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Electrical Coupling among Heart Cells in the Absence of Ultrastructurally Defined Gap Junctions

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Summary. Cells from the ventricles of 7-day chick embryos were aggregated into spheroidal clusters by 48 hr of culture on a gyratory platform. All aggregates beat spontaneously and rhythmically. Microelectrode impalement of widely separated cells within aggregates indicated that they were coupled, as evidenced by a mean coupling ratio ($\Delta V_2/\Delta V_1$) of 0.81 ± 0.09, and by simultaneity of intrinsic electrical activity (action potentials and subthreshold voltage fluctuation). In freeze-fracture preparations, the cell surfaces contained numerous small groups of intramembrane protein (IMP) particles, arranged in macular clusters, and linear and circular arrays. Using the criterion of 4 clustered IMP particles to define a minimal gap junction, 0.27% of the total P-face examined was devoted to gap junctional area. Within such clusters particles were packed at about 8200/μm^2; in nonjunctional regions, particles were scattered at a density of about 2000/μm^2. When exposed to cycloheximide (CHX: 50 μg/ml) for 24–48 hr, coupling ratio declined to 0.44. This decrease could be attributed largely to leakiness of the nonjunctional membrane. Aggregates continued to beat rhythmically and in a coordinated fashion even after 72 hr in inhibitor. However, between 3–21 hr in CHX gap junctional area declined to 0.10%, and all particle clusters disappeared from the P-faces of aggregates in CHX for 24 or 48 hr. Neither macular nor linear particle arrays were seen. We conclude that organized gap junctions are unnecessary for electrotonic coupling between embryonic heart cells. These findings support the idea that low-resistance cell-to-cell pathways may exist as isolated channels scattered throughout the area of closely apposed plasma membranes.

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The plasma membrane that surrounds most excitable cells is relatively impermeable to charged particles. Only a negligible fraction of an ionic current injected into any given cell would pass through the membranes of adjoining cells unless they were connected by direct cell-to-cell permeability pathways. The gap junction is now accepted by many as the morphological site of this low-resistance electrical coupling (DeHaan & Sachs, 1972; Gilula, Reeves & Steinbach, 1972; McNutt & Weinstein, 1973; Bennett, 1978; Loewenstein, 1979; DeHaan, Williams, Ypey & Clapham, 1980; Peracchia, 1980). This structure has commonly been identified in the electron microscope between the apposed surfaces of coupled cells, as 5- or 7-layered regions where the membranes come into close proximity (Revel & Karnovsky, 1967), or as paracrystalline arrays of intramembrane proteins (IMP) particles in freeze-cleave preparations (Chalcroft & Bullivant, 1970; Gilula, 1978). Numerous authors have proposed models of the structure of the gap junction (Loewenstein, 1966; 1974; McNutt & Weinstein, 1973; Goodenough, 1975; Makowski, Caspar, Phillips & Goodenough, 1977; Zampighi, Corless & Robertson, 1980). The low-resistance pathway is thought to be composed of paired "protochannels" (Loewenstein, Kanno & Socolar, 1978) or "connexons" (Goodenough, 1974; 1976), one from each closely apposed cell membrane. These pore-bearing IMP particle pairs are thought to abut in the intercellular cleft to form channels which are insulated from the extracellular space and thus provide a direct pathway for the flow of ions and larger molecules between cells sharing these junctions. The "junctional channel unit" (Loewenstein, 1966; 1979) has an effective bore size in arthropod cells of 1.5–2.0 nm which allows molecules up to 1200 daltons to be transferred directly from cell to cell (Simpson, Rose & Loewenstein, 1977). Junctions between mammalian cells exhibit a

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somewhat lower size limit, and tracer molecules are also restricted in their permeation properties by their electronegativity (Flagg-Newton & Loewenstein, 1979).

Gap junctions can be identified readily in many coupled tissues (adult liver, granulosa and lens cells, adult heart, etc.) because they are comprised of large well-defined macular aggregations of particles (Larsen, 1977; Peracchia, 1977). However, the degree of organization required for a conducting gap junction *in vivo* is not known at present. It is apparent that the lattice configuration, center-to-center distance and size of gap junctional particles vary with the metabolic state of the cell prior to fixation and fracture of the tissue (Peracchia & Dulhunty, 1976; Bennett & Goodenough, 1978; Raviola, Goodenough & Raviola, 1978; Baldwin, 1979), and there is substantial evidence that tightly packed, paracrystalline arrays of particles represent a junction in a nonconducting state (Peracchia, 1980). In certain embryonic and regenerating tissues, moreover, junctions are composed only of small particle clusters which are either loosely arranged (Johnson, Hammer, Sheridan & Revel, 1974; Decker, 1976) or seen in linear or annular arrays (Mazet, 1977; Gros, Mocquard, Challice & Schrevel, 1978; Williams & DeHaan, 1978; Masson-Pevet, 1979). In these cases, it is often difficult to distinguish gap junctions that are comprised of a few aggregated particles from random clusters of particles that may be present in nonjunctional membrane.

Such a condition is notable in smooth muscle. Both the circular and longitudinal smooth muscles of the intestine (Bennett, 1973; Tomita, 1973) and of the uterine myometrium at all stages of pregnancy (Daniel & Lodge, 1973; Kuriyama & Suzuki, 1976) are known to be electrically well coupled. Numerous well-formed macular gap junctions were present between circular muscle cells of the intestine (Daniel, Daniel, Duchon, Garfield, Nichols, Malhotra & Oki, 1976; Gabella & Blundell, 1979) and in the uterus during parturition (Daniel et al., 1976; Garfield, Sims, Kannan & Daniel, 1978). But these structures appeared to be absent from longitudinal intestinal cells (Daniel et al., 1976) and from the mid-term pregnant uterus (Garfield et al., 1978). These results have led some workers to question whether gap junctions are necessary for cell-to-cell electrical conduction in smooth muscle (Daniel et al., 1976; Garfield et al., 1978).

Studies of cloned human/mouse cell hybrids have led Loewenstein (1979; 1980) to raise a related question. Cultures of the human Lesch-Nyhan fibroblast parent line were electrically well coupled, permitted cell-to-cell transmission of fluorescent tracer molecules, and exhibited the seven-layered regions of close membrane apposition (Azarnia, Larsen & Loewenstein, 1974) and macular arrays of IMP particles (Larsen, Azarnia & Loewenstein, 1977) that typically identify gap junctions. In contrast, the mouse C1–1D clone (a derivative of the L-cell line) was genetically incompetent to form gap junctions and showed no measurable current flow between adjoining cells. Upon hybridization some of the segregant clones had intermediate characteristics. These were electrically coupled (i.e, passed ions), but the junctions were impermeable to tracer molecules (Azarnia & Loewenstein, 1977). In these cases, linear or "fibrillar" arrays of IMP particles were the only junctional specializations seen in freeze-fracture replicas of junctional surfaces. Loewenstein (1979; 1980) has suggested that these structures may represent deviant particle aggregates containing aberrant channels that nonetheless can mediate electrical coupling between cells.

In the present investigation, we have used heart cell aggregates from embryonic chick ventricles (Sachs & DeHaan, 1973) to investigate the structure-function relationship between gap junctions and low-resistance electrical coupling. All cells within these spheroidal aggregates are normally joined by ultrastructurally identifiable gap junctions (DeHaan & Sachs, 1972). The cells are electrically well coupled and, for small signals at frequencies less than 10 Hz, they display virtual voltage homogeneity (DeHaan & Fozzard, 1975; DeFelice & DeHaan, 1977; Clay, DeFelice & DeHaan, 1979). These electrical properties are presumed to be mediated by low-resistance intercellular pathways. We report here that after prolonged incubation of heart cell aggregates in cycloheximide, an inhibitor of protein synthesis, identifiable gap junctions disappear yet the cells within the aggregates continue to beat synchronously and remain electrically coupled. A preliminary report of these findings has been presented (Williams & DeHaan, 1979).

