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Development of Electrical Coupling and Action Potential Synchrony between Paired Aggregates of Embryonic Heart Cells

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Summary. Pairs of spheroidal aggregates of embryonic chick heart cells, held in suction pipettes were brought into contact and allowed to synchronize their spontaneous action potentials. Contractions were suppressed with cytochalasin B. Both intracellular and extracellular electrodes were used to analyze the development of synchrony. Electric coupling occurred in three phases. During phase I electrical interactions were absent despite close physical contact. Phase II was characterized by partial synchrony. Action potentials in the faster aggregate (*F*) induced small depolarizations in the other member of the pair (*S*). These depolarizations sometimes triggered action potentials in *S* depending on when during the diastolic depolarization in *S* they occurred. In these cases both the latency between the action potentials (*L*) and the fluctuations in latency (V_L) were large. At the end of phase II the aggregates often passed through a brief period when fluctuation in interbeat interval in both increased noticeably. In phase III, beginning about 8 min after initial contact, action potentials were completely entrained at a certain *L*. During the subsequent 20–40 min *L* fell along an approximately exponential time course from about 130 to < 1 msec, while V_L declined in parallel. When well-coupled aggregates were pulled apart and immediately pressed back together, they re-established synchronization according to the usual three-phase time course. Synchronized aggregates could be partially decoupled by separating them just far enough to reduce the area of mutual contact. Pairs joined only by cellular strands maintained entrained action potentials with long latencies for many minutes. These results indicate that electronic junctions form between the paired heart cell aggregates causing the gradual development of action potential synchrony.

In cultures of embryonic heart cells, synchrony develops upon contact between single isolated myocytes (Cavanaugh, 1955; DeHaan & Hirakow, 1972), as well as among groups of heart cells organized in sheets (Harary & Farley, 1963*a, b*) and other geometries (DeHaan & Fozzard, 1975; Griepp & Bernfield, 1978). Electrical coupling via low-resistance junctions is essential for synchronous firing of pacemaker cells (Mark & Strasser, 1966; DeHaan & Hirakow, 1972; Jongsma *et al.*, 1975; De-

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Mello, 1977) and for intercellular transmission of the cardiac action potential in intact heart tissue (Barr, Dewey & Berger, 1965; Weidmann, 1967; Heppner & Plonsey, 1970; DeHaan & Sachs, 1972). The degree of synchrony and the velocity of impulse transmission in cardiac tissue is thought to depend on intercellular coupling resistance (DeFelice & Challice, 1969; Lieberman *et al.*, 1973; Pollack, 1976; Weingart, 1977; Patton & Linkens, 1978), although Sperelakis and his colleagues have developed a model for electrical transmission between myocardial cells that does not depend upon connection by low-resistance pathways (Mann, Foley & Sperelakis, 1977; Mann & Sperelakis, 1979). Little is known about the role of cell coupling in the synchronization of the cardiac impulse when electrical interactions are weak, as when new coupling junctions are forming.

In recent studies of the electrical properties of the heart cell membrane, a tissue culture model has been used which consists of spheroidal ventricle cell clusters (diameter 50–250 μm) dissociated from embryonic chick hearts (Sachs & DeHaan, 1973; DeHaan & Fozzard, 1975). These aggregates behave electrically like giant single cells in that the deviation in potential that exists within cells of a single aggregate is so small as to be negligible for signals of low frequency and amplitude (DeHaan & Fozzard, 1975; DeFelice & DeHaan, 1977; Clay, DeFelice & DeHaan, 1979). Moreover, these preparations permit long duration intracellular recording and can readily be manipulated under low magnification. Their electrical and pharmacological properties resemble closely those of the intact organ from which they were derived (DeHaan & Sachs, 1972; McLean & Sperelakis, 1976).

We have shown that when two such aggregates, beating at different rates, are brought into contact they synchronize their spontaneous beats in less than an hour (DeHaan *et al.*, 1973). If they are pressed together to increase their shared area of apposition, the delay between contact and synchrony (t_s) can be reduced to 8 min (Williams & DeHaan, 1977).

In the present study we analyze the development of beat synchrony with electrophysiological techniques. We show that just prior to synchronization a period of weak electrical interaction exists during which the fluctuation in interbeat interval (IBI) of interacting aggregates is increased. Moreover, after synchrony, the development of electric coupling continues for 20–30 min as is evident from a continued decline of the latency (phase difference) between the synchronized action potentials. Finally, we demonstrate that the degree of electric coupling can be controlled experimentally by adjusting the degree of contact between

the aggregates. We interpret these phenomena as resulting from the development of nexal junctions between the cells of the paired aggregates.

Materials and Methods

Tissue Culture, Media, and Drugs

The embryonic heart cell aggregates were prepared by techniques described in full elsewhere (Sachs & DeHaan, 1973). In brief, cells were dissociated from embryonic ventricles of 7-day chick (White Leghorn) embryos by trypsinization (DeHaan, 1970), and were then reassociated into spheroidal aggregates of 100–200 μm diameter by 48–72 hr gyration (Moscona, 1961; Sachs & DeHaan, 1973). Aggregates were prepared and experiments carried out in medium 818A1; pH = 7.3; $[K^+]_o = 1.3$ mM (for composition, *see* DeHaan & Fozzard, 1975). Cytochalasin B (cyto-B) (Aldrich Chemical Co., Milwaukee, Wisc.) was added from a stock solution of 0.1 mg/ml in dimethylsulfoxide (DMSO) to the culture 20–24 hr before the experiment to a final concentration of 0.33 μg/ml. At this concentration, myofilaments are disrupted and mechanical contractions cease, with no alterations in action potential generation or impairment of electrical coupling (Sachs, McDonald & Springer, 1974). In some experiments tetrodotoxin (TTX) (Sigma Chemical, St. Louis, Mo.) was added (2×10^{-6} g/ml) to block action potential generation.

Electrophysiology

Both intracellular and extracellular electrodes were used in this study. Intracellular electrodes were pulled from pipettes containing glass fibers (Tasaki *et al.*, 1968). Microelectrodes (10–40 MΩ) were connected to capacity-compensated unity gain preamplifiers (Picometric Model 181, Instrumentation Laboratory, Inc., Lexington, Mass.) by Ag/AgCl electrode holders. The preamplifier outputs were connected to dc- and ac-coupled variable gain amplifiers (ac bandwidth 0.015 Hz to 10 kHz). A 5 to 10 kΩ agar bridge connected the bath to a grounded Ag/AgCl reference electrode in 3-M KCl. Current was injected via a $10^9\Omega$ current-limiting resistor. The tissue culture dish was kept at 37 °C on a warm stage, and evaporation was prevented by a covering layer of nontoxic mineral oil (Klearol, Sonneborn Div., Witco Chemical, New York, N.Y.). The pH was kept constant at 7.3 by continuous gassing of the dish (10% O_2, 5% CO_2, 85% N_2). Aggregates were viewed during the experiments at 50 to 75× through a dissecting microscope.

