CELL-TO-CELL COMMUNICATION AND MYOGENESIS

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ABSTRACT

Cell-to-cell communication was characterized in prefusion chick embryo myoblast cultures, and it was determined that the prefusion myoblasts can interact via gap junctions, ionic coupling, and metabolic coupling. The biological relevance of this communication was supported by the detection of gap junctions between myoblasts in embryonic muscle. Communication was also examined in fusion-arrested cultures to determine its potential relationship to fusion competency. In cultures that were fusion arrested by treatment with either 1.8 mM ethyleneglycolbis-(β-aminoethyl ether)N,N' -tetraacetic acid (EGTA), $3.3 \times 10^{-6}$ M 5- bromodeoxyuridine (BUdR), or $1 \mu g/ml$ cycloheximide (CHX), both gap junctions and ionic coupling were present. Therefore, it is possible to conclude that cell communication is not a sufficient property by itself, to generate fusion between myoblasts. The potential role of communication in myogenesis is discussed with respect to these observations.

KEY WORDS cell-to-cell communication, gap junctions, ionic coupling, metabolic coupling, myoblast fusion, myogenesis

The differentiation of skeletal muscle in vivo and in vitro (in culture) is accompanied by the fusion of mononucleated muscle cells (myoblasts) into multinucleated mature muscle fibers (58). This fusion event, in culture, is preceded by a period of cell multiplication and cellular interaction. During this time, the cells are closely apposed and aligned in multicellular strings; this stage of differentiation has been referred to as the "prefusion lag period" (61). Under standard culture conditions this lag period is quite constant, and only cells which have completed their prefusion changes participate in the formation of multinucleated fibers (59). The fusion process that follows the lag period has been previously regarded as a critical event in myogenesis. However, at present, it appears that it is just one of the important events that contribute to the formation of a differentiated muscle fiber (1, 12, 16, 19, 26, 31, 34, 36, 41, 54, 55, 56, 57, 62). The actual fusion process requires a direct physical interaction of the plasma membranes from adjacent cells, and this had led to a series of investigations of the cell surface elements that may be involved in regulating fusion (5, 14, 20). Other studies have suggested that fusion is not necessarily regulated by cell surface components; it may, in fact, be under the direct control of intracellular elements (62).

Recently, it has been reported that a specific type of cell contact, the gap junction (30), and low-resistance pathways (6) are present between muscle cells during amphibian myotome development in vivo. In both studies, it was suggested that gap junctional communication between the muscle cells is related to the efficient transfer of excitatory stimuli from somite to somite, and not to cell fusion. Data from studies on embryonic rat and chick skeletal muscle cells in culture indicate the possible existence of ionic coupling (measured indirectly) and gap junctionlike structures between fusing cells (42). Also, typical gap junctions have been identified in freeze-fracture replicas of
developing rat muscle in vivo (43). Thus far, junctional communication has not been found between mature innervated muscle fibers. Therefore, from earlier studies it appears that gap junctional communication may exist as a transitory state between muscle cells, before and during the fusion process, and that, subsequently, the communication is no longer expressed between the mature innervated muscle fibers.

Currently, gap junctional communication has been characterized as a mechanism that can permit the flow of current, including inorganic ions (3, 22, 33), and the exchange of metabolites such as nucleotides (40, 53) between a variety of interacting cells. Furthermore, the gap junction probably provides the structural channel or pathway for this movement of material from cell-to-cell (23, 24).

The purpose of the present study was to determine if gap junctional communication does exist during the prefusion lag period of myogenesis, and if so, to completely characterize the communication (ultrastructurally, ionically, and metabolically). Also, we have initiated attempts to study the potential role of this communication in muscle differentiation. A preliminary report of this study has previously appeared (29).

MATERIALS AND METHODS

Muscle Cultures

MATERIALS: Fertilized hen’s eggs were obtained from Shamrock Farms (North Brunswick, N.J.). The following items were utilized for this study. Minimum Essential Medium (Eagle) with Earle’s salts without L-glutamine (MEM); Earle’s Balanced Salt Solution (EBSS); penicillin-streptomycin solution; L-glutamine solution; horse serum, heat inactivated; fetal calf serum, heat inactivated (Grand Island Biological Co., Grand Island, N.Y.); trypsin, 1–300 (hog pancreas), ICN Nutritional Biochemicals Div., Cleveland, Ohio); 5-bromo-2'-deoxyuridine (BUdR); cycloheximide (CHX); ethylpeneglycol-bis-(β-aminomethyl ether)N,N'-tetraacetic acid (EGTA) (Sigma Chemical Co., St. Louis, Mo.). All other reagents were analytical grade. Falcon culture dishes (BioQuest, BBL & Falcon Products, Cockeysville, Md.), and Millipore filters (Millipore Corp., Bedford, Mass.) were utilized.

