MEMBRANE EVENTS INVOLVED IN MYOBLAST FUSION

NURIT KALDERON and NORTON B. GILULA

From The Rockefeller University, New York 10021

ABSTRACT

Myoblast fusion has been studied in cultures of chick embryonic muscle utilizing ultrastructural techniques. The multinucleated muscle cells (myotubes) are generated by the fusion of two plasma membranes from adjacent cells, apparently by forming a single bilayer that is particle-free in freeze-fracture replicas. This single bilayer subsequently collapses, and cytoplasmic continuity is established between the cells. The fusion between the two plasma membranes appears to take place primarily within particle-free domains (probably phospholipid enriched), and cytoplasmic unilamellar, particle-free vesicles are occasionally associated with these regions. These vesicles structurally resemble phospholipid vesicles (liposomes). They are present in normal myoblasts, but they are absent in certain fusion-arrested myoblast populations, such as those treated with either 5-bromodeoxyuridine (B UdR), cycloheximide (CHX), or phospholipase C (PLC). The unilamellar, particle-free vesicles are present in close proximity to the plasma membranes, and physical contact is observed frequently between the vesicle membrane and the plasma membrane. The regions of vesicle membrane-plasma membrane interaction are characteristically free of intramembrane particles. A model for myoblast fusion is presented that is based on an interpretation of these observations. This model suggests that the cytoplasmic vesicles initiate the generation of particle-depleted membrane domains, both being essential components in the fusion process.

KEY WORDS freeze-fracture · membrane fusion · myoblast fusion · myogenesis · plasma membranes · unilamellar vesicles

The multinucleated muscle fiber is generated during myogenesis by fusion of mononucleated myoblasts. Several ultrastructural studies (13, 21, 35, 40) provided the basis for the current appreciation of the fusion process: cytoplasmic continuity is established between two fusing myoblasts as a result of the partial disappearance of the plasma membranes. Consequently, many of the studies related to myoblast fusion were focused on the plasma membrane and its involvement in the process; for example, there have been a number of studies on myoblast fusion that have been focused on the cell surface membrane proteins. The rationale for these studies has been that fusion must be regulated and carried out by cell surface membrane components, and the obvious candidate is a protein or group of proteins (3, 5, 9, 11, 24, 35, 36, 39). Moreover, two processes that may be fundamentally different—(a) recognition between cells and (b) membrane fusion—have not been distinguished in most of these studies. These two processes occur independently in a wide variety of cellular systems; for example, recognition may be an important process in many differentiating multicellular systems where membrane fusion is not a consequence (23). Con-
versely, cells can be fused experimentally without any apparent species or histological specificity (1, 22, 27, 28, 33).

Cell fusion can be induced between a variety of nonmuscle cells that normally do not fuse, forming both homokaryons and heterokaryons. It can be induced by viruses (27), certain lipids (33), phospholipid vesicles (liposomes) (22, 28), and different polyols (1).

Several different reports have indicated that the lipid component of the plasma membrane might play an important role in myoblast fusion (14, 34, 41). Myoblast fusion can be regulated (inhibited or enhanced) by altering conditions that are known to affect membrane fluidity, such as temperature and/or lipids (41). It was also reported that myoblast fusion is preceded by an increase in membrane fluidity (14, 34).

To eliminate confusion, we would like to emphasize that this study has been focused on the localized plasma membrane events that are associated with myoblast fusion, in other words, the fusion of two separate membranes and the subsequent establishment of cytoplasmic continuity between myoblasts. The problem of regulating the initial interactions between these cells (i.e., recognition) is not an issue that has been examined in this study. The present study provides a detailed ultrastructural analysis of the transient membrane events that are involved in the fusion process. A preliminary report of this study has been previously presented (19).

MATERIALS AND METHODS

Unless specifically indicated, all the materials and methods utilized in this study were identical to those described previously (18).

Muscle Cultures

Normal cultures. Primary cultures of 11-d-old embryonic chick thigh muscle were prepared as described by Easton and Reich (7). Myoblasts were plated on either 35-mm (1.5 ml) plastic Petri dishes or the 2-mm Balzers gold carriers that were precoated with collagen (for details, see reference 18). The cells were plated at initial densities of 2–3 × 10⁵ cells/ml on the Petri dishes and at 3–4 × 10⁶ cells/ml on the gold carriers. The cultures were maintained in a 5% CO₂ incubator at 37°C.