Suction pipettes (Clapham *et al.*, 1978) were used for extracellular recording and manipulation of the aggregates. The suction pipettes were pulled by hand from 1.0 mm OD borosilicate glass capillaries (Corning Glass Co., Horsehead, N.Y.). The tips of the pipettes were fire-polished and those with inner tip diameters of 15–30 μm selected for use. Pipettes were then fire-sealed to 1 ml PVC syringes which were connected by stiff tubing to a 50 μl microsyringe. The entire system was filled with 818A1 medium. Slight negative pressure applied via the microsyringe was used to attach an aggregate to each of two pipettes. Held by micromanipulators, the suction electrode could be used to bring a pair of aggregates into apposition and control accurately the area of contact between them. Ag/AgCl wire in the pipette system permitted recording of extracellular potentials from each aggregate. Suction electrodes were coupled to amplifiers (Princeton Applied Research, Model 113, Princeton, N.J.) at a gain of 500 or 1000. The extracellular voltage spike was generally 1–3 mV with a duration of about 1 msec. Extracellular spikes and action potentials were

recorded on FM tape and a pen recorder for later measurement of latencies and beat intervals. The term latency (L) is used here to describe the delay (in msec) between the fastest parts of the upstrokes of two consecutive action potentials from the two paired aggregates. Entrainment means that the action potentials from two aggregates are synchronized with a fixed or slowly changing value of L. Beat intervals (IBI) were measured with an intervalometer that generates a potential proportional to the length of time between successive spikes and records those potentials as a dot display on an oscilloscope (Fig. 6).

Results

Development of Action Potential Synchrony

When two spontaneously beating cardiac aggregates from the 7-day chick embryo were brought into contact by micromanipulation, and allowed to adhere without pressure, their pulsations synchronized within a period of 30–60 min. When suction pipettes were used to hold the aggregates and to manipulate them into contact under slight pressure (Fig. 1*a*), the time between contact and synchrony was reduced to a few minutes as shown previously (Williams & DeHaan, 1978). Aggregates having noticeably different beat rates were selected in order to demonstrate the establishment of synchrony more dramatically and to make it easier to identify early stages of weak coupling. Each aggregate was impaled with an intracellular electrode within a few minutes after apposition (Fig. 1*b*). The spontaneous rate of the faster (F) aggregate (upper traces of all frames) rose to about 100 beat/min as a result of the impalement, and gradually slowed to a rate of about 50 beat/min (Fig. 1*c*). The slower aggregate (S), which was impaled earlier, settled down within 2–3 min after contact to a much lower rate of about 10 beats per min. After 4.5 min of contact, there were no signs of electrical communication between the aggregates; each beat at its own rate with no apparent effect upon the other (Fig. 1*b*). After 7 min (Fig. 1*c*), however, each action potential in F induced a small depolarization in S, which occasionally reached threshold. Thus, at this early stage, electrotonic impulse transmission had already begun, but failed to cause synchrony of most beats. The amount of current flowing from F through the contact area into the cells of S was apparently too small to excite the latter. Although the intrinsic rate of diastolic depolarization of the slow aggregate might have been altered at this time by currents from F, the most significant feature of this initial stage of weak electric coupling was the occurrence of depolarizations induced by the impulses from F. At about 10 min

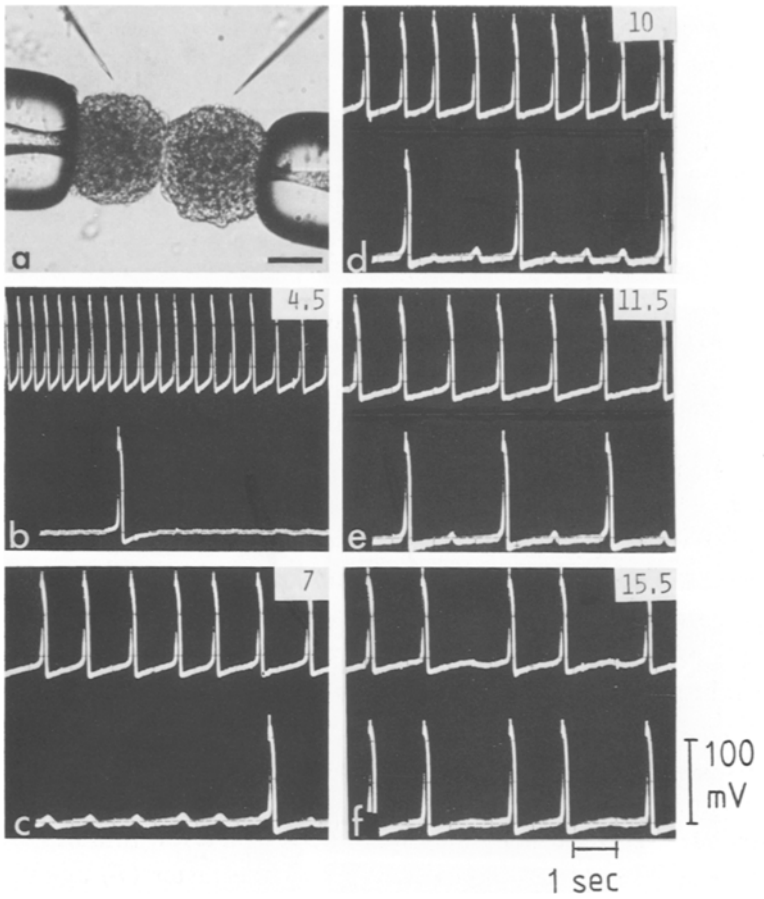

Fig. 1. Action potentials recorded with intracellular electrodes in a pair of aggregates (pair *8*, Table 1) held in contact with suction pipettes. (*a*): Each aggregate is held by a suction pipette attached to a micromanipulator. Microelectrodes are seen poised for impalement. (Scale: 100 μm). (*b–f*): Action potentials recorded from the initially faster (*F*, upper trace) and slower (*S*, lower trace) aggregates are totally uncoordinated 4.5 min after contact (time shown in upper right corner of each panel) but come progressively into phase with increasing time of apposition. (Records shown are from pair *7*, Table 1)

(Fig. 1 *d*) one out of every 3 or 4 beats in *F* on the average was followed by an action potential in *S*. This period is equivalent to the stage of "partial synchrony" (DeHaan & Hirakow, 1972; Jongsma *et al.*, 1975) observed during the synchronization of newly apposed pairs of single cardiac myocytes. The coupling between the aggregates increased further with time (*cf.* Fig. 1 *e*) until every beat in the two aggregates was entrained (Fig. 1 *f*) after a contact period of 15.5 min. With continuous recording the precise time of synchronization was localized to 13 min after contact

