

Membrane Response to Current Pulses in Spheroidal Aggregates of Embryonic Heart Cells

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ABSTRACT Hearts from chick embryos aged 4, 7, or 14 days were dissociated into their component cells, and the cells allowed to reassociate in the form of smooth-surfaced spheroidal aggregates on a gyratory shaker. Records from intracellular electrodes inserted into two widely spaced cells in a spontaneously beating aggregate indicated that the action potentials occurred virtually simultaneously. In aggregates made quiescent with tetrodotoxin, the voltage response to a current pulse injected in one cell could be noted by recording with a second microelectrode at various distances from the current source. The magnitude of the response was found not to vary with distance. It is concluded that the component cells in an aggregate are normally tightly coupled electrically; the cell boundaries do not constitute an appreciable resistive barrier. Such aggregates behave as virtually isopotential systems, with properties similar to those of single spherical cells, as modeled by Eisenberg and Engel (1970. *J. Gen. Physiol.* 55:736-757). Passive membrane time constant ranged from 11 to 31 ms, with a mean value of 17 ms; this value did not vary with aggregate size. Input resistance (V_o/I_o) varied inversely with aggregate size, as predicted, but with much scatter in the measured values. Specific membrane resistance was calculated as either 13,000 or 800 ohm-cm² depending on whether input resistance was attributed to the total cell surface membrane area or to the outer surface of the sphere alone. No systematic difference in passive electrical properties of aggregates composed of 4-, 7-, and 14-day cells was seen. It is concluded that these aggregates may be suitable for voltage clamp analysis of their excitable membrane properties.

INTRODUCTION

Many difficulties in the analyses of electrical properties in excitable cells can be ascribed to the complex organization of the tissues chosen for study (Eisenberg and Johnson, 1970; Rall, 1960). Often the electrical measurements reflect more the tissue geometry than the characteristics of the membranes (Mirolli and Talbot, 1972). The technique of uniform voltage control ("volt-

age clamp") that has been used so effectively in the study of excitable membrane properties of nerve has been difficult to apply to heart muscle tissue because of its multicellular organization (Johnson and Lieberman, 1971; Harrington and Johnson, 1973; Trautwein, 1973). One way to avoid many of such geometrical problems in heart muscle would be to study single cells isolated in tissue culture. A single micropipette can be introduced into individual cells (DeHaan and Gottlieb, 1968; Sperelakis and Lehmkuhl, 1966; Hyde et al., 1969) but these impalements usually last less than a minute, and efforts to control membrane voltage through the single electrode have been unsuccessful.

Such isolated cells readily reassociate into tissue-like masses that can be formed, at will, as monolayer cell sheets (Sperelakis, 1972), cylindrical strands (Purdy et al., 1972), or spheroidal aggregates (Sachs and DeHaan, 1973). Under these conditions, specialized junctions are established between the cells that allow free movement of ions or other small molecules; the weight of evidence indicates that it is the "gap junction" that represents this low-resistance pathway from cell to cell (DeHaan and Sachs, 1972). Gap junctions commonly occur in intact mammalian and avian heart tissue (McNutt and Weinstein, 1973; Martinez-Palomo and Mendez, 1971; Matter, 1973) and form rapidly between newly apposed heart cell surfaces in culture (DeHaan and Hirakow, 1972; Shimada et al., 1974). Furthermore, it has been demonstrated in preliminary experiments that all the cells of a heart-cell aggregate are tightly coupled electrically, and that voltage changes, recorded from widely separated cells during spontaneous action potentials, occur without measurable delay (Sachs and DeHaan, 1973). If the space constant for such an aggregate is large relative to its size, it is possible that virtually isopotential changes could be produced across the surface membranes of all the component cells by a micropipette point source of current. That is, the spheroidal cluster of heart cells might, in fact, be equivalent to a single large spherical cell such as that modeled by Eisenberg and Engel (1970), and thus be useful for quantitative studies of membrane electrical properties that have been difficult or impossible to perform heretofore (Johnson and Lieberman, 1971).

In the present report we confirm that heart cells in spheroidal aggregates are tightly coupled electrically and virtually isopotential during the voltage changes associated with spontaneous action potentials or those achieved by introducing current pulses through a micropipette. Specific membrane resistance and capacitance have been measured. These aggregates, whose pacemaker behavior is much like that of Purkinje fibers, may represent an important tool in the study of the electrical properties and electrophysiological differentiation of heart cells.