Fusion-arrested cultures. PLC-arrested myoblasts (25) were obtained by applying culture medium containing 0.047 U/ml PLC to muscle cultures 20–26 h after plating. Chromatographically pure PLC (94 U/mg; 0.508 mg/ml) was obtained from Worthington Biochemicals Co., Freehold, N. J. BUdR (2), CHX, and ethylene glycol-bis(β-aminoethyl ether)N,N'-tetracetic acid (EGTA) (29) fusion-arrested cultures were obtained as described previously (18). The concentrations of the drugs utilized to inhibit fusion were as follows: 3.3 × 10⁻⁶ M BUdR; 1 μg/ml CHX; 1.8 × 10⁻³ M EGTA.

Ultrastructural Analysis

Samples were fixed in the original culture dishes with 2.5% glutaraldehyde in Dulbecco's phosphate-buffered saline solution (PBS) for 15 min at room temperature, rinsed twice with PBS, and then processed either for monolayer thin sections or for freeze-fracture as described in detail previously (18). Samples that had been cultured in low Ca²⁺ were fixed with 2.5% glutaraldehyde in Ca⁺⁺-, Mg⁺⁺-free PBS. In the case of intact tissue, the fixation and further processing was carried out with the same procedure as that used for the culture samples.

Quantitation of Cells with Particle-free Regions

Muscle cell plasma membranes containing particle-free regions (PFR) in close association with particle-free vesicles were counted by examining freeze-fracture replicas of various muscle cell populations. Fracture faces (either E or P) that were ≥20 μm² were included in the analysis. The PFR was also characterized on the basis of size; a size of ≥0.1 μm in diameter was required for the PFR classification, while a size of >0.3 μm in diameter was regarded as a large PFR (Fig. 1 C).

RESULTS

An ultrastructural study of muscle cells during myogenesis revealed that unilamellar membrane vesicles are found frequently in the cytoplasm of prefusion myoblasts in close proximity to the plasma membrane (Fig. 1). These vesicles exist in clusters or as single elements, and physical contact is often observed between the plasma membrane and the vesicles (Fig. 1 A and B). The distribution of these vesicles varies considerably, for they can be found in any cortical region of the myoblasts, regardless of the presence or absence of another myoblast in the vicinity (Fig. 1 B). In freeze-fracture replicas, the vesicle fracture faces are entirely free of intramembrane particles (Fig. 1 C).

Likewise, the freeze-fracture regions of vesicle membrane-plasma membrane interaction are characteristically smooth, with practically no detectable intramembrane particles (Fig. 1 C). There is a considerable variability in the size of the vesicles; however, the majority of the vesicles have a diameter in the range of 0.1–0.2 μm.

The cytoplasmic vesicles have not been found adjacent to the plasma membrane in thin sections.
of fusion-arrested myoblasts (those treated with either CHX, PLC, or BUdR) or mature myotubes (9 d in culture). The EGTA-treated myoblasts appear to differ in their vesicle content from the other (CHX, PLC, and BUdR) fusion-arrested myoblasts. Occasionally, vesicles have been found in thin sections of EGTA fusion-arrested myoblasts; however, no direct physical contact has been observed between the vesicle membrane and the plasma membrane in the EGTA-treated samples.

To efficiently study the fusion process, it is important to determine the time and/or conditions for optimizing the incidence of the event. Although myoblast fusion in cell culture is considered to be a relatively synchronous event, it spans 5–10 h; and the total number of fusion events that occur at each time point is very low. Therefore, a systematic approach, although tedious, was necessary to define the optimal time for studying fusion events in the culture system. From a previous study (18), it was determined that no fusion images were observed until 44 h after plating. Therefore, it was concluded that the fusion incidence was very low during this time period. Thus, samples were taken at different time points, from 46 to 65 h, and examined for fusion images with the freeze-fracture monolayer technique. This systematic approach proved to be beneficial, and the entire sequence of fusion events could be observed in samples from 47 to 53 h after plating. The surface of the normal muscle cell undergoes a morphological transformation from a smooth continuous appearance during the stage before fusion (Fig. 2A), when gap junctions are found, to the fusion stage (Fig. 2B) where the membranes have an uneven, interrupted appearance. Finally, the newly formed myotube membrane (Fig. 2C) has more curvature with numerous microvilli. In contrast with those of the normal fusing myoblasts, the membranes of fusion-arrested myoblasts (with EGTA, PLC, BUdR, and CHX treatments) do not undergo any gross transformation; the membranes remain smooth and continuous (Fig. 2D, E, and F), and they contain gap junctions, resembling the surface features of a prefusion myoblast (Fig. 2A).