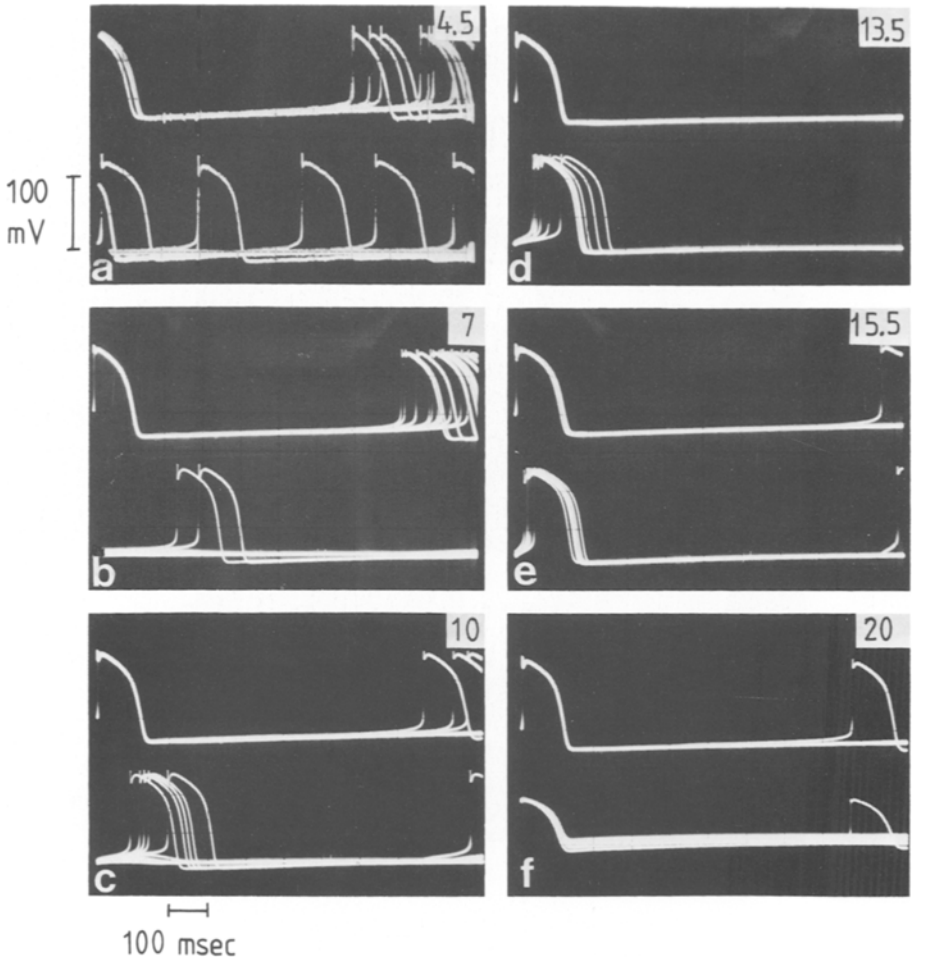

Fig. 2. Changes in latency between the action potentials recorded in the same pair of aggregates as in Fig. 1. About 10 oscilloscope sweeps are triggered by the upper beam. Numbers in the upper corner of each frame are the times since contact of the aggregates (min). From the period when impulse transmission first became apparent (~ 7 min), mean latency ($\bar{L}$) and fluctuation in the latency (V_L) both declined steadily as coupling increased

in the pair illustrated in Fig. 1 *b–f*. This time was easily observed because the transition in rate of F from 40 to 10 beat/min was abrupt. After that time the slope and shape of the subthreshold diastolic depolarization of S was also virtually identical to that of F. The synchronous rate appeared to be dominated by S, the larger member of the pair ($S=180\ \mu m$; $F=150\ \mu m$).

The coupling process is revealed in greater detail with the expanded time scale of Fig. 2. The impulse from F was used to trigger the oscillo-

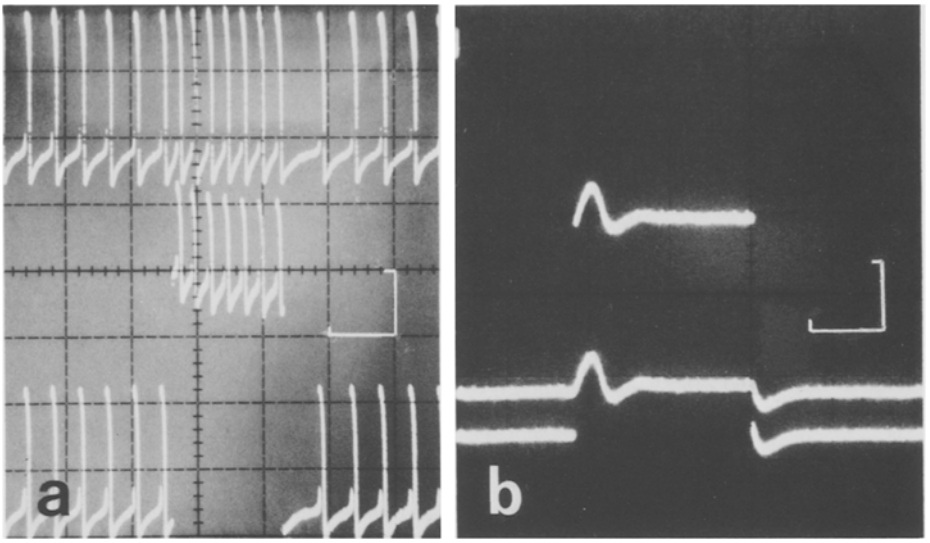

Fig. 3. Injected current flow between aggregates in the final stage of synchrony. (*a*): Well-coupled aggregates 4 hr after contact; spontaneous action potentials are precisely synchronized. A steady 6-nA depolarizing current injected into one aggregate (lower trace, mid-panel) causes a marked synchronous increase in beat rate in both members of the pair. The large offset of about 150 mV in the lower trace results from the current dropping across the 25 MΩ resistance of the injecting electrode. (*b*): A different pair, 1.5 hr after initial contact, in medium containing TTX (1.6×10^{-6} g/ml) to suppress spontaneous firing. A depolarizing current pulse (1 nA, 5 sec) produces a brief oscillation and a steady depolarization (about 0.7 mV) in the injected aggregate, superimposed upon the voltage drop of the electrode resistance (subtracted graphically at high gain). An identical membrane response is seen in the other member of the pair. Scales: (*a*) 50 mV, 2 sec; (*b*) 10 mV, 2 sec

scope (upper beam in all frames) and successive sweeps were superimposed. Shortly after contact, the action potentials of the two aggregates were wholly independent (Fig. 2*a*). Records taken 7 (Fig. 2*b*) and 10 (Fig. 2*c*) min after contact showed partial synchrony in which entrainment of action potentials was variable. In Fig. 2*b* impulses triggered in S by F occur with long variable latencies up to 300 msec. Moreover, some beats in F are missed in S, while F exhibits large fluctuations in its own interbeat interval. As synchronization proceeds (Fig. 2*d*), both the mean latency ($\bar{L}$) and its variancy (V_L) decline. Even when the pair appears to be well coupled and synchronization is 1:1, both $\bar{L}$ and V_L can still be large ($\sim$80 msec; Fig. 2*d*). At this time F clearly leads S. By 15 min after contact, however, as coupling increases (Fig. 2*e*), latency is reduced to 30 msec. In Fig. 2*f*, the electrode impalement in S has degraded. However, the aggregates are well synchronized and

now S leads F by about 10 msec, at a mean interbeat interval determined by S. At this stage V_L approaches 0.

