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Permeability and Structural Studies of Heart Cell Gap Junctions under Normal and Altered Ionic Conditions

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Summary. The permeability and ultrastructure of communicating junctions of cultured neonatal rat ventricular cells are examined under control conditions and during treatments which raise intracellular Ca^{2+}. Lucifer Yellow (487 mol wt) is used to examine junctional permeability. Under normal ionic conditions dye transfer from an injected muscle cell to neighboring muscle cells occurs rapidly (in less than 6 sec) while transfer to neighboring fibroblasts occurs more slowly. Application of monensin, which results in a partial contracture with superimposed asynchrony, or A23187, which results in a partial contracture, do not inhibit the transfer of dye between the muscle cells. A23187 did result in junctional blockade between muscle cells and fibroblasts. Freeze-fractured gap junctions from control and monensin-treated cells exhibit no distinguishable differences. Center-to-center spacing was not significantly different, 9.0 nm ± 1.4 SD versus 9.2 nm ± 1.3 SD, respectively; and particle diameters were virtually unchanged, 8.69 nm ± 0.9 SD versus 8.61 nm ± 1.07 SD, respectively. These results suggest that concentrations of intracellular Ca^{2+} sufficient to support a partial contracture and asynchronous contractile activity do not result in a block of intercellular junctions in cultured myocardial cells. These results are discussed in terms of intracellular Ca^{2+}-buffering and junctional sensitivity to Ca^{2+}.

Key words intercellular communication · dye coupling · gap junctions · A23187 · monensin

Introduction

In recent years, several reports have suggested that Ca^{2+} mediates the control of junctional permeability (Rose & Loewenstein, 1976; Rose, Simpson & Loewenstein, 1977; Dahl & Isenberg, 1980; Weingart, 1977). In *Chironomous* salivary gland cells it has been demonstrated that as the intracellular free Ca^{2+} concentration rises (via Ca^{2+} injection, CN treatment, ionophore application) junctional channels sieve out progressively smaller particles (Délèze & Loewenstein, 1976; Rose et al., 1977). The selective permeability of intercellular junctions decreased over a range of Ca^{2+} of $5 \times 10^{-7} - 5 \times 10^{-5}$ M (pCa 6.3–4.3) (aequorin technique).

As a result of these findings, several studies have examined the intracellular junctions of vertebrate cells for a similar Ca^{2+} sensitivity (*see* Loewenstein, 1981, for review). For the junctions so far examined, all exhibit some degree of Ca^{2+} sensitivity; however, the junctions of cultured mammalian cells appear to be more resistant to Ca^{2+} (Flagg-Newton & Loewenstein, 1979; Gilula & Epstein, 1976).

In the present study, the hydrophilic dye Lucifer Yellow CH (mol wt 457) and freeze-fracture electron microscopy were used to examine the permeability and ultrastructure, respectively, of the intercellular gap junctions of cultured neonatal rat myocardial cells. These studies were performed under control conditions and during exposure of the cells to a Na^+ ionophore, monensin, or a Ca^{2+} ionophore, A23187 (Pressman, 1976). Under control conditions, the muscle cells were well coupled to one another and to neighboring fibroblasts. During exposure to either ionophore, intracellular Ca^{2+} within the muscle cells was increased sufficiently to result in a partial contracture. Under these conditions, the permeability and ultrastructure of the junctions between muscle cells were unchanged. These results are discussed in terms of intracellular Ca^{2+}-buffering and the junctional sensitivity to Ca^{2+} of cultured mammalian cells.

Materials and Methods

Cell Culture

The procedures for establishing confluent cultures of neonatal rat ventricular cells have been previously described in detail (Burt, 1982). Briefly, ventricles were enzymatically digested to yield a single cell suspension. After centrifugation, resuspension in culture medium, and a 90-min preplating period (to reduce fibroblast contamination), the cells were seeded into Rose chambers or petri dishes. After three days in culture, a synchronously beating, confluent monolayer was formed. Culture medium was changed daily starting on day two.

Experiments were performed using culture medium of the following ionic composition: Na^+, 150 mM; K^+, 6 mM; Ca^{2+}, 2.5 mM; Mg^{2+}, 1 mM; Cl^-, 148 mM; PO_4^{3-}, 0.84 mM; CO_3^{2-}, 14 mM. The medium was supplemented with 10% fetal calf serum, essential amino acids, nonessential amino acids, 5.6 mM dextrose,

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vitamins, glutamine, hypoxanthine, and penicillin-streptomycin. Dilutions of monensin (Eli Lilly & Co.) were made from a stock solution of 0.1 M monensin in methanol (used to solubilize the ionophore). The final concentration of methanol in experimental solutions was 0.05%. This concentration of methanol had no effect on the rhythmic electrical and contractile behavior of the cells. Dilutions of A23187 (Calbiochem-Behring) were made from a stock solution of 0.1 M A23187 in dimethyl sulfoxide (DMSO) (used to solubilize the ionophore). Experimental A23187 solutions all contained DMSO at a final concentration of 0.05%. This concentration of DMSO produced no alterations of normal electrical or contractile activity. All compounds were of reagent grade and all solutions were made with double glass-distilled, deionized water.

Selection of Dye

Lucifer Yellow CH was selected for several reasons. First, it is highly lipid insoluble and therefore does not permeate the membrane (Stewart, 1978). Second, the dye has a very high quantum yield similar to fluorescein dyes. This is advantageous because it allows for very brief injection periods. Attempts to do these experiments with Procion Yellow (which has a very low quantum yield) were unsuccessful, probably because impalements could not be held long enough to inject a detectable amount of dye. Finally, Lucifer Yellow CH is stable in position (unlike the fluorescein dyes) after fixation, which means that movement of the dye can be stopped at any time after injection.

Dye Injection

Electrodes for dye injection were made from 1 mm OD "ultratip" Omega dot capillary tubing (Fredrick Haer & Co.). The tips of the electrodes were filled by the capillary method with a 5% solution of Lucifer Yellow CH in water. (Lucifer Yellow was kindly supplied by Harold Koopowitz, courtesy of W.W. Stewart.) The remainder of the electrode was back filled with 2.7 M LiCl. The resulting electrodes had resistances of 40–100 MΩ, tip size <0.5 μm. (These same electrodes, when filled with 3 M KCl, had resistances of 10 MΩ.) These electrodes were used to measure the transmembrane voltage, using standard recording techniques (Burt, 1982), and to inject dye. The indifferent electrode was a Ag–AgCl wire, and the recording-injection electrode was connected to the amplifier by a Ag–AgCl wire. Hyperpolarizing current of between 0.1–1.1 nA was used to inject dye.