A quantitative analysis of the incidence of particle-free regions (PFR) in the muscle cell plasma membrane that are associated with the vesicles was generated by scoring freeze-fracture replicas for the presence or absence of these regions in muscle plasma membranes from different populations (see Materials and Methods). The data are summarized in Table I. Most of the normal myoblasts (87%) that were fixed 48 h after plating contain PFR; 38.4% of these cells have large PFR (>0.3 μm). In some samples, the diameter of these regions was >1 μm. In contrast to the normal fusing myoblast population, the fusion-arrested myoblasts treated with BUdR, PLC, or CHX (data not shown) have practically no detectable PFR. Only 5% of the BUdR-treated myoblasts and 7.9% of the PLC-treated myoblasts (Table I) contained PFR; one cell (PLC-treated) had a “large” PFR. The EGTA fusion-arrested myoblasts significantly differ from the other fusion-arrested cells (CHX, PLC, and BUdR); 46.3% of the cells contain PFR, while most of these (70%) are small in size. The “large” PFR were present in 13.9% of the EGTA-treated cells. When the inhibition of fusion was reversed for 6 h, the plasma membranes of these cells had changed with respect to the PFR. In this sample, the content of the PFR was increased (73.9%) but the most significant change was expressed in the enlargement of these regions; after 6 h in normal medium, 53.6% of the cells contained large PFR that were associated with clusters of vesicles.

**Fusion**

An important event in myoblast membrane fusion appears to be the formation of a single bilayer membrane from the two plasma membranes (Fig. 3). In thin sections (Fig. 3A and B), the plasma membranes of the myoblasts are fused into a single bilayer that is continuous with the membranes of the two adjacent cells. The same image can also be observed in freeze-fracture replicas of fusing myoblasts (Fig. 4). The regions of fusion are detectable as depressions (single bilayer) that join the E-fracture face of cell 2 with the P-fracture face of cell 1; these single bilayer regions are completely devoid of particles (Fig. 4). The regions of fusion in the plasma membrane are small in size ~0.1 μm, and they occupy only a small portion of the total plasma membrane surface area (Figs. 3 and 4).

**The Vesicles and Membrane Fusion**

Occasionally, the unilamellar vesicles can be found associated with the sites of plasma membrane fusion (Fig. 5). A potential role of the unilamellar and particle-free vesicles is indicated in Fig. 6. The vesicle membrane and the two
plasma membranes are mutually connected by only a single bilayer (Fig. 6B); this same vesicle in a different plane of section (Fig. 6A) is clearly interacting with only one of the plasma membranes. This identical arrangement can also be found in freeze-fracture replicas (Fig. 6C); the three membrane systems (vesicle membrane and the two plasma membranes) are fused or connected by a single bilayer (diaphragm) that is particle-free.

The vesicles apparently are not stable structures, for quite often clear spaces or "holes" can be found in the cytoplasm next to regions of fusion (Fig. 3B). These may represent remnants of the vesicles. The vesicles presumably collapse or solubilize after participating in the process. It is not clear whether this tendency to collapse is a normal response or a novel instability to the fixative as a consequence of the fusion process.

The single bilayer together with the vesicles disappears and cytoplasmic continuity is established between the myoblasts (Fig. 7). The regions of the plasma membranes that do not participate in the fusion process are present as oblong membrane sacs or cisternae (Fig. 7B) in the cytoplasm of the newly formed myotube. Remnants of cell-cell contacts can be associated with the cisternal membranes (Fig. 7B), similar to those described previously by Rash and Fambrough (35).

