Junctional Resistance and Action Potential Delay between Embryonic Heart Cell Aggregates

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ABSTRACT   Spheroidal aggregates of embryonic chick ventricle cells were brought into contact and allowed to synchronize their spontaneous beats. Action potentials were recorded with both intracellular and extracellular electrodes. The degree of electrical interaction between the newly apposed aggregates was assessed by measuring the delay or latency (L) between the entrained action potentials, and by determining directly interaggregate coupling resistance (R_c) with injected current pulses. Aggregate size, contact area between the aggregates, and extracellular potassium concentration (K_o^+) were important variables regulating the time-course of coupling. When these variables were controlled, L and R_c were found to be linearly related after beat synchrony was achieved. In 4.8 mM K_o^+ $L/R_c = 3.7$ ms/MΩ; in 1.3 mM K_o^+ $L/R_c = 10.1$ ms/MΩ. We conclude that action potential delay between heart cell aggregates can be related quantitatively to R_c.

The development of synchrony is a striking attribute of embryonic heart cells. Single cells synchronize their visible contractile activity within minutes after initial contact (DeHaan and Hirakow, 1972). Spheroidal aggregates composed of thousands of cardiac cells (Sachs and DeHaan, 1973) also become entrained to a common rhythm when brought together in pairs (Ypey et al., 1977, 1979; Williams and DeHaan, 1978). It is widely held that cardiac tissue behaves like an electrical syncytium. Signals originating at one point traverse the tissue as if ionic currents can flow freely from cell to cell. Since the discovery of cytoplasmic discontinuity at the intercalated disk (Sjöstrand and Anderson, 1954), the mechanism of this electrical coupling between heart cells has been widely investigated (for early reviews, see Weidmann, 1966; DeHaan and Sachs, 1972). Experiments on adult heart preparations have established their conductive properties (Weidmann, 1952; Barr et al., 1965; DeMello, 1975; Pollack, 1976; Weingart, 1977; Bleeker et al., 1980). Moreover, embryonic (Sperelakis, 1969; Goshima, 1971; Lieberman et al., 1973; DeFelice and DeHaan, 1977; Griepp and Bernfield, 1978) and neonatal heart cell systems (Jongsma and Van Rijn, 1972; Jongsma et al., 1975), have been used in a variety of geometries in tissue culture to investigate the spread of action potentials or other electrical signals from cell to cell. An extensive review of the literature and theory of action potential conduction is available based on

electrotonic coupling of heart cells via nexal junctions (Fozzard, 1979). A model for action potential spread that does not depend on low-resistance cell junctions has also been proposed (Sperelakis, 1969; Mann et al., 1977; Mann and Sperelakis, 1979). In the present study we measure directly the decrease in resistance between newly apposed aggregates of embryonic heart cells and correlate that decrease with the onset of synchronous beating.

The development of electrical coupling between a variety of cells has been associated with the formation of gap or nexal junctions (for reviews see DeHaan and Sachs, 1972; Loewenstein, 1975; Sheridan, 1976; Bennett, 1978; DeHaan et al., 1980). The formation of gap junctions between cells results in ionic communication and thus a lowering of intercellular resistance. Recent evidence indicates that nexal units are added quantally and are relatively stable once formed, but these structures may be disrupted by high intracellular calcium concentrations (Loewenstein and Rose, 1978). Decreased resistance between cells associated with newly formed junctions has been found 5 min after cell contact in Novikoff hepatoma cells (Johnson et al., 1974) and within 1–2 min after contact between cells of *Xenopus laevis* embryos (Loewenstein et al., 1978). Nexal junction resistance is thought to be decreased by an accretion (Ito et al., 1974) of individual "junctional units" each having a channel resistance of about $10^{10}\Omega$ (Loewenstein, 1975). The specific nexal coupling resistance is a measure of the ionic permeability referred to a unit area of junction. Recent estimates of specific nexal resistance have been repeatedly revised downward from early estimates of 1–5 $\Omega \cdot cm^2$ (see e.g., Woodbury and Crill, 1961; Weidmann, 1966). When the junctional contact area has been measured, specific junctional resistance has been reported to be 0.009–0.012 $\Omega \cdot cm^2$ in Novikoff hepatoma cells (Sheridan et al., 1978), or 0.05 $\Omega \cdot cm^2$ for rat ventricle (Matter, 1973).

For the studies described here we have used spheroidal aggregates of embryonic chick ventricle cells (Sachs and DeHaan, 1973). Cells within such preparations are in direct electrical communication (DeHaan and Fozzard, 1975; DeFelice and DeHaan, 1977), and within strictly defined limits of frequency and amplitude (Clay et al., 1979) each aggregate approximates an isopotential system. The purposes of the present study were (a) to define the time-course of electrical interaction between a pair of aggregates after they were brought into contact; (b) to relate the degree of electrical interaction to the interaggregate coupling resistance; and (c) to show that the coupling resistance was associated with an impulse delay between synchronized beats of two entrained aggregates. Throughout this paper the term latency (L) will be used to describe the delay in milliseconds between the fastest parts of the upstrokes of two synchronized action potentials. Synchrony, in this context, means that the action potentials from two aggregates are entrained with a fixed or slowly changing time delay between the leading and following spikes.

METHODS

Tissue Culture

After incubation for 7 d, white Leghorn chick embryos were harvested in amniotic fluid and decapitated. Hearts were dissected free and trimmed of extraneous tissue

and the interior was exposed. The apical portions of the ventricles were dissociated into their component cells by the multiple-cycle trypsinization method described previously (DeHaan, 1970) using either crude trypsin (500 μg/ml 1:300, United States Biochemical Corp., Cleveland, Ohio) or DM8, which consists of the following purified components: 50 μg/ml crystalline trypsin (Worthington Biochemical Corp., Freehold, N.J.; 225 U/mg); 5.5 μg/ml deoxyribonuclease (Worthington; 9 × 10^4 U/mg); 1 mg/ml bovine serum albumin (fraction V fatty acid poor, Miles Laboratories, Inc., Elkhart, Ind.). Inocula of 5 × 10^5 cells were allowed to reaggregate for 24–48 on a gyratory shaker in 3 ml of medium 818A (Sachs and DeHaan, 1973). This solution was maintained at pH 7.2 under an atmosphere of 5% CO_2, 10% O_2, and 85% N_2. During gyration spheroidal aggregates were formed which typically ranged in size from 100 to 300 μm in diameter. Aggregates were transferred from the flask to a Falcon plastic tissue culture dish (Becton, Dickinson & Co., Rutherford, N.J.) for electrophysiological recordings.

Media

Medium 818A contained 20% M199 (Grand Island Biological Co., Grand Island, N.Y.), 2% heat-inactivated horse serum (K. C. Biological, Lenexa, Kansas), 4% fetal calf serum (Grand Island), and 0.5% Gentamycin (Schering Corp., Kenilworth, N.J.) in potassium-free Earle's balanced salt solution: (millimolar) NaCl, 116.0; $MgSO_4$, 0.8; NaH_2PO_4, 0.9; $CaCl_2$, 1.8; $NaHCO_3$, 26.3; and glucose, 5.5. The potassium concentration of the final medium was adjusted to 1.3 or 4.8 mM. Tetrodotoxin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in distilled water (1 mg/ml) and diluted to a final concentration of 1 × 10^{-6} g/ml.