CULTURE MEDIUM: Growth medium consisted of 78.5% MEM, 2 mM glutamine, 10% horse serum, 10% chick embryo extract, penicillin (50 U/ml) and streptomycin (50 μg/ml). The concentration of chick embryo extract in the medium was reduced to 5%, 24 h after the initial cell plating.

CHICK EMBRYO EXTRACT: Chick embryos (11 days old) were washed in cold Puck’s Saline G (6.1 mM glucose, 136.9 mM NaCl, 5.4 mM KCl, 0.1 mM CaCl2, 0.6 mM MgSO4, 1.1 mM Na2HPO4, and 1.1 mM KH2PO4, pH 7.4). The embryos were homogenized by passage through a 50 ml syringe, and then stirred in 2 ml EBSS/1 g tissue for 2 h at room temperature and kept at 4°C overnight. The homogenate was spun for 90 min at 20,000 g in a Beckman model J21B centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The supernate was collected and spun for 60 min at 87,000 g in a Beckman model L5–50 ultracentrifuge, and the resultant supernate was collected. This solution was then filtered successively through Millipore filters of 8.0 μm, 1.2 μm, and 0.45 μm, divided into aliquots, and stored at −18°C.

COLLAGEN: Collagen was prepared according to the procedure of Rubin (48), which is a modification of the technique of Bornstein (7).

NORMAL CULTURES: Primary cultures of 11-day-old embryonic chick thigh muscle were prepared as described by Easton and Reich (15). Myoblasts were plated on either 35 mm (1.5 ml) or 60 mm (3 ml) plastic Petri dishes that were coated with collagen and pretreated (conditioned) with MEM containing 1% fetal calf serum according to Bornstein (8). The cells were plated at initial densities of 2–3.5 × 106 cells/ml. The cultures were maintained in a 5% CO2 incubator at 37°C.

FUSION-ARRESTED CULTURES: (a) EGTA-arrested myoblasts were obtained by applying culture medium containing 1.8 mM EGTA to the cultures 20–26 h after plating (37). (b) BUdR fusion-arrested myoblasts were obtained by addition of BUdR (to a final concentration of 3.3 × 10−4 M) to a myoblast suspension before initial plating (4). The BUdR-treated cultures were minimally exposed to light in order to reduce the breakdown of BUdR. (c) CHX fusion-arrested myoblasts were obtained by changing the normal growth medium to a medium containing 1 μg/ml CHX, 16–22 h after the initial plating.

Ultrastructural Analysis

MATERIALS: The following materials were utilized. Glutaraldehyde solution 50% wt/wt, biological grade; uranyl acetate (Fisher Scientific Co., Pittsburgh, Pa.); osmium tetroxide (4% aqueous) (Polysciences, Inc., Warrington, Pa.); Ladd Research Industries, Inc., Burlington, Vt.); tannic acid, analytical grade (Mallinckrodt, St. Louis, Mo.).

FIXATION: Samples were fixed in the original culture dishes with 2.5% glutaraldehyde in Dulbecco’s phosphate-buffered saline solution (PBS) (136.9 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl2, 0.5 mM MgCl2, 1.5 mM KH2PO4, and 6.4 mM Na2HPO4) for 15 min at room temperature, rinsed twice with PBS, and then processed either for monolayer thin sections or for freeze-fracture. Ca2+, Mg2+-free PBS was utilized for samples that had been cultured in low Ca2+. In the case of intact tissue, the fixation was carried out for 1 h with
the same initial fixative, and the subsequent processing was similar to a previously described procedure (21).

**Monolayer Thin Sections:** After the glutaraldehyde fixation, the cells were treated for 1 h with 1% osmium tetroxide in veronal-acetate buffer (pH 7.4), followed by buffer rinses. This was followed by treatment en bloc with 0.1%-0.5% tannic acid in 0.05 M cacodylate buffer (pH 7.0) for 30 min (51), washing with 1% Na$_2$SO$_4$ in 0.1 M cacodylate buffer (pH 7.3) for 10 min, and then treated with uranyl acetate in veronal-acetate buffer for 1 h. All of the above treatments were performed at room temperature. The samples were dehydrated through a graded series of ethanol (70%, 95%, and 100%), and embedded in Epon 812 in the original plastic culture dish in which they were grown. The embedded monolayers (after removal of the plastic dish) were examined with a phase-contrast microscope and suitable areas for examination were selected and marked with a slide-marking objective (C. W. French Div., Etec Corp., Bedford, Mass.). Selected areas were then re-embedded and oriented for sectioning in either vertical or horizontal planes (24). Thin sections were cut with a diamond knife on a Sorvall Porter-Blum MT2-B ultramicrotome (DuPont Instruments, Sorvall Operation, Newtown, Conn.) and mounted on copper grids. The sections were stained with uranyl acetate for 2 min and lead citrate for 1.5 min (45) before examination in a Philips 300 transmission electron microscope (TEM) at 80 kV.

