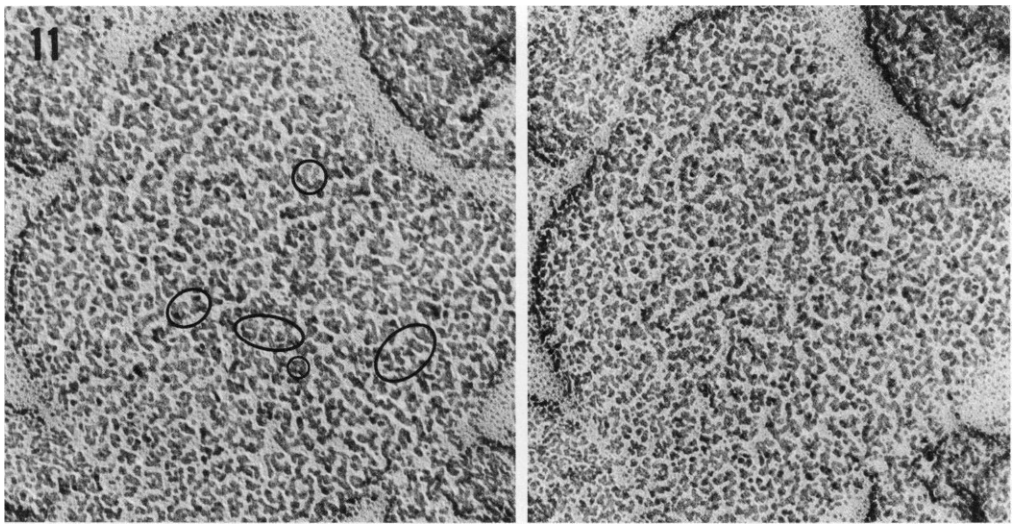
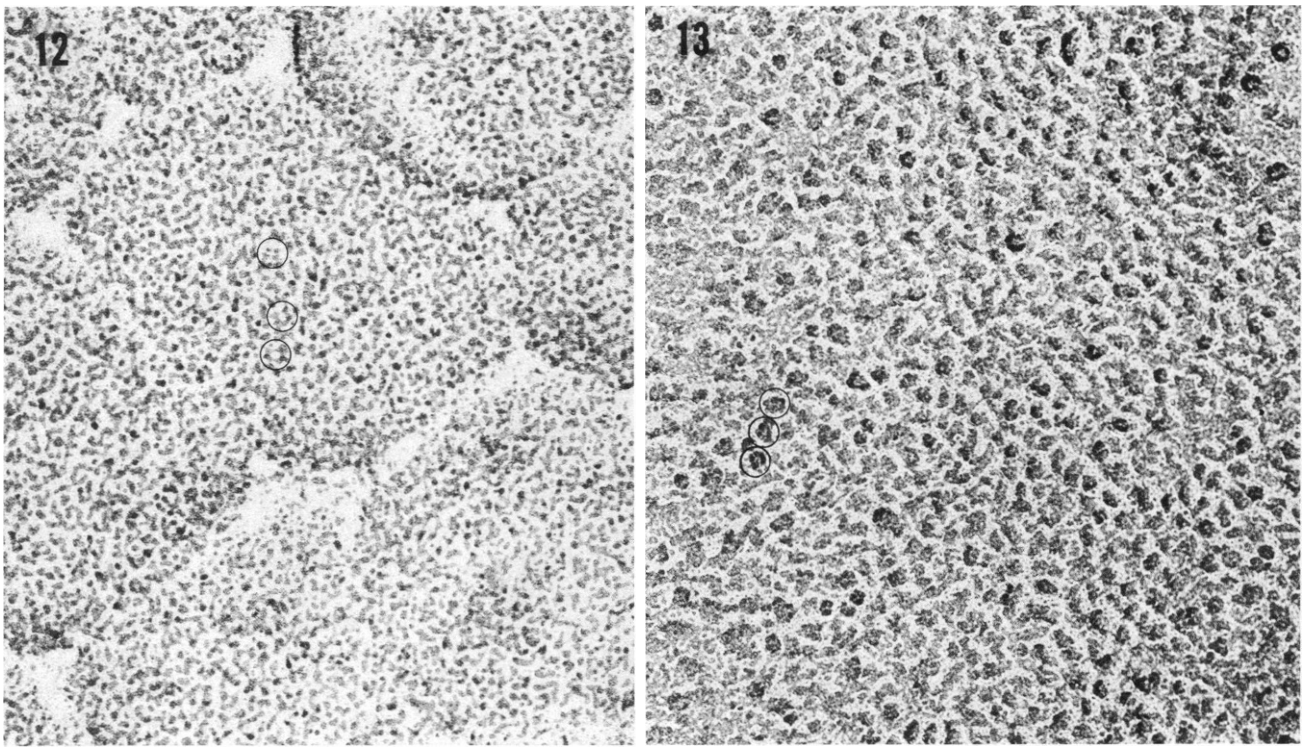