**DISCUSSION**

In the present study we have attempted to obtain ultrastructural data that can be utilized to understand the basis for the membrane events that are involved in myoblast fusion. This type of a phenomenon is extremely difficult to study since the relevant events occur at a relatively low frequency in such a differentiating population, occupying a small fraction of the total plasma membrane surface, and the events are metastable or transitory. Due to these characteristic features of this phenomenon, it should be indicated that the "static" images that have been obtained must be interpreted with a proper respect for the difficulty in stabilizing or "fixing" short-lived events. Therefore, we have recognized that there are a number of possible interpretations of the data that have been obtained, as well as sources for artefacts. With these reservations, we have proposed a hypothetical scheme or model that is consistent with our current interpretation of the available data. The model that has been devised as a result of our interpretation of the ultrastructural data (Fig. 8) contains a cytoplasmic component (the vesicles) and a sequence of events that are associated with the fusion of myoblast plasma membranes. According to this hypothetical scheme, the cellular component that initiates fusion is the unilamellar, particle-free vesicle. The initial stage in the sequence of events leading to fusion is destabilization of the plasma membrane, i.e., formation of regions that are phospholipid enriched. This destabilization in the plasma membrane is induced by the interaction of a vesicle with the plasma membrane, and it is expressed as a particle-free domain in the region

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**Fusion In Vivo**

Thin sections were examined of developing muscle in vivo from 10-14 d-old chick embryos. Extensive fusion was observed in muscle tissue from 13-d samples. Fusion images similar to those described above for the culture preparations were found. These include the intermediate fusion stage (Fig. 3) where two plasma membranes are fused into a single bilayer, and the association of vesicles with the fusant plasma membranes (Fig. 5). An example of the latter is present in Fig. 7A; vesicles are present in cell 2 that may be involved in the fusion of cells 1 and 3 with cell 2.

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**Figure 1** Thin section (A and B) and freeze-fracture (C) images of vesicles in prefusion myoblasts. (A and B) The myoblasts on the collagen substrate (S) were sectioned in a vertical plane to the dish. The myoblasts contain unilamellar vesicles in the cytoplasm adjacent to the plasma membrane (arrowheads). The vesicles are present in clusters (arrow) (A), or isolated (B). The vesicles are randomly distributed in B, and a vesicle (arrow) also exists next to the surface membrane apposed to the substrate (S). (C) Freeze-fracture replica of two interacting myoblasts (43 h in culture). There is a cluster of vesicles (V) in the cytoplasm of cell 1; the vesicle membranes are particle-free and the plasma membrane interacting with the vesicles is also particle-free (arrowheads). This is considered to be a large PFR as compared to the small PFR indicated by the arrowhead on the extreme right of this image. The inner membrane half (P-fracture face) of cell 1 and the outer membrane half (E-fracture face) of cell 2 are also indicated. (A-C) Bar, 0.2 µm. × 45,900.
FIGURE 2 Freeze-fracture replicas of the plasma membranes of normal and fusion-arrested myoblasts at low magnification (A–F). (A, B, and C) These images illustrate the striking differences in plasma membrane morphology that exist at different stages of the fusion process. (A) Prefusion myoblasts (27 h in culture); the membranes are smooth and continuous. (B) Fusing myoblasts (47 h after plating); the fracture face is not continuous since the fracture plane moves frequently from the membranes of the fusing cells. (C) Newly formed myotube (65 h after plating); the membrane fracture face is interrupted by numerous microvilli. (D, E, and F) Plasma membranes of fusion-arrested myoblasts. (D) BUdR-treated myoblasts (48 h after plating). (E) PLC-treated myoblasts (48 h after plating). (F) EGTA-treated myoblasts (70 h after plating). In all three fusion-arrested populations, the plasma membranes have an appearance similar to the prefusion myoblasts in A. (A–F) Bar, 2 \( \mu m \). \( \times 4,530 \).
of vesicle-plasma membrane interaction. Subsequently, the vesicle membrane fuses with a plasma membrane particle-free domain to form a single particle-free bilayer. Two plasma membrane particle-free regions of adjacent cells can then fuse to form a single bilayer membrane that is devoid of particles. The single bilayer as well as the fused vesicles disappear because of their instability, and cytoplasmic continuity is estabished between the myoblasts. The model, as described above, was generated by integrating the static ultrastructural observations in this study with data derived from fusion studies on other systems, both artificial and biological.