Experimental Procedure

A four to six day old culture, which exhibited rhythmic beating, was selected for experimentation. The top coverslip of the Rose chamber was removed and the bottom of the chamber (containing the cells) was placed on the heated stage of the inverted microscope used for electrophysiology. A cluster of beating cells was selected, and normal electrical activity was recorded. At this time the same cell could be injected with dye. Alternatively, an experimental condition could be imposed by exchanging the normal bathing medium for an experimental solution. In the latter case, a new cell was then selected, its electrical activity recorded and then injected with dye. At this time, the cells could be fixed with 4% formaldehyde in balanced saline solution or left in the experimental medium. The top coverslip of the chamber was replaced, and the illuminated circle containing the injected cell was marked. The chamber was then placed on the stage of a Zeiss epi-fluorescence microscope, and the injected cell relocated. (The time from injection to cell relocation was 3–4 minutes minimum.) A 50-W Hg lamp with Zeiss filter set 487707 was used to view the fluorescence. This filter set includes a G 407/436 excitation and LP 515 barrier filter which is compatible with the 430-nm stimulation and 540-nm emission maxima for the dye (Stewart, 1978). Phase microscopy could be accomplished by switching to trans-illumination with a halogen lamp.

Photography

Tri-X was used to photograph the fluorescent image. Exposure times ranged between 15 and 40 sec at 400× magnification and 30–60 sec at 160× magnification. The film was developed with either Acufine or Diafine, which pushes the ASA to 1200 or 1600, respectively. Ektachrome 400 could also be used and was pushed two times (ASA 1600) by a commercial laboratory.

Freeze-Fracture Electron Microscopy

After a 25-min exposure of 3-day old synchronously beating cells to either HEPES-buffered saline solution (HEPES-BSS) (control) or HEPES-BSS with 25 μM monensin added, the cells were fixed with 2% glutaraldehyde in cacodylate buffer for 15 min. The cells were then rinsed with 12% glycerol and treated for 1 hr with 25% glycerol. The cells were then gently lifted from the dish, placed in gold alloy, double replica hats (3 mm diameter, Balzer High Vacuum), and rapidly quenched in liquid freon 22 cooled to its freezing point by liquid N_2. The fracturing and shadowing were done as previously described by Frank, Beydler, Kreman and Rau (1980). Replicas were cleaned in bleach and distilled water before mounting on 300-mesh grids. Electron microscopic observations were made on either a Siemens 1A microscope or a JEOL 100 CX.

Quantification of Gap Junction Particles

All gap junctions in the replicas were photographed at approximately 30,000× in the electron microscope. Magnifications were calibrated with a carbon grating replica, the negative films were projected at a 10-fold magnification (final magnification 300,000×) onto the screen of a Nikon Profile Projector (6C). An acetate sheet was placed on the viewing screen, and either the centers of particles were marked by placing a small dot on the acetate sheet over the particles or particle size was marked by circling the particle. The acetate sheet was then xeroxed and center-to-center spacing or particle size measured using a metric calipers. At least 60 measurements of center-to-center spacing were made for each junction and 20 measurements for particle size. Five different junctions of areas greater than 0.1 μm^2 were quantified in control and in monensin-treated myoblasts. Values are expressed as the mean ± SD. Statistical significance was assessed using Student's *t*-test for pooled variances of unequal sample sizes. *P* values of less than 0.05 are assumed significant.

Results

Under control conditions cells exhibit synchronous, rhythmic contractile activity. As might be expected, these cells are electrically coupled (Hyde et al., 1969) through low resistance junctions. It will be demonstrated here that these low resistance junctions also allow transfer of the dye Lucifer Yellow CH (mol wt 457). In the experiment illustrated in Fig 1, a single muscle cell within a cluster of cells was injected with Lucifer Yellow. Three minutes later dye could be detected throughout the entire cluster of muscle cells and in some of the neighboring fibroblasts. Cells incubated in a solution containing free-dye (0.1 mM, 30′) were never observed to have incorporated the dye

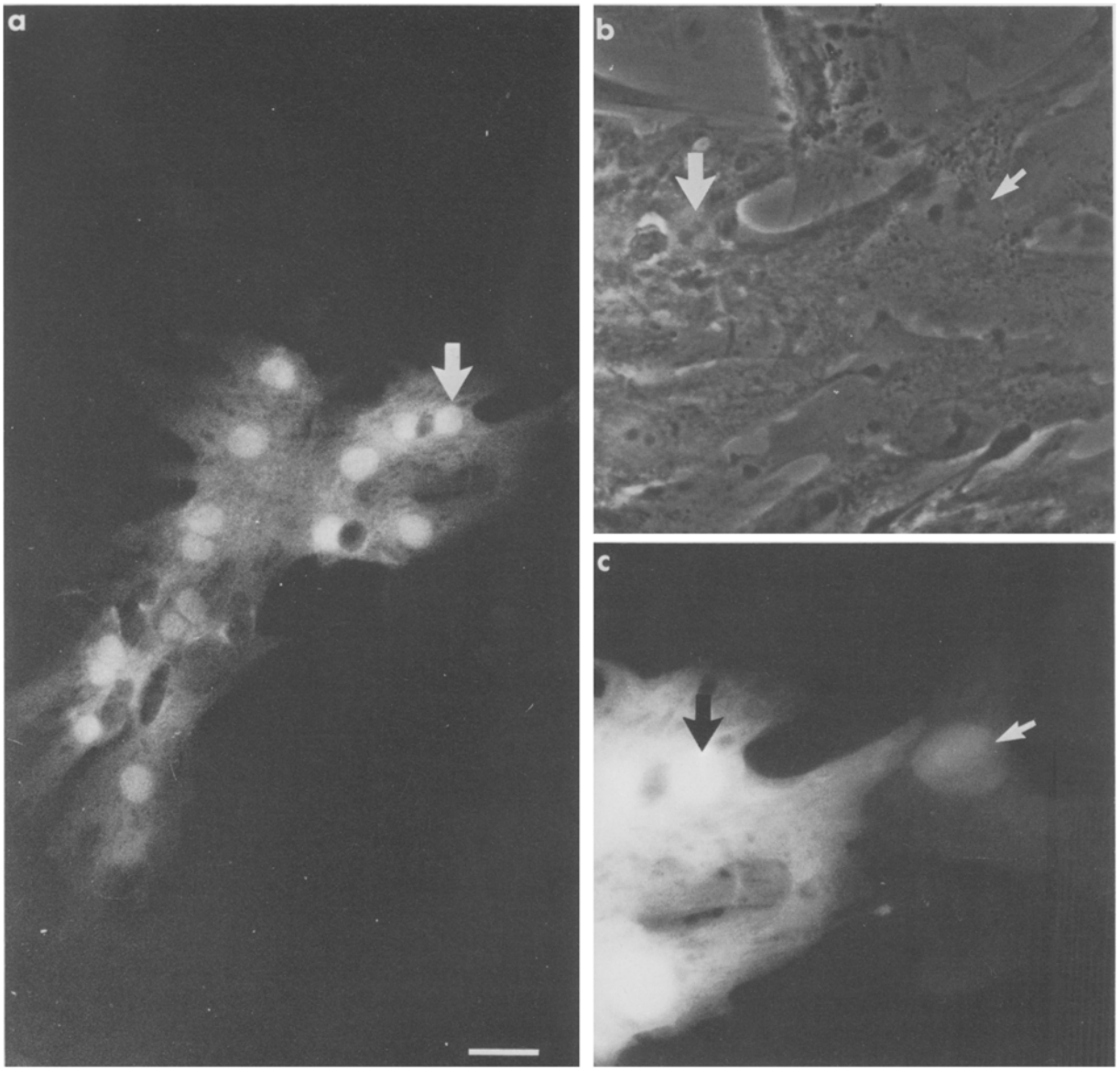

Fig. 1. Dye-filled cluster of muscle cells and surrounding fibroblasts. (*a*): Cluster of muscle cells filled with dye and visualized by fluorescence microscopy. Arrow indicates the cell which was injected with dye. (*b* and *c*): Phase and fluorescence micrographs of injected cell (large arrows) and neighboring fibroblast, demonstrating transfer of dye from muscle cell to neighboring fibroblast (small arrows). Calibration = 24 μm in *a*, 13 μm in *b* and *c*