To confirm that action potential synchrony in the final stage was associated with electric coupling between aggregates, 5-sec pulses of current were injected via an intracellular electrode into one member of a well-coupled pair ($L < 1$ msec). Depolarizing current produced simultaneous increases in beat rate of both aggregates (Fig. 3*a*); hyperpolarizing currents slowed the rate of both. When spontaneous synchronized beats were suppressed with TTX, injection of a depolarizing current of 1 nA into one aggregate caused virtually identical responses with a dc component in both aggregates (Fig. 3*b*), indicating that electric coupling is – at least – resistive and that the coupling resistance in the final synchrony stage is less than the input resistance R_{in} (1–2 mΩ) of either aggregate.

Decline in Latency during Action Potential Synchronization

The action potentials recorded in Fig. 2 revealed large variable latencies in the initial stage of synchrony and a decrease of both $\bar{L}$ and V_L with time after contact. To avoid the possibility that these phenomena reflected artifacts of impalement (gradual "healing in", stabilization of membrane, etc.), beating was recorded through extracellular suction electrodes which were also used to hold and manipulate the aggregates. Because the extracellular spike is short (about 1 msec), it serves as a precise time mark for the corresponding action potential in each aggregate. These recordings (Fig. 4) show more clearly than the action potentials themselves the progressive development of synchrony after apposition.

With the two aggregates held apart in separate suction electrodes, 30 interbeat intervals of F are displayed (Fig. 4*a*). The superimposed oscilloscope sweeps triggered by spikes from F (upper trace), show the total lack of coordination between the spikes of F and S. One minute after F and S are pressed together (Fig. 4*b*), the IBI of F increases slightly but the two aggregates remain asynchronous. Three minutes after contact (Fig. 4*c*) the IBI of F fluctuates markedly, and at 4 min (Fig. 4*d*) the two aggregates are in a state of partial synchrony, in which some beats of S follow those of F, but with a lag of 100–450 msec. Two minutes later (Fig. 4*e*) complete synchrony is achieved. Every beat in F is followed by one in S, with a mean interaggregate latency of about 200 msec. With longer times of contact (Fig. 4*f–h*) both $\bar{L}$ and

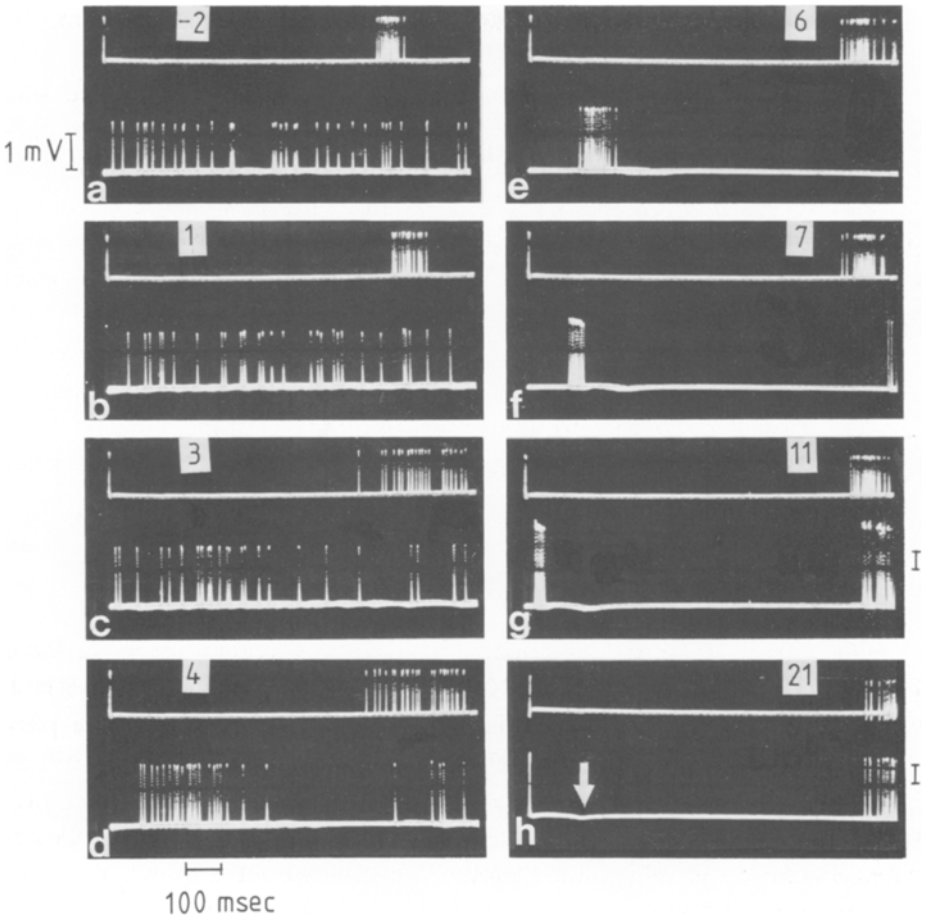

Fig. 4. Extracellular spikes recorded from coupling aggregates (pair *1*, Table 1). The faster aggregate (*F*, upper trace) was 135 μm in diameter and had a spontaneous rate of 77.6±2.7 beat/min (mean ±SD, calculated from 410 IBI's) before pairing (*a*); that of the slower (*S*, lower trace, 190 μm diam.) was 66.8±2.0 beat/min (mean ±SD (from 360 IBI's). About 30 successive sweeps are superimposed in each frame at different times after initial contact. Pressing the aggregates together had little effect on beat rate (compare frames *a* and *b*). The apparent randomness of the beats in *S* in panels *a–c* results from triggering the oscilloscope sweep by events in the upper trace and illustrates the lack of coordination between the aggregates prior to coupling. After entrainment (panel *d*) the gradual increase in synchrony and decrease in latency is evident (panels *e–h*). Repolarization deflections are visible (arrow) after the fast spike in *S* (panels *f–h*). Scales: 1 mV; 100 msec

V_L decrease. Also the mean interbeat interval ($\overline{\text{IBI}}$) of *F* continues to increase.

