

# Phase resetting of spontaneously beating embryonic ventricular heart cell aggregates

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GUEVARA, MICHAEL R., ALVIN SHRIER, AND LEON GLASS. *Phase resetting of spontaneously beating embryonic ventricular heart cell aggregates*. *Am. J. Physiol.* 251 (Heart Circ. Physiol. 20): H1298–H1305, 1986.—The influence of isolated 20-ms duration current pulses on the spontaneous rhythm of embryonic chick ventricular heart cell aggregates was studied. A pulse could either delay or advance the time of occurrence of the next action potential, depending on whether it fell early or late in the cycle. As the stimulus amplitude was increased, the transition from delay to advance occurred over a narrower range of coupling intervals. At low-stimulus amplitudes the transition from delay to advance occurred in a smooth continuous fashion; at medium-stimulus amplitudes the transition was discontinuous; at high-stimulus amplitudes graded action potentials were seen. It was impossible to annihilate spontaneous activity in aggregates with a single stimulus. The phase-resetting response to hyperpolarizing pulses was qualitatively the reverse of that produced by depolarizing pulses. A very high-amplitude depolarizing or hyperpolarizing pulse could produce rapid repetitive activity. Theoretical aspects of these phenomena are discussed.

all-or-none depolarization; graded action potentials; annihilation; embryonic chick

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DELIVERY OF A SINGLE premature stimulus to the heart can result in dramatic changes in its rhythmic activity. An appropriately timed stimulus delivered to a normal healthy heart can abolish rhythmicity by inducing fibrillatory activity. In isolated cardiac tissue a single premature stimulus can annihilate spontaneous activity (14, 20, 21) and can also terminate as well as start triggered activity (10). However, the effect of a premature stimulus is usually not quite so drastic; generally, there is a "resetting" of the cardiac rhythm.

Several recent studies have analyzed the phase resetting of cardiac tissue by brief depolarizing and hyperpolarizing inputs. The magnitude of the phase resetting obtained depends on the phase in the cycle at which a current pulse is injected as well as its polarity, amplitude, and duration (8, 15, 17, 19–23, 27–30). For example, in response to a brief depolarizing input there is a delay to the next action potential if the pulse is delivered early in the cardiac cycle and an advance if the stimulus is delivered late in the cycle. The transition from delay to advance occurs over an increasingly narrow range of stimulus phase as stimulus amplitude increases. The response to a single stimulus delivered at various phases in the cycle can be used to predict the effect of periodically delivered stimuli (15, 17, 19, 22, 23, 27, 31).

In this paper we describe the effects of single stimuli on the spontaneous rhythm in aggregates of ventricular cells from embryonic chick heart. In this virtually isopotential preparation (7, 12, 13, 24), propagation effects are minimized. This facilitates interpretation and modeling of the data. We pay particular attention to phase-resetting behavior where transition from delay to advance is a sensitive function of stimulation parameters. These transitional regions have not been adequately characterized in previous work. In some preparations, delivery of a single stimulus can lead to a long delay as a result of several skipped beats; in others, there is a transient acceleration of the cardiac rate. We discuss theoretical mechanisms underlying some of these phenomena.

## MATERIALS AND METHODS

*Tissue culture.* Aggregates were prepared following techniques of DeHaan and Fozzard (13) as previously described (9). White Leghorn chick embryos were incubated for seven days at a temperature of 37°C and a relative humidity of 85%. The embryos were decapitated, and the apical portions of the heart ventricles were isolated, fragmented, and dissociated into single cells. Dissociation was carried out by a multiple-cycle procedure in an enzyme-containing solution (11). The cell suspension was filtered through a membrane with a 12.0- $\mu\text{m}$ -diameter pore size and centrifuged at about 170 *g* for 15 min. The cells were resuspended, counted, and aliquoted into 25-ml Erlenmeyer flasks each containing 3 ml of maintenance medium at a density of  $5 \times 10^5$  to  $7 \times 10^5$  cells per flask. The flasks were gassed with a mixture of 5%  $\text{CO}_2$ -10%  $\text{O}_2$ -85%  $\text{N}_2$ , sealed with a silicone rubber stopper, and placed on a gyratory table (70 rev/min) for 48–96 h at 37°C to allow spheroidal aggregates to form.

