Freeze-Cleave Demonstration of Gap Junctions between Skeletal Myogenic Cells In Vivo

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Developing muscle masses from hind limbs of 19-day fetal rats were freeze-cleaved, platinum and carbon replicated, and examined electron microscopically. Gap junctions were observed linking cell pairs clearly identified as myogenic by the presence of easily recognized and characteristic arrays of cross or longitudinally fractured myofibrils. Occasionally gap junctions were also observed between identified nonmyogenic cells, but none were observed between myogenic-nonmyogenic cell pairs. Because the recently formed conjoint myogenic cells were already encapsulated by developing basal laminae and normally would have fused to form discrete myofibers, we suggest that this report provides additional evidence that gap junctions normally form immediately before and thus perhaps mediate the initial events of myogenic cell fusion in vivo as well as in vitro.

INTRODUCTION

In a recent report detailing the use of combined electrophysiological and electron microscopic techniques in the investigation of muscle cell fusion in culture, Rash and Fambrough (1973) presented evidence that the cells developed low resistance electrical coupling immediately before fusing and that all coupled but not yet fused cell pairs exhibited regions of close membrane apposition similar to images obtained from thin sections of gap junctions (Revel and Karnovsky, 1967) in other tissues. Since high resolution goniometric analysis of the complete serial section records revealed that noncoupled cells lacked the “close junctions” and since no other types of membrane appositional complex were observed between either coupled or noncoupled cell pairs, Rash and Fambrough (1973) concluded that the close (or “gaplike”) junctions were the sites of electrical coupling, and thus functionally equivalent to true gap junctions. (Gap junctions are now almost universally accepted as specialized areas of close membrane apposition which permit the direct intercellular exchange of ions (electrical or ionic coupling) and low molecular weight substances (metabolic coupling). For reviews and recent references see DeHaan and Sachs, 1972; Goodenough and Revel, 1970; McNutt and Weinstein, 1973; Satir and Gilula, 1973; Staehelein, 1974.) Because the gaplike junctions coupling myogenic cells in vitro (a) were formed or at least became functional just before cell fusion, (b) were present in the immediate vicinity of the initial fusion pore, and (c) disappeared shortly after fusion, and because cytoplasmic fusion occurred without detectable ionic leakage, Rash and Fambrough proposed that the gaplike junctions may also participate in the normal process of myogenic cell fusion in vivo, perhaps as the closely apposed sites thought to be required for the initiation of membrane fusion.

In contrast to the situation in tissue culture, the difficulties of finding and of clearly demonstrating gap junctions in thin sections of developing skeletal muscle masses in vivo is indicated by the fact that only one report (Kelley and Zacks, 1969) has appeared in which areas of close membrane apposition (there termed “close” and “tight” junctions) are clearly demonstrated. To circumvent this problem in the present study, we have used the freeze-
Cleave technique (as interpreted by Branton, 1966), which has the advantage over ultrathin sectioning of both tremendously increasing the examinable membrane area and providing different criteria for the positive identification of true gap junctions. Accordingly, we selected a stage during embryonic development when muscle cell fusions are most frequent and the developing myogenic cells are easily recognized by the presence of rudimentary myofibrils. As a result of this investigation, we now present evidence for the general occurrence of true gap junctions between normal myogenic cells of 19-day fetal rat hind limbs and propose that they represent the same structures identified as "gaplike" junctions between electrically coupled myogenic cells in vitro (Rash and Fambrough, 1973) and as "close" or "tight" junctions in thin-sectioned embryonic intercostal muscle (Kelley and Zacks, 1969). We interpret the present results as suggesting the need for further investigations probing possible roles of gap junctions in the initiation and regulation of myogenic cell fusion. We also suggest that the in vitro myogenic system could provide a useful model for studying the passage of electrolytes and informational or regulatory molecules between differentiating cells in vivo and in vitro.

Material and Methods

Freeze-cleave preparations. Hind limbs from 19-day fetal rats were dissected into 0.1 M cacodylate-buffered 2% glutaraldehyde (pH 7.3), minced rapidly into 0.5 mm cubes, allowed to remain in the fixative for 1 h, and then slowly equilibrated to 25% glycerol. After storage at 4°C in 25% glycerol for an additional hour, the tissue samples were placed on standard Balzers specimen holders and quickly frozen in liquid Freon 12 cooled to −150°C with liquid nitrogen. Samples were stored in liquid nitrogen until fractured and replicated in a Balzers freeze-etch apparatus (Balzers, Principality of Liechtenstein) at −105°C. The platinum-shadowed carbon replicas were freed from the cells in Clorox, rinsed in distilled H2O, cleaned with chromic acid, rinsed, and mounted on 200-mesh copper grids and examined with a JEM-100B electron microscope.