across the cell membrane. This result indicates that dye transfer occurs through permeable junctions. In order to investigate the time course of dye transfer, it was necessary to devise a procedure in which the transfer of the dye could be stopped at appropriate intervals (less than the limiting time of 3 min described in methods). This was accomplished by fixing the cells with 4% formaldehyde at the desired time interval. That fixation inhibits or stops movement of the dye is illustrated in Fig. 2. In this experiment the cells were fixed prior to dye injection. The nucleus of a muscle cell was impaled and dye was injected. Photographs made immediately after injection and 4 hr later revealed the same localized confinement of the dye; the dye did not even spread throughout the injected cell.

By using this procedure, the time course of dye transfer was examined. In the two experiments illustrated in Fig. 3, a muscle cell was impaled and normal activity recorded for a period of time long enough to establish the stability of the impaled cell. At this time injection commenced. Fixative was added to the bathing solution either after 11 sec of injection (Fig. 3*a–c*) or after 6.5 sec of injection (Fig. 3*d–f*).

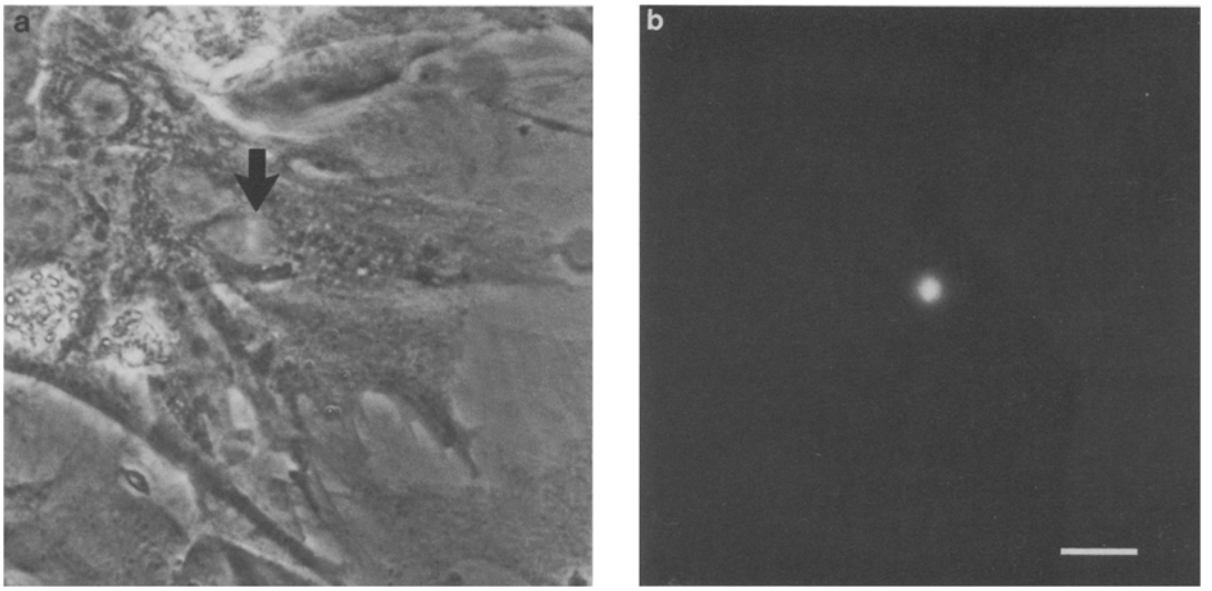

Fig. 2. Dye injection after fixation. Phase and fluorescence micrographs of a cell injected after it was fixed. Arrow indicates the nucleus injected. Note that the dye did not spread from the point of injection. Calibration = 13 μm for both *a* and *b*

The addition of fixative immediately stops the spread of injected dye and dislodges the electrode. Figure 3(*a–c*) demonstrates that within 11 sec considerable dye transfer has occurred. Similarly, Fig. 3*d–f* demonstrates that 6.5 sec is still long enough for dye transfer to occur.

Dye transfer between muscle cells and fibroblasts occurs more slowly. In Fig. 4, micrographs taken at 4.5 and 10 min after injection are illustrated. At 4.5 min no dye is detectable in neighboring fibroblasts, whereas at 10 min dye can definitely be detected in the nuclei of neighboring fibroblasts.

Intracellular recording in fibroblasts or injection of dye into fibroblasts is much more difficult because the cells are very thin. (The percentage of successful impalements is considerably less with fibroblasts than with muscle cells.) Fibroblasts that are surrounded by muscle cells generally exhibit action potentials, which are indistinguishable from the typical nonpacemaker potential observed in the neighboring muscle cells. The presence of action potentials in these cells is an indication of electrical coupling. In two experiments in which dye was successfully injected into a fibroblast, no dye could be detected in the neighboring muscle cells at any time after the injection (Fig. 5), in spite of measureable action potentials (during the injection period) in the fibroblast.

Dye Transfer during Monensin or A23187 Treatment

It was recently reported (Burt, 1982) that treatment of cells with monensin results in a partial contracture with, depending on the monensin concentration, superimposed asynchronous subcellular contractile activity. The range of intracellular [Ca^{2+}] which supports partial contractures and asynchronous contractile activity in adult rat myocardial cells is *p*Ca 6.25–6.0. Above this level (i.e., between 6.0 and 5.5) partial contracture without asynchrony occurs (*see* Fig. 3 in Fabiato & Fabiato, 1978). The Ca^{2+} concentration associated with partial activation varies from one species to the next. Most other estimates are higher than that of Fabiato and Fabiato, so the *p*Ca 6.25–6.0 should be regarded as a lower limit (Solaro, Wise, Shiner & Briggs, 1974). This range of intracellular Ca^{2+} concentrations is expected to lead to a block of cell coupling (Rose et al., 1977). This hypothesis was tested as follows. During exposure to 25 μM monensin (9 experiments) or 10 μM monensin (2 experiments), dye was injected into a muscle cell within a cluster of cells. In eight of the nine experiments performed in 25 μM and in both of the experiments performed in 10 μM monensin, the dye spread from the injected cell to neighboring cells with approximately the same time course found under normal ionic conditions. In Fig. 6*a–c* a muscle cell injected for 17 sec while in the presence of 25 μM monensin and immediately fixed is illustrated. Dye can easily be detected in neighboring muscle cells. Similarly, Fig. 6*d–f* illustrates a cell injected for 11 sec during monensin treatment and immediately fixed. Again, dye can be detected at low levels in the nuclei of neighboring cells.