Electrophysiological Measurements

A tissue culture dish with 100–200 aggregates was placed on a controlled (37°C) warm stage of a dissecting microscope. Nontoxic mineral oil (Klearol, Sonneborn Division, Witco Chemical Co., New York) layered over the medium prevented evaporation, and a 10% O_2, 5% CO_2, 85% N_2 gas mixture was directed from a toroidal gassing ring over the dish to control pH. Aggregates in 1.3 mM potassium beat vigorously for hours or even days at a regular beat rate under these conditions. Aggregates in 4.8 mM potassium did not beat spontaneously, but action potentials could be evoked by mechanical or electrical stimulation.

Measurement of Transaggregate Resistance

Individual embryonic heart cells in aggregates have diameters averaging 11 μm.[1] Microelectrode recording in aggregates with one or even two electrodes can be accomplished routinely, but sustained impalements with three or four microelectrodes simultaneously are not practical for a large series of experiments. Two electrical configurations were used in measuring the transaggregate resistance. (*a*) In most cases, current was injected through one of two suction electrodes while transmembrane potential was measured in each aggregate (V_1, V_2) via intracellular microelectrodes (Fig. 1*b*). Each aggregate was thus connected to one current-injecting suction electrode and one voltage-measuring microelectrode. Essentially, the measurement was the same as the four-microelectrode experiments of Ito et al., (1974), except that in our experiments a smaller fraction of injected current flowed into the cells. The rest of the current passed via low resistance clefts to ground. (*b*) In a few experiments we adopted a three-microelectrode configuration in which one of the aggregates was penetrated by two microelectrodes while the third was used to record potentials from the second

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[1] Atherton, B. T., and R. L. DeHaan. Unpublished observation.

aggregate. Suction electrodes were used in this configuration only for manipulating the aggregates.

In both configurations a complete experimental sequence consisted of (a) measurement of input resistance in each aggregate (R_1 and R_2) before contact; (b) continuous measurement of coupling resistance (R_c), and input resistance for 2-3 h after contact;
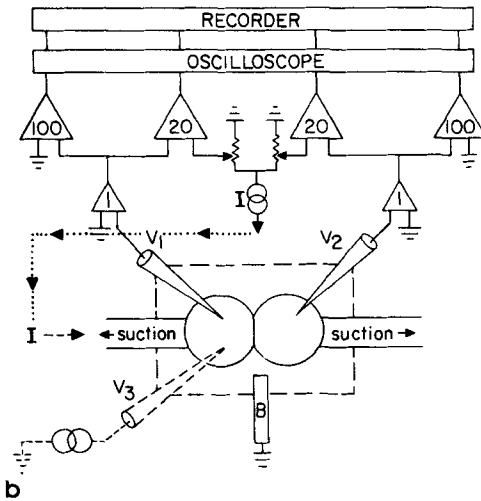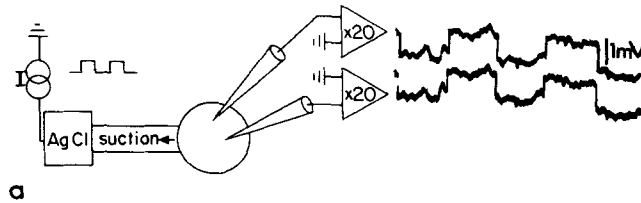

FIGURE 1.   (a) Uniformity of voltage response in a single aggregate to a series of 5-s current pulses injected via a suction electrode. Two voltage electrodes in the aggregate measure the same potential, regardless of orientation or separation distance. One microelectrode was then used to inject current to obtain the input resistance of the isolated aggregate. Aggregate input resistance varied with size and K_o^+ and ranged from 0.5 to 4 MΩ for the preparations studied here. (b) Diagrammatic representation of measurement system. Suction electrodes were used for current injection and micromanipulation of aggregates. Two microelectrodes (V_1, V_2) were used to measure response to current pulses applied via one suction electrode in most cases. In some experiments, a third microelectrode was used (V_3) for current injection in lieu of the suction electrode. Slight offset from current drop across the agar bridge (B) to ground was balanced as shown. High gain (×100) amplifiers are AC-coupled.

and (c) redetermination of input resistance R_1 and R_2 after separation of the aggregates. With a constant current pulse, R_c could be calculated from the expression

$$R_c = \frac{R_2(V_1 - V_2)}{V_2} \qquad (1)$$

Other expressions exist for R_c, but this equation incorporates the parameters with least measurement error in our experimental configuration.

Suction Electrode/Microelectrode Experiments

An aggregate was attached by gentle suction to a 15 μm i.d. firepolished glass electrode filled with normal medium. Suction was provided by a 100 μl syringe connected by teflon tubing to the suction port of the electrode holder. Aggregates were allowed to attach to the suction electrodes for ~ 30 min. The seal around each aggregate (~ 100 kΩ) was monitored and the aggregates were brought close together after a steady-state sealing resistance was reached (generally in a few minutes). Current was injected through a 10 MΩ current-limiting resistor connected to a battery-powered pulse generator (Pulsar 4, Frederick Haer & Co., Brunswick, Maine). Pulses were given in either hyperpolarizing, or depolarizing directions for 5-s cycles. Before each experiment, two microelectrodes were placed in each aggregate to measure input resistance (R_1, R_2). Microelectrodes were held in Ag/AgCl electrode holders attached to micromanipulators. Leads were connected to unity gain buffer amplifiers (Picometric 181, Instrumentation Laboratory, Inc., Lexington, Mass.), the outputs of which were connected to low noise preamplifiers (Princeton Applied Research Corp., Princeton, N.J., model 113, X20 gain). Aggregates were gently pushed together after input resistances had been measured, using the suction electrodes attached to micromanipulators. Microelectrodes were then placed in each aggregate. Current (I), and voltages (V_1, V_2) were measured continuously as coupling progressed (Fig. 4 a). Input resistances R_1, R_2 were again measured ~30 min after completion of coupling and subsequent separation of the aggregates. Only experiments in which input resistance had not changed more than 15% were accepted for further analysis. Nonjunctional input resistances (R_1, R_2) were assumed to stay constant during coupling since total input resistance of the coupling pair changed with junction formation (see Ito et al., 1974).

In all experiments, pulses injected during the coupling experiment by suction electrodes resulted in uniform voltage deflections regardless of the positions of the two microelectrodes within the aggregate (see Fig. 1 a). The aggregate is made of 2,000–3,000 cells and most (~99%) of the current from the suction electrode is shunted through the low resistance intercellular space (Clapham, 1979). The intracellular current is distributed across all membranes to result in the uniform voltage deflection.