Materials and Methods

Preparation of Heart Cell Aggregates

Ventricles from 7-day chick embryo hearts were dissociated into a single-cell suspension by multiple-cycle trypsinization (DeHaan, 1970). The dissociation medium contained 50 μg/ml crystalline trypsin and 4.5 μg/ml DNAse (Worthington Biochemical Corp., Freehold, N.J.) in a dextrose-phosphate buffered saline (PBS) solution. After the initial 8-min trypsin-digest cycle, the supernatant was removed and discarded and replaced with fresh dissociation medium. Ventricles were again incubated with stirring, and supernatant containing single cells was removed and collected every 8 min, this procedure being repeated until only acellular stroma remained. The collected cell suspension was filtered under positive-pressure through bolting silk (Tetco, Inc., Elmsford, N.Y.) and the cells were pelleted at $800 \times g$ for 10 min at room temperature. The supernatant was discarded and the pellet resuspended in flasks containing culture medium 818A (DeHaan, 1970) with 1.3 mM K_o^+ (2×10^6 cells/ml) on a gyrating platform (70 rpm; 37 °C) under an atmosphere of 5% CO_2, 10% O_2, and 85% N_2. During 24 hr of gyration culture, the cells re-adhered to form spheroidal aggregates 60–250 μm in diameter. For observation and experiments

the aggregates were transferred after 48–72 hr to plastic culture dishes (#3001, Falcon Plastics, Oxnard, Calif.) on a temperature-controlled stage of an inverted microscope.

Inhibition of Protein Synthesis

Aggregates were preincubated with cycloheximide (CHX: Sigma Chemical, St. Louis, Mo.) at 50 μg/ml for 1 hr or 16 hr before being collected and pelleted at 200 × *g*. The rate of protein synthesis was determined by measuring the uptake by the pelleted aggregates of a ^{3}H-amino acid mixture (New England Nuclear) into trichloroacetic acid (TCA) precipitable material. For long-term experiments, CHX was added to the medium after 24 hr of gyration culture, and culture medium containing CHX was renewed every 16 hr.

After a 1-hr incubation in medium containing labeled amino acids, pellets were homogenized in 10% TCA and recentrifuged. The sediment was heated in fresh TCA to 90 °C for 15 min and dissolved in 150 μl 0.4 N NaOH. TCA was again used to precipitate the protein which was pelleted and washed in 1:1 ether alcohol. The final pellet was washed with 100% ether and allowed to dry overnight. The precipitate was weighed, dissolved in hyamine and transferred to scintillation vials. Tubes were washed 10× with scintillation fluid which was also transferred to the scintillation vials. Each CHX preincubation point was obtained in triplicate.

Freeze-Fracture Preparation

For freeze-fracture analysis, aggregates were fixed for 2 hr in 2% gluteraldehyde in a phosphate-buffered saline solution (PBS; pH 7.3, 300 mOsm), washed 4× in PBS and soaked in 25% glycerol in PBS for 0.5 hr. Aggregates were transferred to gold carriers, frozen in the solid-liquid interface of Freon 22 and stored in liquid nitrogen. The preparations were fractured at −115 °C in a Balzer's device (BA360M). Replicas were cleaned with bleach, collected on copper grids and examined and photographed with a Philips 400 transmission electron microscope. For the data presented in Tables 2 and 3, gap junctional area was estimated from micrographs (100,000×) of more than 400 membrane areas taken from 36 replicas. From these prints, the perimeters of total sampled P-face and of gap junction particle aggregations were measured with a digitizing planimeter (Hewlett-Packard 986A into a 9810 calculator) programmed to calculate the ratio of gap junctional area to total sampled area. Gap junctions were identified as clusters of four or more particles in close proximity. The minimum of four particles was arrived at by examining nonappositional or peripheral plasma membranes, known not to possess functional gap junctions. These areas frequently contained close pairs of particles and occasional triads, but never were four or more particles observed together. Furthermore, only clusters of particles which were in the size range of 7–10 nm in diameter were counted. This is the size range of IMP seen in well-defined gap junctions in control preparations (*see below*).

Electrophysiology

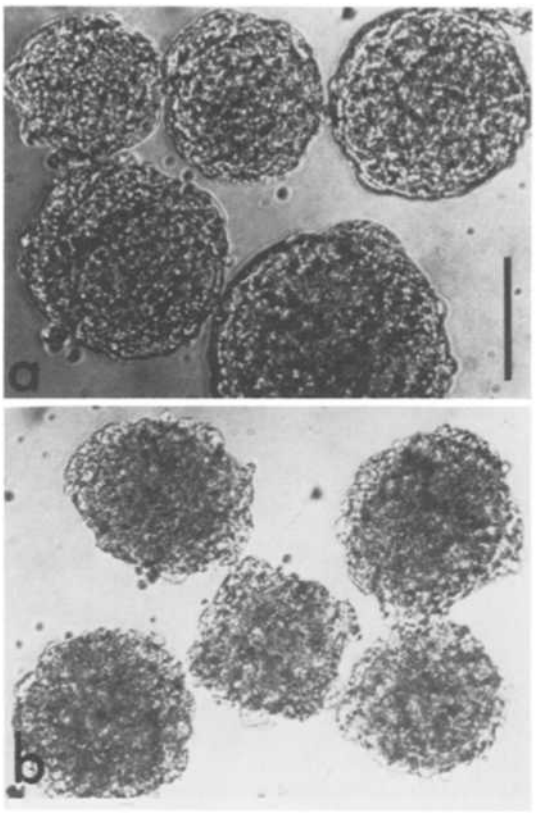
Aggregates incubated in medium 818A with or without CHX (50μg/ml) were poured into a plastic tissue culture dish (Falcon #3001) and placed on a warming stage (37 °C) of an inverted microscope. A layer of nontoxic mineral oil (Klearol, Witco Chemical Co.) was added over the medium and the dish was gassed with a mixture of 5% CO_2, 10% O_2 and 85% N_2 from a toroidal gassing ring. After 20–30 min, the preparations adhered to the bottom of the culture dish. In this condition, in medium containing 1.3 mM potassium, they beat vigorously and regularly for several days, unless spontaneous activity was suppressed with tetrodotoxin (TTX). For intracellular recordings, two glass microelectrodes (8–35 MΩ), filled with 2M KCl were coupled to amplifiers via Bioelectronic Ag/AgCl half-cells, and controlled by Leitz micromanipulators. Separate cells at distant points within an aggregate were impaled. One microelectrode was used for simultaneous recording of membrane potentials and injection of current, with the chopped-current injection device described by Wilson and Goldner (1975). We have used a switching frequency of 1000 Hz with each cycle divided into three phases: current injection, 470 μsec; potential decay, 445 μsec; voltage recording, 85 μsec. With this regime, signals up to 40 Hz could be measured reliably (±2% error). This technique is superior to the balanced bridge method; measured potential is insensitive to electrode resistance (that is, "bridge-balance" errors are avoided), and no series convergence resistance or point source effects (Eisenberg, Barcilon & Mathias, 1979) are encountered. The second microelectrode, for monitoring the membrane potential in a distant cell, was connected to a high impedance unity gain preamplifier (Picometric 181, Instrument Labs.). A grounded aluminum-foil shield was positioned between the two electrodes to reduce capacitive coupling. The membrane potentials and current signals were monitored on a Tektronix oscilloscope and the data were recorded at 15/16 or 3-3/4 inches/sec on a magnetic tape recorder (H-P Model 3964A).