The progressive decline in interaggregate latency is plotted as a function of time after contact for three aggregate pairs (Fig. 5). In pair

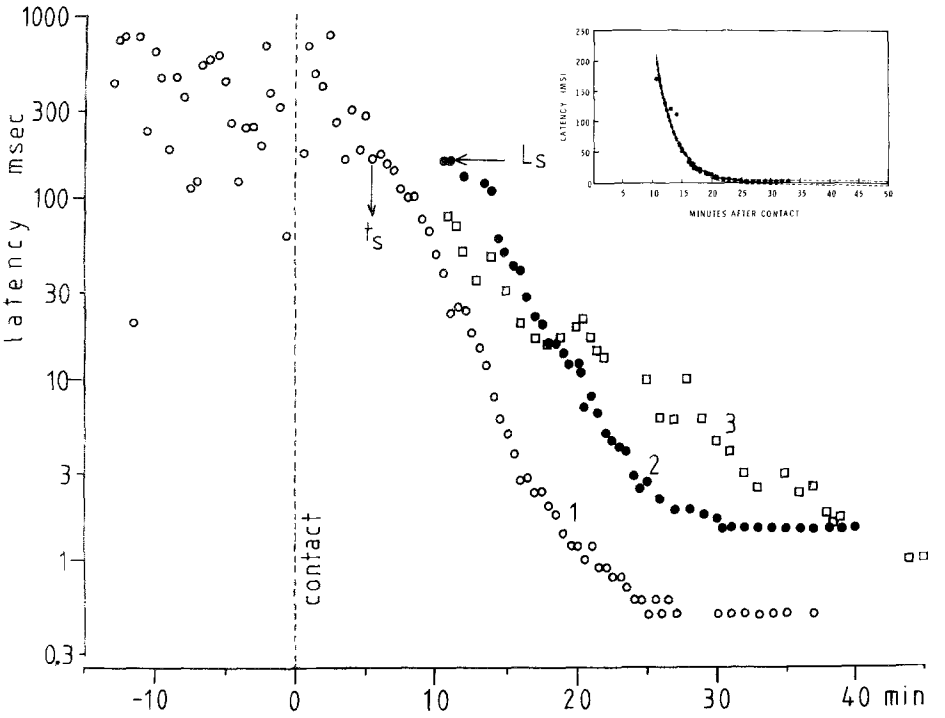

Fig. 5. Decrease in interaggregate action potential latency. Three pairs of coupling aggregates plotted on a semilog scale. Each point represents the time lag between a single beat in *F* and the induced beat in *S*. Aggregate diameters, contact areas, and other parameters of the three aggregate pairs are listed in Table 1 (pairs *1–3*). The experimental points from pair *2* are fit with an exponential curve (Eq. (1)) plotted on linear scales (inset). The dashed lines show the 95% confidence limits

1 measurements of L were begun 13 min before the aggregates were pressed together. At this time, and for about 5 min after contact, L fluctuated between 20 and 800 msec (*see* Fig. 4*a*). In pairs 2 and 3 measurements were begun as soon as it was clear that every beat in the two aggregates was in phase. The time to first synchrony (t_s) in the three pairs illustrated (Fig. 5) were 5.5, 10.5 and 11 min. At these times, latencies at first synchrony (L_s) ranged from 80 to 200 msec; L fell with an exponential time course to very small values (< 1 msec) after 20–40 min. The fall in L with time can be described by the exponential equation:

$$L(t) = L_{\min} + L_o e^{-t/\tau_L} \tag{1}$$

where $L(t)$ is the latency as a function of time after synchrony, τ_L is the time constant characterizing the decline, $L_{\min}$ is the minimal latency

at the apparent final plateau (here taken as constant) and L_o is the point of intercept of the exponential slope with the Y axis.

The parameters used to characterize the latency decline (Table 1) include the initial latency L_s (occurring at first synchrony), the time to first synchrony t_s, and the time to reach a latency of 1 msec (t_1). The value of t_1 was determined graphically by finding the time at which the extrapolated linear curve of the exponential intersects the horizontal 1 msec line drawn on semi-log scales. Values for the final latency plateau ($L_{\min}$) are not given in the table. The "plateau" after the exponential decline may actually represent a period of continuing decline in latency with a small, but nonzero slope.

The mean value of L_s in nine aggregate pairs treated with cyto-B was 127 ± 53 msec (Table 1, 9 experiments). First synchrony occurred 8.1 ± 3.7 min after contact. This value is consistent with that observed in aggregates pressed together between glass blocks (Williams & DeHaan, 1977). Latency fell to 1 msec (t_1) on average 26.2 min after contact. A second group of 10 pairs, not exposed to cyto-B, synchronized somewhat more slowly ($t_s = 18 \pm 9$ min and $t_1 \simeq 40$ min.) Thus, the degree of electrical coupling between a pair of aggregates continues to increase exponentially for a period about 3 times as long as the time required to reach first synchrony. The time constant ($\tau_L = 3.6 \pm 1.4$ min) is presumably a reflection of the decline in coupling resistance due to the continuous insertion

Table 1. Latency and synchronization parameters of aggregates treated with cytochalasin B

Aggregate pair	Aggregate diameters (μm)		Diameter contact area (μm)	Latency at first synchrony L_s (msec)	Time to first synchrony t_s (min)	Time to 1-msec latency t_1 (min)	Y-axis intercept L_o (sec)	Time constant of latency decline τ_L (min)
	F	S						
1	135	190	130	170	5.5	19.5	2.6	2.5
2	170	180	130	170	10.5	29	4.6	3.4
3	180	180	110	80	11	41	0.7	4.9
4	115	115	90	200	2	19	0.5	3.5
5	190	190	180	35	11	22	5.9	2.2
6	130	190	180	90	9	37	0.6	6.8
7	150	180	110	120	13	24	5.0	3.0
8	180	190	115	160	4	19	0.7	3.0
9	170	190	130	115	7	25	0.7	3.5
Mean ± SD	158 ± 26	178 ± 24	131 ± 31	127 ± 53	8.1 ± 3.7	26.2 ± 8.0	2.4 ± 2.2	3.6 ± 1.4

of nexal channels between cells at the newly apposed surfaces of the coupling aggregates (DeHaan *et al.*, 1980). Variability in t_s may be partly due to the fact that the intrinsic frequency differences of the members of the pairs were different between pairs (Linkens & Datardina, 1977).

Irregular Beating just before Synchrony

Single aggregates normally beat with a regular rhythm. Standard deviation of the fluctuation in IBI (σ_{IBI}) was usually a few % of $\overline{IBI}$ (*see* legend Figs. 4 and 7). During the first minutes after contact, σ_{IBI} of the independently beating members of a pair remained small. However, just before the onset of synchrony each aggregate usually became more irregular, during a partial-synchrony phase, and then resumed rhythmic beating after action potential entrainment was achieved. The phase of large fluctuation in IBI was a reliable early sign of the onset of coupling. In some pairs synchrony developed with a relatively constant $\overline{IBI}$ throughout the whole process (Fig. 6). In the pair illustrated (pair *1*, Table 1) the period of significantly increased fluctuations began in *F* about 3 min after contact and ended when synchrony was achieved (t_s = 5.5 min). During the 2.5 min of partial synchrony, however, IBI of *F* increased to a value similar to that of *S*, while *S* did not change its beat rate; neither increased its σ_{IBI} clearly. In other aggregate pairs, coupling was associated with a slow drift of IBI. In the example shown in Fig. 7*a*, a 20% shift in IBI both before and after synchrony (t_s = 10.5 min) occurred. Nonetheless, σ_{IBI} began to increase about 7 min after contact (Fig. 7*b*) and reached a maximum just before synchrony. At $t = 10$ min, when $\overline{IBI} = 800$ msec, σ_{IBI} (60 msec) = 7.5% of $\overline{IBI}$.

