FENESTRATIONS OF SARCOPLASMIC RETICULUM

Delineation by Lanthanum Acting as A Fortuitous Tracer and *In Situ* Negative Stain

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INTRODUCTION

The sarcoplasmic reticulum (SR) of striated muscle has an intricate geometry that makes anatomical analysis difficult; even in fortunate *en face* views, tubules of the SR pass in and out of the plane of sectioning and, because of the thickness of the section, morphological details of the SR are often obscured by the inevitable superimposition of adjacent myofibrils and other cellular components. The present communication reports new structural features in the SR of muscle fibers of the mouse diaphragm that were revealed when lanthanum gained access, quite fortuitously we presume, not only to transverse tubules (TT), but

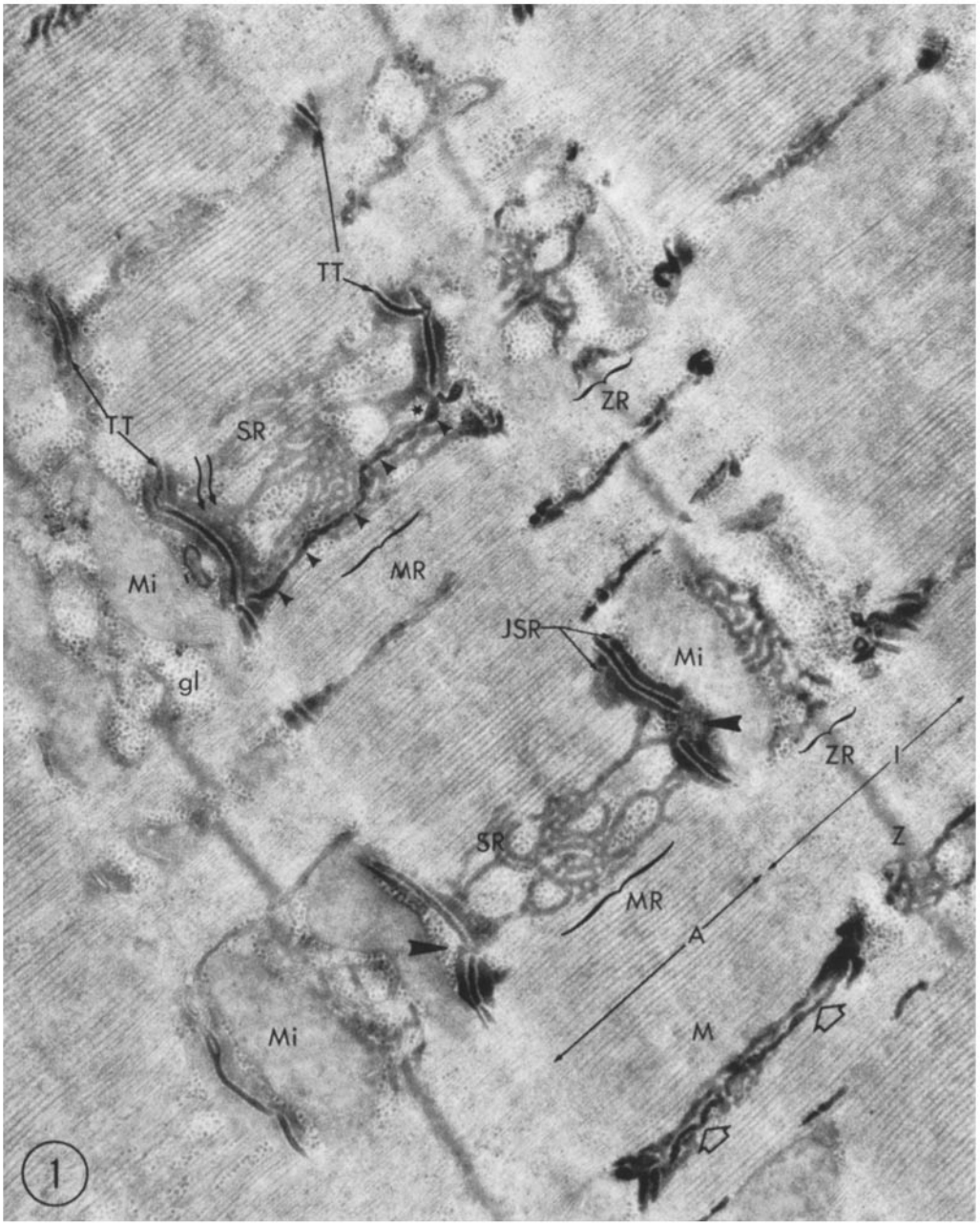


FIGURE 1 The transverse tubules (*TT*), located at the A-I junction, are filled with lanthanum and are generally darker than adjacent sarcoplasmic reticulum (*SR*) which is also filled with lanthanum. A longitudinal tubule is present (small arrowheads) and is probably a longitudinal extension of the *TT* system although it is not continuous with the TT in this micrograph. The similarity in densities between the longitudinal tubule and the junctional SR (JSR) (asterisk) gives the impression that the two systems are connected, but in stereo projections the longitudinal tubule is out of the plane of the adjacent SR. The tubule complex at the lower right (open arrows) emphasizes the difficulty in distinguishing longitudinal TT from adjacent SR. The free SR is distributed in two tubular networks or retes overlying the M line (*MR*) and Z disk (*ZR*) regions of the sarcomere. Fenestrations occur in both retes and in the JSR. The latter fenestrations are generally smaller than the rete fenestrations and are aligned in rows parallel to the long axis of the coupling. A double row of fenestrations (curved arrows) is seen in the junctional SR on the upper left. The continuity of the SR is evidenced by the areas where JSR is continuous around the TT (large arrowheads). Mitochondria (*Mi*), lightly stained glycogen (*gl*), and regions of the sarcomere (M line, *M*; Z disk, *Z*; A band, *A*; and I band, *I*) are labeled. ×27,000.