Fusion and the Vesicles

The unilamellar vesicles as observed in thin sections (Fig. 1A and B) are particle-free in freeze-fracture replicas (Fig. 1C). It is generally believed that the intramembrane particles are proteins whereas the smooth surfaces in the replicas represent the lipid bilayer portion of the membrane (for reviews, see references 4, 8). Thus, regions that are particle-free are probably lipid enriched. Therefore, it is reasonable to suggest that the particle-free vesicles are lipid enriched or protein depleted. From this point of view, these vesicles resemble artificial phospholipid vesicles (liposomes) (16). With a similar rationale, the regions in the plasma membrane that are particle-free (Fig. 1C) can also be considered to be lipid enriched. Moreover, the formation of these particle-free regions in the plasma membrane appears to be induced by the localized interaction of vesicles with the plasma membrane. There are two reasons for postulating this role for the vesicles: (a) particle-free regions in the plasma membrane are always associated with the sites of vesicle interaction or apposition; and (b) the plasma membranes of fusion-arrested myoblasts (those treated with either PLC, BUDR, or CHX) contain few, if any, of these particle-free regions. In thin sections of fusion-arrested myoblasts, the vesicles have not been detected in close proximity to the plasma membrane, with the exception of the EGTA-treated myoblasts. Vesicles are present in EGTA-treated myoblasts, but no physical contact has been observed between the vesicle membrane and plasma membrane as can be seen in normal myoblasts.

The plasma membrane of EGTA fusion-arrested myoblasts also contains particle-free regions, although the majority of these are small in size and associated with only a single vesicle. The presence of these elements in the EGTA fusion-arrested cells, and not in the other arrested populations, may be related to the fact that the EGTA treatment blocks fusion at a much later stage than the other arrest treatments. Since the cellular Ca++ concentration of EGTA-treated myoblasts is unknown, it is impossible to determine whether any relationship exists between vesicle-plasma membrane interactions and Ca++ concentrations. Nevertheless, Mg++ ions are still present during the EGTA treatment, and certain membrane interactions can be affected by high concentrations of Mg++ ions (15). Finally, the fact that there is a dramatic increase in the size of the vesicle-associated particle-free regions after the release of EGTA-arrest for 6 h strongly suggests that this is an important event in membrane fusion that appears to be somewhat calcium dependent.

The proposed role of lipid-enriched membranes (vesicles) in the myoblast fusion process is consistent with observations that have been made on a variety of biological and model systems. The idea that membrane fusion is primarily lipid-mediated (requiring the initial depletion of membrane proteins) was originally suggested by Akhong et al. (1). Recently, the formation of

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### TABLE I

**Distribution of Particle-Free Regions in Various Muscle Cell Populations**

| Treatment                    | No. cells | % with PFR | Total | Large |
|------------------------------|-----------|------------|-------|-------|
| Normal (48 h in culture)     | 78        | 68         | 30    | 87.0  | 38.4  |
| BUDR (48 h in culture)       | 40        | 2          | 0     | 5.0   | 0     |
| PLC (48 h in culture)        | 127       | 10         | 1     | 7.9   | 0.8   |
| EGTA                         | 108       | 50         | 15    | 46.3  | 13.9  |
| EGTA + 6 h reverse           | 69        | 51         | 37    | 73.9  | 53.6  |

See Materials and Methods for procedural details.
FIGURE 3 Thin sections of fusing myoblasts (46 h in culture). (A) The myoblasts already contain myofibrils (MF), and the plasma membranes are joined in two places by a single bilayer (clear arrows). The region of fusion is small in comparison to the total length of plasma membrane between the two cells. The nuclei (N) are indicated in both myoblasts. Bar, 0.2 \( \mu \text{m} \)  \( \times \) 64,260. (B) A single bilayer (asterisks) is present between these myoblasts (cell 1 and cell 2), and this bilayer is continuous with both plasma membranes. The empty cytoplasmic regions (arrowheads) adjacent to the single bilayer may result from the breakdown of unilamellar vesicles. The nucleus (N) is indicated in cell 2. Bar, 0.1 \( \mu \text{m} \)  \( \times \) 130,000.
**FIGURE 4** Freeze-fracture replicas of fusing myoblasts. (A and B) In both images cell 2 is situated on top of cell 1, the outer membrane half of cell 2 (E) is located on top of the inner membrane half (P) of cell 1. The fusion regions between cell 1 and cell 2 are characterized by the small depressions (arrows) that extend from the E face towards the P face. Note that here also, like in the thin section images (Fig. 3), the regions of fusion comprise only a small portion of the plasma membrane surface, although most of the E-fracture face in (B) is particle-free. Also, the particle-free regions in the plasma membrane (arrowheads) (B) are associated with some bulging (protruding) components from below. (A) 51 h in culture. (B) 47 h in culture. (A and B) Bar, 0.2 μm. × 64,260.