Experimental Manipulation of Synchrony

If a pair of well-coupled aggregates was pulled apart and all previous adhesions severed, each aggregate stopped beating temporarily and then resumed its rhythmic activity with a rate nearly equal to its original, precontact rate, unrelated to the other member of the pair. When two such recently separated aggregates were brought back into contact, coupling was re-established according to the usual time course. Synchronized

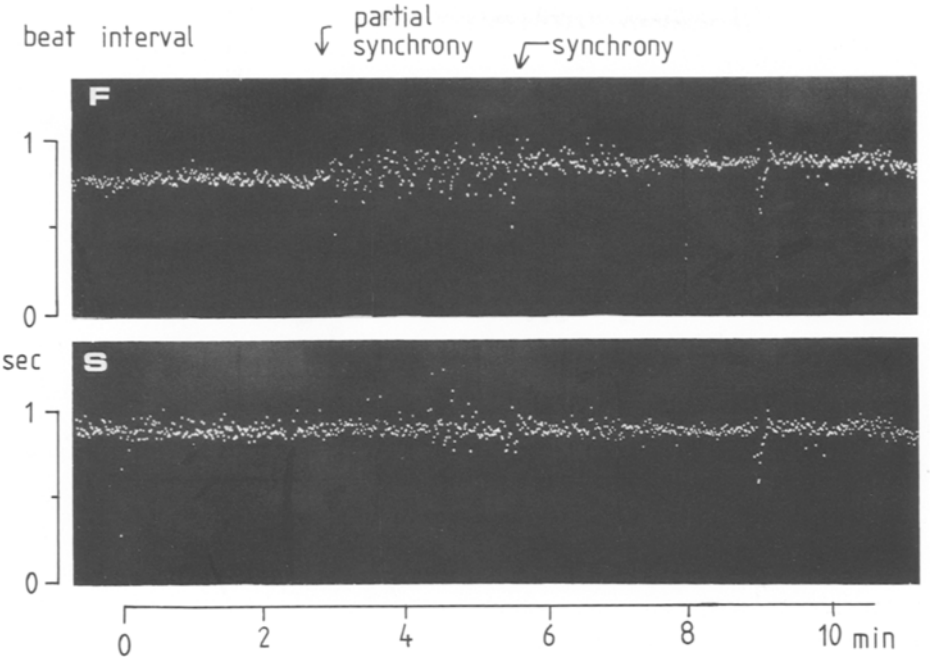

Fig. 6. Increase in fluctuation of IBI during partial synchrony phase. Intervalometer records from a pair of aggregates brought into contact at $t=0$. Each white dot is a measured IBI. In this pair (pair *1*, Table 1), $\overline{IBI}$ of *S* remained constant throughout the course of coupling. An increase in fluctuation around $\overline{IBI}$ in *F* during the phase of weak coupling prior to synchrony is associated with a shift in IBI. (*F*, 135 μm diam, initial IBI = 773 msec; *S*, 190 μm diam, initial IBI = 898 msec)

aggregates could also be partially decoupled by separating them just far enough to tear some of the cellular adhesions and reduce the area of mutual contact, after the method of Ito, Sato and Loewenstein (1974). The results of such an experiment are illustrated in Fig. 8 with a pair of aggregates that established synchrony 14 min after first contact (curve *1*) at $L_s = 140$ msec; t_1 was 42 min. These aggregates were then separated completely and brought back together three successive times (latency curves 2–4) to demonstrate their ability to recouple. Near the end of latency curve 4, the aggregates were carefully separated to reduce the diameter of their contact area from 115 to 66 μm. Latency rose abruptly from 4 to 100 msec without loss of synchrony and then fell in the usual exponential fashion (curve *5*). At $t = 223$ min, the aggregates were pulled 30 μm apart, leaving only two thin cellular strands (< 10 μm in diameter) bridging the gap between them. After a short pause, the aggregates