To explore further the effects of increased intracel-

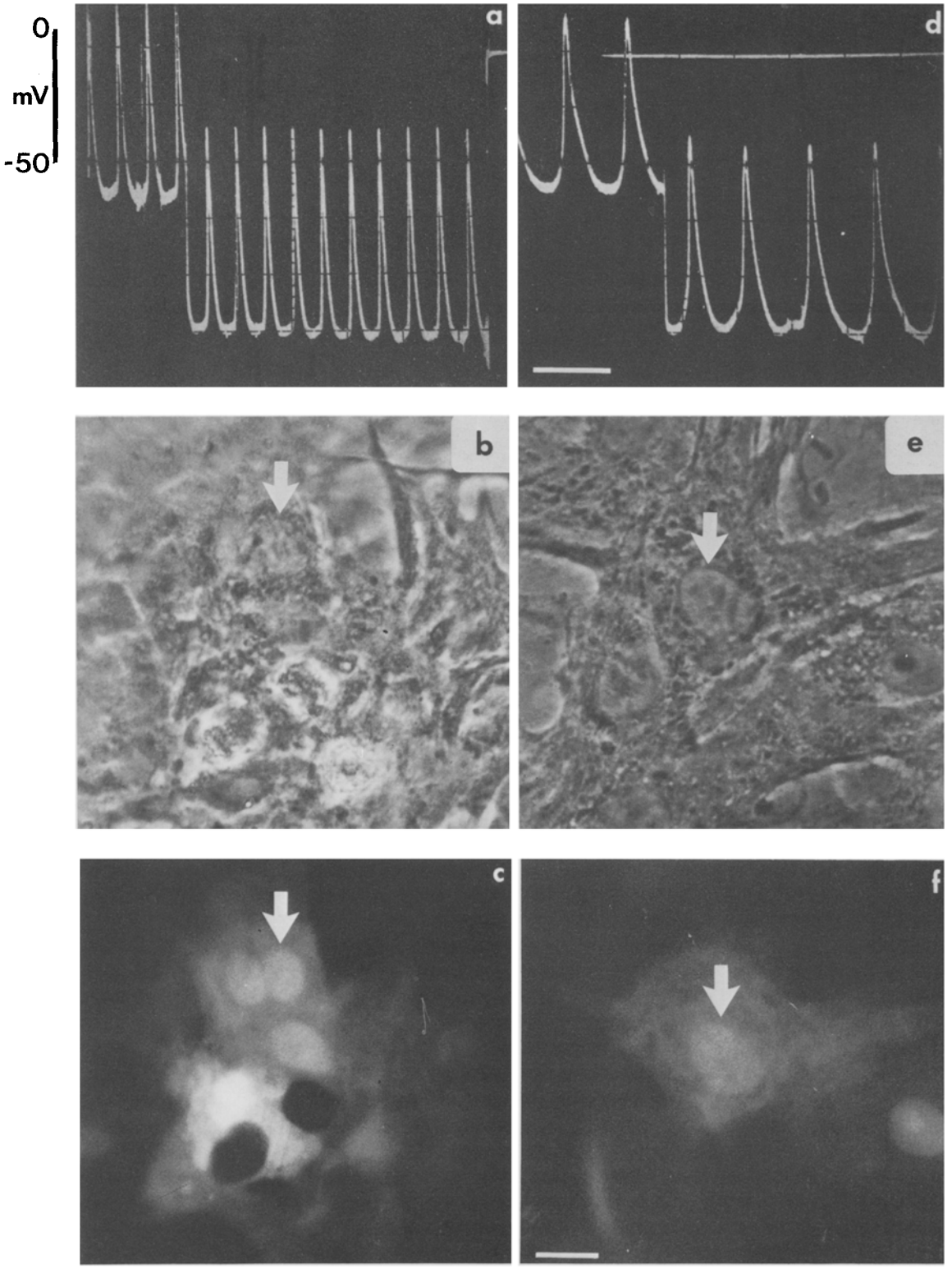

Fig. 3. Time course of dye transfer to neighboring muscle cells under normal ionic conditions. (*a–c*): Cell injected for 11 sec and immediately fixed. (*d–f*): Cell injected for 6.5 sec and immediately fixed. (*a* and *d*): Electrical record before and during injection. (*b* and *e*): Phase micrographs of injected clusters of cells; arrow indicates the specific cell injected. (*c* and *f*): Fluorescence micrographs of injected clusters of cells. Note that enough time elapsed during injection for dye transfer to occur. Calibration for all micrographs = 13 μm. Time calibration bar = 3 sec for *a*, 1.5 sec for *d*