**FIGURE 5** Unilamellar vesicles (V) interacting with the plasma membranes (clear arrows) of adjacent fusing myoblasts (46 h in culture). (A) The myoblasts are not fused, and a vesicle (V) is present in the region of contact between the plasma membranes. × 130,000. (B) The plasma membranes are fused; both membranes are continuous (arrowhead), and a vesicle (V) is physically involved in the region of fusion. Bar, 0.1 μm. × 110,160. (C) The myoblasts are connected by a single continuous bilayer (arrowhead), and a vesicle (V) is next to the bilayer. × 125,070.
particle-free areas in the plasma membrane has been observed as a common feature in several systems where membranes fuse: during exocytosis of secretory vesicles (15, 20, 26, 30, 32), and during the acrosomal reaction in guinea pig sperm (10). The myoblast fusogenic vesicles, in fact, may participate in fusion in the same manner as liposomes that are capable of artificially inducing cell fusion (22, 28).

In the proposed model, the second step in the sequence of events leading to myoblast fusion is the fusion of the vesicle membrane with the plasma membrane to form a single bilayer. The assumption that this intermediate step exists is based upon the direct observation that vesicles are fused with the plasma membrane, without resulting in cell fusion. This observation may also simply represent a random event that will not necessarily result in cell fusion. The next event, in the proposed fusion sequence, is the fusion of two particle-free plasma membrane regions into a single particle-free bilayer. Although we have, arbitrarily, divided the fusion process into a series of sequential events, these events apparently can occur simultaneously. For example, fusion can apparently occur at the same time between at least three membrane systems (the vesicle membrane and the two plasma membranes) since all three can be connected by a single particle-free bilayer (Fig. 6). At present, it is not clear whether two vesicles, one in each myoblast, are required to generate fusion or only one vesicle is sufficient to initiate the whole process. There is some evidence (Fig. 3B) that two vesicles may participate in the fusion process; however, there is no experimental basis to rule out the possibility that only a single vesicle is required. This is described schematically in the left portion of the model (Fig. 8).

The first step in the formation of particle-free regions in the plasma membrane would not be sufficient to generate fusion, and probably active participation of the vesicle membrane is required. Although the surface area of the particle-free regions is usually quite large (Fig. 4B), fusion is confined to only a small portion of these "smooth" domains (Figs. 3 and 4). The fusion of the vesicle membrane with the plasma membrane can be hypothesized as an insertion of some essential fusogenic phospholipids into the lipid portion of the plasma membrane, and these lipids are responsible for facilitating the fusion between the two plasma membranes. Some physical measurements carried out on fusing myoblasts indicate that there is a change in the fluidity of the plasma membrane during fusion (14, 34). These results are consistent with our morphological observations of insertion or incorporation of the vesicle membrane into the plasma membrane at the same time.

The results presented in this study were primarily obtained from cultures of fusing muscle cells; however, we also examined thin sections of fusing muscle cells in vivo. The results were very similar; vesicles, as well as fused plasma membranes, were found at different stages. Therefore, the myoblast fusion events studied in culture are probably closely related to those that exist in vivo. The culture system was exceedingly more desirable for defining these events because of its simplicity and accessibility.

**Fusion and Gap Junctions**

It has been suggested by several investigators that gap junctions might be involved directly in the fusion process. In particular, Rash and Stachelin (36) suggested that gap junctions normally form immediately before myoblast fusion, and thus perhaps mediate the initial structural events of fusion. Our data indicate that gap junctions...
FIGURE 7 Thin section of fused muscle cells in 13-d-old chick embryo thigh muscle. (A) Small regions of cytoplasmic continuity are present between cell 1 and cell 2 (large arrows). Note the row of small membrane sacs or cisternae between the arrows. Also, two vesicles (V) are found in cell 2 interacting with the plasma membranes of cells 1 and 3. Bar, 0.5 μm. × 28,950. (B) High magnification image of the cytoplasmic continuity (arrows). Note the closed membrane sacs (S) and the attached remnants of cell-cell contact elements (clear arrows). Bar, 0.2 μm. × 45,900.
FIGURE 8 Myoblast fusion model. Fusion is initiated between cell I and cell 2 by the cytoplasmic unilamellar particle-free vesicles (V). Fusion may result from a single vesicle residing in one of the two cells (left hand side), or from vesicles in both cells (right hand side). ——— particle-free membrane; membrane with particles. Nucleus (N) and lipid droplets (LD) are also indicated.