The dissociation medium consisted of  $5.25 \times 10^{-5}$  g/ml crystalline lyophilized trypsin (Worthington Biochemical, 245 U/mg) and  $5 \times 10^{-6}$  g/ml deoxyribonuclease I (Worthington,  $9.1 \times 10^4$  U/mg) in a  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -free, phosphate-buffered balanced salt solution with concentrations (mM) as follows: NaCl 116.0, KCl 5.4,  $\text{NaH}_2\text{PO}_4$  0.44,  $\text{Na}_2\text{HPO}_4$  0.95, dextrose 5.6. The pH of the dissociation medium was adjusted to 7.3 with either 1 N HCl or 1 N NaOH.

The maintenance medium, a modification of medium 818A (13), consisted of 2% horse serum (Kansas City Biological), 4% fetal bovine serum [Grand Island Biolog-

ical (GIBCO)], and 20% medium 199 (GIBCO) in a bicarbonate-buffered balanced salt solution. The final concentrations (mM) were approximately as follows: NaCl 116.0, KCl 1.3, CaCl<sub>2</sub> 1.8, MgSO<sub>4</sub> 0.8, NaH<sub>2</sub>PO<sub>4</sub> 0.9, NaHCO<sub>3</sub> 20.0, dextrose 5.5. The antibiotic gentamicin sulfate (Schering, Garamycin, 10 mg/ml) was added to the medium to yield a final concentration of  $5 \times 10^{-5}$  g/ml.

The enzyme-inactivating medium was the same as the maintenance medium but with the following exceptions: 0% fetal bovine serum, 10% horse serum, and ~4 mM KCl. All solutions were filtered with a sterile filter having a 0.45- $\mu$ m-diameter pore size.

**Electrophysiology.** After 2–4 days of gyration culture, the reaggregates of cardiac cells were poured into a 35  $\times$  10-mm plastic tissue culture dish (Falcon 3001). Mineral oil was layered out on top of the medium to prevent evaporation. The medium was gassed from above by a toroidal gassing ring at a flow rate of 200 ml/min with a gas mixture of 5% CO<sub>2</sub>-10% O<sub>2</sub>-85% N<sub>2</sub> (13). The bicarbonate buffer in the medium maintained the pH at ~7.2–7.3. Temperature was maintained at  $36 \pm 1^\circ\text{C}$ . Phenol red in the maintenance medium (0.04 mg/ml) provided a rough continuous estimate of the pH. Under these conditions, >98% of the aggregates in a dish beat spontaneously. Mean aggregate diameter, taken to be the mean of the minor and major axes in the horizontal plane, could be estimated accurately and repeatedly to within one-half of a minor division of the graticule (i.e., to  $\pm 19 \mu\text{m}$ ).

Electrical activity was recorded intracellularly using microelectrodes filled with 3 M KCl (electrode resistance 20–100 M $\Omega$ ). Transmembrane potential was registered using an amplifier with negative capacitance compensation. The bathing medium was maintained at virtual ground by being coupled to a current-to-voltage converter (10–100 mV/nA) through an agar-salt bridge and a chlorided silver wire. Pulses of current were injected into the aggregate through the same microelectrode used for recording the membrane voltage and their amplitudes measured by the current-to-voltage converter. Currents were measured to the nearest 0.5 nA. Two programmable stimulators (Frederich Haer, Pulsars 4i and 6i) connected to external logic circuitry were used.

Voltage and injected current waveforms were monitored on a digital oscilloscope (Nicolet model 206) and recorded on an FM instrumentation recorder at a tape speed of 3 $\frac{3}{4}$  in./s (Hewlett-Packard model 3964A; 3-dB frequency response at 3 $\frac{3}{4}$  in./s: DC-1250 Hz) for later off-line analysis.

The protocol involved a 20-ms duration current pulse delivered at various coupling intervals after every 10 spontaneous beats. The coupling interval was automatically incremented (by a multiple of 1 or 10 ms) scanning a part of or, in some cases, the entire spontaneous cycle. The above protocol was then repeated for a different current amplitude, duration, or polarity.