FIGURE 11 Stereo micrograph of rat SR vesicle. Note that the vesicle has a flat surface and steep sides. Grouping of particles is emphasized by uranyl acetate treatment and single, double, and triple sets of dimers (circled) are seen. (Uranyl acetate.) Tilt ± 6°, ×300,000.



FIGURES 12 and 13 A comparison of the size and disposition of heads on the surface of freeze-dried SR vesicles (Fig. 12) and of intramembranous particles on the cytoplasmic leaflet of fractured SR (Fig. 13). Circles closely surrounding four heads on the surface of the vesicles exactly enclose large particles on the fractured surface. This suggests that the large intramembranous particles on the cytoplasmic leaflet of fractured SR membranes represent tetramers. ×350,000.

counts from a large number of vesicles, after ensuring that the areas to be counted were not tilted. We are thus confident that the numbers obtained are quite reliable. It is reassuring that the counted density is consistent with that calculated from vesicles with completely polymerized ATPase, knowing that ATPase-free lipid patches are rare. The higher density is also in accordance with the very high protein-to-lipid ratio in native SR, which cannot be increased by very much using enrichment procedures (Martonosi, 1964; Wang et al., 1979). However, such a density is not consistent with the approximate molecular size determined by Le Maire et al. (1976). The reason for this discrepancy is not clear, but recent calculations (Catellani and Hardwicke, 1983; Taylor et al., 1984) indicate that previous determinations of molecular domain may have been too large.

Our estimate that the large intramembranous particles contain tetramers is at variance with a dimeric interpretation of the intramembranous particle (Napolitano et al., 1983). Two possible reasons for this discrepancy are: (*a*) Napolitano et al. (1983) base their calucation on an average particle size of 100–120 nm^2, while we consider only the large particles, with a diameter of 138 nm^2; and (*b*) our estimate of the surface area occupied by one molecule is based on unit cell dimensions in polymerized ATPase, while Napolitano et al. (1983) use less direct considerations from x-ray and neutron diffraction data.

If the total ATPase density is ~30,000/μm^2, and the large particles are tetramers, while the smaller represent fewer ATPase molecules, than the average particle density should be >7,500/μm^2. The fact that published counts of intramembrane particle densities are much lower (2–6,000; see Franzini-Armstrong, 1976, for review, and also Wang et al., 1979) is a clear indication that particle counts are not a reliable means of obtaining an estimate of ATPase density. The method used by Napolitano et al. (1983) gives a considerably higher density, and it may be more appropriate. The interpretation of large particles as tetramers may appear to agree with previous estimates of a 3–4 ratio between counts of intramembranous particles and estimates of ATPase density. However, we must point out that the agreement is fortuitous, based as it is on past underestimates, both for the ATPase density and for the particle counts.

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