METHODS

Tissue Culture

After incubation for 4, 7, or 14 days, White Leghorn chick embryos were harvested in amniotic fluid and decapitated. Hearts were dissected free, trimmed of extraneous tissue, and snipped with iridectomy scissors to release blood and expose the interior. The apical portion of the ventricles, or in some cases, whole hearts, were dissociated with trypsin by techniques now standard in this laboratory (DeHaan, 1967, 1970). Aggregates were prepared as previously described (McDonald et al., 1972; Sachs and DeHaan, 1973). Briefly, an inoculum of 5×10^5 cells was added to 3 ml of culture medium (818A) contained in a 25-ml Erlenmeyer flask. The flask was gassed with 5% CO₂, 10% oxygen, 85% nitrogen mixture, sealed with a silicone rubber stopper, and placed on a gyratory shaker at 37°C. Aggregation took place during 24–48 h gyration at 70 rpm. Aggregates were transferred from the flask to a Falcon plastic tissue culture dish (35-mm diameter) and allowed 2 h in a culture incubator (water saturated atmosphere of 5% CO₂, 10% O₂, 85% N₂) to attach to the dish bottom, after which they were washed with fresh medium.

Media

Aggregates were prepared and maintained in medium 818A (DeHaan, 1970). This medium contains 20% M 199 (Grand Island Biological Co., Grand Island, N. Y.), 2% heat-inactivated selected horse serum (Colorado Serum Co., Denver, Colo.), 4% fetal calf serum (Grand Island), and 0.5% Gentamycin (Schering Corp., Bloomfield, N. J.) in potassium-free Earle's balanced salt solution: (mM) NaCl 116.0, MgSO₄ 0.8, NaH₂PO₄ 0.9, CaCl₂ 1.8, NaHCO₃ 26.2, and glucose 5.5. The fetal calf serum had previously been dialyzed against potassium-free balanced salt solution. The potassium concentration of the final medium was adjusted to 1.3 or 4.3 mM as needed. For electrophysiological studies, medium 818A was replaced with a richer medium, 1126A, which in addition to the components of 818A contained 25% nutrient solution 8X4X (Ham, 1965). Tetrodotoxin (Sankyo, Tokyo, Japan) was dissolved in distilled water (1 mg/ml) and diluted 100-fold for use at a final concentration in the medium of 1×10^{-5} g/ml.

Electrophysiological Measurements

The electrical activity of cells in aggregates was recorded using techniques modified from DeHaan and Gottlieb (1968). A tissue culture dish with aggregates adhering firmly to the bottom was placed on the warm stage (37°C) of a dissecting microscope. Nontoxic mineral oil (Klearol, Sonneborn Division, Witco Chemical Co., New York) layered over the medium prevented evaporation, and a gas mixture of 10% O₂, 10% CO₂, 80% N₂ was directed from a toroidal gassing ring over the dish. The mixing that took place with room air maintained CO₂ and pH at 5% and 7.3, respectively. Under these conditions aggregates continued beating vigorously for hours or even days, unless their spontaneous activity was reversibly suppressed with tetrodotoxin.

Aggregates could be viewed at a magnification of $\times 50$ –75. Sizes and interelectrode distances were measured with an ocular reticle on which the smallest divisions represented $33 \mu\text{m}$. Measurements could be made accurate to about half a division. Total cell surface area of aggregates was estimated by assuming that all the cells were spherical in shape and had volumes of $820 \mu\text{m}^3$ and surface areas of $423 \mu\text{m}^2$. The number of cells was estimated on the assumption that 80% of the aggregate volume was occupied by cells and 20% intercellular space, based on the data of McDonald and DeHaan (1973). No allowance was made for membrane folding, transverse tubular membrane, or shapes of cells other than spherical. Since gap junctional area represents less than 4% of total cell surface in adult mammalian ventricle (Page and McCallister, 1973; Matter, 1973) and is probably much smaller in embryonic avian heart (Hyde et al., 1969), the surface participating in nexal junctions in our aggregates was assumed to be a negligibly small fraction of the total. The aggregate volume was calculated from measurements of the major and minor hemiaxes with the equation for volume of a prolate spheroid (DeHaan and Sachs, 1972).

Glass microelectrodes were drawn on a mechanical puller and filled with 2 M KCl by the glass-fiber method of Tasaki et al. (1968). The DC resistance of these electrodes ranged from 10–50 M Ω . The electrode-filling solution was connected via a Ag-AgCl junction to a unity-gain electrometer amplifier (Picometric or Bak). Current was injected into cells by dropping an applied voltage across a 22-M Ω resistor in series with the microelectrode. Injected current was measured by an operational amplifier (Tektronix type O, Tektronix, Inc., Beaverton, Ore.) in current-to-voltage configuration, which clamped the solution surrounding the aggregates to virtual ground. Voltage and current signals were displayed on a storage oscilloscope (Tektronix model 5103/D13).