Aggregate input resistance ($\Delta V_2/I$) and coupling ratio ($\Delta V_2/\Delta V_1$) were obtained by injecting 6-sec step pulses of 0.1–2.4 nA of hyperpolarizing current (I) into one cell of an aggregate made quiescent by 1 μM TTX, while simultaneously recording the change in membrane potential in that cell (ΔV_1) and another cell 80–100 μm away in the same aggregate (ΔV_2). Both ΔV_1 and ΔV_2 were measured as the difference between the mean potential during the 2 sec just prior to the initiation and termination of the current pulse. A high value of the ratio $\Delta V_2/\Delta V_1$ in most systems indicates good coupling between cells (Ito, Sato & Loewenstein, 1974; Clapham, Shrier & DeHaan, 1980). With both electrodes positioned in the intercellular space within an aggregate (i.e., at ground potential), or with one impaling a cell and the other in the cleft space, no perceptible voltage deflection in V_2 accompanied a 2–3 nA current pulse through V_1. This test for "pseudocoupling" (Potter, Furshpan & Lennox, 1966; Socolar & Loewenstein, 1979) was performed on every aggregate impaled. In some experiments a steady d–c current was also applied through the switching current electrode to shift the membrane potential over a range ± 8 mV from rest. The recorded current injection data included the sum of any d–c current being imposed and the hyperpolarizing test pulses from the square-wave generator (Wavetech 164). Values of specific membrane resistance (R_m, Table 5) were calculated as the product of measured input resistance and total cell surface area (S_a). Since 20% of total aggregate volume (V_a) was extracellular space, and mean cell radius (r_c) and volume (V_c) of freshly dissociated cells were 5.4 μm and 660 μm^3, respectively, S_a could be determined from 3.2 πr_c^2 (V_a/V_c). V_a was taken as $2(ab)^{1/2}$, where a and b were the major and minor hemiaxes measured from a top view of the aggregate with an ocular reticle (Clay et al., 1979; Nathan & DeHaan, 1979).

Results

Aggregates

After 48 hr of gyration culture, spheroidal aggregates ranged in size from about 60 to 250 μm diameter (Fig. 1*A*). In 1.3 mM K_o^+, all aggregates beat spontaneously and rhythmically. Under 125× magnification, each aggregate contracted in a coordinated fashion, all parts beating in unison. After 1 hr incubation in CHX (50 μg/ml), protein synthesis in aggregates was reduced to 3.9% of control level. Lower concentrations of inhibitor were less effective (Table 1). The



residual incorporation of amino acids into TCA precipitable material presumably reflected mitochondrial protein synthesis, which was insensitive to CHX, and inherent cytoplasmic protein synthesis "leakage" (Lansman & Clayton, 1975). After 16 hr incubation in CHX, protein synthesis in aggregates was 6.5% of control values (Table 1). Despite prolonged absence of protein renewal, aggregates continued to beat spontaneously and regularly in CHX for as long as 72 hr and appeared normal under light microscope observations (Fig. 1*B*). Toward the end of this period, aggregates exhibited some cell sloughing from their surfaces and could be more readily dislodged from the plastic Petri dishes. However, they appeared to retain normal adhesivity to each other.

When fixed after 48–96 hr of gyration culture and prepared for transmission electron microscopy, aggregate structure was that of a loose tissue, 80–90% myocytes, with nonmuscle cells scattered within and at the surface of the spheroid (DeHaan & Sachs, 1972; Sachs & DeHaan, 1973). No necrotic or un-

Fig. 1. Microscope field of heart cell aggregates. (*a*): Aggregates prepared from 7-day embryonic ventricle cells after 48 hr in gyration culture. The aggregates were generally spheroidal in shape and beat spontaneously. (*b*) Similar aggregates after 72-hr exposure to CHX (50 μg/ml). (Magnification bar: 100 μm)

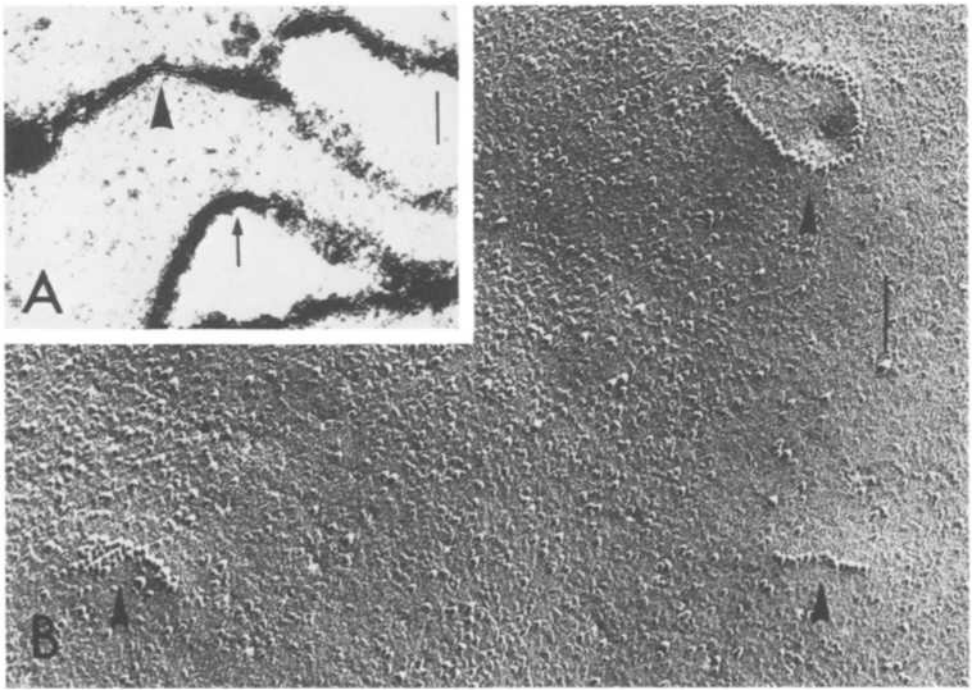


Fig. 2. Gap junctions between adjoining cells in a heart cell aggregate. *A*. Transmission electron micrograph of an aggregate stained with ruthenium red. The gap junction (arrowhead) has a characteristic 2–4 nm cleft between the apposed plasma membranes. The thick surface glycocalyx is indicated by the small arrow. *B*. Freeze-fracture preparation showing the three typical configurations of junctional IMP particles. Shadowing is oriented from bottom to top. (Magnification bars: $A=100$ nm; $B=80$ nm)

healthy cells were seen in aggregates of 250 μm diameter or less. Cells were joined by numerous zones of close apposition, penetrable by ruthenium red, and having the typical seven-layered configuration of gap junctions (Fig. 2*A*). Occasional desmosomes, but no tight junctions or other junctional specializations were seen.

Gap Junction Ultrastructure

In freeze-fracture preparations, the gap junctions between the cells of control aggregates exhibited three general particle configurations: a) small macular arrays, b) linear chains, and c) circular or annular rows of particles (Fig. 2*B*). The particles that comprised these configurations were mainly in the 7–10 nm size range (Fig. 3). In macular regions, these particles were packed at a density of 8163 ± 648 particles/μm^2 ($n = 11$). Gap junctions in control preparations occupied 0.27% of the total P-face area of the plasma membrane (Table 2, col. 4).

Table 1. Incorporation of ^{3}H-amino acid mixture into TCA precipitable material by heart cell aggregates in the presence of cycloheximide. Aggregates were incubated with label for 1 hr prior to assay.

Incubation time (hr)	Cycloheximide (μg/ml)	Average DPM/mg Protein ($\times 10^3$)	% Incorporation
Control	0	139.3	100.0
1	10	14.6	10.5
1	50	5.5	3.9
16	50	9.1	6.5

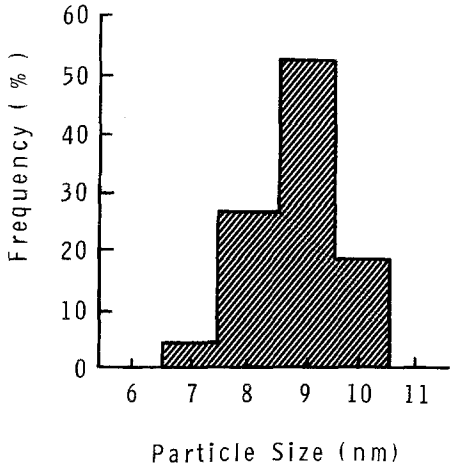


Fig. 3. Size class distribution of intramembranous particles within the gap junctions of aggregates prepared from 7-day embryonic chick ventricle. A total of 1008 particles were counted to obtain this size frequency distribution