**Scanning Electron Microscopy:** Glutaraldehyde-fixed samples were dehydrated through a graded series of ethanol (70%, 95%, and 100%), passed through acetone for 15 min, and dried with a critical point drying system (Sorvall). They were then mounted on aluminum stubs and coated with gold in an Edwards Vacuum Coater (Model 306, Edwards High Vacuum, Inc., Grand Island, N.Y.). Samples were examined in an ETEC Autoscan scanning electron microscope (SEM).

**Freeze-Fracture With a Monolayer Device:** Freeze-fracture replicas of cell culture monolayers were obtained with a modification of previously reported procedures (9, 10, 38). Myoblasts were plated on collagen-coated flat gold alloy specimen carriers (Balzers High Vacuum Corp., Santa Ana, Calif.) which were placed in a well of a multi-well unit (Falcon). A suspension of myoblasts (1.5 ml), which were prepared as described above, were plated at a density of $5 \times 10^6$ cells/ml. The myoblasts settled down, attached, and developed normally on the gold carriers as can be seen with the SEM in Figs. 1A and C. The samples were fixed, as described above, and treated with 25% glycerol in 0.1 M cacodylate buffer (pH 7.3) for 2 h at room temperature, or overnight at 4°C. After removal of most of the solution, each of the gold carriers was covered with a brass disk (2.1 mm in diameter, 0.06 mm thick), as can be seen with the SEM in Fig. 1B. The sandwich was frozen immediately in the solid-liquid interface of Freon-22 and stored in liquid nitrogen. Fracturing was carried out in a Balzers device (Balzers High Vacuum Freeze-Etch Unit BA 360M) at −115°C by knocking off the brass disk with a razor blade mounted in the microtome arm. The replicas obtained by evaporating platinum and carbon were cleaned by treatment with bleach and then examined in the Philips 300 TEM. The freeze-fracture micrographs have been mounted with the shadow direction from the bottom to the top.

**Metabolic Coupling**

**Materials:** The tritium-labeled nucleosides [H]uridine (25 Ci/mmol) and [H]thymidine (6 Ci/mmol), sterile aqueous solutions, were obtained from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N.Y.). Autoradiography emulsion, Ilford K2 emulsion, was obtained from Ilford Ltd. (Essex, England); Kodak D-19 developer and Kodak fixer from Eastman Kodak Co. (Rochester, N.Y.); and Papanicolaou hematoxylin stain (Harris) from Fisher Scientific Co. (Pittsburgh, Pa.).

**Intercellular Nucleotide Transfer:** For the metabolic coupling studies (40), the myoblasts were plated at 1.5-2 × 10^6 cells/ml; the donor cells were plated in 60 mm dishes and the recipient cells in 35 mm dishes. The donor myoblasts were labeled for 4 h before co-culturing with [H]uridine (8.3 μCi/ml) and then washed three times with MEM containing 1% fetal calf serum. The recipient cell nuclei were labeled by treatment with [H]thymidine (3.3 μCi/ml) at the time of the initial plating. Next, medium was added to the dishes, and the donor cells were scraped with a Teflon policeman and spun down with a serological centrifuge at 900 rpm for 5 min. The cells were then suspended in half the original volume and added to dishes containing recipient cells at a ratio of 2:1 (donor:recipient). After 2 h of co-culture, the medium was aspirated to remove the unattached cells, and fresh medium was added. The co-culturing was terminated by fixation with glutaraldehyde (see above). The fixed samples were then dehydrated through a graded series of ethanol and air dried. The dishes were coated with the K2 emulsion (diluted 1 g/ml water) and placed vertically to dry; the samples were then maintained at 4°C in a light-proof container in the presence of a desiccant. The autoradiographs were developed for 4 min with freshly prepared D-19 developer, followed by 5 min in the fixer solution. The cells were then stained with hematoxylin (Harris) for 1 min before examination with bright field optics in a Zeiss Photomicroscope II.

**Electrophysiology**

Electrophysiological studies were performed on myoblasts plated on 35 mm dishes. The culture dishes were mounted on an inverted microscope stage (Leitz Dialvert), and electrophysiological recordings were carried out.
Scanning electron micrographs of a myoblast culture (42 h after plating) that has been prepared for monolayer freeze-fracturing. (A) Gold specimen carrier with the cultured myoblasts. × 20. (B) The gold carrier with the cells covered with a brass disk. The entire "sandwich" is frozen, and the disk is subsequently knocked off by the microtome arm. × 20. (C) Image of the cells that have been cultured on the gold carrier in Fig. 1A. The cells are virtually identical to those cultured on either plastic or glass substrates. Bar, 2.5 μm. × 4,800.
out at room temperature and ambient CO₂ levels. The cells that were selected for impalement were initially photographed and then penetrated with microelectrodes that were filled 2.8 M KCl. The microelectrodes had resistances of 40–100 MΩ. Electrical coupling between myoblasts was determined with the procedure and apparatus that was previously utilized for cultured cells (18).