For measurement of the delay between signals recorded from two different positions within a spontaneously beating aggregate, electrodes were introduced into two widely spaced cells under direct observation at a magnification of $\times 75$. When recorded action potentials from the two cells had stabilized, a 400-Hz sine wave was passed from ground through both recording systems (electrode–electrometer–cell). At a high oscilloscope sweep speed (0.5 ms/cm) the capacitance feedback circuits of the two amplifiers were adjusted until the two sine waves became superimposed. When the imposed sinusoidal signal was removed, the degree of superimposition of the rapidly rising action potential spikes could be observed. With this method for matching the response time of the two recording systems, time differences between action potentials greater than about 40 μs could be measured reliably.

RESULTS

Spontaneous Action Potentials

All aggregates derived from 4- or 7-day whole-heart or ventricle, and most of those prepared from 14- to 15-day whole-heart beat spontaneously, unless their pacemaker behavior was deliberately suppressed by adding excess potassium or tetrodotoxin to the bathing medium. Records from two intra-

cellular electrodes in a spontaneously beating aggregate (Fig. 1 *a*) illustrate that the action potentials from two widely spaced cells occur simultaneously, within the limits of the measurement methods here employed. When response times for the two recording systems were matched (Fig. 1 *b*) and the action potentials from cells 223 μm apart were recorded at high sweep speed, virtually no delay was seen between their rapidly rising phases (Fig. 1 *c*). Moreover, when one recording electrode was moved to a much closer cell, and the two systems rebalanced, no change in apparent delay was seen (Fig. 1 *d*). More than 30 two-electrode impalements in eight different aggregates yielded similar results.

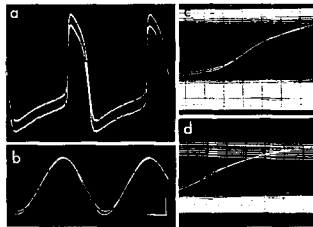


FIGURE 1. (*a*) Simultaneous recording of action potentials through microelectrodes in two cells, 223 μm apart, near opposite ends of a 4-day spheroidal aggregate. The two traces are displaced vertically in order to distinguish them. (*b*) At high sweep-speed a 400-Hz sine wave applied to both recording systems through ground is superimposed by adjustment of the capacitance feedback circuits of the two amplifiers. (*c*) At high sweep-speed the regions of maximal upstroke velocity occur without measurable delay. Records in *a-c* taken with electrodes 1 and 2 remaining in the same two cells. (*d*) Electrode 1 moved to a cell 60 μm from electrode 2, and the recording systems rebalanced. Calibration scales: Voltage *a-d*, 20 mV/division; time (*a*) 100 ms/division. (*b-d*) 500 μs /division.

Applied Current Pulses

Passage of a brief hyperpolarizing current step during the pacemaker potential produced a response in all cells of the aggregate with a time constant for the voltage change on the order of 20 ms (Fig. 2 *a*). Varying the amplitude of the stimulus altered the subsequent course of the pacemaker potential (Fig. 2 *b*), as has been reported previously for the Purkinje fiber by Weidmann (1952).

Prolonged depolarizing current accelerated the spontaneous pacing rate in a graded fashion (Fig. 3 *a, b*). Hyperpolarizing steady currents slowed the spontaneous beating altogether (Fig. 3 *c, d*). In all cases, current flow altered the rate of depolarization of the pacemaker potential. Where short hyperpolarizing currents were introduced at different times during the pacemaker potential (Fig. 4 *a*), the voltage responses to the current were greater when the pulses were later in the cycle. This also resembles the experimental result

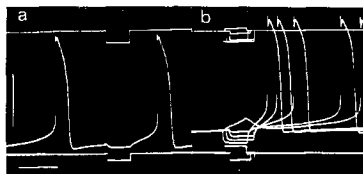


FIGURE 2. Double impalement of an aggregate of 7-day heart, $100 \times 120 \mu\text{m}$ in diameter. (a) A single hyperpolarizing current pulse (6×10^{-9} A) is injected into one cell about midway between two spontaneous action potentials recorded from a second cell $80 \mu\text{m}$ distant. (b) A subthreshold depolarizing pulse introduces a substantial delay in the appearance of the next action potential while that delay is proportionately decreased by passage of progressively larger hyperpolarizing currents. Calibration scales: Vertical = 50 mV, 5×10^{-8} A; horizontal = 400 ms.