Effect of CHX

In aggregates exposed to CHX, the area occupied by identifiable particle clusters became progressively smaller as a function of incubation time in the inhibitor (Table 2). The time-course of decrease was exponential (Fig. 4), and had a half-time ($t_{1/2}$) of 4.8 hr. The data points (0–21 hr) fit a semilog regression line of slope -1.221 with a correlation coefficient of 0.944. After 21 hr in CHX the area occupied by gap junctions fell to 0.01% of the total P-face plasma membrane, and after 27 or 48 hr in CHX neither linear, macular, or circular IMP configurations were

Table 2. Gap junction area in intra-aggregate P-faces after preincubation in CHX (50 μg/ml)

CHX (hr)	Junction area (μm^2)	Total P-face sampled (μm^2)	Fractional area of gap junctions/total sampled area (%)	Areas predicted from slope $t_{1/2}=4.8$ hr
0	0.5023	187.53	0.27	0.300
1	0.1849	53.86	0.34	0.265
3	0.0594	48.23	0.12	0.200
7	0.0333	26.25	0.13	0.115
17	0.0223	41.84	0.05	0.028
21	0.0189	131.78	0.01	0.016
27	0	53.20	0.00	0.0066
48	0	84.55	0.00	0.0003

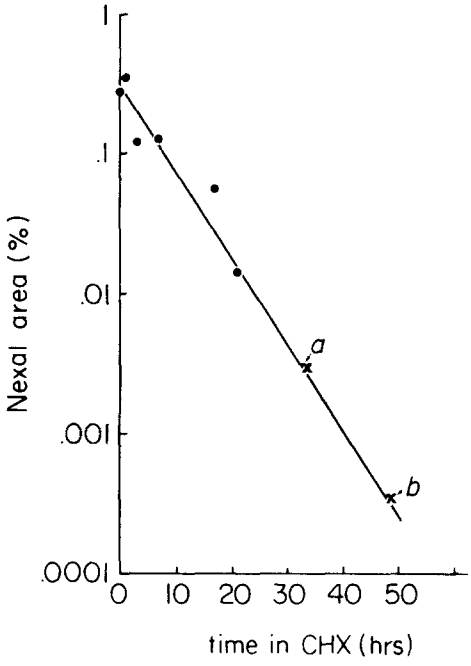


Fig. 4. Gap junctional area as a percentage of total sampled P-face in aggregates exposed to CHX (data from Table 2). The semilog regression line fitted to the data points has a slope of -1.221 ($r = 0.944$) which yields a decay rate ($t_{1/2}$) of 4.8 hr. The total cell junctional area at *a* would be equivalent to a single macular cluster of 80 IMP particles and at point *b*, to a cluster of 4 particles

Table 3. Intramembranous particle density in the nonjunctional P-face of heart cell aggregate myocardial cell membranes incubated in cycloheximide (50 μg/ml)

Incubation in cycloheximide (hr)	IMP density ($\times 10^2/\mu m^2$)
0	16.4 ± 0.8
1	22.4 ± 2.0
3	16.2 ± 2.8
7	25.8 ± 3.3
17	22.2 ± 2.1
21	23.1 ± 3.7
27	17.3 ± 2.0
48	16.6 ± 2.3
Grand mean	20.0 ± 3.8

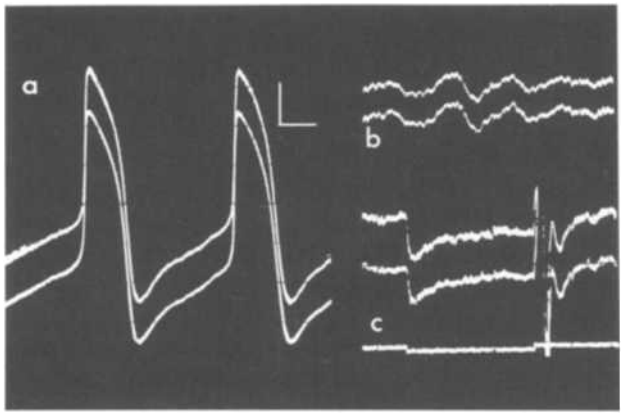


Fig. 5. Intracellular microelectrode recordings from widely separated cells (~100 μm) in heart cell aggregates incubated in cycloheximide (50 μg/ml). *a.* Action potentials from a spontaneously beating aggregate incubated in cycloheximide for 27 hr. Aggregate diameter = 155 μm. Scale: Vertical = 20 m; Horizontal = 0.1 sec. *b.* Voltage noise from cells in an aggregate in CHX for 48 hr, maintained in a quiescent state at $V_m = -51$ mV by 1.4 nA of steady hyperpolarizing current and 1 μM tetrodotoxin. Aggregate diameter = 165 μm. Scale: Vertical = 4 mV; Horizontal = 1 sec. *c.* Voltage responses (upper two traces) from two cells within an aggregate in cycloheximide for 48 hr to a 0.5 nA injected current pulse superimposed on 1.4 nA of d-c hyperpolarizing current. Current (shown in the bottom trace) was injected into the cell represented by the upper trace. Aggregate in 1 μM tetrodotoxin. Aggregate diameter = 155 μm. Scale: Vertical = 4 mV, 5 nA; Horizontal = 2 sec

Table 4. Action potential characteristics of heart cell aggregates in the presence and absence of cyloheximide (50 μg/ml). These values were obtained from intracellular microelectrode recordings in spontaneously beating aggregates. Values reported are averages from at least three stable impalements.

Incubation in CHX (hr)	Peak-peak (mV)	Maximum diastolic potential (mV)	Overshoot (mV)	Upstroke velocity (V/sec)
0	125	98	26	180
27	123	96	27	73
48	110	86	24	52

present in the areas sampled; gap junctions (i.e., clusters of four or more particles) could no longer be observed. During this period in CHX, however, the density of randomly distributed IMP in the nonjunctional P-face showed no systematic changes (Table 3).

Electrical Effects

Even after 48–72 hr exposure to CHX, aggregates continued to beat rhythmically (Fig. 5*a*). None fibrillated nor were other signs of disorganization of the beat observed. Nonetheless, their electrical parameters changed systematically (Table 4). Maximal upstroke velocity of the action potentials ($\dot{V}_{max}$) became progressively slower, falling to less than half the control value at 27 hr, while action potential (AP) amplitude remained essentially unchanged. During the period 27 to 48 hr in CHX, AP amplitude decreased significantly mainly as a result of the less negative maximum diastolic potential (MDP) values (Table 4, col. 3). The spontaneous beat of control aggregates was reproducibly suppressed by 1 μM TTX. Aggregates in CHX for extended periods became less sensitive to CHX; even at a concentration of 10 μM they displayed bursts of spontaneous contractile activity, visible microscopically without impalements. Microelectrode impalements of aggregates always caused a period of spontaneous beating even in the presence of TTX (1 μM) presumably due to the introduction of an injury current. In control preparations in TTX, this spontaneous burst normally stopped after a train of 20–30 beats, and the membrane potential stabilized at -56 ± 2 mV as the electrodes healed in. Aggregates exposed to CHX for 24–48 hr and then treated with TTX did not stop firing repetitive AP after impalement. These preparations could be made quiescent only by continuous injection of hyperpolarizing currents of 0.4–2.0 nA. After 48 hr in CHX, the potential at which spontaneous beating was suppressed by a combination of hyperpolarizing 1.4 nA current and TTX was -50 ± 2 mV, approximately 6 mV less negative than the control resting potential. Larger currents produced little additional hyperpolarization.