to portions of the SR as well; locked within the lumen of the SR, the lanthanum provided additional contrast permitting visualization of structural details that otherwise might have escaped detection. As a result, we have noted so-called SR fenestrations in locations other than those in which they have previously been described in skeletal (1–3) and cardiac muscle (4). In addition, the very prominence of the lanthanum-filled SR has given us more insight into its over-all distribution and morphology, especially within the space that spreads between A and I triads of adjacent sarcomeres and, thus, surrounds the Z disks.

MATERIALS AND METHODS

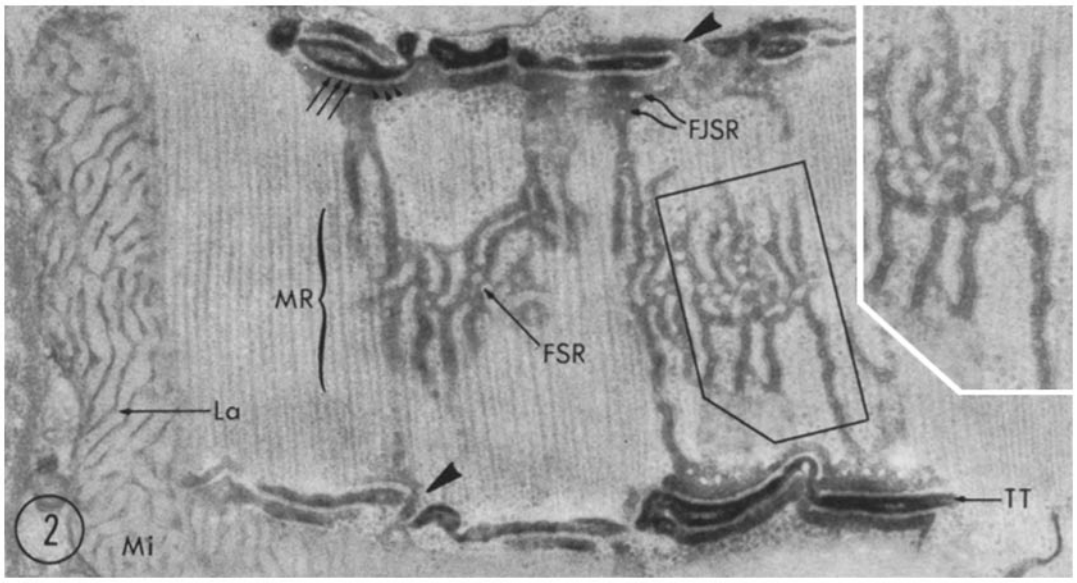
Both hemidiaphragms from an anesthetized (pentobarbital) C3H mouse were removed and pinned under slight tension in a wax-coated petri dish filled with cold (4–6°C), oxygenated, Krebs-Henseleit solution (5). Within 2–3 min of excision of the tissue, the salt solution was replaced with cold 3% glutaraldehyde (diluted from 50% biological grade from Fisher Scientific Co., Pittsburgh, Pa.) in 0.175 M (300 mosM) cacodylate buffer, pH 7.2, containing 2% polyvinylpyrrolidone (6) and 0.5% Alcian blue (7). The total osmolarity of the fixative solution was 755 mosM. After 1 h, the tissue was cut into small pieces suitable for embedding and was left in cold fixative for 20 h. The tissue was then washed for 5 min in 0.175 M cacodylate buffer, pH 7.2, and postfixed for 3 h in 2% osmium tetroxide in 0.15 M cacodylate buffer, pH 7.2, containing 0.8% potassium ferrocyanide (8). The total osmolarity of the postfixing solution was 325 mosM. After washing for 1 h in 0.175 M cacodylate buffer, pH 7.2, the tissue was soaked in the cold in a 1% solution of lanthanum in 0.175 M cacodylate buffer, pH 8.05. The lanthanum was prepared by titrating a 4% solution of lanthanum nitrate to slight opalescence with 0.01 N sodium hydroxide (9), followed by dilution with the buffer. After 1½ h the tissue was dehydrated in ethanol, cleared in propylene oxide, and embedded in Epon 812. Control tissue was treated similarly except that it was not exposed to lanthanum and was postfixed both with and without the addition of potassium ferrocyanide to the osmium tetroxide solution. Gray to silver sections were cut and the grids examined both with and without double staining by 4% aqueous uranyl acetate and lead citrate (10). The accompanying micrographs are all from sections which were double stained on the grid. The magnifications are approximations within the limits of routine photographic processing and calibration of the JEM 100B electron microscope.