Inasmuch as the suction electrodes were filled with medium and the microelectrodes with 2 M KCl, both referenced to a common ground, the tip potential of the microelectrodes was dialed to 0 with the buffer amplifier. The pulse current drop across the low series resistance (5 kΩ) agar bridge to ground was also balanced off at the differential input to the low noise amplifiers (X20 gain).

In one series of preliminary experiments, $R_i (= V/I)$ was measured repeatedly over a period of 2 h in single aggregates. Current pulses were applied either through a suction electrode, through a second intercellular electrode in the aggregate, or through the voltage recording electrode connected to a balanced bridge circuit. R_i did not vary perceptibly with time when measured with two intracellular electrodes, even though repeated impalements were required. When current was injected through a suction electrode, variations in R_i up to 20% could be obtained by applying sufficient suction to deform the aggregate. With only the suction needed to produce a good seal, measured R_i varied with time by less than 5%. With current injected via a balanced bridge through the voltage recording electrode, measured R_i varied by up to 50%, presumably as a result of drift in electrode resistance. Thus we report no experiments with the balancing technique.

Three-Microelectrode Experiments

Two intracellular microelectrodes, each connected to pass current or record voltage, were placed in one aggregate; a third, voltage-recording electrode, was placed in a second aggregate of equal size that had been brought into contact with the first. Current pulses were held to about 1 nA to keep the voltage response in the linear range.

Spontaneously beating aggregates in 1.3 mM K_o^+ were made quiescent with 10^{-6} g/ml TTX. In 4.8 mM, K_o^+ aggregates were quiescent without TTX. Microelectrode impalements were considered acceptable only if the resting membrane potential was stable at near -50 mV (1.3 mM K_o^+) or -80 mV (4.8 mM K_o^+). Microelectrode voltages, amplified at a low gain ($\times 20$) or at high gain ($\times 100$) were recorded on line through two storage oscilloscopes; data were also recorded on a seven-channel FM tape recorder (Sangamo, Sabre II, Springfield, Ill.) for off-line analysis.

Latency Measurements

During an action potential, a small (1-5 mV) short duration (1 ms) extracellular voltage spike was measured by the suction electrodes from each aggregate (Fig. 2, *inset*). Extracellular voltage spikes occur at $\dot{V}_{max}$ and therefore can be used as a precise time marker of the action potential upstroke. The latency (L) between synchronized aggregates was measured as the delay between the extracellular voltage spikes from each aggregate. In quiescent aggregates, latencies were obtained from a train of stimulated action potentials initiated by a pulse of current injected through one suction electrode. The latencies in these cases were measured between action potentials firing after termination of the stimulating current.

RESULTS

Experiments measuring the decline in L and in coupling resistance (R_c) between newly apposed aggregates were made at two different potassium concentrations: 1.3 and 4.8 mM. Aggregates beat spontaneously in 1.3 mM K_o^+ but not in 4.8 mM. Measurements of L could be made from aggregate pairs whether they were spontaneously active or not, by stimulation of action potentials in the latter case. However, R_i and R_c could only be determined accurately in nonspontaneous preparations with stable membrane potentials. To measure the voltage response to small injected pulses of current, the spontaneous activity of aggregates in 1.3 mM K_o^+ was suppressed by blocking fast Na channels with tetrodotoxin (TTX). In this case, slowly rising action potentials ($\dot{V}_{max} = 5$-10 V/s) could still be stimulated (Nathan and DeHaan, 1978) to permit measurements of L.

In most experiments, either L or R_c was determined separately with time after contact, except in a few cases, where both measurements were obtained on the same aggregate pair throughout coupling.

Before pairing, R_i was measured routinely on each single aggregate by injecting current pulses through one intracellular electrode and measuring voltage deflection with the other from a widely separate cell. Sufficient current was then injected through the suction electrode to achieve a similar voltage response (~ 1 mV) in both intracellular electrodes (Fig. 1 *a*). In all cases that exhibited criteria of good impalements, the voltages recorded at the two intracellular points did not differ perceptibly, irrespective of their distance from each other or their position relative to the suction electrode.

Latency

A total of 44 experiments was performed to measure the decrease in L with time after action potential synchrony; 25 in 1.3 mM K_o^+ and 19 in 4.8 mM K_o^+. Extracellular potentials used to monitor L were related to the first derivative of the action potential (Clapham, 1979) and served as precise time markers of $\dot{V}_{max}$ with a resolution of ~ 100 μs for spontaneously beating aggregates and about 2 ms for slow action potentials fired in TTX. A measurement of L is demonstrated in Fig. 2 (*inset*). The top trace has been
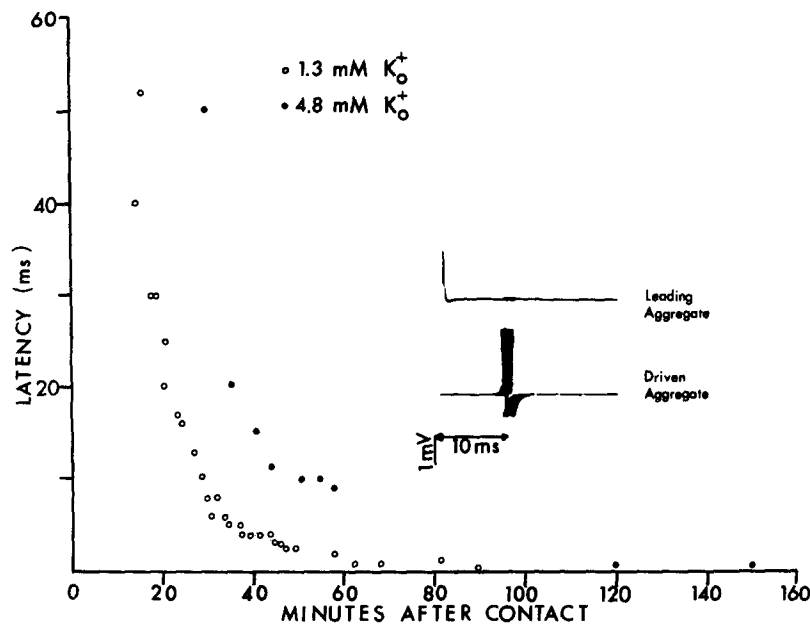

FIGURE 2. Two experiments to show the decline in latency after apposition of a pair of aggregates. Latencies were measured after synchrony was achieved at 16 (1.3 mM K_o^+) and 30 (4.8 mM K_o^+) min after initial contact. A latency measurement is shown in *inset*. Extracellular spikes of about 3 mV amplitude were recorded from each aggregate with suction electrodes. The oscilloscope was triggered by the upper trace and 10 consecutive sweeps were stored. The interval between the spikes from the two aggregates was defined as L. Aggregates beat spontaneously in 1.3 mM K_o^+. In 4.8 mM K_o^+ latencies were obtained from trains of action potentials that followed a short depolarizing current pulse through the suction electrode. Latencies were measured between action potentials initiated after termination of the depolarizing current.

used to trigger the oscilloscope while 10 consecutive sweeps were recorded. The traces show a mean L between the action potentials of the leading and driven aggregate of 10 ms. This record also indicates that the aggregates were well synchronized, as evidenced by the small fluctuation about that mean.