The relatively depolarized state of the CHX-treated aggregates, their hyperexcitability and the decay in MDP and $\dot{V}_{max}$ all suggested that, after 24–48 hr in the inhibitor, aggregate cell membranes became leaky. Direct measurements of membrane resistance, using small-pulse analysis, confirmed this prediction (Table 5). Membrane resistance normally is steeply voltage-dependent (Clay et al., 1979). In 5 control aggregates, mean membrane resistance, measured at -53 ± 2 mV in medium containing 1.3 mM K^+ was 81.4 $K\Omega \cdot cm^2$. When measurements were made at

Table 5. Membrane-resistance and coupling ratio between distant cells in nonbeating aggregates before and after exposure for varying time to CHX (50 μg/ml)

Incubation time in CHX (hr)	R_m[a] (KΩ·cm²)	Coupling ratio (0.1–1.0 nA)[d]	Coupling ratio (1.2–3.0 nA)[d]
0[b]	81.4±43.5 (55)[c]	0.81±0.09 (63)[c]	0.66±0.11 (43)[c]
0.1– 4.5	77.8±34.0 (44)	0.79±0.01 (40)	0.63±0.05 (56)
8–10	24.1± 6.2 (9)	0.72±0.17 (20)	–
12–13	24.3± 4.4 (9)	–	0.68±0.11 (10)
28–30	53.2± 9.3 (5)	0.44±0.18 (15)	–
46–48	16.0± 3.2 (6)	–	0.77±0.20 (9)

[a] Specific membrane resistance determined at $V_m = 53 \pm 2$ mV.
[b] Control values are derived from the analysis of 6–22 current pulses in each of 5 aggregates.
[c] Mean ± standard deviation for the number of pulses given in parentheses, measured at $V_m = 42$–58 mV. Data taken from impalements of a representative aggregate at each incubation time (*see text*).
[d] Sum of test pulses plus d-c current.

comparable membrane potentials in aggregates exposed to CHX, R_m fell substantially even after 8 hr in the inhibitor, and by 48 hr R_m was only about one-fifth of control value (Table 5). At each CHX incubation time, 2–5 aggregates were impaled in TTX. In most cases, the electrical parameters did not vary substantially among these. However, the pulse analyses shown in Table 5 were taken in each case from the impalement that gave the highest values of R_m, to try to exclude data from "leaky" impalements.

The cells that comprised the aggregates in this study (125–223 μm diameter) were electrically well coupled (DeHaan & Fozzard, 1975; Clay et al., 1979). The mean coupling ratio (CR) determined from 63 small current pulses in five control aggregates was 0.81 (Table 5, col. 3). When the test pulses were superimposed on an applied d-c current of 1 nA or greater, such that the sum of the pulse and steady current was $\geqq 1.2$ nA, measured coupling ratio in control aggregates declined to 0.66 (Table 5, col. 4). Despite the decrease in R_m and relative depolarization of the aggregate cell membranes, after prolonged exposure to CHX spontaneous voltage perturbations in widely separated cells were still experienced simultaneously (Fig. 5*B*), injected pulses showed the oscillatory overshoot characteristic of this excitable membrane (DeHaan & DeFelice, 1978; Clay et al., 1979) with little decrement (Fig. 5*C*), and CR declined only slightly from the control value. In those preparations in which V_m had to be shifted with d-c current to bring it near −53 mV, measured CR did not decline at all in the presence of CHX, despite the substantial decrease in R_m.

Discussion

In previous studies in this laboratory, heart cell aggregates were found to be essentially isopotential; that is, within measurement error no appreciable deviation in intracellular potential existed between the cells within an aggregate for intrinsic signals of low frequency and amplitude (DeFelice & DeHaan, 1977; DeHaan & DeFelice, 1978) or in response to small injected currents (DeHaan & Fozzard, 1975; Clay et al., 1979). This high degree of electrical coupling (coupling ratio near 1.0) was presumed to be mediated by low-resistance ionic channels, i.e. gap junctions between the cells within an aggregate (for review *see* DeHaan et al., 1980).

The difference between a CR of 1.0 in previous work and that seen in the present control measurement (CR = 0.81) results from our use here of the chopped-current injection circuit. In our earlier work (DeHaan & Fozzard, 1975; Clay et al., 1979; Nathan & DeHaan, 1979) current was driven into one cell while membrane potential was compared in two or more different cells, close to and far from the injection site. In that configuration no appreciable difference existed. In the present study, using the chopped-current circuit, V_1 represented the potential from the cell into which current was passed. This improvement in technique permits us to interpret our results in terms of a model similar to that used by Masson-Pevet, Bleeker and Gros (1979) to describe current flow in the rabbit sinus node. Thus, our values of CR here include the voltage drop across the junctions from the source cell into the rest of the aggregate while our previous measurements excluded that drop. Our calculations, as well as those by Clapham (1979) based on a more comprehensive model of spherical syncytia (Eisenberg et al., 1979) indicate that the voltage gradient between the source cell and the surrounding cells in a standard aggregate is very steep. A measureable voltage differential would not extend beyond the immediate neighbors of the source cell. Thus, even the small deviation from isopotentiality represented by CR = 0.8 would exist only in a few cells in an aggregate (probably < 10). Moreover, this gradient may account for the decrease in CR with larger injected currents (Table 5). Spray, Harris and Bennett (1979) have reported that nexal conductance diminishes with transjunctional voltage differentials.

In the present study we show that organized gap junctions comprise 0.27% of the total P-face of heart cell plasma membranes within an aggregate (Table 2). This value corresponds well with that found in other studies of embryonic heart tissue (Mazet, 1977; Gros, Mocquard, Challice & Schrevel, 1978; 1979; Masson-Pevet, 1979), and with the amount of gap

junctional area on the membranes of leading pacemaker cells in the adult rabbit sinus node (Masson-Pevet et al., 1979) and of circular smooth muscle of the guinea pig ileum (Gabella & Blundell, 1979). Gap junctions in heart cell aggregates are comprised of as few as four IMP to an upper limit of approximately 150 IMP. Small nexuses were also found in the intact embryonic chick heart. Mazet (1979) found gap junctions containing 3–17 particles in the 5-day embryonic ventricle. The size range of IMP associated with gap junctions is 7–10 nm with the predominant size being 9 nm (Fig. 3). These IMP were arranged in pleiomorphic configurations: macular clusters, linear chains and partially on fully closed annuli (Fig. 2). They were packed in these clusters at a density of about 8200 particles/μm^2. This agrees with the nexal particle packing density of 7263/μm^2 in intestinal smooth muscle (Gabella & Blundell, 1979) and 7625/μm^2 in ventricular muscle of the 21-day fetal rabbit (Shibata, Nakata & Page, 1980). It is not known whether there is any specific functional significance associated with the different configurations of clusters (for discussion *see* Larsen, 1977; Loewenstein, 1979; Peracchia, 1980).

In the nonjunctional P-face, IMP particles were scattered at a density of about 2000/μm^2 (Table 3). Sjostrand (1979) has recently criticized the conclusions of some early freeze-cleave studies on the grounds that membranes with different lipid/protein contents may not exhibit identical cleavage planes. Nonetheless, the appearance and density (IMP/μm^2) of background P-face particles in our preparations were remarkably similar to those seen in 18-day chick corneal fibroblasts (Hasty & Hay, 1977), adult guinea pig smooth muscle (Gabella & Blundell, 1979), mammalian red blood cells (Kirk & Tosteson, 1973) and fetal and adult rabbit ventricular sarcolemma (Frank, Beydler, Kreman & Rau, 1980; Shibata et al., 1980).

In our control aggregates in the present work, the total number of nonjunctional IMP particles per cell was about 720,000 (2000 $\mu m^2 \times 360$ μm^2/cell), while those clustered into identifiable nexuses on each cell numbered approximately 8000 (0.27% $\times$ 360 μm^2 per cell $\times$ 8200 particles per μm^2). Coupling ratio was 0.81. Using the value commonly estimated for unit channel resistance of $1 \times 10^{10} \Omega$ (Loewenstein, 1975; Bennett, 1978; DeHaan et al., 1980), and a specific nonjunctional membrane resistance of 80 $K\Omega \cdot cm^2$ (Table 5), we have calculated that a coupling ratio of 0.8 would be achieved if every cell in the aggregate were coupled to each of its adjoining neighbors by 800 junctional channels. These calculations and the electrical equivalent circuit on which they were based will be published in a subsequent paper (DeHaan & Williams, *in preparation*). If all of these channels were obliterated as the macular nexuses disappeared in CHX, CR between the cells in the aggregate should have fallen to near zero. The observed CR of 0.44 at 28–30 hr and 0.77 (with applied current) at 46–78 hr in CHX indicates that cells remained connected by substantial numbers of low-resistance pathways. A macular gap junction composed of 80 IMP particles would occupy about 0.01 μm^2. At the rate of decrease shown (Fig. 4) there would be fewer than one such particle cluster per total cell surface after 33 hr in CHX, and only one cluster of 4 particles per cell would be present after 48 hr in CHX. That is, if clustered particles were required for functional coupling any given cell in an aggregate could be weakly connected to only one of its several surrounding neighbors after 48 hr. Nonetheless, the aggregates continued to beat in an organized fashion after 48 hr and even after 72 hr in the inhibitor the activity showed no sign of disorganization. Intracellular electrode recordings from widely separated cells within these aggregates verified that they remained well coupled (Fig. 5*A*,*B*). Moreover, current injected at one point produced voltage changes in distant cells as if all cells were still connected by low-resistance junctions (Fig. 5*C*). These results are consistent with the view that low-resistance electrotonic coupling channels remain patent between the cells that comprise an aggregate despite the disappearance of organized nexuses.