RESULTS

Characterization of Gap Junctional Communication between Myoblasts before Fusion

Gap junctional communication was examined in muscle cell cultures before fusion (27-44 h after plating). The myoblasts were examined for the presence of (a) gap junctional contacts; (b) ionic coupling, and (c) metabolic coupling.

Ultrastructural Detection of Gap Junctions

THIN SECTIONS: The muscle cells were fixed and processed for thin sectioning as described in Materials and Methods. Clusters of closely apposed myoblasts were selected from the Epon embedded samples, and they were examined in sections parallel to the dish (Fig. 2A) and in sections vertical to the dish (Fig. 2B). In the culture dish, the myoblasts were piled on top of one another into two or three layers so that all of the cells did not appear to interact directly with the substrate. This property can be visualized in both scanning (Fig. 1C) and transmission microscopy (Fig. 2B). In thin sections, gap junctions together with desmosomes were detected between the interacting myoblasts (Fig. 3). The gap junctions were characterized by a close apposition of the two plasma membranes of adjacent cells, and the entire width of this structure is about 16 nm (Fig. 3, inset). In most instances, the space or intervening “gap” is extremely difficult to resolve with conventional staining procedures. In some regions of junctional contact, the cytoplasmic surface contained an accumulation of microfilaments (Fig. 3), similar to regions of junctional contact in various epithelia. Also, some dense amorphous material was frequently associated with the cytoplasmic surfaces of the gap junctions (Fig. 3, inset). The gap junctions were generally small, and they ranged in size from 20-300 nm in length. The average gap junctional size was about 70 nm. Several gap junctions were found between two apposed myoblasts, and the junctions were present between somatic portions of the cells, as well as between cell processes.

Intact chick embryo thigh muscle (8-12 days) was also examined with thin sections for the presence of gap junctions. Small gap junctions were detected between the myoblasts in this tissue (Fig. 4); in this case, from 9-day-old chick embryo thigh muscle. Although gap junctions were detected between different cell types in the embryonic tissue, it was still possible to accurately identify the myoblast junctions since the characteristic myofilaments were already assembled into bundles in the myoblast cytoplasm (Fig. 4). Without serial sectioning, it was impossible to determine if the myoblasts with myofilament bundles were already involved in the fusion process. However, it was possible to identify gap junctional contact on multinucleated muscle fibers. The gap junctions that were found in vivo are virtually identical in thin section appearance and size to those that have been detected in culture (Fig. 4, inset). This strongly suggests that the gap junctions between myoblasts in culture are indeed a relevant feature of myogenesis.

FREEZE-FRACTURE: Typical gap junctions were found in the same prefusion myoblast cultures in freeze-fracture replicas. The replicas were obtained by using a monolayer freeze-fracture approach (see Materials and Methods) that permitted the preservation of the original morphology and alignment of the interacting cells in culture (Figs. 1A and C, and 5). This approach facilitated unequivocal identification of the cell types in the cultures; in this case, it was possible to distinguish between myoblasts and fibroblasts. The gap junctions were detected as small plaques of tightly packed particles, in some cases polygonal, on the inner membrane half (fracture face P) (Figs. 5 and 6). Complementary arrangements of pits or depressions were detectable on the outer membrane half (fracture face E) (Fig. 6A and D). Linear strands and loosely packed particles were also observed in regions of contact (Fig. 6B and C). These particles are larger in size (10 nm) than the junctional particles, and they may be related to the formation of gap junctions between myoblasts. Similar large “precursor” particles have been described and related to the junctional formation process in other systems (2, 11, 28, 44). In general, the size, frequency, and distribution of gap junctions between myoblasts in the freeze-fracture replicas were consistent with
FIGURE 2 Thin section micrographs of interacting myoblasts at low magnification. (A) The cells (40 h after plating) have been sectioned in a plane that is horizontal to the dish. The collagen substrate (C) is detectable as dense, amorphous fibrils in this image. Bar, 2 μm. × 9,090. (B) The cells (43.5 h after plating) have been sectioned in a vertical plane to the dish. The collagen substrate (C) is prominent as a thick, dense carpet on the bottom of the dish. Note the significant amount of overlap between the cells, and the interacting cell processes (cf. Fig. 1 C). Two sites of potential gap junctional interaction are indicated (arrowheads). Bar, 2 μm. × 9,800.
the same information that was obtained in thin sections.

There are a few relevant details that should be mentioned as a result of the ultrastructural studies. (a) Gap junctions were found between myoblasts in cultures 20 h before extensive fusion took place (Fig. 5). (b) It appeared that the gap junctional size was a function of time in culture; the size was frequently larger in 71-h cultures (Fig. 6A) than in younger myoblast populations. (c) Gap junctions were observed between myoblasts and myotubes in thin sections.