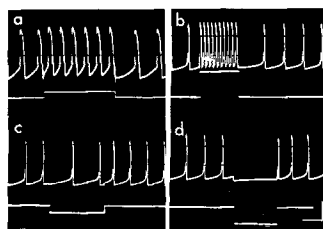


FIGURE 3. Influence of steady depolarizing currents (a, b) and hyperpolarizing currents (c, d) on spontaneous rate of action potentials in an aggregate. In addition to the modification of rate seen during current passage, the spontaneous rate was also altered after termination of the current. Transient acceleration occurred when the current had depressed the rate (c, d) and transient slowing occurred when the rate had been accelerated (a, b). Calibration scale (shown in panel d); ordinate: 50 mV and 10 nA/division, abscissa: (a) 0.5 s/division; (b-d) 2 s/division.

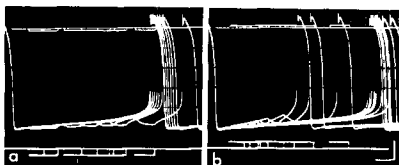


FIGURE 4. Small pulses of current imposed during diastolic depolarization. In panel a the current was hyperpolarizing; the voltage response was increased when the pulse was applied later in diastole. Note the influence of pulses on subsequent timing of the spontaneous action potential. In panel b the current was depolarizing. Even small currents early in diastole would bring the aggregate to threshold when applied later. Subthreshold depolarizing pulses also altered the timing of the subsequent action potential. The upper trace is a time and direction marker for the lower current input. Calibration scale in b 20 mV and 10 nA/division; 200 ms/division.

of Weidmann (1952), and is consistent with a fall in total membrane conductance during the pacemaker potential. However, it should be noted that the later pulses also influenced the onset of the subsequent action potential, suggesting that the change in voltage itself altered the membrane conduct-

ances. The same experiment was not easily done with depolarizing currents, since currents that barely produced a passive response early in the pacemaker potential were sufficient to bring the aggregate to threshold when delivered later (Fig. 4 *b*).

Spatial Uniformity within the Aggregate

Experiments reported above were consistent with a high degree of electrical coupling between cells in the aggregate, but gave no indication of the amount of nonuniformity within the cell group. An aggregate might be considered electrically as a single unit in which the active membrane may be represented either by the surface membranes of all the cells in the aggregate, or alternatively, only by the outer surface membranes of the peripheral cells. In the former case the internal resistance would reside partly in the cytoplasm and partly in the cell-to-cell junctions. With the latter model, cell-to-cell junctional resistance would be considered negligible. If the junctions impeded current flow significantly from one cell to another, the amplitude of the passive voltage response to current injected in one cell would vary with distance from that cell.

The extent of passive spread within cells versus leakage out of the membrane in the case of a long cylindrical cell or cell column can be defined as the length constant. An equivalent space constant can be defined for spread in tissues having other geometries, such as a sheet of cells. In such cases the space constant represents the distance at which the potential is found to be $1/e$ of its value at the site of injection. In the spherical case, the concept of a space constant is not applicable. Eisenberg and Engel (1970) have calculated the distribution of potential in a spherical cell as a function of the angle of the recording site from the injecting site. The relationship of voltage displacement to angle or recording was found to be a function of the radius and the ratio of membrane (inside-outside) resistance to internal resistance. For this sort of analysis, 0° represents the site at which current is introduced and other angles are measured between the recording micropipette and the injecting point. For these experiments with heart-cell aggregates the extracellular K^+ was maintained at 4.3 mM and tetrodotoxin was used at a concentration of 1×10^{-5} g/ml, to inhibit action potentials and to cause the aggregates to assume steady resting potentials (McDonald et al., 1972). Brief small currents were injected in a hyperpolarizing direction in order to produce transmembrane voltage changes in the range of 5–10 mV. The response at various recording angles from 10 to 115° was then tested. Because this required removal and reinsertion of the micropipettes many times in an aggregate, the currents measured were not always the same. An experiment including results at five angles is illustrated as a current-voltage plot in Fig. 5. Although scatter in the results would mask differences smaller

than about 15%, the responses at the different angles showed no systematic variation. If the best fit line is drawn for each angle measured, and the voltage response expected at a standard current is plotted, the result (Fig. 6) does not deviate from that predicted by Eisenberg and Engel (1970). A similar result was found in two other aggregates with four and five angles