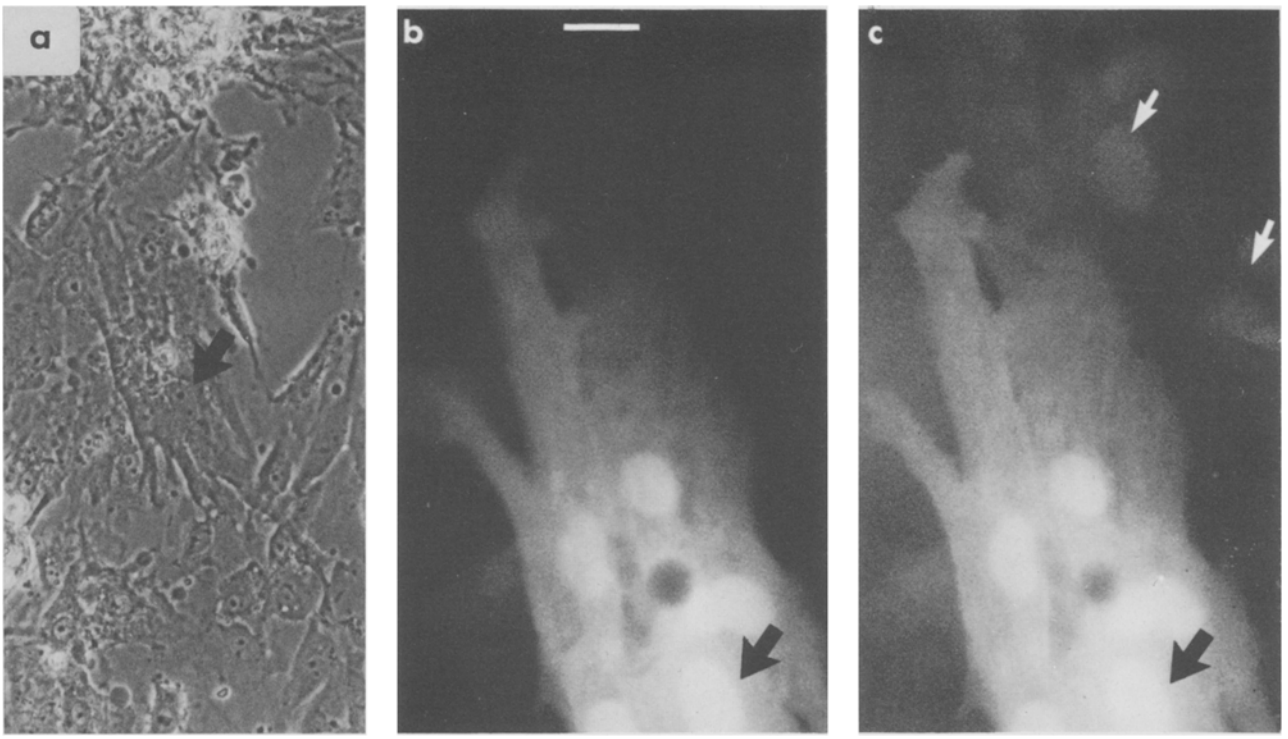

Fig. 4. Time course of dye transfer to neighboring fibroblasts under normal ionic conditions. (*a*): Phase micrograph of injected cluster of cells and surrounding cells. (*b*): Fluorescence micrograph taken 4.5 min after injection. (*c*) Fluorescence micrograph taken 10 min after injection at similar exposure and printing times. Large arrows indicate injected cell. Note that at 10 min, nuclei of neighboring fibroblasts (small arrows) show detectable dye while at 4.5 min no dye can be detected. Calibration bar for $a=24$ μm and for *b* and $c=13$ μm

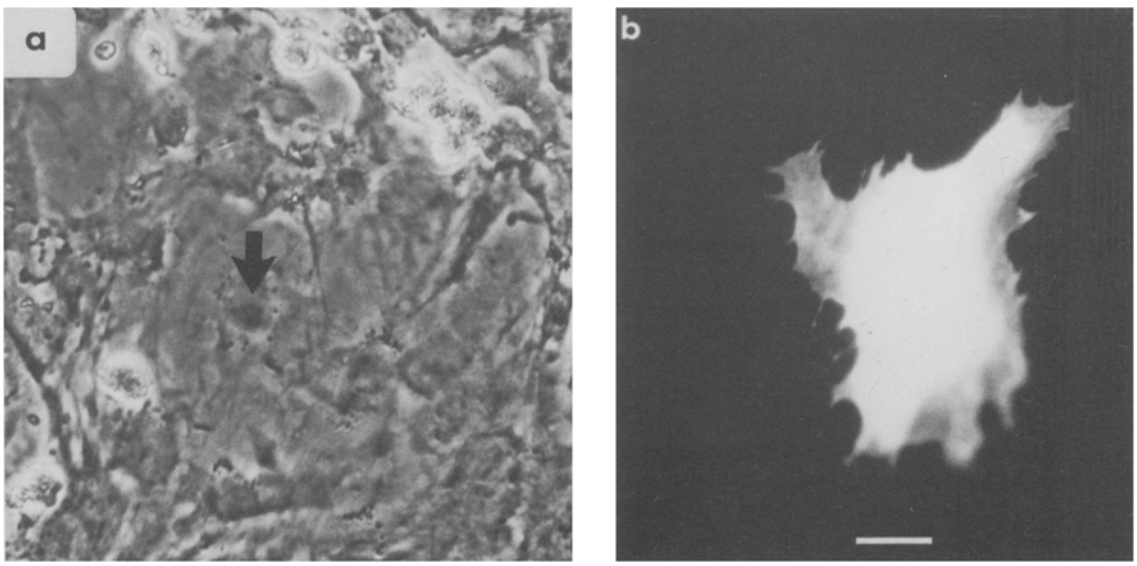

Fig. 5. Absence of dye transfer from an injected fibroblast to neighboring muscle cells under normal ionic conditions. In spite of electrical coupling prior to and during injection (measureable action potentials in the fibroblast), no dye transfer occurs from the fibroblast to neighboring muscle cells. (*a* and *b*): Phase and fluorescent micrographs of injected cell (arrow indicates fibroblast nucleus) and surrounding cells. Calibration = 13 μm for *a* and *b*

lular levels of free Ca^{2+} on junctional communication, A23187 was employed. This ionophore has been shown to induce a partial contracture in this preparation (a 29% decrease in intracellular particle separation during diastole is observed: *see* Burt, 1982), and it also results in the death of many fibroblasts in