Results

Nineteen-day fetal rat hind limbs were selected for the present study in order to maximize the chances for observing gap junctions between myogenic cells. In the fetal rat, the hind limb bud does not appear until about day 9, premuscular mesoderm is not recognizable until day 11, and separate muscle precursor masses are not recognizable until day 13 (Millaire, 1965). Most of the cells comprising these masses remain in an undifferentiated, proliferative state until about day 16. From day 16 to day 19, both proliferation and differentiation of cells occurs, so that by day 19, many developing myofibers are recognized, each surrounded by a developing basal lamina (cf. Kelley and Zacks, 1969; and Bash, unpublished observations). At this stage, the developing basal laminae have already enclosed many discrete groups of differentiating mono- and multinucleate myogenic cells. During the following 24 hr most of the myogenic cells within a single basal lamina fuse, forming a single central myofiber. Those mononucleate cells which do not fuse to the central myofiber apparently are trapped in a nonproliferative, nondifferentiated state, and are known as "satellite" cells. After parturition (day 21), cell proliferation and fusion continue to occur but at a much reduced rate.

Although developing muscle masses from the 19-day fetal rat hind limb contain several types of tissue in addition to myogenic cells, including developing nerves, arterioles, venules, capillaries, and fibrocytes, it was quickly recognized that most nonmyogenic cell types are easily recognized by characteristic arrays of membrane particles, pits, and vesicles and
by internal (cytoplasmic) organization (A description of differentiating cell types based on appearance in freeze-cleave preparations is not within the scope of this report.) Developing myocytes, however, often can be unambiguously identified by the presence of the unique hexagonal array of cross-fractured thick and thin filaments (Fig. 1, cf. also Rayns, 1972). In order to avoid confusion concerning the identification of cell types, the data we present below is derived only from cell pairs in which at least one cell contained well formed myofibrils and the other, either rudimentary or well formed myofibrils. Further, the presence of an encapsulating basal lamina usually was detected around groups of myogenic cells. In Fig. 2, for example, a freeze-cleaved bi- or oligonucleate myogenic cell was seen associated via gap junctions to a well developed multinucleate, myofibril-packed myotube (not shown). The most prominent and easily recognized feature is the nucleus with its characteristic nuclear pores. Surrounding the nucleus is the very granular cytoplasm, which contains several unidentified membrane profiles and a single rudimentary myofibril (inscribed area). At higher magnification (Fig. 2b), the unique 400 Å spacing and regular hexagonal array of thick filaments plus the faintly resolved hexagonal pattern of fractured thin filaments (circled area) is more readily discerned. (The few filaments and the general low level of cytoplasmic organization within the limited area of cleaved cytoplasm, however, suggests that this region of the bi- or oligonucleate cell may have begun to synthesize contractile proteins only very recently.) Most of the remaining area of Fig. 2a is occupied by the A face (by definition the cleaved internal face of the cytoplasmic leaflet) of the immature myocyte plasma membrane, on which numerous small pits (thought to represent the openings of pinocytotic vesicles) and small, randomly scattered particles can be observed. Closer examination of the A-face, however, reveals three relatively large gap junctions, composed of irregular arrays of 8–9 nm particles (arrows), the arrays corresponding to the “type I” gap junction of Staehelin (1972). Typically, the particles are arranged into clusters of 2–8 particles (Fig. 2c), which only rarely assume the close packed hexagonal configuration.

On the complimentary B faces of myogenic cell membranes, the gap junction regions were recognized as arrays of small pits, either in the close-packed hexagonal configuration (Fig. 3a) or in the more common “cluster” pattern. In the cluster pattern the gap junction B faces often have a mottled appearance (Fig. 3a), with groups of pits surrounded by minute areas of nonpitted B-face membrane. Gap junctions intermediate in packing density between the hexagonal array and the cluster array were also observed (Fig. 3b). On several fortuitous occasions, gap junctions were observed in areas where the fracture plane passed from within the plasma membrane of one cell to that of the closely apposed cell (Fig. 3b), thereby demonstrat-
FIG. 3. (a) B-face view of a gap junction linking two myogenic cells. Notice that the pits can be arranged either in clusters (small arrow) or in closely packed arrays (large arrow). (b) Micrograph of two gap junctions linking myogenic cells. The adjacent plasma membranes have been cleaved so as to reveal the association of particles on the A face and pits on the B face within each gap junction. (a) x 135,000; (b) x 185,000.

In the present study we have demonstrated that true gap junctions occur routinely between prefusion myogenic cells in vivo. Future, more detailed studies will be required in order to establish the role of these junctions, their number and disposition during limb and/or muscle morphogenesis, and their possible similarities to other gaplike junctions frequently observed between cells of the premuscular mesenchyme (Hay, 1968).