The decrease in L for representative pairs of aggregates is plotted in Fig. 2 at the two different K_o^+ concentrations. At time 0, two aggregates held in suction electrodes were pushed together while their extracellular potentials

were continuously recorded. For the first few minutes each aggregate continued beating at its own intrinsic rate, and the action potentials showed no correlation or definable latency. 16 (1.3 mM K_o^+) or 30 (4.8 mM K_o^+) min after contact, in the two pairs shown, the aggregates achieved synchrony, in that a definable mean L could be measured (52 and 50 ms, respectively). At this early stage of synchrony, latencies exhibited large fluctuations. With continued time after contact, L declined rapidly, reaching a plateau of about 1 ms after about 1 h in low-K medium. As shown in Table I, aggregates consistently achieved initial synchrony later in 4.8 mM K_o^+ than did similar-sized preparations in 1.3 mM K_o^+, whereas the time constant (τ) of the decline in L was not significantly different. (Behren's test, 5% level, Fisher and Yates, 1967).

Coupling Resistance

The input resistance of aggregates scales with total membrane area ($\propto r^3$) (Clay et al., 1979) and with K_o^+. In general, for the same size aggregate, the input resistance is three to four times lower in 4.8 mM K_o^+ (1–2 MΩ) than in 1.3 mM K_o^+ (4–5 MΩ). In the simple circuit of Fig. 4 a, R_i and R_c alone

TABLE I

LATENCY AND SIZE PARAMETERS OF COUPLING
AGGREGATES IN DIFFERENT K_o^+ CONCENTRATIONS

Concn	r_1	r_2	r_{ac}	n	t_s	τ
	μm	μm	μm		min	min
1.3 mM K_o^+	87±13	79±13	61±5	11	16.5±8.9	10.3±9
4.8 mM K_o^+	79±13	79±15	55±13	12	34.5±13	19.1±12

Representative latency data: r_1 and r_2, radius of aggregates, r_{ac}, radius of area of contact, t_s, time to first synchrony, τ, time constant of exponential decline in latency.

determine the amount of current exchanged between aggregates across the newly formed coupling junctions. The input resistances R_1 and R_2 are assumed to remain constant during electrical coupling since the area of nonjunctional membrane converted to junctional membrane in the coupling process is probably a small percentage of the total nonjunctional membrane (Matter, 1973; DeHaan et al., 1980). The coupling process should depend directly on the coupling resistance and inversely on the input resistance of the driven aggregate. Since coupling resistance must depend on area of contact (more cell area for junction formation) and input resistance depends on aggregate total membrane, we expected a correlation of coupling time with both of these parameters. In Fig. 3 we have plotted the time between contact and initial synchrony (t_s) vs. the ratio of contact area ($\sim r_a^2$) to the total membrane surface area of the driven aggregate ($\sim r_2^3$). The substantial scatter results mainly from the aggregate size measurement which has a resolution of ±10 μm. Furthermore, the relation between visible area of contact and actual area of nexal junction is not known for the aggregates. Nonetheless, an increase in relative apparent junctional area is seen to have a dramatic effect in reducing t_s.

Fig. 4 *a* represents diagrammatically the experimental configuration used to measure the change in coupling resistance with time when current was injected through a microelectrode. In Fig. 4 *b*, the results of one such experiment are plotted. A 3-nA constant current pulse was injected for 5-s hyperpolarizing cycles during the entire experiment. The resulting changes in V_1 and V_2 are plotted against time along with the coupling ratio, V_2/V_1 and R_c. The calculated coupling resistance falls from approximately 5 to 2 MΩ over a period of 90 min. Due to the considerable difficulty of maintaining three microelectrodes in two aggregates for several hours, only one complete experiment of this kind was obtained. However, the many incomplete experiments with microelectrodes and those with suction electrodes (described below) were corroborative of these results.
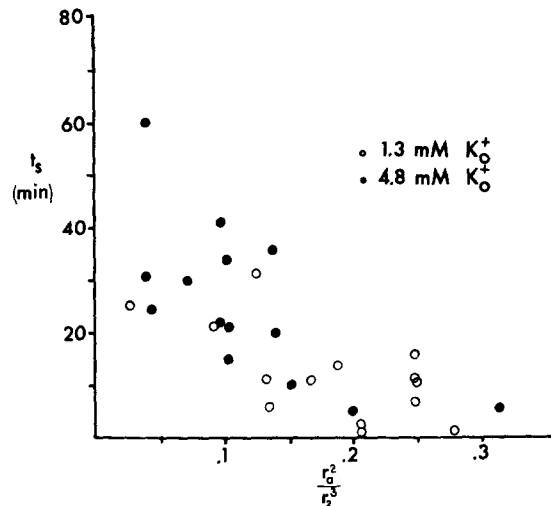

FIGURE 3. Relation of size and area of shared contact to time to synchrony (t_s). r_a, radius of apparent circular area of contact; r_2, driven aggregate radius. Coupling resistance is directly proportional to area of contact ($\propto r_a^2$) and inversely proportional to aggregate input resistance ($\propto r_2^3$).

In all experiments in which suction electrodes were used for current injection, voltage homogeneity of each aggregate was verified during the measurement of input resistance. Linearity of the voltage response was confirmed at the beginning of each experiment by varying input current over a suitable range. Of 21 experiments with the microelectrode-suction electrode combination, six experiments contained sufficient information for complete analysis. Representative records from one experiment in 1.3 mM K_o^+ are shown in Fig. 5. The results from an experiment in 4.8 mM K_o^+ are summarized in Fig. 6. Both microelectrodes had been inserted and achieved stable potentials by 15 min after contact but there was no appreciable pulse transfer until 40 min. R_c declined from 10 MΩ to less than 2 MΩ in 80 min. The slower onset of coupling seen here, compared with pulse transfer in 8 min in 1.3 mM K_o^+ (Fig. 5), was typical of coupling in high K_o^+. Furthermore, coupling ratios obtained

in 4.8 K_o^+ (avg = 0.5) in the steady state were lower than those obtained in 1.3 mM K_o^+ (avg = 0.85). The lower coupling ratio in 4.8 mM K_o^+ would be expected even if R_c were the same in both K_o^+ levels, because the input resistance is lower in 4.8 mM K_o^+.