**Ionic Coupling:** Myoblasts with clearly distinguishable single cell characteristics, i.e., spindle shape mononucleated cells, were selected for microelectrode impalements, and ionic coupling was examined between these interacting cells 30–36 h after plating. The interacting myoblasts that were selected for impalements frequently had a very small visible area of cell-cell contact (Fig. 7). After impalement with microelectrodes, a pulse of current injected into one cell produced an electrotonic potential in the adjacent cell that indicated the presence of ionic coupling (Fig. 7). In 24 cell pairs examined, 50% were ionically coupled. The resting membrane potential values that were recorded from the myoblasts averaged −7 mV. This value is in agreement with a mean value of −8 mV that was previously reported for similar cells (46).

**Metabolic Coupling:** The presence of metabolic coupling between myoblasts was examined by monitoring nucleotide transfer (40) from "donor" to "recipient" myoblasts. The donor myoblasts were loaded with [3H]uridine, and subsequently added to a population of recipient myo-
FIGURE 4 Thin section of interacting myoblasts in 9-day-old thigh muscle. These myoblasts are already assembling myofibrils (MF), and they are joined by gap junctions (large arrow) and numerous desmosomes (D). × 36,720. Bar, 0.5 μm. Inset: high magnification image of the gap junctional region. The cells are joined by one large gap junctional plaque and two small focal gap junctions (arrowheads). Bar, 0.1 μm. × 110,160.

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batches with [3H]thymidine prelabeled nuclei (see Materials and Methods). After 35 h in culture, the donor cells were loaded with [3H]thymidine (3.3 µCi/ml) for 4 h. The recipient cell nuclei were labeled by adding [3H]thymidine (3.3 µCi/ml) at the time of the initial plating and incubating for a 34 h period. After this time the [3H]thymidine was chased with 0.32 mM thymidine for 6 h.
Figure 6  Freeze-fracture replicas of gap junctional membrane specializations between normal prefusion myoblasts. (A) A large gap junctional particle aggregate is present at the site of interaction between two cells (arrow). This plaque contains both P-face particles and complementary E-face pits or depressions. Another gap junctional plaque (asterisk) is present on the E-face. These cells were fusion-arrested with EGTA (26 h after plating), released from the arrest 39 h later, and examined 6 h after the reversal. Bar, 0.2 μm. × 45,900. (B and C) These two images represent possible stages in gap junctional formation between myoblasts (27 h after plating). In Fig. 6B, a loose arrangement of large "precursor" particles are present (arrows); while in Fig. 6C, a similar population of large particles co-exists with a polygonal aggregate of smaller gap junctional particles. (B) × 104,230. (C) Bar, 0.1 μm. × 104,230. (D) High magnification image of the gap junctional plaque present in Fig. 6A. Note that complementary particles and pits are present. × 104,230.

Subsequently, the donor cells were co-cultured with recipient cells, and samples were assayed with autoradiography for nucleotide transfer after 3, 4, 5, 8, and 17 h of co-culture.

Although metabolic coupling was initially detected in the autoradiographs after 4 h, the amount of transferred label was elevated significantly above background by 8 h. Metabolic coupling is clearly indicated in Fig. 8A, C, and E. The donor cells are heavily labeled, and the recipient cells are distinguished by their labeled nuclei (Fig. 8 B and D). The recipient cells that are metabolically coupled are those cells in which both the nucleus and cytoplasm are labeled (Fig. 8A, C, and E). The label was transferred only by cell-to-cell contact and not via the me-
FIGURE 7 Ionic coupling between prefusion myoblasts (36 h after plating). Photomicrograph of interacting myoblasts that were impaled with microelectrodes to determine ionic coupling. The two cells that were impaled to obtain the coupling information (below) are indicated by the microelectrodes. Note that these two cells are joined by a limited region of contact. Bar, 25 μm. × 410. In the electrophysiological records, the bath potentials for V₂ and V₁ are indicated by the solid undeflected lines. After electrode impalement of the two cells, the resting membrane potentials could be observed as vertical changes from the bath potentials for V₂ and V₁. A pulse of current (I) injected into one cell (V₁) produced a voltage deflection of the bridge, and an electrotonic potential, indicative of ionic coupling, was recorded in the adjacent cell (V₂). Calibration pulse is 20 ms and 5 mV. The vertical line represents 2.5 × 10⁻⁹ A.

dium, as non-interacting cells were not labeled (Fig. 8 B). Also, metabolic coupling was not detected in every interacting donor-recipient pair.