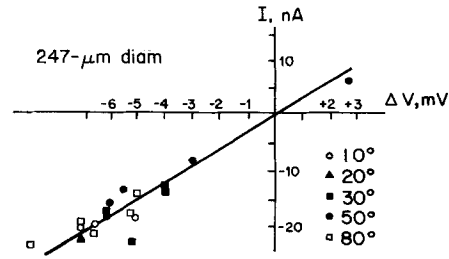


FIGURE 5. Current-voltage relationship determined from multiple impalements of a single tetrodotoxin-treated aggregate. Each point represents the result of a two-electrode impalement during which a small current was passed into a cell through one electrode having a specified angular separation from the voltage recording electrode. An angular separation of ϕ degrees corresponds to a curvilinear separation of $S = \pi r \phi / 180$. For an aggregate 247 μm in diameter, the angles specified correspond to electrode separations of 22, 44, 65, 110, and 175 μm , respectively.

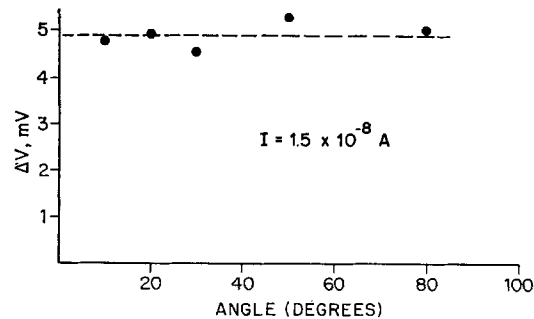


FIGURE 6. Membrane potential response to a current pulse recorded from electrodes having different degrees of angular separation. The data shown in Fig. 5 have been normalized to a constant current, averaged, and replotted according to angle ϕ .

examined over the same range. Within the limits of the error of the measurements, the interior of the entire aggregate appeared to be virtually isopotential.

Measurement of Passive Electrical Properties

Input resistance and time-course of potential change across the membranes were measured in a series of aggregates of various sizes and ages (Table I).

TABLE I
MEASURED INPUT RESISTANCE AND CALCULATED SPECIFIC PARAMETERS
OF AGGREGATE MEMBRANES

Hemixes		Age	Estimated, no. of cells	V_o/I_o	r_{in}	T_m	R_m^o	R_m^t	C_m^o	C_m^t
<i>a</i>	<i>b</i>									
μm	<i>days</i>			$K\Omega$	<i>ms</i>	Ωcm^2	Ωcm^2	$\mu f/cm^2$	$\mu f/cm^2$	
17.5	20.0	14	25	2,500	12.0	110	260	109.0	46.20	
58.0	58.0	4	800	1,350	11.5	574	4,490	20.0	2.56	
57.0	66.0	4	880	810	11.5	364	3,070	31.6	3.82	
75.0	91.0	15	2,100	1,000	12.0	868	8,870	13.8	1.37	
82.0	82.0	4	2,440	1,050	31.0	850	10,960	36.0	2.80	
82.5	102.0	7	2,793	470	13.0	480	5,550	27.1	2.34	
90.0	99.0	4	3,280	1,170	25.0	1,276	16,270	19.6	1.54	
99.0	99.0	4	3,920	800	19.0	990	13,300	19.0	1.43	
97.5	120.0	7	4,580	280	11.0	393	5,430	28.0	2.02	
116.0	132.0	7	7,060	500	21.0	921	14,950	22.8	1.41	
124.0	124.0	7	9,770	320	13.0	622	10,360	20.9	1.26	
137.5	148.0	14	11,480	380	18.0	974	18,400	18.4	0.98	
140.0	140.0	4	11,270	180	14.0	450	8,590	31.0	1.63	
165.0	165.0	4	18,100	174	23.0	544	13,300	42.3	1.73	

S^o = Area of the outer surface of the sphere ($4\pi r^2$).

S^t = Total cell surface area [$(4/3\pi r^3 \times 0.8 \times 423)/820$].

R_m^o = Specific membrane resistance referred to the outer membrane area (S^o).

R_m^t = Specific membrane resistance referred to S^t .

The time constant (T_m) was measured as the time required for membrane potential to reach 63% of its steady-state value in response to a step voltage input. Only those records in which membrane potential responded with an exponential change were employed. The mean value of these measurements was 17 ms. Time constant was not apparently related to either the diameter of the aggregates or the age of their component cells.

Input resistance (r_{in}) was greater in smaller aggregates, as one would expect. It appeared to vary in a complex way both with aggregate radius (Fig. 7) and with log volume (Fig. 8).