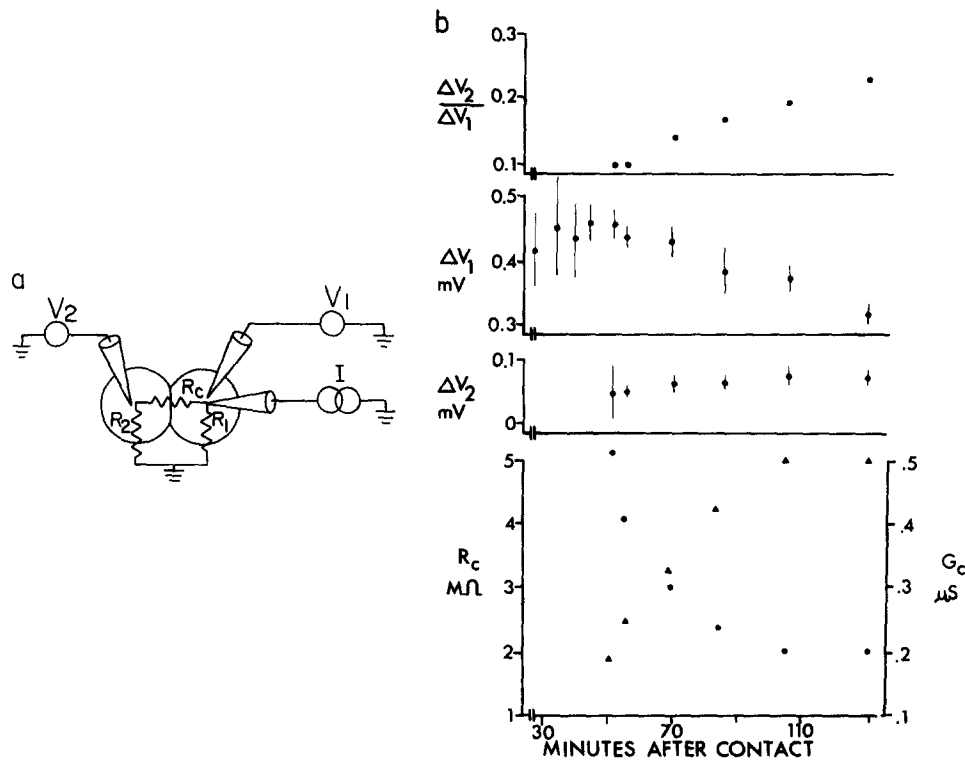The decline in coupling resistance with time for all complete experiments

FIGURE 4. (a) Schematic diagram of the three-microelectrode measurements of coupling parameters used to obtain data shown in b. This simple three-resistor circuit is assumed in all subsequent measurements. (b) Coupling parameters obtained from continuous pulse injection for a period of 2 h after pairing of aggregates. 10-s hyperpolarizing pulses (1 nA, 50% duty cycle) were injected by the I electrode while monitoring with voltage electrodes V_1 and V_2, K_o^+ = 4.8 mM. Resting potential −75 mV. r_1 = 80 μm, r_2 = 90 μm. R_1 = 0.87 MΩ; R_2 = 0.9 MΩ. r_a = 65 μm. Error bars ± 1 standard deviation. R_c is calculated as shown in text. Coupling conductance (G_c) is $1/R_c$ in units of Siemens (Ω^{-1})· 10^{-6}.

using suction electrodes and microelectrodes is summarized in Fig. 7. R_c has been normalized for contact area (measured under the dissecting microscope). Junctional resistance apparently begins to decline earlier in 1.3 mM K_o^+ than in 4.8 mM K_o^+. This implies a direct effect of potassium concentration on the rate of formation of gap junctions; i.e., 4.8 mM K_o^+ seems to delay junction formation.

Correlation between L and R_c

Action potentials were stimulated periodically during the course of some of the experiments in high K_o^+ to obtain both latency and resistance data at the same time (Fig. 8). The parallel time-course of decline in both L and R_c (semilog scales) is apparent, although the methods for determining these parameters were completely independent; R_c was calculated from pulse transfer whereas L was measured from action potential delays. Synchrony of action potentials at $t = 10$ min occurred before R_c could be resolved. At ~50 min one of the microelectrodes came out of the cell resulting in the gap in the data. The disruption was fortunate since it produced a shift in the decline in L, which was reflected precisely by a similar shift in R_c. Apparently, the damage and resulting leakage from attempts to repenetrate caused a setback in the
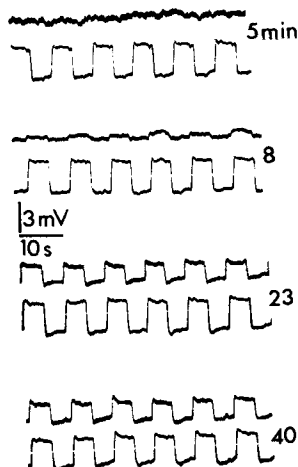

FIGURE 5.   Representative voltage responses from a pair of coupling aggregates to 5-s hyperpolarizing current pulses injected by suction electrode into the aggregate represented by the lower trace. Aggregates were placed in contact at time zero. Coupling progressed rapidly to a coupling ratio (V_2/V_1) of 0.8 at 40 min. $K_o^+ = 1.3$ mM.

time-course of coupling, perhaps due to a rise in intracellular Ca^{++} (DeMello, 1975; Rose and Loewenstein, 1976). Both R_c and L were equally affected, confirming the validity of the correlation. A more careful examination of the two curves shows correlation of changes in R_c and L over even shorter time intervals. Once the aggregates were successfully reimpaled, the size and shape of the action potentials were found to be the same as those recorded immediately after contact, and they showed no appreciable changes as R_c continued to fall. The correlation between R_c and L is shown in Fig. 9 (solid lines) at the two different levels of K_o^+. There is a striking degree of linearity for the entire duration of the coupling process. The simple linear relationship between R_c and L, with no changes in action potential parameters as coupling progressed, suggests that the junctional resistance had a dominant effect in causing action potential delays across the junction in this preparation.

The slope of L/R_c was 3.7 ms/MΩ in 4.8 mM K$^+$ (with no TTX) and 16 ms/MΩ in 1.3 mM K$^+$ (with TTX). To determine how much of the increase in L at a given level of R_c resulted from alterations in the electrical parameters of the aggregates in TTX rather than from the difference in K_o^+, 13 separate aggregate pairs in 1.3 mM K_o^+ with no TTX were apposed and allowed to achieve various values of L. These preparations are arranged in order of decreasing latency in Table II (column 2). At the indicated value of L, TTX
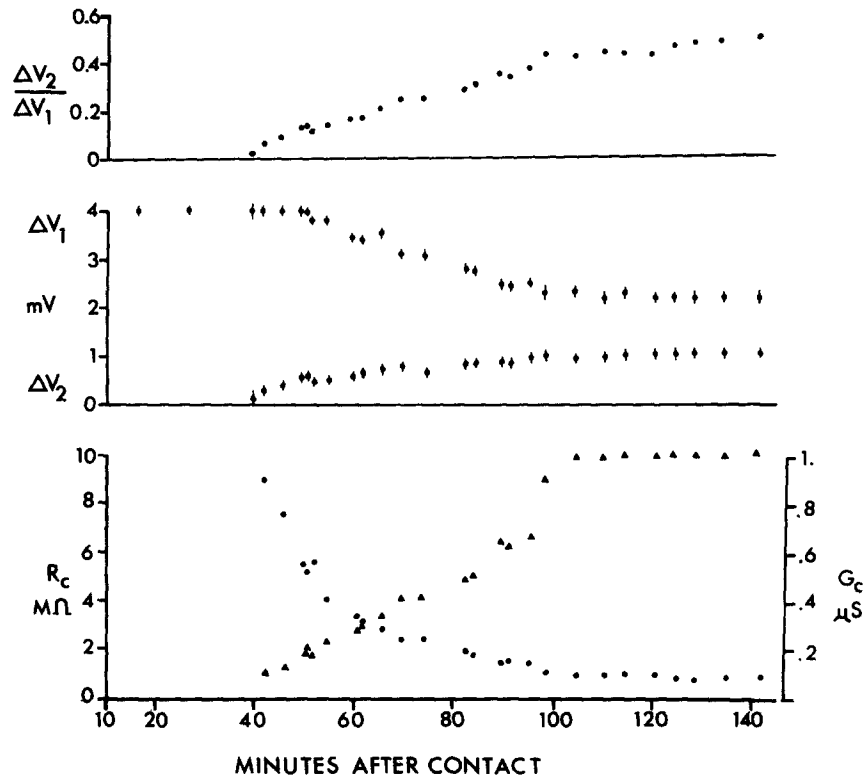