The total amount of a cellular protein species depends on the sum of its synthesis and degradation rates (Schimke, 1975). Upon inhibition of protein synthesis, a decrease in membrane protein may be expected. Membrane-associated IMP particles seen in replicas of many cell types (Kirk & Tosteson, 1973; Ellisman, Rash, Staehelin & Porter, 1975) are generally thought to be globular proteins (Pinto da Silva & Branton, 1970), and there is strong evidence that the major component of gap junctions is a protein (Duguid & Revel, 1975; Goodenough, 1976; Hertzberg & Gilula, 1979; Kensler & Goodenough, 1980). The fact that the number of IMP particles in the nonjunctional P-face did not decrease after 48 hr of inhibition of protein synthesis (Table 3) suggests either that the turnover rate of nonjunctional membrane proteins is exceedingly slow, or that both the synthetic and degradative enzymes involved in membrane turnover were blocked by CHX (Franke, Moore, Deumling, Cheetham, Kartenbeck, Jarasch & Zentgraf, 1971; Schimke, 1975; Dean, 1980; Wildenthal & Crie, 1980). Interestingly, under conditions of severe anoxia nonjunctional membrane particle

density in rabbit ventricle fell only moderately from control values (2300 particles/μm^2 to 1763 particles/μm^2; Frank et al., 1980).

The disappearance of macular gap junctions from the freeze-fractured membranes of well-coupled heart cell aggregates suggests at least three possible explanations. (1) The low-resistance intercellular pathway is not associated with the nexus as morphologically defined, but with some different structure. This possibility must not be discounted; it has been explored by Daniel et al. (1976) and by Loewenstein (1979). But there is presently little supportive evidence. (2) Prolonged exposure to CHX does not cause the disappearance of gap junctions in the live tissue. It merely makes these structures more susceptible to artifactual disruption by the preparative procedures required for freeze-fracture analysis. This hypothesis has not been tested critically. Although no differences in gap-junctional structure were noted between preparations fixed in gluteraldehyde and those quick-frozen in liquid helium (Shibata et al., 1980), this question can probably best be approached with the ultra-rapid freezing technique that is purported to preserve cell structures in their native state (Raviola et al., 1978; Heuser, Reese, Dennis, Jan & Evans, 1979; Heuser & Kirschner, 1980). (3) The low-resistance intercellular pathway is represented by gap junctional particles (as supported by the evidence reviewed above), but these particles need not be organized into macular configurations to remain functional. That is, only a small number of patent individual channels between apposed cell membranes may be sufficient to mediate electrical coupling (Loewenstein, 1979). The effect of CHX may be to cause the digestion or internalization of some junctional particles, leaving only a few behind to serve as intercellular pathways. Or the macular clusters of particles may simply disperse, as individual junctional units dissociate from the clusters and migrate centrifugally into the surrounding membrane.

The contention that cells may be coupled by small numbers of junctional units not organized into macular clusters is supported by a growing body of recent evidence. It is now well documented that macular nexuses can form rapidly by clustering of previously isolated IMP particles (for reviews *see* Gilula, 1978; Sheridan, 1978; DeHaan et al., 1980). Under some normal conditions junctions rapidly disappear, but it is not known whether by dispersal or degradation. For example, during liver regeneration (Yee & Revel, 1978) and in hormonally regulated uterine myometrial smooth muscle (Garfield et al., 1978; Garfield, Kannan & Daniel, 1980) small nexuses appeared, enlarged and then disappeared. In the latter case, however, the uterine smooth muscle exhibited electrical continuity even before parturition, at a time when no gap junctions were evident.

Electrical coupling but not the intercellular passage of tracer molecules between human/mouse cell hybrids was mediated by linear arrays of IMP particles in the absence of macular clusters (Larsen et al., 1977; Loewenstein, 1979; 1980). Moreover, when isolated Novikoff hepatoma cells were brought into contact, electrical coupling (Johnson et al., 1974) and intercellular transfer of fluorescein (Sheridan, 1971) became detectable at the same time that particle clusters appeared in closely apposed membrane regions, between 10 and 30 min after initial apposition. However, Sheridan (1978) has argued that the earliest coupling in the first minutes after contact may have occurred across membranes in which putative junctional particles had not yet aggregated. Thus, macular clusters of channel-bearing IMP particles appear to form by the progressive recruitment of individual junctional units. The electrical correlate of this process may be the sequential "quantum jumps" in junctional conductance seen between newly apposed *Xenopus* cells (Loewenstein et al., 1978).

A similar inference comes from studies of nexus formation during normal embryonic development. At early stages of mammalian embryogenesis (Magnuson, Demsey & Stackpole, 1977) and during neurulation in the amphibian embryo (Decker & Friend, 1974), the first sign of nexuses between cells was the appearance in freeze-fractured preparations of small groups of IMP particles closer together than those in nonjunctional membrane. With development, nexal plaques formed and enlarged by a continuous process of recruitment and close packing of nearby particles (Benedetti, Dunia & Bloemendal, 1974). Similar events have been reported during cardiac development. In the chick heart, a coordinated beat begins after two days of development. But even in the 5-day chick myocardium, Mazet (1979) observed only tiny nexuses with as few as 3–17 particles spanning from the P-face of one cell to the E-face of an adjoining cell. The cells of the embryonic mouse heart established synchrony of contraction (i.e., electrical coupling) at about 8 days after fertilization. At that stage only a few loose clusters and linear arrays of IMP particles distinguish apposed surfaces from nonjunctional membrane (Gros et al., 1978). Small macular gap junctions became apparent two days later and gradually increased in size and relative area with continued development (Gros et al., 1979). Shibata et al. (1980) have suggested that junctions (in fetal rabbit heart) increase their size by insertion of new membrane and particles within the early junctional plaque rather than by fusion or peripheral expansion.

We have recently obtained independent evidence supporting the idea that only a few junctional channels are required for coupling, by measuring directly the junctional resistance between apposed heart cell aggregates. When two spontaneously beating aggregates were brought into contact under slight pressure each member of the pair continued to beat at its own rate for the first few minutes, but soon thereafter they synchronized their beats to a common rhythm (mean time to synchrony was 8 min; Ypey, Clapham & DeHaan, 1979). When the aggregates were first brought into contact, the coupling resistance (R_c) across the apposed surfaces was very high (>100 MΩ), but began almost immediately to fall (Clapham et al., 1980). When the aggregates first synchronized their beats, R_c was about 20 MΩ. During the subsequent hour or more, synchrony and action potential entrainment improved as R_c continued to decrease to <1 MΩ.

On the basis of the time course of the decline in R_c, Clapham et al. (1980) estimated that junctional channels, each with $1\times10^{10}\Omega$ resistance, were inserted into the surfaces of the cells joining two newly apposed aggregates at the rate of 1 channel per cell per minute. At 8 min after initial contact, when the aggregates first synchronized their beats at $R_c=20$ MΩ, only 500 channels were required to join the 100 apposed cells in the junctional region. Thus, about 5 channels per cell served to carry enough current to synchronize two aggregates. An hour later, when the aggregates were synchronized into better beat-to-beat fidelity and a short action potential latency, and R_c had declined to 1 MΩ, Clapham et al. (1980) estimated that 10,000 parallel channels joined the aggregates, or about 100 per apposed junctional cell. One hundred junctional channel particles scattered in the apposed cell surfaces would not readily be distinguishable from the background IMP particles.