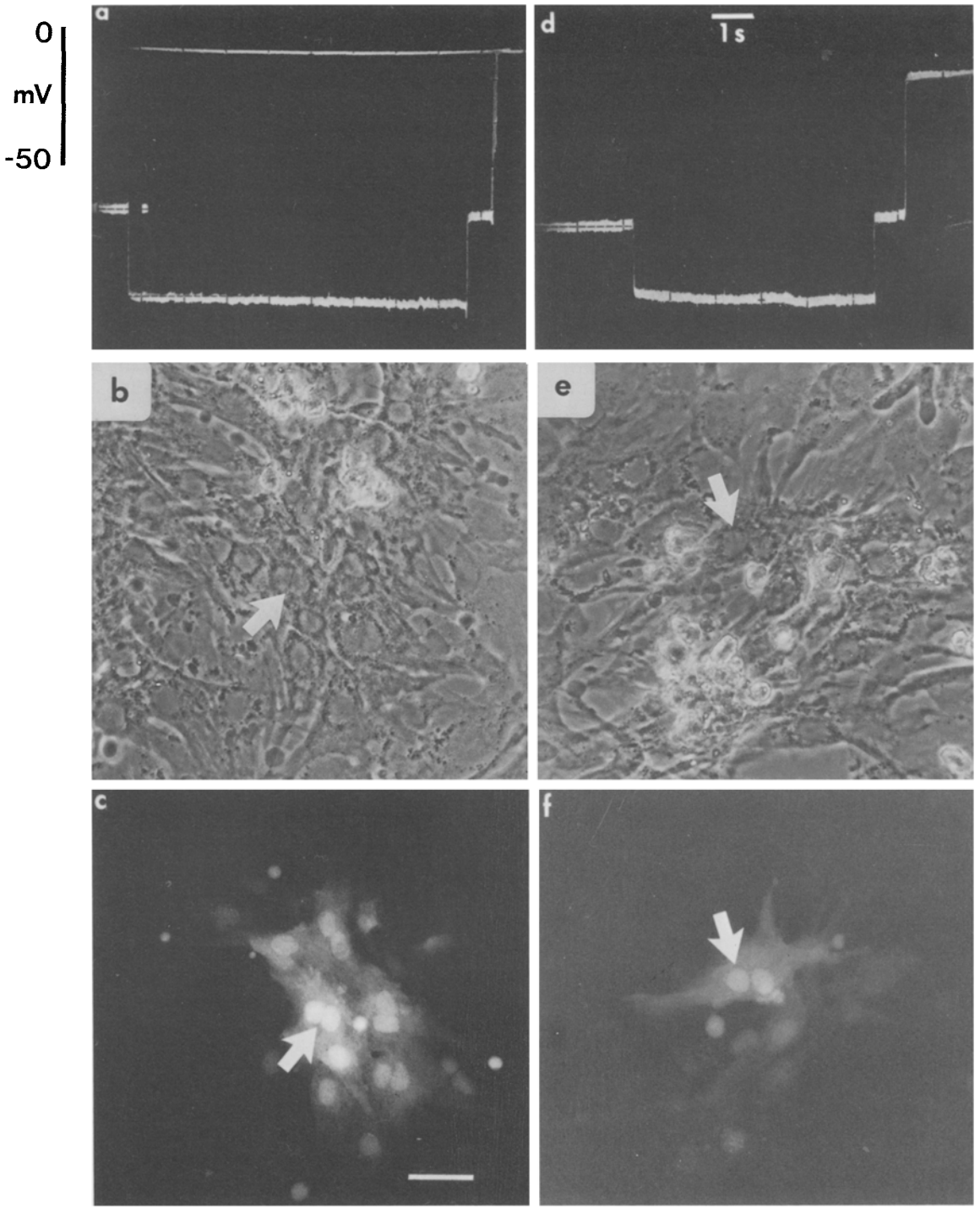

Fig. 6. Dye transfer from an injected muscle cell to neighboring cells during exposure to monensin. (*a–c*): Cell injected for 17 sec after 5 min in 25 μM monensin and immediately fixed. (*d–f*): Cell injected for 11 sec after 22 min in 25 μM monensin and immediately fixed. Note in both cases that dye transfer occurred. Arrows indicate the injected cells; calibration bar for all micrographs = 24 μM. Time calibration for *a*: equivalent bar length to that seen in *d* (1 sec) would equal 2.4 sec

a treated culture. The spread of dye from an injected muscle cell to neighboring muscle cells was unimpeded by application of 10 μM A23187 (5 experiments) or 25 μM A23187 (5 experiments) (Fig. 7). In contrast, no dye could be detected in neighboring fibroblasts in any of these same 10 experiments.

Gap Junction Ultrastructure

The appearance of freeze-fractured gap junctions in neonatal rat hearts after three days in culture was typical of that reported for gap junctions in neonatal rabbits by Shibata, Nakata and Page (1980) (*see*

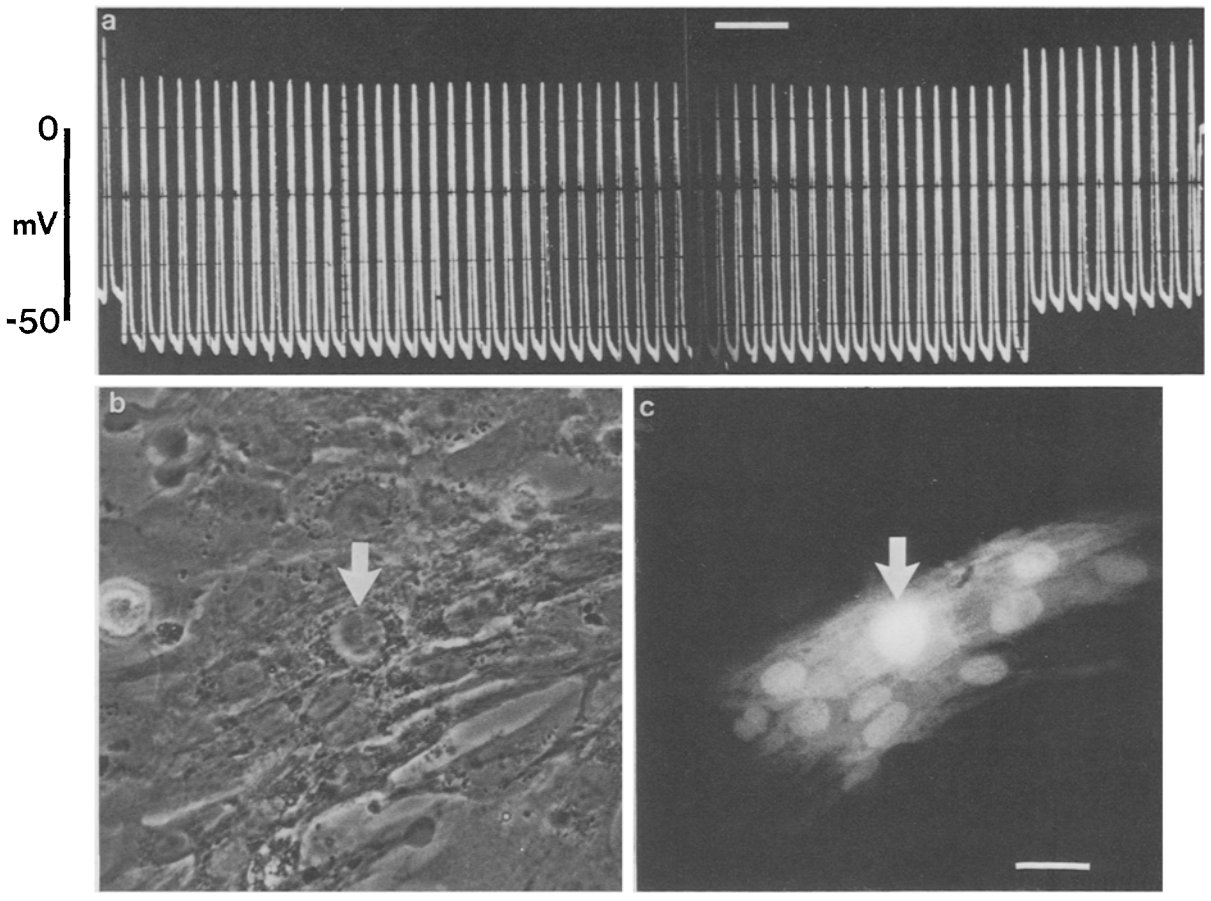

Fig. 7. Dye transfer from an injected muscle cell to neighboring cells during exposure to A23187. (*a–c*): Cell injected after a 10-min exposure to 25 μM A23187 and not fixed. Note that dye transfer has occurred to neighboring muscle cells but not to neighboring fibroblasts. Arrows indicate the injected cell nucleus. Calibration bar in *a* represents 3 sec. Calibration for *b* and *c* = 24 μm

Fig. 8). Both particles (in the *P* face) and pits (in the *E* face) were frequently arranged in the "aisle configuration" characterized by a large area of lipid bilayer. This appearance is common in developing hearts and appears to be related to growth spurts of the junctions (Shibata et al., 1980). Junctions from control myoblasts and monensin-treated cells (25 μM for 15 min) were similar (*see* Fig. 8). Measurement of center-to-center spacing and particle size from both control and experimental groups demonstrated that the junctions were not sufficiently different to be statistically significant. Center-to-center spacing in control junctions was 9.0 nm ± 1.4 SD ($n=308$) and in monensin treated cells was 9.2 nm ± 1.3 SD ($n=266$) ($0.1 > P > 0.05$). In these measurements we avoided measuring interparticle distances across a particle-free aisle. Particle size in control junctions was 8.7 nm ± 1.3 SD ($n=99$) and in monensin-treated cells was 8.6 nm ± 1.07 SD ($n=102$) ($P > 0.5$).