Gap Junctional Communication Between Fusion-Arrested Myoblasts

Three different treatments were utilized to obtain fusion-blocked myoblasts: (a) 1.8 mM EGTA (36), (b) 3.3 × 10⁻⁶ M BUdR (4, 52), and (c) 1 μg/ml CHX (60). All three treatments effectively inhibit the fusion process (Fig. 9). 65 h after plating, fusion is observed in normal cultures (Fig. 9 B), while in fusion-arrested cultures the majority of cells are mononucleated (Fig. 9 D, F, and H).

In the three arrested samples, gap junctions and ionic coupling were detected between myo-
FIGURE 8 Metabolic coupling between prefusion myoblasts. (A, B, C, and E) Autoradiograph of donor-recipient co-cultures (8 h). The heavily labeled cells (with [3H]uridine) are the donor myoblasts. The myoblasts with labeled nuclei ([3H]thymidine) are the recipient cells. Transfer of label or metabolic coupling has occurred only between the interacting cells as seen in A, C, and E; the recipient cells that are metabolically coupled have been indicated R. On the other hand, in Fig. 8B, non-interacting recipient myoblasts are present; note the small number of grains in the cytoplasm which represents the background level. The amount of label in the interacting pair in B (cell indicated with R) is slightly above background, and it might represent a low level of metabolic coupling. (D) Control-autoradiograph of recipient myoblasts. Note that no grains are detectable in the cytoplasm of the [3H]thymidine labeled cells. (A–E) Bar, 25 μm. × 480.

blasts (Table I). The gap junctions were similar in thin section and freeze-fracture appearance to those characterized in normal cultures (Fig. 10A and B). For example, Fig. 10A is a thin section of myoblasts that have been treated with 1 μg/ml CHX 16 h after plating, for a 24 h period; gap junctional interactions are present between the cells. Multiple gap junctional contacts were frequently detected in freeze-fracture replicas of the fusion-arrested cells (Fig. 10B), in this case EGTA-treated myoblasts. In EGTA-arrested cells, the junctional size appeared to increase with
FIGURE 9 Photomicrographs of fusion-competent and fusion-blocked muscle cultures. The cultures were photographed at 41 h (A, C, E, and G) and at 65 h (B, D, F, and H) after plating. The composite contains a normal culture (A and B), EGTA-arrested culture (C and D), BUdR-arrested culture (E and F), and CHX-arrested culture (G and H). Note that fusion (myotube formation) has only occurred in the normal 65 h culture (B). (A–H) Bar, 100 μm. × 128.
**Table I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. sampled</th>
<th>No. coupled</th>
<th>% coupled</th>
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<tr>
<td>Normal</td>
<td>24</td>
<td>12</td>
<td>50</td>
</tr>
<tr>
<td>$3.3 \times 10^{-6}$ M BUdR</td>
<td>29</td>
<td>21</td>
<td>72</td>
</tr>
<tr>
<td>1 μg/ml CHX</td>
<td>22</td>
<td>10</td>
<td>45.2</td>
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<tr>
<td>1.8 mM EGTA</td>
<td>45</td>
<td>13</td>
<td>28.9</td>
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the length of the arrest period. In BUdR-arrested populations, the junctional size was not detectably affected by the drug treatment. However, there were no systematic attempts made in this study to relate junctional size to a specific drug treatment or the length of the arrest period.

Ionic coupling was detected in all three cases, as summarized in Table I. In the BUdR-arrested population, ionic coupling was detected in 72% of the 29 cell pairs that were examined. No qualitative difference could be distinguished between resting membrane potentials recorded from fusion-arrested and fusion-competent populations. After treatment with CHX (1 μg/ml), coupling was observed in 45% of the 22 cell pairs sampled. In the EGTA-arrested cultures, coupling was detected in only 29% of the cells sampled, and the input resistance and the resting membrane potential of these cells were very low. This low incidence of coupling is probably related to the enhanced fragility of the cells in the absence of calcium, as indicated by their response to microelectrode penetration.

**DISCUSSION**

In the present study we have determined that gap junctional communication is present during myogenesis in culture. The communication in the culture system has been extensively characterized with several approaches (ultrastructural, electrophysiological, and metabolic transfer), and it was demonstrated that myoblasts can interact: (a) via typical gap junctions; (b) via low-resistance electrotonic pathways; and (c) via a cell contact mediated metabolic exchange (transfer). Since cell contact is a requirement for myoblast fusion and gap junctions are one type of specialized cell contact, we have also examined the gap junctional communication properties of myoblast cultures that were reversibly arrested from fusion by treatments with a variety of agents. In the fusion-arrested populations, the gap junctional communication was present and virtually identical to that detected in the fusion-competent populations. Therefore, it is possible to conclude that gap junctional communication, by itself, is not sufficient to generate myoblast fusion.