DISCUSSION

Rash and Fambrough (1973) recently demonstrated that most (if not all) myogenic cells in culture develop low resistance electrical coupling immediately prior to

FIG. 2. (a) Prefusion myogenic cell. Three gap junctions (GJ) may be recognized as arrays of particles on the A face of plasmalemma. A single rudimentary myofibril (upper inscribed area and Fig. 2b) is observed in the limited area of cleaved cytoplasm. The characteristic 400 Å hexagonal array of thick filaments (Fig. 2b) and the faint hexagonal array of thin filaments (circle) are partially obscured by the very low local shadowing angle. A portion of one gap junction (lower inscribed area) is enlarged (Fig. 2c), revealing the 8-9 nm subunit particles in the “cluster” configuration. Nucleus (N), pinocytic vesicle pits (PV). (a) x 25,000; (b) x 80,000; (c) x 95,000.
cytoplasmic fusion and in thin sections observed regions of close membrane appositions similar to gap junctions between all such coupled cells. Because fusion appeared to occur only after electrical coupling, they proposed that the gaplike junctions may participate in the normal process of myogenic cell fusion, either as sites for the transfer of information molecules (cf. Gilula, et al., 1972), or as the fusion initiation site (cf. Satir et al., 1973). It was also suggested that if these observations were of general significance, then gap junctions should also be observed between in vivo myogenic cells undergoing fusion. Because of the ease with which gap junctions can usually be seen in freeze-fracture replicas, this technique was chosen for the present study, during which we have identified more than 30 gap junctions between 12–15 identified myogenic cells in muscle masses of 19-day fetal rat hind limbs. Since each developing myotube is surrounded by a developing basal lamina, it seems unlikely that any gap junctions could be formed between an enclosed myogenic cell and a cell of another type (fibroblasts or capillary endothelia, for example) and, in fact, none were observed. Thus, we have obtained strong evidence for the normal occurrence of true gap junctions between myogenic cells of fetal rat limbs, both in vivo (the present report) and in vitro (Rash and Fambrough, 1973).

Since many cells develop electrical coupling and the associated gap junctions at some time during development (see DeHaan and Sachs, 1972, and Furshpan and Potter, 1968, for reviews) and since such connections have been implicated in the transfer of molecules thought to regulate the differentiation of diverse cell types (Gilula et al. 1972), it is possible that the gap junctions observed during myogenesis in vitro serve no role in the fusion process, but only reflect the terminal phase of some other obligatory coupling phase occurring normally in vivo. Perhaps this is the mechanism operating during coupling in myogenesis, but we must note (Rash and Fambrough, 1973) that gap junctions are routinely formed between postmitotic myofibril containing cells which are already clearly committed to a single differentiated state (Bischoff and Holtzer, 1969). Thus, the formation of gap junctions shortly before myogenic cell fusion (in vitro, at least) and their almost immediate breakdown during fusion suggest to us that these junctions may not serve solely as channels for the exchange of routine informational molecules, but that they may participate either directly or indirectly in the process of cell fusion. Stated differently, we suggest that the gap junctions may either (1) function directly in the passage of molecules needed in order to initiate and/or coordinate the fusion events in both cells, (2) participate directly in the process of initial membrane attachment and fusion, or (3) contribute in a yet unknown way to the normal process of myogenesis.

Since no one has yet observed the initial ultrastructural events in the fusion of skeletal myogenic cells, either in thin sections or in freeze-cleave preparations, perhaps it is premature to speculate about possible ultrastructural mechanisms of membrane attachment and fusion. Yet we must note that many examples of membrane fusion occur in nature, and surely we would be remiss if we failed to consider the similarities evidenced by certain of these. For example, recent freeze-etch studies of mucocyst secretion in *Tetrahymena* (Satir et al., 1973) and synaptic vesicle release from vertebrate nerve terminals (Pfenninger et al., 1972) may be particularly informative. Although it can be argued that fusion of cytoplasmic vesicles to the inside of surface membranes may not necessarily be similar to the fusion of exterior membrane leaflets (as in muscle cell fusion), it must also be suggested that basic mechanisms for attaching and stabilizing the apposed prefusion membranes must occur before the intermembrane molecular interactions of
membrane fusion can follow. It is thus tempting to speculate that the gap junction particles which form a physical link between the prefusion myoblast membranes may be functionally similar to the particles observed at the sites of mucocyst-plasma membrane fusion in *Tetrahymena* (Satir et al., 1973). Further studies must be completed before it can be ascertained whether the initial pore for myogenic cell fusion is, in fact, initiated within areas linked by gap junctions. To investigate this possibility, we contemplate combined electrophysiological and freeze-etch studies of myogenic cell fusion in *vitro* using the techniques of Rash and Fambrough (1973) and Pfenninger (1972).

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