FIGURE 6.   Coupling parameters obtained from current pulse injection through suction electrode, K_o^+ = 4.8 mM. r_1 = 50 μm; r_2 = 60 μm. R_1 = 1.5 MΩ; R_2 = 1.0 MΩ. Radius of apparent area of contact 35 μm. No coupling was detected until 38 min after contact although electrodes were in place by 15 min after apposition of the aggregates.

(3 μM) was added. This stopped spontaneous beating, but a brief stimulus was used to initiate a train of action potentials, from which a new value of L was measured (Table II, column 3). In every case except expt. 8, TTX caused an increase in L (column 4). If we assume that R_c is not changed by the addition of TTX to the medium, and that at zero R_c, L is also zero, we can correct for the TTX-induced increase in L. To this end, new points were extended from the solid 1.3 mM K_o^+ (TTX) line in Fig. 9 corresponding to the values of L_{TTX}-L in Table II (column 4). A least squares fit line through these

points yields a derived L/R_c relation for 1.3 mM K_o^+ in the absence of TTX (Fig. 9, dashed line). It has a slope of 10.10 ms/MΩ.

To demonstrate the effect of electrical interaction on pacemaker behavior, two quiescent (4.8 mM K_o^+) aggregates (A_1, A_2) were placed in contact and stimulated periodically (Fig. 10). At 34 min after contact, A_2 was stimulated briefly (0.5 s), giving rise to a train of beats. Initially, A_1 was driven by A_2 only intermittently at the higher firing rate. Five beats in A_2 corresponded to one in A_1 (5:1 conduction block). As firing slowed, the rate-dependent block gradually changed to 1:1 conduction. The latency at 34 min was 50 ms. At 55 min, when the sequence was repeated, there was 1:1 conduction at all firing rates, and latency had decreased to 20 ms. Thus, the rate-dependent block appeared to vary with L, and thus also with R_c.
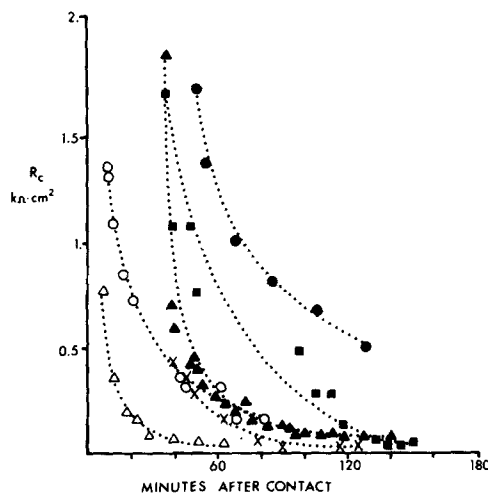

FIGURE 7.   Six complete coupling experiments that illustrate the change in coupling resistance, R_c, with time after contact between pairs of aggregates. R_c has been normalized for apparent contact area. True contact area can only be measured by electron microscopy and may be 1-2 orders of magnitude smaller. Open symbols: 1.3 mM K_o^+ (TTX). Closed symbols: 4.8 mM K_o^+.

DISCUSSION

The data presented in this study relate the delay in electrical events between two aggregates to the measured resistance across their junctional area. We have shown that aggregates synchronize their beats when their coupling ratio is low (<0.1) and their coupling resistance is high, and that R_c is linearly related to latency over the range in which both can be measured. Our interpretation of coupling is that the continuous insertion of gap junctional channels is responsible for lowering the resistance between heart cell aggregates.

In this investigation, we have modeled the aggregates as single large cells coupled through a declining junctional resistance. The geometry of the culture

system allows us to simplify considerably the treatment of R_c. Junctional resistance is defined at a precise location between the aggregates, rather than being distributed along a cable as in an intact cardiac fiber. The concept of latency is thus defined by the simple three-resistor circuit of Fig. 4 a. One question that naturally arises concerns the justification for comparing the aggregate to a single cell. Work over the last several years has led us to the conclusion that, although the aggregate is not perfectly isopotential, deviation from isopotentiality is small at low frequencies. A high degree of voltage homogeneity has been found by pulse and action potential analysis (DeHaan and Fozzard, 1975) and by intrinsic voltage membrane noise analysis (De-
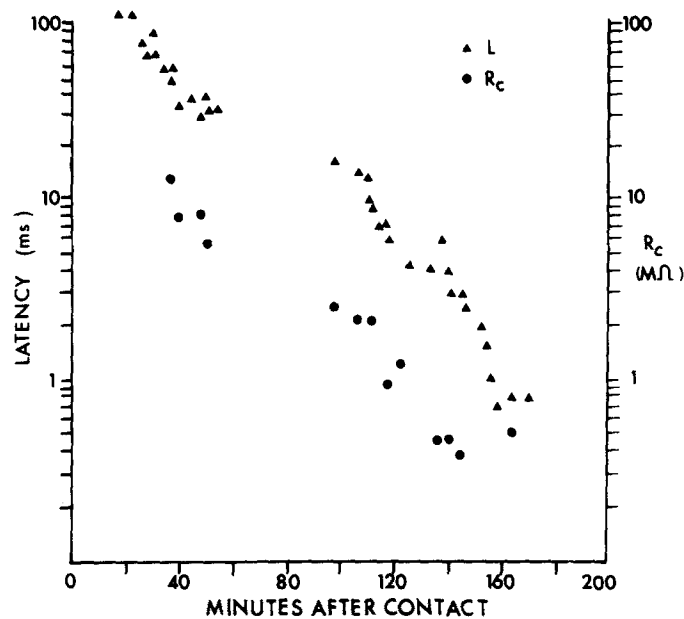

FIGURE 8. L and R_c plotted against time after contact for one aggregate pair (K_o^+ = 4.8 mM). Note semilogarithmic scale. The gap in the data between t_s = 50–90 min represents multiple penetrations with a microelectrode to regain a puncture. Coupling has been "set back" but is regained at the same slope. r_1 = 80 μm, r_2 = 80 μm. R_1 = R_2 = 1.5 MΩ. r_a = 60 μm.