In order to establish that gap junctional communication exists during myogenesis, it has been extremely important to recognize and effectively deal with several problems that are endogenous to the muscle cell system. Some of these problem areas are discussed below. (a) **Distinguishing between myoblasts that are fused vs. myoblasts that are communicating.** Since fused myoblasts are also ionically coupled as a result of cytoplasmic continuity, it was necessary to carefully select cells from early prefusion stages (27–44 h). Cells were selected for electrophysiological analysis on the basis of their single cell appearance with phase microscopy. Similar cells were examined with thin-section electron microscopy, and none of the selected cells were fused or in the process of fusion as observed previously (25, 32, 50). Therefore, it was possible to establish, with confidence, that the communication was a result of gap junctional low-resistance pathways and not cytoplasmic continuity between fusing cells (42). The measurements were not made on earlier stages since the alignment of the myoblasts starts at 20 h after plating and the incidence of cell clusters is very low at that time. (b) **Distinguishing between membranes of adjacent myoblasts that are involved in fusion vs. those involved in gap junctional communication, i.e., two fusing membranes have a similar appearance to gap junctions in thin sections.** After initiating this study, it became clear that fusing cells are practically never encountered with the sampling procedure that was used in the 27–44 h cultures. In addition, it was extremely difficult to study fusion even when it was the primary objective of the selection procedure. Furthermore, in recent studies on cultures of synchronized fusing cells we have found that there is no difficulty in distinguishing between membrane fusion and gap junctional membranes,1 in freeze-fracture replicas, the intramembrane differentiation associated with gap junctions is completely different from the sites of membrane fusion that are characterized by smooth particle-free regions. (c) **Distinguishing the specific cell types, i.e., myoblasts or fibroblasts, that are participating in the gap junctional communication.** This difficulty has

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FIGURE 10 Thin section and freeze-fracture appearance of gap junctions between fusion-arrested myoblasts. (A) Thin section of two myoblasts that have been treated with 1 μg/ml CHX (16 h after plating), and fixed after 40 h in culture. The nuclei (N) are close to the region of cell-cell interaction, where a number of possible gap junctional contacts are present (arrows). The contact at the top of the micrograph is definitely a gap junction, while the other two are more difficult to resolve clearly. Bar, 0.2 μm. × 91,800. (B) Freeze-fracture appearance of gap junctions (arrows) between myoblasts that have been treated with EGTA. EGTA-containing medium was applied 21 h after plating, and the culture was fixed after 49 h in the arrested state. The junctions are detectable on both P- and E-fracture faces. Bar, 0.2 μm. × 73,440.
been overcome by carefully integrating the information from monolayer thin section and freeze-fracture techniques. By utilizing this approach, it has been possible to unequivocally distinguish between myoblasts and the contaminating fibroblasts. In general, the two cell types can be distinguished with this approach on the basis of cell shape, cytoplasmic content, freeze-fracture plasma membrane features, and gap junctional structures. With regard to gap junctional elements, the fibroblastic gap junctions are characteristically distinguishable from those between myoblasts; the fibroblastic structures are usually larger, and the plaques contain many particle-free aisles.

Our sampling was primarily focused on myoblast-myoblast interactions as opposed to myoblast-myotube interactions. The latter were the focus of a previous study (42). Rash and Fambrrough (42) deduced from their studies that most of the electrical coupling between the cell pairs was established a few minutes before fusion and therefore might be the initiator step for fusion. Since their method for detecting ionic coupling was based on the presence of acetylcholine receptor activity, this method had limited their measurements to a later stage of myogenesis when the receptor activity is expressed (36), i.e., ~50 h in culture. From our studies on younger cultures, it was clear that gap junctions were present at least 20 h before fusion. From our in vivo studies, gap junctions were observed between presumptive myoblasts in 8-day-old thigh muscle and between myoblasts in 9-day-old thigh muscle. The only difference which was detected in the culture system was that the size of the gap junctions appeared to be dependent on the duration of cell contact. For example, in fusion arrested (EGTA treatment) and released cultures which have had cell contacts prolonged for about 30 h, the gap junctional size was detectably increased above the sizes expressed under normal conditions (Fig. 6A).

After establishing the existence of gap junctional communication between myoblasts, in our efforts to clarify the role of this communication in myogenesis we initially examined the possibility that gap junctional communication is directly involved in the fusion process. A logical approach was to inhibit fusion, and to determine whether the inhibition mechanism operates via breaking down gap junctional communication. Since the fusion process can be manipulated in the culture system, it was possible to characterize communication in cultures that had been fusion arrested by several different treatments. (a) Removal of calcium. Calcium ion is required for myoblast fusion (49); cultures treated with a low-calcium medium (49) or with the Ca**-chelator, EGTA (37), do not fuse. There have been several recent reports that fusion can be selectively blocked by calcium depletion from the medium without any apparent inhibition of the synthesis of several major components that mark the course of muscle differentiation, i.e., the appearance of muscle proteins such as the acetylcholine receptor (36), acetylcholinesterase, and adenylate cyclase (41), contractile proteins (16, 19, 34, 57), creatine phosphokinase activity (31, 55, 56, 62) and the elevation of cAMP levels (62). (b) BUdR treatment. Treatment of the myoblasts with the thymidine analogue, BUdR, has various effects on myoblast differentiation, and the expression of the inhibitory effect is a function of the BUdR concentration (27). At a low concentration, 3.3 x 10^-6 M, it has been reported that BUdR primarily causes an arrest of fusion (27, 31). (c) Inhibition of protein synthesis. Protein synthesis is also a requirement for myoblast fusion. CHX, a protein synthesis inhibitor, blocks fusion if added at the proper time (60).