FIGURE 2 The tubular nature of the SR rete overlying the M line region (*MR*) is apparent. The fenestrations in both the SR rete (*FSR*) and the junctional SR (*FJSR*) are readily visible. The FJSR (curved arrows) are aligned in rows and, in one location, a single fenestration of a second row is seen. The small arrowheads denote dimples of the junctional SR membrane which appear to be filled with lanthanum and are in register with the junctional processes of the adjacent area of the coupling (arrows). A mitochondrion (*Mi*) near the cell surface shows lanthanum (*La*) within the outer mitochondrial compartment. The *inset* is an enlargement of the SR rete. Note the particulate substructure of the SR and/or its contents. The lucent space separating the SR membrane from the lanthanum-filled space is apparent as are the membrane boundaries of the fenestrations. Again, several areas are seen (large arrowheads) where the junctional SR is continuous around the transverse tubules. ×53,000. Inset ×75,000.

FIGURE 3 The membrane boundaries of the fenestrations within both the junctional (*JSR*) and free sarcoplasmic reticulum (*SR*) are apparent. The 20–30-Å lucent space beneath the SR membrane is continuous from free to junctional SR and includes that portion of the SR which faces the transverse tubule (*TT*). A similar lucent line is present adjacent to the lanthanum-filled TT. Note the particulate substructure of the SR in both locations. × 146,000.

FIGURE 4 Two triads composed of junctional sarcoplasmic reticulum (*JSR*) flanking central transverse tubules (*TT*) are seen in cross section. The right hand junctional SR appears to be sectioned at the level of the fenestrations (arrowheads). This is more apparent in the lower triad. A complete gap thru the JSR is not seen because of the section thickness relative to the small size of the fenestrations. × 146,000.