Felice and DeHaan, 1977). These studies do not show that all the cells of an aggregate experience the same transmembrane potential since voltage gradients must exist in the intercellular cleft. However, by studying the dependence of aggregate impedance on aggregate volume, Clay et al. (1979) have provided evidence that the transmembrane potential is uniform for frequencies <10 Hz and potentials <2 mV. At high frequencies and potentials, such as during the upstroke of the action potential (170 V/s) when nonjunctional membrane resistance also drops precipitously, the delay across a single 160 μm aggregate was 43 ± 20 μs (Clapham, 1979). This delay is three orders of magnitude smaller than latencies found between newly paired aggregates.

Even after 11 h of contact, the shortest interaggregate latency achieved was 500 μs. Recently, Eisenberg et al. (1979; EBM model) have derived the closed-form solutions for voltage distribution in a spherical syncytium. Calculations using heart cell parameters with the EBM model (Clapham, 1979) show deviations from homogeneity of <5% (for frequencies to 10 Hz) beyond the immediate vicinity ($\sim$10 μm) of a current-injecting microelectrode. Since we were unable to measure deviation from isopotentiality experimentally, we conclude that voltage gradients within the aggregate are small, perhaps as small as the theoretically derived deviations. Thus, for analysis of our coupling experiments aggregates may be treated as single large cells with an effective surface area equal to that of all the cells within the aggregate.
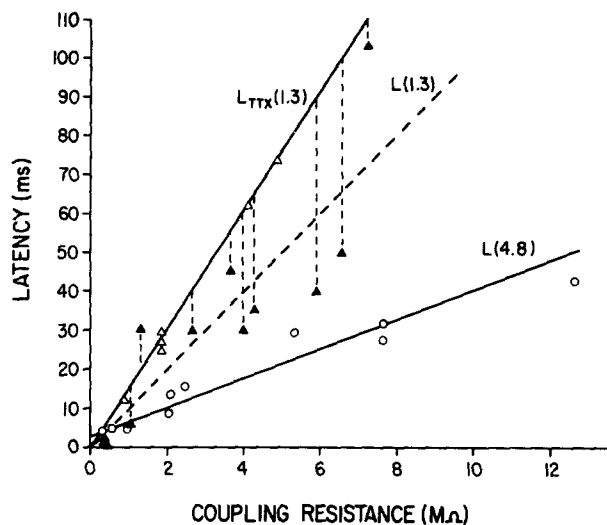

FIGURE 9. Correlation of R_c and L for one pair of aggregates in 4.8 mM K_o^+ (O) and a different pair in 1.3 mM K_o^+ (TTX) ($\triangle$). The solid lines represent least squares fits of the data. The correlation expected from aggregate pairs in low-K_o^+ medium without TTX (dashed line) has been calculated ($\blacktriangle$) by subtracting the values for L_{TTX}-L in Table II (column 4) from the corresponding points in the L_{TTX} (1.3) curve (see text).

The synchronization of aggregates depends on the amount of current that crosses the junctional resistance from the lead aggregate to depolarize its driven neighbor. For example, synchrony for the pair of aggregates measured in Fig. 8 occurred when R_c was about 20 MΩ, about 20 min after first contact. Thus, R_c was about 20 times R_2. At first synchrony, if the lead aggregate initiates an action potential when the driven aggregate is in diastolic depolarization (with an input resistance of $\sim$1 MΩ), the current that flows across the junctional resistance into the driven aggregate is approximately equal to the instantaneous potential difference between the aggregates divided by R_c. If R_c = 20 MΩ and (during the plateau of an action potential in the lead aggregate) $V_1 - V_2$ = 60 mV, then about 3 nA of current flows from the lead aggregate

across the coupling junctions into the driven aggregate, to produce a 100-200 ms depolarizing pulse. This pulse is just sufficient, in the case shown, to entrain the action potentials of the two aggregates. As R_c drops from 20 MΩ to <1 MΩ, more and more current flows across the junction into the driven aggregate and both L and the variability in L decrease progressively (Ypey et al., 1979). These results suggest that low-resistance coupling junctions begin to be formed between aggregates soon after they are brought into contact and that they enlarge continuously thereafter.

Our finding in aggregate pairs is that L is related linearly to R_c (Figs. 8 and 9). Continued addition of junctional channels and decline in R_c leads to a proportionate fall in L without substantial changes in action potential (AP)

TABLE II

EFFECT OF TETRODOTOXIN ON LATENCY

Expt. no.	Latency	Latency in TTX	$L_{TTX} - L$
	ms	*ms*	
1	100	110	10
2	50	100	50
3	45	55	10
4	40	90	50
5	35	65	30
6	30	60	30
7	30	40	10
8	30	20	-10
9	5	15	10
10	2	4	2
11	1.5	4	2.5
12	1.5	4	2.5
13	0.5	5	4.5

13 different pairs of aggregates were apposed for progressively longer times to achieve various latencies (column 2). At the indicated value of L, TTX (10^{-6} g/ml) was added and a new latency was measured within 3-4 min from extracellular voltage spikes (column 3). In every case except expt. 8, TTX caused an increase in L (column 4). The values for $L_{TTX} - L$ are subtracted from the upper curve in Fig. 9 (L_{TTX} [1.3]), to yield the derived L/R_c slope (L[1.3]) corrected for the delaying effect of TTX.

upstroke, shape, or size during coupling. Since the AP in the driving aggregate serves as a relatively constant low-impedance excitatory source, the proportionality between L and R_c is predicted by a simple strength-duration relationship (Lapique, 1907; see Fozzard, 1979 for a lucid discussion and recent references). That is, as R_c declines, a stronger stimulating current is exchanged between aggregates, resulting in shorter latencies.

At early stages of entrainment, L is both long and highly variable (Ypey et al., 1979). Part of that variability results from the fact that the specific membrane resistance of an aggregate increases from 18 to about 80 kΩ·cm^2 between −70 and −59 mV (Clay et al., 1979). Thus, R_2 should increase proportionately during each diastolic depolarization. Since the amplitude of the voltage pulse produced in the driven aggregate by a given junctional

current depends on R_2, the effect of that current will depend on when, in the diastolic depolarization of the driven aggregate, the upstroke of the lead aggregate action potential happens to fall.