If the interior of all the cells in an aggregate was nearly isopotential during flow of these small currents, the specific membrane resistance (R_m) and capacitance (C_m) can be calculated. These calculated values are shown in Table I, in two separate columns, according to the method used to calculate the reference membrane area. One is based on the total cell surface area (R_m^t ; C_m^t); the other only on the outer surface area (R_m^o ; C_m^o) of the aggregate. The range of values in both sets of calculations is large, especially for the smallest aggregates. There was no apparent relation between age of cells and resistance. The relation between specific resistance values and aggregate radius is plotted in Fig. 9.

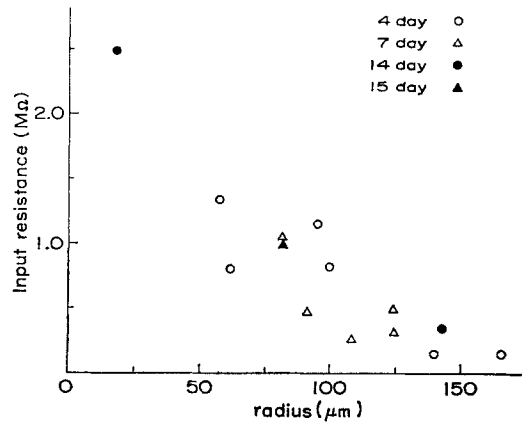


FIGURE 7. Input resistance (r_{in}) measured as V_o/I_o , as a function of aggregate radius. For nonspherical aggregates "radius" is shown on this graph as the mean hemiaxis $(a + b)/2$. Aggregates composed of cells from different aged hearts are identified by specific symbols.

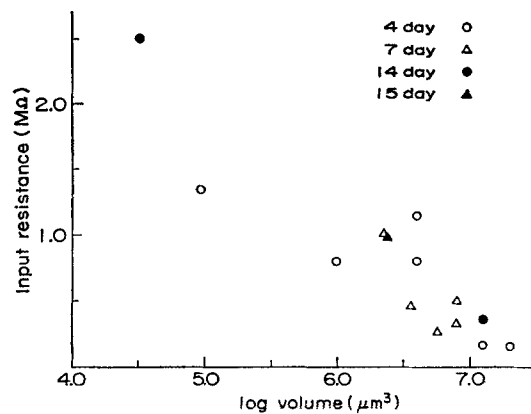


FIGURE 8. Input resistance as a function of aggregate volume, plotted on a log scale.

DISCUSSION

Conduction velocities measured from adult and fetal mammalian myocardial tissues have ranged well below 1 m/s (Weidmann, 1970; Gennser and Nilsson, 1970); in the chick embryo heart, Lieberman and Paes de Carvalho (1965) have recorded values of 0.4–0.5 m/s. Heart cell preparations in tissue culture propagate action potentials even more slowly; 1–30 cm/s being the reported range of values (Olivo and Ricamo, 1949; Lieberman et al., 1973).

If an action potential were to arise in a single pacemaker cell at one edge of an aggregate and be conducted at a velocity of 10 cm/s (100 μm/ms) from cell to cell throughout the sphere, the delay between the action potentials recorded from two electrodes 223 μm apart (as in Fig. 1 *c, d*) would

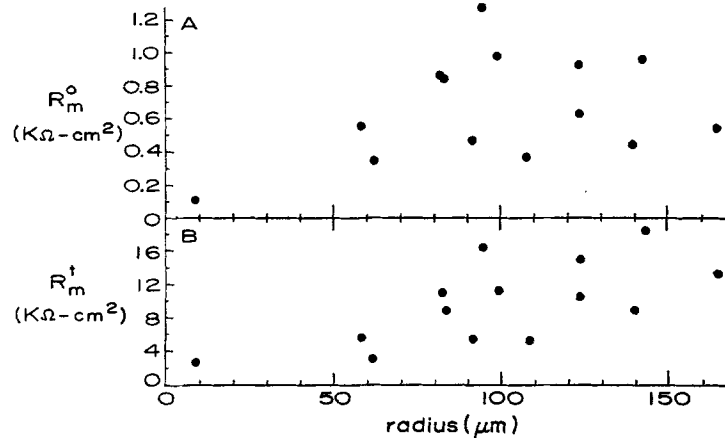


FIGURE 9. Specific membrane resistance as a function of aggregate radius. (A) Resistance (R_m^o) calculated with reference to the outer surface area (S^o) of the aggregates. (B) Resistance (R_m^t) calculated with reference to the total cell surface area (S^t) in the aggregates.

be as much as 2 ms, depending on the position of the primary pacemaker cell relative to the electrodes. Only under the unlikely condition that the dominant pacemaker region were always located near the center of the sphere would all cells in the periphery (those we most often impaled) fire simultaneously. Barring this possibility, one important conclusion is that within the size range of aggregates employed (60–400- μm diameter) changes in membrane potential associated with spontaneous action potentials are distributed virtually simultaneously to all parts of the aggregate.