FIGURE 5 *En face* view of a triad. Fenestrations are present on both sides of the transverse tubule (*TT*). In places, periodic densities or junctional processes (arrows) appear to completely span the gap between the junctional SR and the TT. The TT membrane is scalloped and/or has periodic densities (bracket) which are in register with the junctional processes noted by the arrows. × 115,000.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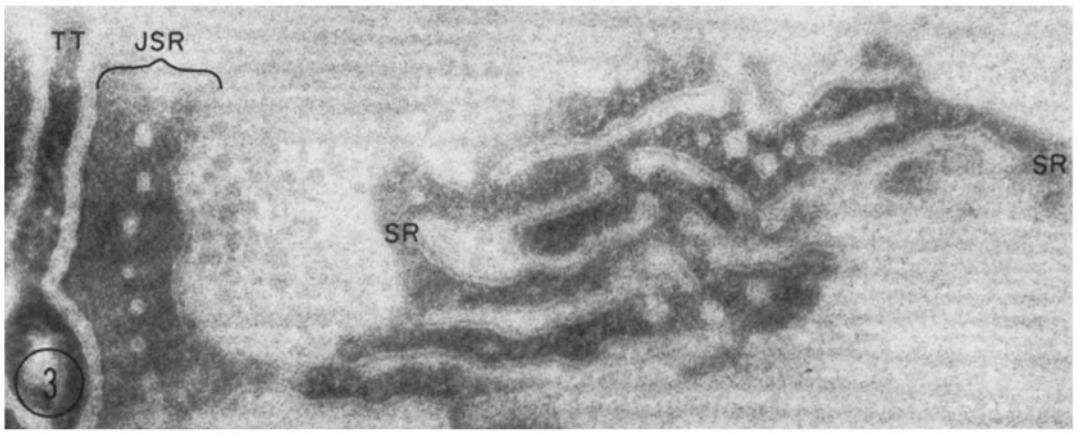
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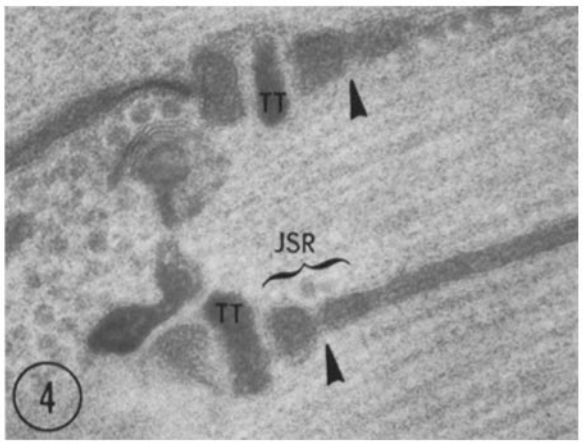
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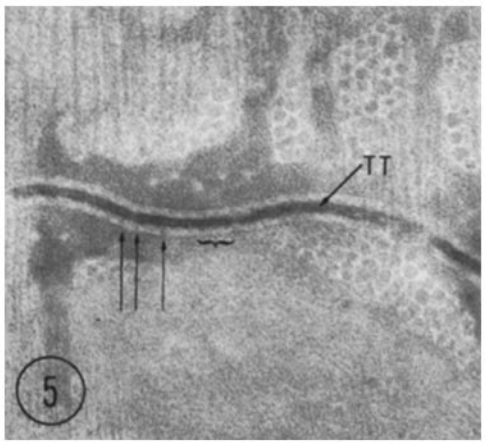
TT
5

RESULTS AND DISCUSSION

In scattered areas of the muscle, lanthanum filled both TT and SR, the former more consistently (Figs. 1 and 2). The TT were at the A-I junction; longitudinally oriented TT were also present (Fig. 1). Often the TT appeared denser than adjacent junctional SR and free SR, suggesting that perhaps lanthanum had freer access to TT than to SR. The central portions of each SR rete over the M line region contained numerous fenestrations which appeared as holes or cylinders with axes normal to the longitudinal axes of the sarcomeres (Figs. 1–4). The M line SR rete had a tubular geometry in contradistinction to the fenestrated collar of frog sartorius muscle in which SR tubules coalesce to form large, flat cisternae surrounding the M line region of the sarcomere (3). Assuming 250 Å as the average diameter of the SR fenestrations and further assuming that there is one fenestration per 1,000 Å of SR tubule and letting d equal the average diameter of the SR, then the ratio $(\pi \cdot 250 \text{ Å} \cdot d)/(\pi \cdot d \cdot 1{,}000 \text{ Å})$ shows that the fenestrations can contribute a 25% increase in total membrane area; this rough approximation supports Peachey's contention that fenestrations exist for that reason (11). In addition to the M line SR rete, a second SR rete surrounded the Z-I region of the sarcomere (Fig. 1). The two retes were sometimes continuous with one another via junctional SR at a transverse tubule (Figs. 1 and 2). While SR in the Z-I region has been well documented in bat cricothyroid muscle (12), our observations establish, in addition, that at least in mouse diaphragm the SR in the Z-I region is differentiated into a full rete complete with fenestrations equivalent to that in the M line region. The fenestrations found in the Z rete, viewed as a form of discrete differentiation of the free SR, suggest that the similar fenestrations in the SR of the M region do not serve a function peculiar to that one location. One may tentatively conclude, therefore, that the SR retes and their fenestrations are an evolutionary response to similar functional demands in both locations. By way of contrast, the location of junctional SR, another discrete differentiation of the SR, seems much more restricted in that it is prominently associated with Z-I regions, even when not associated with TT. That the TT are not the sole factor determining the location of junctional SR derives from the fact that junctional SR exists in the Z-I region in the absence of TT in the form of peripheral couplings in all cardiac and some skeletal muscles (13) and in the form of extended junctional SR in birds (14).