Although gap junctional channels have not been isolated in pure form from membrane preparations, there is substantial evidence that the "junctional unit" (Loewenstein, 1966) is formed by a pair of proteinaceous pore-bearing particles, one from each membrane, which penetrate the apposed membrane lipid bilayers and abut in the intercellular cleft (for reviews, see McNutt and Weinstein, 1973; Loewenstein, 1975; Gilula, 1977). The two pores in register form a continuous tubular channel about 15 nm long and 2 nm i.d. connecting the cytoplasm of the apposed cells (Loewenstein and Rose, 1978; Sheridan et al., 1978). A cylindrical tube of these dimensions containing fluid with a
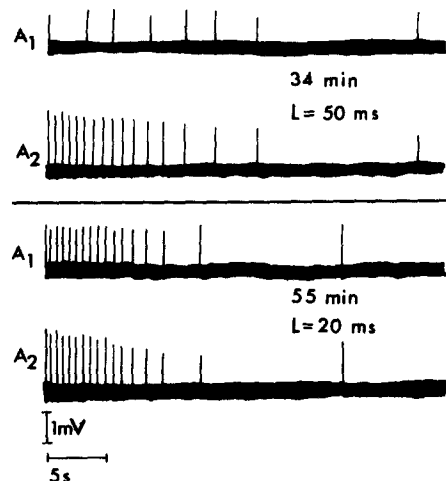

FIGURE 10. Example of rate-dependent "heart block" exhibited by a pair of aggregates in 4.8 mM K_o^+. $r_1 = 50\ \mu m$; $r_2 = 75\ \mu m$; $r_a = 16\ \mu m$. A train of action potentials was stimulated and the extracellular potentials measured via suction electrodes. At 34 min after contact there is a 5:1 block which converts gradually to 1:1 conduction at slow beat rates. By 55 min after contact, even higher beat rates show 1:1 conduction.

resistivity of 150 $\Omega \cdot$cm would have a resistance of $10^{10}\ \Omega$. This is the estimate for unit junctional channel resistance most commonly used (Loewenstein, 1975; Bennett, 1978) in lieu of an accurately measured value. When a pair of aggregates were pressed together and a single junctional channel formed between any of the apposed cells, about 10 pA of current would flow across the junction when an action potential fired in one aggregate while the other was at rest. On the basis of the time-course of the decline in R_c (Fig. 7), we can make an approximate estimate of the subsequent rate of insertion of junctional units. An area of contact of 8×10^{-5} cm^2 ($r_a = 50\ \mu m$) is large enough to include about 100 apposed cells in the junctional zone. If, after an initial delay of 3-4 min to permit membrane proximity,[2] channels were established among

[2] Williams, E. H., and R. L. DeHaan. Unpublished observation.

these 100 cells at a constant average rate of one channel per cell per minute, 500 channels would be formed during the first 8–9 min of contact and would yield a junctional resistance of 20 MΩ. As noted above, this was the value of R_c at first entrainment for the pair represented in Fig. 8. That is, an average of only five channels per cell, by these calculations, could have been sufficient to permit synchrony between two 160-μm diam aggregates. Smaller preparations would require even fewer channels to achieve synchrony, and a pair of isolated cells would readily entrain across a single channel (even if we have underestimated the unit channel resistance by one order of magnitude).

At the same continued rate of addition between two apposed aggregates, 10,000 parallel channels would have formed in <2 h of contact and would result in $R_c = 1$ MΩ, which was the condition in which aggregates were synchronized with short latencies and good beat-to-beat fidelity. These calculations assume a constant rate of insertion of junctional channels (after an initial delay). The approximately linear increase in coupling conductance (G_c) from 40 to 90 min after apposition for the pairs illustrated in Figs. 4 and 6 is consistent with that assumption. However, the time dependence of G_c for the pair represented in Fig. 8 is more nearly exponential, implying that junctional units were added at increasingly greater rates with time after initial contact. Among the six aggregate pairs represented in Fig. 7, for which G_c was measured continuously, plus three other pairs in which less complete records were obtained, the rate of increase was constant in three pairs (with variable delays) approximately exponential in four pairs, and intermediate in the remaining two pairs. We do not know whether a linear or exponential increase in G_c reflects a similar difference in rate of insertion of channels, or whether it may result from other factors such as differences in initial delay, or in rate of increase in appositional area.

The factors that determine conduction velocity in the intact heart have recently been reviewed (Fozzard, 1979). The rate of spread through any region of cardiac tissue depends largely on the local excitability characteristics of the membrane and the cable characteristics (which include serial cell-cell coupling resistance), complicated by spatial and temporal inhomogeneities along the conduction path (Cranefield, 1975; Fozzard, 1977). A large body of literature dating back to Weidmann's early measurements (1952, 1966, 1970), demonstrates the importance of internal fiber resistance R_i to conduction velocity. Numerous workers, using ultrastructural techniques, have shown that areas of slow conduction exhibit a paucity of gap junctions (James and Sherf, 1968; DeFelice and Challice, 1969; Masson-Pevet et al., 1978; Bleeker et al., 1980). Moreover, Pollack (1976) described a general correlation between junctional permeability and conduction velocity in five regions of the heart; tissues with high conduction velocity (atrial fibers, e.g.) permitted faster diffusion of dye from cell to cell than did the atrioventricular (AV) node, by about three orders of magnitude. Weingart (1977) observed that a twofold reduction in conduction velocity in bovine ventricular muscle exposed to ouabain could be attributed to alterations in upstroke velocity and amplitude of the action potential and to the observed changes in membrane capacity and in longitudinal resistance, but he estimated that about 60% of the decrease in conduction

velocity could be accounted for by the measured increase in R_i alone. Wojtcjak (1979) has recently reported that internal longitudinal resistance in bovine ventricular trabeculae rose threefold under anoxia and 20-fold when the tissue was exposed to both low O_2 and epinephrine, and that these conditions were associated with decreased conduction velocity. On the other hand, Carmeliet and Willems (1971) showed that conduction velocity in Purkinje fibers can be slowed dramatically by exposing the preparation to Na-free Sr-Tyrode's solution, which reduced upstroke velocity to 3–5 V/s without altering longitudinal resistance or other cable properties. Moreover, slow conduction through the sino-atrial (SA) and AV nodes is usually explained by the smaller inward current density or low diastolic potentials of the component cells (Cranefield, 1975; Bonke, 1978). Therefore, the mechanisms that underlie conduction disturbances have generally been sought in terms of altered excitability or membrane parameters (for recent literature, see Cranefield and Wit, 1979; Senges et al., 1979; James et al., 1979; Allessie et al., 1977).

In the present experiments, we have shown that newly apposed pairs of heart cell aggregates exhibit changes in action potential delay resulting purely from alterations in junctional resistance. L and R_c were linearly related over a wide range of values (Fig. 9); that is, the decrease in L with time after initial contact could be accounted for entirely by the measured decrease in R_c. In these preparations, junctional resistance is located mainly at the newly apposed surfaces. R_c and R_i can be measured directly. The resting potential, upstroke velocity, and other action potential parameters are uniform in each aggregate, separately measurable, and largely under experimental control. Thus, we suggest that this preparation of embryonic cells may provide a useful model with which to explore further the role of uncoupling in cardiac tissue.

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