Discussion

When dissociated embryonic or neonatal ventricular myocytes are grown in monolayer culture they display synchronous beating (Crill, Rumery & Woodbury, 1959; Harary & Farley, 1963), which has been demonstrated to be the result of electrical coupling mediated by low resistance junctions between myocytes ($M-M$ junctions), between myocytes and fibroblasts ($M-F$ junctions) and between fibroblasts ($F-F$ junctions) (Crill et al., 1959; Goshima, 1969, Hyde et al., 1969; Jongsma & van Rijn, 1972; Sachs, 1976). In this study, it has been shown that under normal ionic conditions both $M-M$ and $M-F$ junctions will also allow the transfer of the dye Lucifer Yellow CH (mol wt 457). The dye transfers quite rapidly between myocytes as illustrated in Fig. 3 but less rapidly to neighboring fibroblasts as illustrated in Fig. 4. This difference in degree of coupling between myocytes

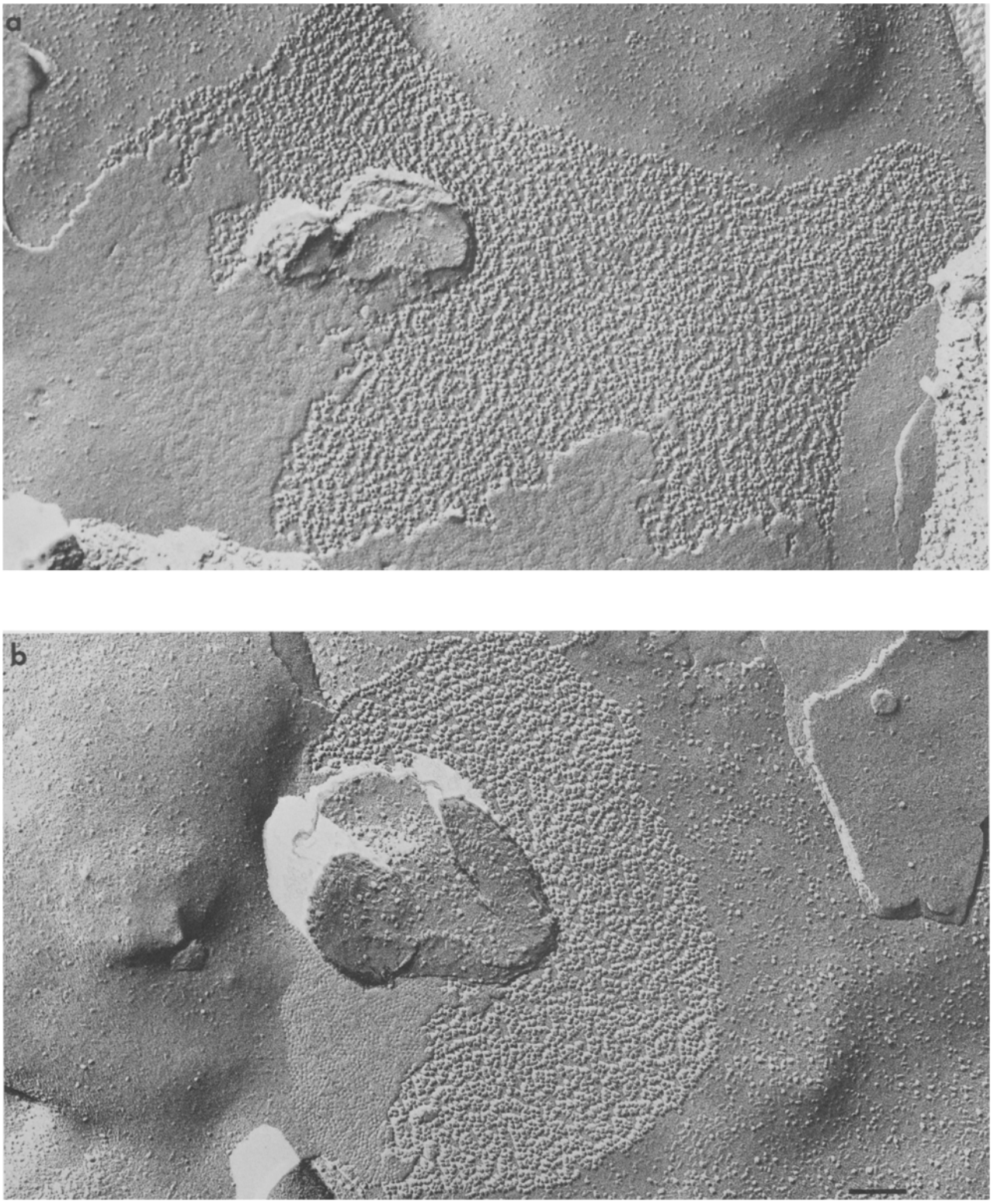

Fig. 8. Freeze-fracture replica of gap junctions between control (*a*) myoblasts and monensin-treated (*b*) myoblasts. Fracture plane crosses both $P+E$ faces in both junctions. The arrangement of particles is similar in control and treated cells. Particle-free aisles, commonly seen in junctions of developing hearts, are present. Calibration = 0.11 μm

vs. myocytes and fibroblasts most likely reflects a greater junctional area between myocytes *vs.* between myocytes and fibroblasts; however, the possibility that the permeability of homo- *vs.* heterotypic junctional channels differs cannot be eliminated. The failure to detect transfer from an injected fibroblast to surrounding myocytes to which it is electrically coupled is most likely due to cell damage and elevated $[Ca^{2+}]$ within the fibroblast upon impalement of the fibroblast.

The intracellular Ca^{2+} concentration range, which defines marginal permeability to full permeability of *Chironomous* salivary gland intercellular junctions, has been estimated by Rose et al. (1977) as 5×10^{-5} (pCa=4.3) and 5×10^{-7} (pCa=6.3), respectively. In heart tissue intracellular Ca^{2+} rises and falls with each cycle of contraction and relaxation exactly in this range (Solaro et al., 1974). This observation raises the question of how heart cells prevent uncoupling on a beat-to-beat basis (*see* Weidmann, 1970). The available evidence indicates that the junctions of cells in heart tissue are Ca^{2+} sensitive (Délèze, 1970; DeMello, 1975; Weingart, 1977; Dahl & Isenberg, 1980). Dahl and Isenberg (1980) estimated the Ca^{2+} levels associated with the initial increase in longitudinal resistance of sheep Purkinje fibers during exposure to high levels of ouabain, ouabain and low $[Na^+]_o$, or DNP to be 4 ± 1.5 μM (pCa 5.6–5.25). This threshold level may well differ from one species to the next and between Purkinje *vs.* ventricular muscle. As such, it remains to be seen as to whether a similar threshold pCa^{2+} level would be necessary for junctional blockade to occur in ventricular cells.