Very orderly rows of fenestrations of rather equal size were also seen in the junctional SR (Figs. 1–5) of the coupling although they were less numerous on the side toward the Z line. In general the fenestrations were smaller than those of the M and Z retes, and their axis of alignment was always parallel to the long axis of the coupling. In favorable sections, two and occasionally three such parallel rows could be seen extending into that portion of the free SR which connects the rete with the junctional SR.

Junctional processes (12) or SR feet (15) were readily apparent in sections stained on the grid and, to a lesser degree, in unstained tissue. It is of interest that they were so prominent despite the absence of block staining with uranyl acetate (15). Sometimes the junctional processes appeared to completely span the gap between the junctional SR and TT (Fig. 5). Whether this represents contrasting of these processes by lanthanum functioning either as a tracer or as a stain cannot be determined from this preparation. Fig. 2 does suggest, however, that lanthanum fills dimples extending from the SR membrane towards the TT; as reported by Franzini-Armstrong (15), these dimples are in register with foot processes that may be noted in other areas of the same coupling.

Fig. 2 (inset) and Fig. 3 are higher magnifications of the lanthanum-filled SR and reveal a particulate substructure of both free and junctional SR. Also apparent is a linear, lucent area measuring approximately 20–30 Å in width that separates the lanthanum-filled space within the SR from a single cytoplasmic lamina of the SR membrane. This space was continuous from free SR to junctional SR (Fig. 3) and was also present in areas around the TT (Figs. 3–5). Although this space may represent the center of the SR membrane, this possibility cannot be evaluated because it was not possible to resolve both layers of the unit membrane in this preparation.

In addition to lanthanum and the conventional processing techniques, the tissue was treated with two other substances (7, 8) capable of enhancing contrast in the final image. The question therefore arises as to whether the SR was labeled by lanthanum or one of the other compounds. Control tissue not exposed to lanthanum was fixed in

glutaraldehyde containing Alcian blue and post-fixed in osmium tetroxide with and without potassium ferrocyanide. No electron-opaque substance within the SR was demonstrated in these controls. This observation and those of Fahimi and Cotran (16) and Sommer and Jewett (17) which showed lanthanum within the SR of tissues that had been treated with lanthanum alone leads us to conclude that lanthanum was the primary source of labeling of the SR in our preparation.

We consider the filling of the SR with lanthanum fortuitous. In particular, we do not believe that our findings provide any evidence for the notion, however attractive, that the SR is by design open to the extracellular space such that it would be accessible to an extracellular tracer (17). Filling of the SR by lanthanum to the extent seen in the present illustrations happened in only one out of many specimens that received identical treatment. Even in that one specimen, the filling of the SR was spotty at best and was always confined to the superficial layers of the muscle fibers. The lanthanum presumably gained entrance to the SR through a communication, artifactual or otherwise, between the extracellular space and the SR and, from that spot, expanded as far as diffusion would carry it. In several instances the nuclear envelope and outer mitochondrial compartment (Fig. 2) were blackened as well. The possible artifactual nature of direct communications between the extracellular space and the SR or other cellular compartments is underscored by the recent observations of Fahimi and Cotran who found filling of the SR and, significantly, staining of the cytoplasm by lanthanum after thermal injury to muscle cells (16). We also have noted cytoplasmic staining by lanthanum both in the present experiment and in other preparations. In such cases the cytoplasmic staining never approached the density seen in some TT and in the SR, presumably because the dilution of lanthanum possible in a large volume of cytoplasm is simply much greater than that which can be obtained within the restricted spaces of either the TT or SR. The implications are compelling, indeed, and carry over to all experiments in which extracellular tracers have been found in intracellular organelles, including SR. Consequently, experiments such as those of Rubio and Sperelakis (18) warrant cautious interpretation.

Aside from the fact that, at least for the moment, the lanthanum tracer must be judged to be inconclusive in demonstrating in vivo continuity of tissue and cellular compartments, it has nevertheless afforded the advantage of displaying the geometry of the SR retes in all their intricacy. In addition, it is apparent that this kind of preparation, even if it occurs but occasionally, can be exploited to investigate the intraluminal structure of the SR by capitalizing on the *in situ* negative staining effect that lanthanum produces.

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