In fusion-arrested myoblast cultures treated separately with EGTA, BUdR, and CHX, the myoblasts interacted with gap junctions, and they were ionically coupled, even though they failed to fuse. The communication in the EGTA fusion-arrested cultures may initially seem unexpected, since calcium is critically involved in regulating communication between insect epithelial cells (47). However, a similar role for calcium has not been well established for communication between vertebrate cells (23, but see also references 13 and 35). The fact that BUdR, which is incorporated into the myoblast DNA (4, 52) did not affect gap junctional communication might indicate that this myoblast property is not acquired during the later stages of myoblast differentiation, but that it is a pre-existing property probably expressed in many embryonic cells. The fact that CHX, which was added to the cultures 16 h after plating, did not affect gap junctional communication indicates that the gap junctional proteins are present at this early time in the myoblast since no protein synthesis occurred. Similar observations...
were reported recently in another system (17).

The variable incidence of ionic coupling observed in normal (50%), BUdR (72%), and CHX (45%)-treated myoblast cultures may reflect poor electrode penetration or uncoupling produced by damage from electrode impalements. Also, the low coupling incidence must be related to the fact that all measurable impalements were included for the final calculations even though the impalements may not have been adequate for optimal demonstration of coupling. The difficulty and instability of the impalements is further supported by the fact that the resting membrane potentials were very low. Myoblasts treated with EGTA showed a lower percentage of coupling, and this may be related to a lower input resistance, which would reduce the electrotonic potential below the limits of detection.

Since the three different blocking treatments did not inhibit or interfere with the gap junctional communication, it is clear that the presence of gap junctions is not sufficient to generate myoblast fusion. In addition, the junctions are probably not an integral part of the membrane components involved in the fusion process as suggested by Rash and Staehelin (43). 1

In attempting to clarify the role of gap junctional communication in myogenesis, we decided to examine the possibility that the myoblasts are metabolically coupled, i.e., capable of transferring metabolites. For this purpose, we utilized the well-characterized system of nucleotide transfer (40). Pitts and Simms (40) used the radioactively labeled nucleoside $[^{3}H]$uridine as a marker for metabolic coupling, and they found a transfer of the labeled material between communicating cells. In addition, they demonstrated that the radioactively labeled material that was transferred was the incorporated $[^{3}H]$uridine nucleotide compounds and not a high molecular weight molecule such as RNA. In our study, we have found that the myoblasts are capable of transferring the $[^{3}H]$uridine-related nucleotides presumably via the gap junctions and not through the medium (Fig. 8).

Since we have found gap junctions, ionic coupling, and metabolic coupling between prefusion myoblasts, during their lag period interaction, it is possible to conclude that cell communication is a characteristic property of these cells. Furthermore, the cells are potentially capable of utilizing this mechanism for exchanging a variety of cytoplasmic metabolites that may play a significant regulatory role in myogenesis. The structural pathway for this metabolic exchange is most likely the gap junction, as has been demonstrated previously in other systems (24, for review, see reference 23).

At the present time, it is impossible to determine the potential role of cell communication in myogenesis. From the observations in this study, it is reasonable to conclude that cell communication does not provide the only regulatory mechanism for myoblast fusion, since the fusion-arrested cells are still communication-competent. However, it is certainly conceivable that communication, as a secondary event, can regulate the timing and synchrony of the fusion process in such a multicellular system. This could occur by transmitting intracellular regulatory signals, such as cyclic AMP, that have been reported during myogenesis (63). In preliminary studies, it appears that cell communication may be utilized to confer fusion competency to a fusion-arrested myoblast population. 1 In this regard, it will be extremely important in the future to examine the regulatory properties of signals that may be transmitted between presumptive and prefusion myoblasts during the early stages of myogenesis. For example, via communication the cells could receive signals that would identify them as: (a) a proliferating cell (presumptive myoblasts); (b) a cell committed to differentiation and cease division (prefusion); or (c) a heterologous cell type (fibroblast, endothelial cell). In essence, the precise role of communication in muscle fiber formation may become clear when either: (a) communication can be experimentally inhibited between prefusion myoblasts, or (b) a fusion-incompetent myoblast population is discovered that is also communication defective.

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