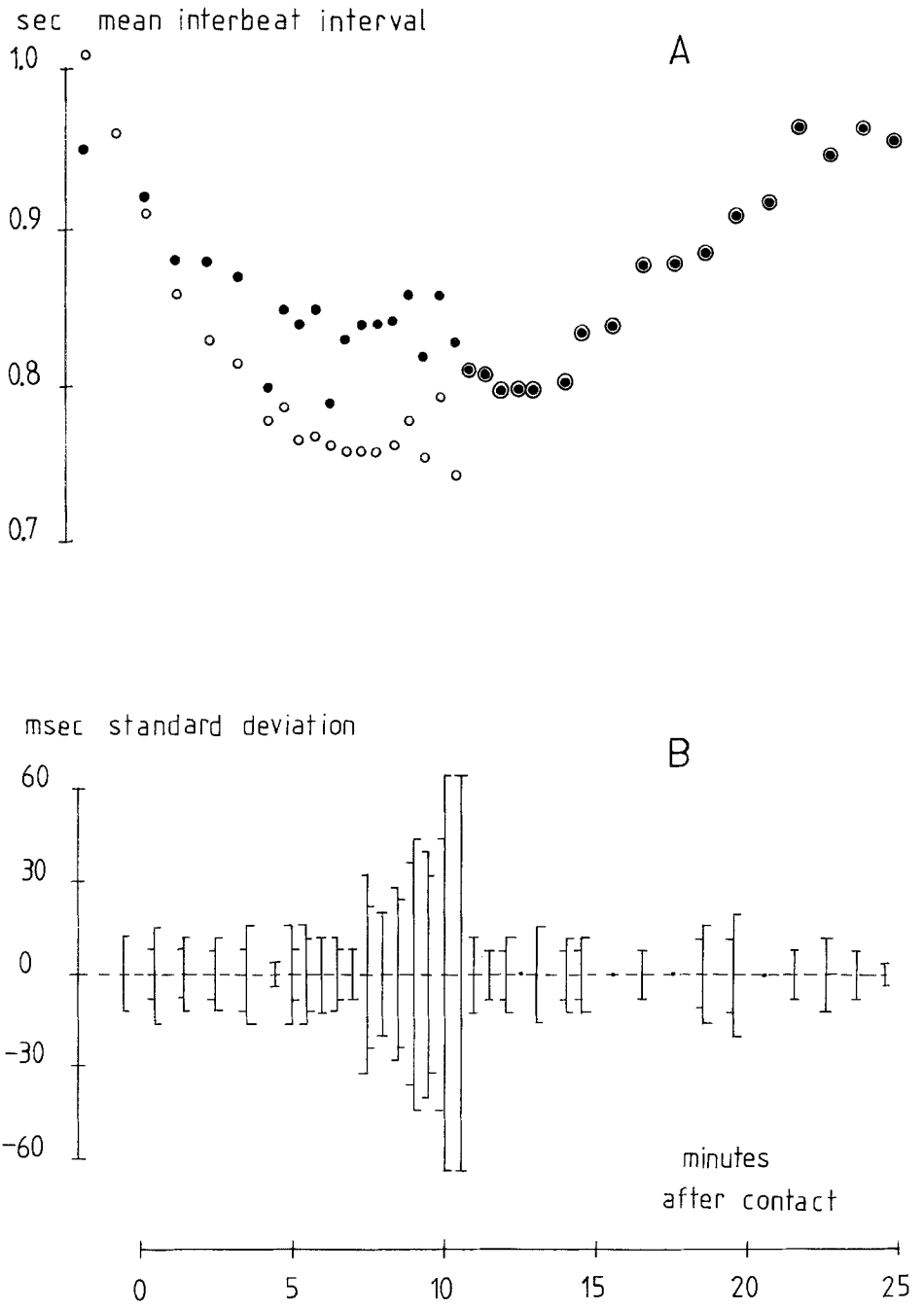

Fig. 7. Changes in $\overline{IBI}$ and σ_{IBI} during entrainment. (*A*): $\overline{IBI}$ calculated from the first 30 sec (30 to 40 IBI's) of each consecutive minute after contact (pair *2*, Table 1; t_s= 10.5 min). (*B*): Horizontal line segments to the left of each σ_{IBI} line (30 to 40 IBI's) refer to *F*, those to the right refer to *S*. (F=180 μm diam; S=170 μm diam)

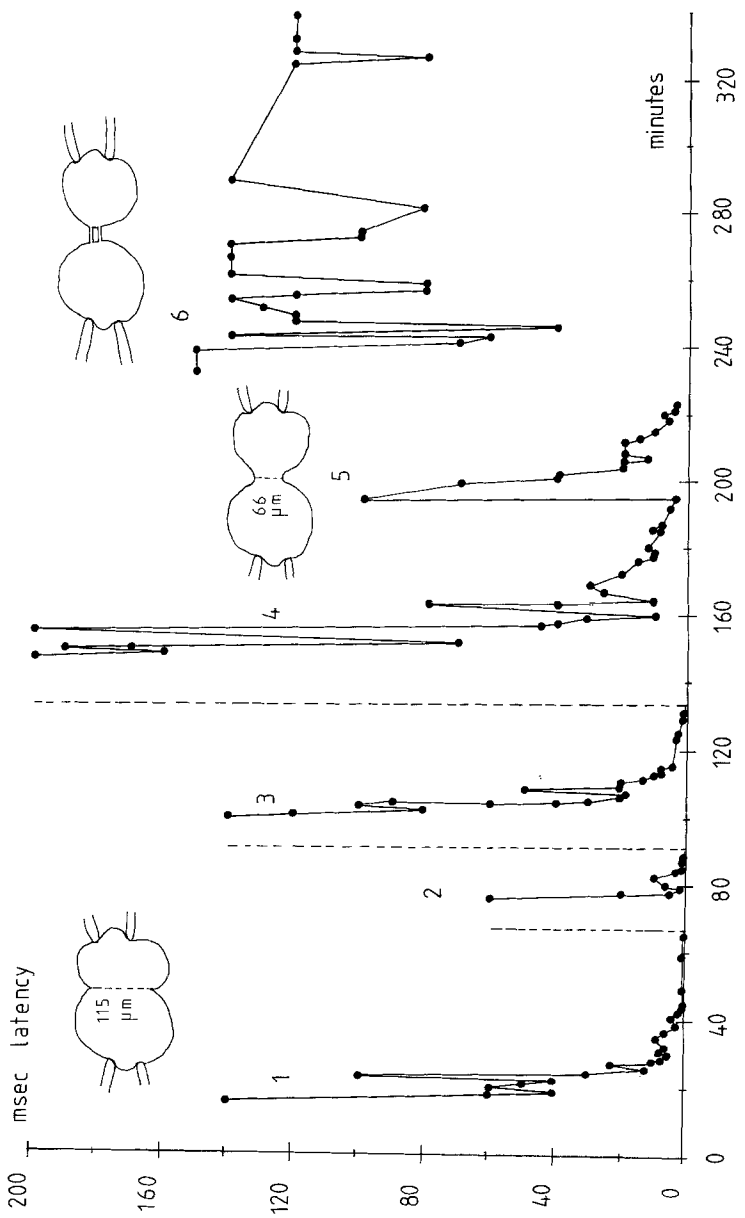

Fig. 8. Synchronization after repeated separation in a single pair of aggregates. ($F = 140$ µm, $S = 165$ µm). No cyto-B was present in this experiment. For latency curve (*1*) contact area was adjusted to 115 µm diameter. For the next three latency curves, contact between the aggregates was disrupted just before the dashed vertical lines and was readjusted to 115 µm diameter at the time indicated by the dashed lines. For coupling cycle (*5*) contact area was reduced to 66 µm by pulling the aggregates slightly apart, without disrupting synchrony. For coupling cycle (*6*) aggregates were pulled apart to an interdistance of 30 µm, leaving two cellular strands intact

resumed beating, but with complete loss of synchrony. Ten minutes later, with the aggregates held in the same position, they again synchronized, but in this case latency did not immediately decline exponen-

tially; instead, it continued to fluctuate widely (40–150 msec) for almost 2 hr until the experiment was terminated (latency curve *6*). Experiments of the type illustrated in Fig. 8 have been repeated with other aggregate pairs, with and without exposure to cyto-B, with similar results.

Discussion

Development of Synchrony

We have seen that coupling of a pair of newly apposed heart cell aggregates occurs in three distinct phases. Phase I is the period immediately after contact when the two preparations maintain their initial independent rates. Although they are in physical contact, the aggregates appear to be electrically insulated from one another, i.e., Z_{cp} seems to be large compared to Z_1 or Z_2.

We have referred to coupling phase II as the period of "partial synchrony" (DeHaan & Hirakow, 1972; Jongsma *et al.*, 1975) during which electrical interactions were weak. At the beginning of this phase, a 100 mV (*p–p*) action potential in *F* produced only a 3–4 mV depolarization during the diastolic phase of *S* (Fig. 2*c*). This transient depolarization was coincident with the plateau phase of the driving action potential, when the input resistance of both aggregates was high (1–2 mΩ) (Goldman & Morad, 1977; DeHaan & DeFelice, 1978b). From these observations we calculate roughly that Z_{cp} at this time must be on the order of 25–50 MΩ. This degree of electrotonic transmission was insufficient in most cases to bring *S* to threshold, and therefore action potentials remained asynchronous. Z_{cp} apparently further declined (Fig. 2*d–f*) and transmission increased until synchrony was achieved. Just prior to this time, we recorded a period of fluctuation in *L* and IBI (Figs. 4, 6 and 7). We attribute these fluctuations to membrane noise and to weak interactions between *F* and *S*.