Cultured neonatal rat myocardial cells exhibit a partial contracture (15%) with superimposed subcellular asynchrony when $[Na^+]_o$ is reduced or $[Na^+]_i$ raised via treatment with ouabain or monensin (Burt, 1982). Application of A23187 also results in a partial contracture (29%), but with superimposed weak, synchronous beating (Burt, 1982). A partial contracture indicates that the free-Ca^{2+} concentration within the muscle is elevated to the μM range (Solaro et al., 1974; Fabiato & Fabiato, 1978). Neither drug resulted in a block of dye transfer between myocytes. However, in all of the experiments with A23187, dye was never detected in neighboring fibroblasts.

The similar behavior of $M-M$ junctions and $M-F$ junctions during exposure to monensin *vs.* a differential behavior during exposure to A23187 most likely reflects the mechanism by which Ca^{2+} is elevated by the two drugs. In heart cells a rise in intracellular Na^+ induces, via Na^+-Ca^{2+} exchange, a rise in intracellular Ca^{2+} (Mullins, 1979; Langer, 1980; Bers & Ellis, 1982; Burt, 1982; Burt & Langer, 1982). If the Na^+-Ca^{2+} exchanger is absent, or if the density of the exchanger in the fibroblast membrane is less than in the myocyte, then intracellular Ca^{2+} would not be elevated in the fibroblast. In this case the $M-F$ junction would remain open unless Ca^{2+} levels from the myocyte side were sufficiently high to result in blockade. A23187 is expected to result in an elevated Ca^{2+} level directly in all cell types. While muscle cells are quite capable of dealing with an increased Ca^{2+} influx, via sequestration (sarcoplasmic reticulum, mitochondria) and efflux mechanisms, fibroblasts are not. As a consequence, with A23187 application the Ca^{2+} concentration in the fibroblast would rise to a higher level and result in decreased permeability of $M-F$ junctions. This explanation is consistent with the frequently observed fibroblast necrosis upon exposure to A23187.

For an ultrastructural comparison of the coupled and uncoupled states, gap junctions are typically quantified in terms of center-to-center particle spacing and particle size. Values for center-to-center spacing of 9.4 ± 2 nm in sheep Purkinje fibers (Dahl & Isenberg, 1980) and 10.9 ± 0.1 nm in one day old rabbit ventricle (Shibata et al., 1980) are typical. Particle size has been best quantified by Dahl and Isenberg (1980) who found an average of 8.3 ± 0.5 nm for sheep Purkinje fibers. In the uncoupled state, center-to-center spacing and particle size change, although the extent and nature of the changes is still controversial (*see* Page & Shibata, 1981, for review). In general, center-to-center spacing decreases while particle diameter increases.

The structure of control gap junctions of cultured rat myocardial cells, as observed in this study appeared similar to that of gap junctions from monensin-treated cells. Measurements of center-to-center spacing and particle diameter from control junctions are similar to those found in the studies by Dahl and Isenberg (1980), Shibata et al. (1980), Peracchia (1977), and Peracchia and Peracchia (1980). Similar measurements from monensin-treated cells reveal no differences, indicating no change in junction structure as a result of monensin treatment.

On the basis of the observed changes in contractile state of the cells during monensin or A23187 treatment, a block of dye transfer between myocytes was expected. Several explanations might account for the absence of junctional blockade. First, the concentration of Ca^{2+} at the myofibrils may not reflect the concentration at the nexus due to compartmentalization. Second, the sensitivity of gap junctions to Ca^{2+} may be age dependent (neonate *vs.* adult), species dependent (rat *vs.* sheep *vs.* invertebrate), or tissue dependent (heart *vs.* salivary gland *vs.* liver), or a

unique property of cultured cells (Gilula & Epstein, 1976; Flagg-Newton & Loewenstein, 1979). Or third, mammalian gap junctions may exhibit an "all-or-none" response to Ca^{2+} instead of a graded permeability response. In this case, dye transfer would continue (albeit at a slower rate) until all channels were closed.

Other authors have also had difficulty uncoupling cultured mammalian cells (Gilula & Epstein, 1976; Flagg-Newton & Loewenstein, 1979). Gilula and Epstein examined the effects of A23187 on electrical coupling of 3T3 and cultured neonatal mouse ventricular cells. Since the muscle cells continued to beat synchronously in the presence of A23187, they concluded that electrical communication was maintained and questioned whether A23187 was effective as a Ca^{2+} ionophore in their cells. It has previously been shown that A23187 results in a partial contracture in cultured neonatal rat ventricular cells, which indicates that the ionophore is active (Burt, 1982). Flagg-Newton and Loewenstein (1979) examined the effect of A23187 on junctional permeability in a rat epithelial and a rat fibroblast cell line. Although the permeability to large molecules was decreased, intermediate size molecules (400 mol wt or less) remained permeable. To explain the comparatively high resistance of these cells to junctional blockade by Ca^{2+}, they suggested that the junctions were shielded from the high $[Ca^{2+}]$ present elsewhere in the cell. They proposed that shielding could result from "(i) special, powerful Ca^{2+} sequestering processes in the vicinity of the junctional channels, or (ii) special conditions of cell geometry where the channels are far enough away from the sites of $[Ca^{2+}]_i$ elevation, with ordinary sequestering processes intervening, or (iii) a combination of the two." For the epithelial and fibroblast cells, they provided evidence for option (ii) by demonstrating that the junctions occur on bilateral fine cell processes. In the case of cultured myocardial cells, junctions are quite large and located between cell bodies rather than on processes. Thus, it cannot be assumed, on the basis of cell geometry, that the junctions do not see the elevated Ca^{2+} concentration. Nevertheless, the concentration of Ca^{2+} at the junction may not be equivalent to that at the myofilaments due to sequestration. Cultured neonatal rat myocardial cells have a structurally well-developed sarcoplasmic reticulum (Langer, Frank & Nudd, 1979), numerous mitochondria, a sarcolemmal Ca^{2+}-pump, and presumably multiple cytosolic Ca^{2+} binding sites. One or several of these Ca^{2+} sequestering processes could be acting to shield the junction from the increased Ca^{2+} available to the myofilaments. The compartmentalization of Ca^{2+} in cultured myocardial cells has been well characterized and can be easily manipulated (Langer et al., 1979; Langer & Nudd, 1980; Ponce-Hornos, Langer & Nudd, 1982). These studies should provide a background for the analysis of the role of sequestration in shielding the junctions in future studies.

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