More convincing evidence of the coupling of cells and the virtual isopotentiality of the aggregates stemmed from experiments in which stimuli were injected into one impaled cell and recorded from a distant cell. Steady currents so injected at any point in the aggregate produced alterations in spontaneous beat rate, cessation of pulsation with hyperpolarizing currents, and overdrive suppression of the beat (Vassalle, 1971), as would be expected if the entire cell population were being depolarized or hyperpolarized simultaneously. Moreover, no systematic variation in voltage responses to an imposed current could be found when recordings were made with an exploring microelectrode inserted at various distances from the current source (Fig. 6). All of these results are consistent with the idea that the cell boundaries in an aggregate do not constitute an appreciable electrical barrier; the cells are normally tightly coupled electrically across gap junctions. The body of evidence in support of this conclusion has been reviewed recently (DeHaan and Sachs, 1972; Berger, 1972; McNutt and Weinstein, 1973; Matter, 1973).

An isopotential system may be defined as one in which zero voltage gradient

is produced by the three-dimensional spread of current from a point source. In an ideally isopotential spherical cell, the change in voltage (V_m) just under the surface, which is produced by injecting current, is defined by the expression: $V_m = i_o R_m / 4\pi r^2$, where i_o = current applied; r = radius of the sphere; R_m = resistance of 1 cm² of membrane in ohms-cm². Expected deviations from this ideal state can be derived from the theoretical analysis of voltage distribution in a spherical cell by Eisenberg and Engel (1970). With a current source located just under the outer surface membrane, the steady-state distribution will be dependent on the cell radius (r) and the ratio: $\Lambda = R_m/R_i$, where R_i is the volume resistance of the cell interior.

In the case of the single spherical cell considered by Eisenberg and Engel (1970), R_m is calculated by multiplying measured input resistance by the surface area of the sphere. A similar calculation for the aggregates listed in Table I, referring their calculated membrane resistance only to the outer spherical surface area, yields values averaging about 800 ohm-cm² (Fig. 9). Assuming the interior of the aggregate to have a resistance (cytoplasm plus junctions) of about 470 ohm-cm, similar to that measured by Weidmann (1970) for mammalian trabecular muscle, the ratio (Λ) would be 1.7 cm. For an aggregate of 100- μ m radius, the generalized space constant r/Λ (as defined by Eisenberg and Engel, 1970) would be 0.006. Entering that value in their Table 2, one obtains expected variations in voltage of 7% at 10°, 2% at 30°, and -1% at 180°. If the measured input resistance is referred to the total cell surface area instead of the outer surface only, values of Λ would range an order of magnitude larger, and the predicted voltage variations would be negligible at all measurement angles.

Some of the observed variation in the data given in Table I may have resulted from real differences in membrane properties. For example, since the current-voltage relationship for excitable cells is nonlinear, measurements of resistance made at different resting potentials may not be compared. The assumptions that average cell size was uniform or that cell packing in the aggregate was always the same could be in error. Finally, the impalement of cells with multiple electrodes may have led to some current leakage because of inadequate sealing, or to partial decoupling of the impaled cells from their neighbors. Nonetheless, input resistances and membrane time constants obtained with our heart cell aggregates fell in the same range as those reported for mammalian Purkinje fibers (Weidmann, 1952; Fozzard, 1966) and trabecular muscle (Weidmann, 1970) and for embryonic heart cells in culture (Sperelakis, 1972; Lieberman, 1973). Girardier et al. (1967) reported an r_{in} for single isolated rat heart cells, measured with a bridge technique, of 4.6 M Ω , and Sperelakis (1972) gives values ranging between 6 and 20 M Ω . Extrapolating the curve shown in Fig. 8 back to the volume of a single ventricle cell (820 μ m³) yields an r_{in} value of 4 M Ω . For small

groups or confluent sheets of coupled cells Girardier et al. (1967) reported an r_{in} of 2.8–3.3 M Ω , which corresponds well with our measured value of 2.5 M Ω for the smallest aggregate in Table I which is estimated to contain only 25 cells. Nonetheless this value of r_{in} yields a calculated C_m which is out of line with the remaining values by at least an order of magnitude. Preparations with small numbers of cells are most susceptible to damage upon impalement. This would result in low measured values of r_{in} , but should have less effect on membrane time constant. The specific membrane capacitance calculated from such values would be too high.