We believe that these arguments, and the results described in the present study showing that the cells in an aggregate can remain coupled in the absence of organized nexuses, support the idea that in our system, and perhaps in others, low-resistance pathways may exist between cells as isolated channels scattered throughout the area of closely apposed plasma membranes.

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References

Azarnia, R., Larsen, W.J., Loewenstein, W.R. 1974. The membrane junctions in communicating and non-communicating cells, their hybrids and segregants. *Proc. Natl. Acad. Sci. USA* **71**:880

Azarnia, R., Loewenstein, W.R. 1977. Intercellular communication and tissue growth. VIII. A genetic analysis of junctional communication and cancerous growth. *J. Membrane Biol.* **34**:1

Baldwin, K.M. 1979. Cardiac gap junction configuration after an uncoupling treatment as a function of time. *J. Cell Biol.* **82**:66

Benedetti, E.L., Dunia, I., Bloemendal, H. 1974. Development of junctions during differentiation of lens fiber. *Proc. Natl. Acad. Sci. USA* **71**:5073

Bennett, M.V.L. 1973. Function of electrotonic junctions in embryonic and adult tissues. *Fed. Proc.* **32**:65

Bennett, M.V.L. 1978. Junctional permeability. *In*: Intercellular Junctions and Synapses. J. Feldman, N.B. Gilula and J.D. Pitts, editors. pp. 23–36. Chapman-Hall, London

Bennett, M.V.L., Goodenough, D.A. 1978. Gap junctions, electrotonic coupling and intercellular communication. *Neurosci. Res. Program Bull.* **16**:373

Chalcroft, J.P., Bullivant, S. 1970. An interpretation of liver cell membrane and junction structure based on observation of freeze-fracture replicas of both sides of the fracture. *J. Cell Biol.* **47**:49

Clapham, D.E. 1979. A whole tissue model of heart cell aggregates: Electrical coupling between cells, membrane impedance, and the extracellular space. Ph. D. Thesis, Emory University, Atlanta, Georgia

Clapham, D.E., Shrier, A., DeHaan, R.L. 1980. Junctional resistance and action potential delay between embryonic heart cell aggregates. *J. Gen. Physiol.* **75**:633

Clay, J.R., DeFelice, L.J., DeHaan, R.L. 1979. Parameters of current noise derived from voltage noise and impedance in chick embryonic heart cell aggregates. *Biophys. J.* **28**:169

Daniel, E.E., Daniel, V.P., Duchon, G., Garfield, R.E., Nichols, M., Malhotra, S., Oki, K. 1976. Is the nexus necessary for cell-to-cell coupling of smooth muscle? *J. Membrane Biol.* **28**:207

Daniel, E.E., Lodge, S. 1973. Electrophysiology of myometrium. *In*: Uterine Contraction. J.B. Josimovich, editor. Wiley-Interscience, New York

Dean, R.T. 1980. Protein degradation in cell cultures: General considerations on mechanisms and regulation. *Fed. Proc.* **39**:15

Decker, R.S. 1976. Hormonal regulation of gap junction differentiation. *J. Cell Biol.* **69**:669

Decker, R.S., Friend, D.S. 1974. Assembly of gap junctions during amphibian neurulation. *J. Cell Biol.* **62**:32

DeFelice, L.J., DeHaan, R.L. 1977. Membrane noise and intercellular communication. *IEEE Trans. Biomed. Eng.* **65**:796

DeHaan, R.L. 1970. The potassium sensitivity of isolated embryonic heart cells increases with development. *Dev. Biol.* **23**:226

DeHaan, R.L., DeFelice, L.J. 1978. Electrical noise and rhythmic properties of embryonic heart cell aggregates. *Fed. Proc.* **37**:213

DeHaan, R.L., Fozzard, H.A. 1975. Membrane response to current pulses in spheroidal aggregates of embryonic heart cells. *J. Gen. Physiol.* **65**:207

DeHaan, R.L., Sachs, H.G. 1972. Cell coupling in developing systems: The heart cell paradigm. *Curr. Top. Dev. Biol.* 7:193

DeHaan, R.L., Williams, E.H., Ypey, D.L., Clapham, D.E. 1981. Intercellular coupling of embryonic heart cells. *In*: Mechanisms of Cardiac Morphogenesis and Teratogenesis. T. Pexieder, editor. pp. 299–316. Raven Press, New York

Duguid, J.R., Revel, J.-P. 1975. The protein components of the gap junction. *Cold Spring Harbor Symp. Quant. Biol.* **40**:45

Eisenberg, R.S., Barcilon, V., Mathias, R.T. 1979. Electrical properties of spherical syncytia. *Biophys. J.* **25**:151

Ellisman, M., Rash, J., Staehelin, L.A., Porter, K.R. 1975. Studies of excitable membranes. *J. Cell Biol.* **68**:752

Flagg-Newton, J., Loewenstein, W.R. 1979. Experimental depression of junctional membrane permeability in mammalian cell culture. A study with tracer molecules in the 300 to 800 dalton range. *J. Membrane Biol.* **50**:65

Frank, J.S., Beydler, S., Kreman, M., Rau, E.C. 1980. Stucture of the freeze-fractured sarcolemma in the normal and anoxic rabbit myocardium. *Circ. Res.* **47**:131

Franke, W.W., Moore, D.J., Deumling, B., Cheetham, R.D., Kartenbeck, J., Jarasch, E., Zentgraf, H. 1971. Synthesis and turnover of membrane proteins in rat liver: An examination of the membrane flow hypothesis. *Z. Naturforsch.* **26b**:1031

Gabella, G., Blundell, D. 1979. Nexuses between the smooth muscle cells of the guinea pig ileum. *J. Cell Biol.* **82**:239

Garfield, R.E., Kannan, M.S., Daniel, E.E. 1980. Gap junction formation in myometrium: Control by estrogens, progesterone, and prostaglandins. *Am. J. Physiol.: Cell Physiol.* **7**:C81

Garfield, R.E., Sims, S.M., Kannan, M.S., Daniel,E.E. 1978. Possible role of gap junctions in activation of myometrium during parturition. *Am. J. Physiol.: Cell Physiol.* **4**:C168

Gilula, N.B. 1978. Structure of intercellular junctions: *In*: Intercellular Junctions and Synapses. J. Feldman, N.B. Gilula and J.D. Pitts, editors. pp. 3–22. Chapman-Hall, London

Gilula, N.B., Reeves, O.R., Steinbach, A. 1972. Metabolic coupling, ionic coupling, and cell contacts. *Nature* **235**:262

Goodenough, D.A. 1974. Bulk isolation of mouse hepatocyte gap junction. Characterization of the principal protein, connexin. *J. Cell Biol.* **51**:557

Goodenough, D.A. 1975. Methods for the isolation and structural characterization of hepatocyte gap junctions. *Methods Membr. Biol.* **3**:51

Goodenough, D.A. 1976. In vitro formation of gap junction vesicles. *J. Cell Biol.* **68**:200

Gros, D., Mocquard, J.P., Challice, C.E., Schrevel, J. 1978. Formation and growth of gap junctions in mouse myocardium during ontogenesis: A freeze-cleave study. *J. Cell. Sci.* **30**:45

Gros, D., Mocquard, J.P., Challice, C.E., Schrevel, J. 1979. Formation and growth of gap junctions in mouse myocardium during ontogenesis: Quantitative data and their implications on the development of intercellular communication. *J. Mol. Cell. Cardiol.* **11**:543

Hasty, D. J., Hay, E.D. 1977. Freeze-fracture studies of the developing cell surface. I. The plasmalemma of the corneal fibroblast. *J. Cell Biol.* **72**:667