Vesicles: Reality or Artefact

Particle-free unilamellar vesicles are not uniquely found in myoblasts before fusion. Similar vesicles are found, for example, in the nerve growth cone (31). These vesicles in the growth cone are believed to serve as a source of lipid for the expanding plasma membrane. It is important to indicate, however, that particle-free membrane protrusions and vesicles have been regarded by some investigators as an artefact of glutaraldehyde fixation, for example see the recent study of Hasty and Hay (12). It has not been within the scope of this study to resolve this difficult problem of fixation artefacts. Fixation “reality” is difficult to define; for example, is the “optimal” fixation condition one that preserves or eliminates certain structures? Therefore, we have based our conclusion that the unilamellar vesicles and the particle-free regions in the myoblast plasma membrane are not artefactual, but rather important elements in the fusion process, on the following experimental data: (a) The
vesicles have been detected only in fusion-competent cells and not in either fusion-arrested cells (BuDR, PLC, or CHX) or mature myotubes, although all these different muscle populations were fixed identically (see Materials and Methods). It is important to stress that the cells were fixed with a physiological buffer (2.5% glutaraldehyde in PBS) rather than sodium cacodylate. (b) The incidence of the particle-free regions and their size is directly related to the fusion competency of the myoblasts (Table I, EGTA-reversed myoblasts). (c) The vesicles and the particle-free regions are found in freeze-fracture replicas of unfixed unglycerinated normal myoblasts (17) that were frozen by the same procedure utilized for fixed and glycerinated cells (18). (d) We have fixed myoblasts (46–51 h after plating) with a combined fixative (12), i.e., glutaraldehyde and osmium tetroxide, a procedure that was reported to eliminate the artefactual blisters and vesicles (12). Unfortunately, this procedure did not lead to an improved resolution of the muscle ultrastructure; most of the unilamellar vesicles disappeared from the myoblasts leaving "holes" in the cytoplasm, many portions of the plasma membrane were also missing, and the vesicles that were detectable contained only one leaflet of the trilaminar membrane image. Similar images were apparently also obtained by Hasty and Hay (12). This could be explained on the basis that certain phospholipids are not fixed by treatment with OsO4; for example, egg lecithin, after reaction with OsO4 is unstable and dissolves (6). Therefore, it is possible that the lipid regions in the plasma membrane adjacent to the vesicles in fusing myoblasts, as well as the vesicles themselves, are extremely sensitive to the glutaraldehyde-osmium fixative. This could result in the appearance of the "lesions" in the fixed cells. On the other hand, glutaraldehyde, which is a cross-linking reagent, reacts with and cross-links amines (37) in proteins and also will cross-link phosphatidyethanolamine and phosphatidylserine in membranes (38). Thus, if the vesicles are enriched with these two phospholipids, they could be cross-linked by the glutaraldehyde and preserved during the processing for ultrastructural analysis. (e) Finally, we have isolated a subcellular fraction of low density vesicles (d \leq 1.065) from prefusion myoblasts (unpublished results). The vesicles in this fraction are not necessarily the same fusion-related vesicles, but they are particle-free, of similar dimensions, and they are obtained without exposure to any of the fixation procedures.

In conclusion, it should be noted that the precise relationship of the vesicles to the fusion competency of the myoblasts cannot be resolved in this study. In fact, it is possible that these vesicles might be involved in a completely independent event that occurs simultaneously with fusion.

In this study, we have presented some ultrastructural evidence for the membrane events associated with myoblast fusion. Further, we have utilized this information to generate an interpretative model for the fusion process that is centrally focused on the importance of the particle-free vesicles and the particle-free plasma membrane domains. This model has been presented because it is consistent with the available data (albeit dependent on interpretation), and it provides a constructive framework for future experimentation on myoblast membrane fusion.

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