Calculation of specific membrane resistance and capacitance from the measured values of r_{in} and τ_m also depends on one's estimate of the area of membrane interposed between the coupled cytoplasmic compartment of an aggregate and the extracellular medium. According to one model, it may be assumed that current flows from the cytoplasm of a centrally located cell mainly through low-resistance junctions to the cytoplasm of peripheral cells, and thence across the outer cell boundary to the extracellular medium. In this case the membrane acting as the major resistive path for current would approximate the spheroidal outer surface of the aggregate (S^o), and the appropriate specific membrane resistance of the internal cells plus the longitudinal resistance of the intercellular clefts connecting to the extracellular medium is tacitly assumed to be large relative to either R_m^o or the internal longitudinal resistance (R_i).

According to an alternative model, a substantial fraction of the current flowing from central cells would pass across the membranes of those cells to the intercellular clefts. In this case, these clefts are visualized as connecting directly to the bulk medium without major resistive barriers. With this model the membrane acting as the major resistive path for current would approximate the total cell surface area (S^t), and the appropriate specific membrane resistance will be given by R_m^t .

According to the first model, embryonic heart cell membrane has a resistance averaging around 800 ohm-cm² and a capacitance of about 25 μ F. According to the second model, these values are, respectively, about 13,000 ohm-cm² and 2 μ F/cm². The dilemma posed by these alternatives is not restricted to the spheroidal preparations employed in the present study. For monolayer sheets of cultured rat heart cells values of several thousand ohm-cm² have been reported (Hyde et al., 1969; Jongasma and Van Rijn, 1972), whereas the estimate of R_m for a single isolated myocyte was approximately 60 ohm-cm² (Girardier et al., 1967). The latter workers estimate C_m values of 0.3–6.5 μ F/cm². For chick cells, Sperelakis (1972) reports an R_m of 630 ohm-cm² and C_m of about 11 μ F/cm². Estimates of membrane resistance and capacitance in adult heart muscle have been confounded by the same difficulties. As pointed out by Fozzard recently (1972), whether 1 cm² of

cardiac cell membrane has a "true" capacitance of approximately $1 \mu\text{F}$, or $10\text{--}30 \mu\text{F}$ depends largely upon which set of assumptions prove correct regarding the membranes that provide a pathway for current flow between inside and outside. Stereological measurements which provide accurate estimates of the actual amounts of membrane surrounding heart cells (Mobley and Page, 1972; Page and McCallister, 1973), are of fundamental significance. But the problem can be solved only when a realistic model representing the electrical equivalent circuit of heart tissue is devised (Weidmann, 1974).

The present results show no systematic differences in passive properties between aggregates composed of 4-, 7-, or 14-day cells. Resistance values and measured time constants were not distinguishably different, and electrical coupling was equally good in the three groups. While there are large differences in excitable behavior between younger and older cells (DeHaan, 1968; McDonald et al., 1972) these results suggest that the passive membrane properties are not responsible for these differences.

Quantitative study of active membrane properties of heart cells could best be accomplished by controlling membrane voltage. In some respects the aggregates described here are suited for such voltage clamp experiments. They can be made arbitrarily small, from about 50 to $400 \mu\text{m}$ in diameter. This small size favors fairly uniform potential control from a point source of current. It is not difficult to introduce two micropipettes into aggregates of this size, and such double impalements often last up to 30 min. In many cases pipettes of resistance as low as $10 \text{M}\Omega$ can be introduced with effective sealing and no loss of resting potential; these allow substantial currents to be passed. For accurate control of membrane potential in a voltage clamp, the surface membrane must have little resistance in series with it (Johnson and Lieberman, 1971; Trautwein, 1973). The series resistance in an aggregate according to either model described above should be low. We conclude that spheroidal aggregates of embryonic heart cells should be useful for studying the active electrical properties of heart muscle, and especially for the analysis of the differentiation of electrical parameters.

Supported in part by the Carnegie Institution of Washington and USPHS General Research Support Grant RR05364 to Emory University School of Medicine (Dr. DeHaan), and USPHS grant HL-11665 and Myocardial Infarction Research Unit Contract PH-43681334 (Dr. Fozzard).

Received for publication 7 June 1974.

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