Hertzberg, E.L., Gilula, N. B. 1979. Isolation and characterization of gap junctions from rat liver. *J. Biol. Chem.* **254**:2138

Heuser, J.E., Kirschner, M.W. 1980. Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. *J. Cell Biol.* **86**:212

Heuser, J.E., Reese, T.S., Dennis, M.J., Jan, Y., Evans, L. 1979. Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *J. Cell Biol.* **81**:275

Ito, S., Sato, E., Loewenstein, W.R. 1974. Studies on the formation of a permeable cell membrane function. I. Coupling under various conditions of membrane contact. Effects of colchicine, cytochalasin B, dinitrophenol. *J. Membrane Biol.* **19**:305

Johnson, R., Hammer, M., Sheridan, J., Revel, J.-P. 1974. Gap junction formation between reaggregated Novikoff hepatoma cells. *Proc. Natl. Acad. Sci. USA* **71**:4536

Kensler, R.W., Goodenough, D.A. 1980. Isolation of mouse myocardial gap junctions. *J. Cell Biol* **86**:755

Kirk, R.G., Tosteson, D.G. 1973. Cation transport and membrane morphology. *J. Membrane Biol.* **12**:273

Kuriyama, H., Suzuki, H. 1976. Changes in electrical properties of rat myometrium during gestation and following hormonal treatments. *J. Physiol. (London)* **260**:315

Lansman, R.A., Clayton, D.A. 1975. Mitochondrial protein synthesis in mouse L-cells: Effect of selective nicking of mitochondrial DNA. *J. Mol. Biol.* **99**:777

Larsen, W.J. 1977. Structural diversity of gap junctions. A review. *Tissue Cell* **9**:373

Larsen, W.J., Azarnia, R., Loewenstein, W.R. 1977. Junctional membrane structure of hybrids between communication-competent and communication-incompetent cells. *J. Membrane Biol.* **34**:39

Loewenstein, W.R. 1966. Permeability of membrane junctions. *Ann. N.Y. Acad. Sci.* **137**:441

Loewenstein, W.R. 1974. Cellular communication by permeable junctions. *In*: Cell Membranes: Biochemistry, Cell Biology and Pathology. G. Weissman and R. Clairborne, editors, pp. 105–114. HP Publishing Co., New York

Loewenstein, W.R. 1975. Permeable junctions. *Cold Spring Harbor Symp. Quant. Biol.* **40**:49

Loewenstein, W.R. 1979. Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta* **560**:1

Loewenstein, W.R. 1980. Junctional cell-to-cell communication and growth control. *Ann. N. Y. Acad. Sci.* **339**:39

Loewenstein, W.R., Kanno, Y., Socolar, S.J. 1978. The cell-to-cell channel. *Fed. Proc.* **37**:2645

Magnuson, T., Demsey, A., Stackpole, C.W. 1977. Characterization of intercellular junctions in the preimplantation mouse embryo by freeze-fracture and thin-section electron microscopy. *Dev. Biol.* **61**:252

Makowski, L., Caspar, D.L.D., Phillips, W.C., Goodenough, D.A. 1977. Gap junction structures. II. Analysis of the X-ray diffraction data. *J. Cell Biol.* **74**:629

Masson-Pevet, M. 1979. The fine structure of cardiac pacemaker cells in the sinus node and in tissue culture. Ph. D. Thesis, University of Amsterdam

Masson-Pevet, M., Bleeker, W.K., Gros, D. 1979. The plasma membrane of leading pacemaker cells in the rabbit sinus node. *Circ. Res.* **45**:621

Mazet, F. 1977. Freeze-fracture studies of gap junctions in the developing and adult amphibian cardiac muscle. *Dev. Biol.* **60**:139

Mazet, F. 1979. Etude ultastructurale des jonctions presente dans la myocarde de la Poulet. *Biol. Cellulaire Francais* **29**:27a

McNutt, N.S., Weinstein, R.S. 1973. Membrane ultrastructure at mammalian intercellular junctions. *Prog. Biophys. Mol. Biol.* **26**:45

Nathan, R.D., DeHaan, R.L. 1979. Voltage clamp analysis of embryonic heart cell aggregates. *J. Gen. Physiol.* **73**:175

Peracchia, C. 1977. Gap junctions. Structural changes after uncoupling procedures. *J. Cell Biol.* **72**:628

Peracchia, C. 1980. Structural correlates of gap junction permeation. *Int. Rev. Cytol.* **66**:81

Peracchia, C., Dulhunty, A.F. 1976. Low resistance junctions in crayfish. Structural changes with functional uncoupling. *J. Cell Biol.* **70**:419

Pinto da Silva, P., Branton, D. 1970. Membrane splitting in freeze-etching. Covalently bound ferritin as a membrane marker. *J. Cell Biol.* **45**:598

Potter, D.D., Furshpan, E.J., Lennox, E.S. 1966. Connections between cells of the developing squid as revealed by electrophysiological methods. *Proc. Natl. Acad. Sci. USA* **55**:328

Raviola, E., Goodenough, D.A., Raviola, G. 1978. The nature structure of gap junctions rapidly frozen at 4°K. *J. Cell Biol.* **79**:299a

Revel, J.-P., Karnovsky, M.J. 1967. Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* **33**:C7

Sachs, H.G., DeHaan, R.L. 1973. Embryonic myocardial cell aggregates: Volume and pulsation rates. *Dev. Biol.* **30**:233

Schimke, R.T. 1975. Turnover of membrane proteins in animal cells. *Methods Membr. Biol.* **3**:201

Sheridan, J.D. 1971. Dye movement and low-resistance junctions between reaggregated embryonic cells *Dev. Biol.* **26**:627

Sheridan, J.D. 1978. Junction formation and experimental modification. *In*: Intercellular Junctions and Synapses. J. Feldman, N.B. Gilula and J.D. Pitts, editors. pp. 37–59. Chapman-Hall, London

Shibata, Y., Nakata, K., Page, E. 1980. Ultrastructural changes during development of gap junctions in rabbit left ventricular myocardial cells. *J. Ultrastruct. Res.* **71**:258

Simpson, I., Rose, B., Loewenstein, W.R. 1977. Size limit of molecules permeating the junctional membrane channels. *Science* **195**:294

Sjostrand, F.S. 1979. The interpretation of pictures of freeze-fractured biological material. *J. Ultrastruct. Res.* **69**:378

Socolar, S.J., Loewenstein, W.R. 1979. Methods for studying transmission through permeable cell-to-cell junctions. *Methods Membr. Biol.* **10**:123

Spray, D.C., Harris, A.L., Bennett, M.V.L. 1979. Voltage dependence of junctional conductance in early amphibian embryos. *Science* **204**:432

Tomita, T. 1973. Electrical properties of mammalian smooth muscle. *In*: Smooth Muscle. E. Bulbring, A. Brading, A. Jones, and T. Tomita, editors. pp. 197–243. Williams and Wilkins, Baltimore, Maryland

Wildenthal, K., Crie, S. 1980. The role of lysosomes and lysosomal enzymes in cardiac protein turnover. *Fed. Proc.* **39**:37

Williams, E.H., DeHaan, R.L. 1978. Alteration in synchronization time of newly apposed heart cell aggregates by pretreatment with trypsin and cycloheximide. *J. Cell Biol.* **79**:239a

Williams, E.H., DeHaan, R.L. 1979. Changes in IMP particle distribution in gap junctional and non-junctional membrane of heart cells after prolonged inhibition of protein synthesis. *J. Cell Biol.* **83**:81a

Wilson, J.A., Goldner, M.M. 1975. Voltage clamping with a single microelectrode. *J. Neurobiol.* **6**:411

Yee, A.G., Revel, J.-P. 1978. Loss and reappearance of gap junctions in regenerating liver. *J. Cell Biol.* **78**:554

Ypey, D. L., Clapham, D.E., DeHaan, R.L. 1979. Development of electrical coupling and action potential synchrony between paired aggregates of embryonic heart cells. *J. Membrane Biol.* **51**:75

Zampighi, G., Corless, J.M., Robertson, J.D. 1980. On gap junction structure. *J. Cell Biol.* **86**:190

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