If the latency between an action potential in *F* and the induced potential in *S* is long, such that the delayed impulse begins as that in *F* starts to repolarize, that repolarization may be retarded. The rising phase of an action potential in *F* may increase the slope of the diastolic depolarization in *S*, whereas the repolarization may decrease that slope (Fig. 1*d*). These interactions interfere with the original rates of *F* and *S*. Regular time sequences may occur in the IBI-fluctuations (Gollub, Brunner & Danly, 1978), but were not noticed, probably because coupling

resistance is changing continuously and membrane noise introduces additional irregularity when interactions are weak (Verveen & Derksen, 1968). An important experimental fact that undoubtedly influences impulse transmission especially during the phase of weak interactions is that the input impedance of the heart cell varies in a characteristic way during each action-potential cycle. During diastolic depolarization impedance is high and increases about fivefold between maximal diastolic potential and threshold (Clay *et al.*, 1979) while during the brief instant of the upstroke it drops precipitously (Weidmann, 1951). As noted above, during the plateau phase the membrane is again in a high-resistance state. Thus aggregate interactions during the development of synchrony must represent a complicated function of the cyclic changes in Z_1 and Z_2 and the decline with time in Z_{cp}.

In phase III, coupling is sufficient to establish a synchronous rhythm, with every beat in the two aggregates in phase. Even subthreshold events ultimately were transmitted and became equal (Figs. 2*f* and 10*a*). Clearly, however, the onset of beat synchrony is only a measure of some threshold level of interaggregate conductance, not of the initiation of junction formation itself (Sheridan, 1976). The remaining fluctuations in L just after synchrony are of the same range as normal IBI fluctuations of the independent aggregates (Fig. 4). They are, therefore, probably due to membrane noise (DeFelice & DeHaan, 1977; DeHaan & DeFelice, 1978*a*) interfering with threshold crossing of the depolarizations evoked by F in S (Verveen & Derksen, 1968).

The final synchronized beat rate of an aggregate pair was generally intermediate between the prior independent rate of each. F, which was usually the smaller aggregate (DeHaan & Sachs, 1972) always led S (Fig. 5). However, S (the larger one) usually had a dominant influence in determining the final beat rate (Fig. 6) as predicted by Jongsma *et al.* (1975). Thus, differences in Z_1 and Z_2, the value of Z_{cp}, as well as other aspects of the physiological state of both aggregates apparently are important in determining the synchronous rate (Patton & Linkens, 1978; Berkinblitt *et al.*, 1974).

Structural Interpretation of Progressive Coupling

Linkens and Datardina (1977) coupled Hodgkin-Huxley type models of pacemaker cells and observed a decline in action potential latency when lowering Z_{cp}. Such an effect of Z_{cp} on latency has also been

found for aggregate synchronization by Clapham *et al.* (1979). It is clear, however, that other parameters may also be important in determining synchronization. Ito *et al.* (1974) have commented upon the importance of the leakage resistance through imperfectly sealed abutting nexal channels via the intercellular clefts to the bulk medium in determining coupling. In the present experiments, there was no way to discriminate between nexal resistance and shunt resistance. Since coupling junctions develop between embryonic heart cells within minutes (DeHaan & Hirakow, 1972) and changes in shunt resistance are presumably relevant only during the early stages of coupling, it is difficult to envision how changing shunt resistance could be more than a minor component in the observed coupling phenomena.

If changes in leakage resistance are ignored, the decline in latency during coupling must reflect a decrease in interaggregate resistance. Recent experiments on the development of electrical coupling between *Xenopus* embryo cells (Loewenstein, Kanno & Socolar, 1978) suggest that each additional nexal channel adds a stable, constant transcellular conductance. As more nexal channels are formed between aggregates, more current is exchanged with each action potential in F to drive the membrane voltage of S to threshold and shorten latency. This is the basic interpretation of the present work. Cardiac aggregates have at their periphery an irregular layer of fibroblasts, 1–2 cells thick (Steinberg, 1970; Sachs & DeHaan, 1973). However, coupling junctions appear to form as well between myocytes and fibroblasts as between myocytes, and such nonexcitable cells can mediate synchronization between pacemaker cells (Goshima, 1975). Thus, the presence of those cells does not influence our conclusions.

Minimal Required Contact Area

We have shown (Fig. 8) that the decline in latency that normally follows synchrony can be interrupted merely by reducing the area of contact. But only a remarkably small remaining area is required to maintain synchrony; even though with large latencies (Fig. 8, curve *6*). Effective coupling between two aggregates with input resistance of about 1 MΩ can occur when Z_{cp} falls to about 20 MΩ (Clapham, 1979). Estimates of the specific resistance of nexal membranes range from 1 $\Omega \cdot cm^2$ (Spira, 1971; Weidmann, 1966) to 0.01 $\Omega \cdot cm^2$ (Sheridan, *et*

al., 1978). Thus, the minimal area of nexal contact between two aggregates must be at least $1\text{–}100 \times 10^{-9}$ cm^2 or 0.1–10 μm^2. This corresponds to a circular appositional area with a diameter of 0.4–4 μm. Depending on the ratio of nexal to non-nexal membrane in the contact region and the longitudinal resistance of thin strands such as those produced by pulling aggregates apart, the effective contact area would probably be larger than these minimal estimates. In experiments such as that represented in Fig. 8 (curve *6*), latency was prevented from falling below 40 msec for long periods. This suggests that interaggregate coupling resistance could not decrease either because contact area in the connecting strands was so small as to limit the number of nexal channels, or because the longitudinal cytoplasmic resistance of the strands was too great.

Relevance to the Intact Heart

The normally functioning heart has a well-known pacemaker rate gradient with cells of the *SA* node having the highest intrinsic beat rate and pacemaker control of the rest of the heart. The ventricle has a lower intrinsic beat rate than the atrium (Goldman, 1976). This condition may be compared to the coupled beating of two aggregates, one of which has a much lower beat rate than the other. In the intact organ, the atrium drives the ventricle with a delay (*PR*-interval) of about 180 msec, and the synchronized rate is determined by the atrial pacemaker (*SA*-node). In the experiments described here, *S* may slow *F* to an intermediate rate or even to a rhythm close to its own, depending on the relative volume (R_i) of the two aggregates. Here too, the conduction delay may be 100–200 msec early in phase III. But a profound difference between the intact heart and the aggregate pair model exists. In the normal heart, *PR*-interval fluctuations are remarkably small and *AV* conduction occurs with a large safety factor. In aggregates at the stage when latency is 100 msec or more, fluctuations in the delay are considerable and the safety factor is small. At later stages of coupling, when latency no longer fluctuates and R_{cp} is low, interaggregate latencies fall to < 1 msec. It may be that the long delay in the intact *AV* node results from the summation of many successive small latencies at each cell junction. The sequential fluctuations around *L* at each junction would then average out.

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