**Data analysis.** Off-line analysis was carried out with the digital oscilloscope and by an automated system. Magnetic tapes were played back at a tape speed of 15/16 in./s (i.e., 1/4 real time), low-pass filtered, and the

voltage waveform then sampled at 250 Hz by a Z80-based microprocessor system (Cromenco System III fitted with a California Data, AD-100 12-bit analog-to-digital converter). The digitized waveform was transferred over an RS-232 serial line at 9,600 baud to a minicomputer (Hewlett-Packard model HP1000 series F) and stored on digital magnetic tape. Interbeat intervals were extracted out of the digitized waveform by a pattern recognition program.

All of the experimental voltage traces in this report (with the exception of Fig. 1A) were obtained by playing back the tape-recorded signal to the digital oscilloscope through a low-pass filter (to avoid aliasing). The contents of the oscilloscope memory were then reconverted to an analog signal and sent to an analog X-Y plotter (Hewlett-Packard model 7015B).

## RESULTS

**Spontaneous activity of the aggregate.** Heart cell aggregates generally beat with a regular rhythm (Fig. 1, A and

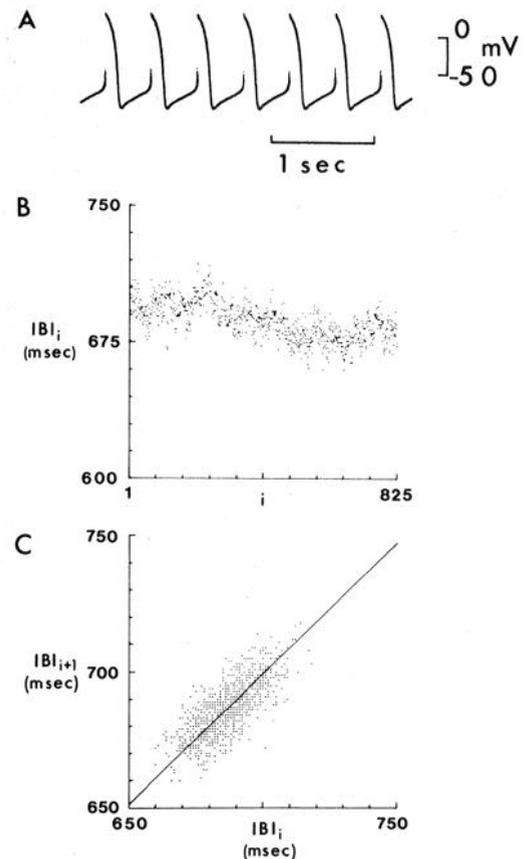


FIG. 1. A: tracing of transmembrane voltage recorded during spontaneous unperturbed activity in an aggregate illustrating regularity of beating. Unlike voltage tracings in other figures in this paper, this tracing was obtained by sending output of tape recorder directly to plotter. Top and bottom of vertical calibration bar in this and all subsequent figures indicate 0 mV and -50 mV, respectively. (Aggregate no. 1: diameter = 114  $\mu\text{m}$ .) B: interbeat interval (IBI<sub>i</sub>) plotted vs. interval number (i) from a period of unperturbed activity. No. of beats = 826. Precision in measuring an interbeat interval is  $\pm 1.0$  ms. Mean, SD, and coefficient of variation were 686 ms, 10.2 ms, and 0.015, respectively. C: scattergram of same data shown in B with each of 824 interbeat intervals plotted as function of immediately preceding interbeat interval. Straight line plotted through the data is least-squares fit to data; it has slope of 0.92 and coefficient of determination ( $r^2$ ) of 0.922. (Aggregate no. 2: diameter = 105  $\mu\text{m}$ ).

B), with a coefficient of variation (SD/mean) of the (electrical) interbeat interval of  $\sim 0.02$  (see also Ref. 7). We define the (electrical) interbeat interval to be the time from the crossing of 0 mV on the upstroke of one action potential to the zero crossing on the upstroke of the following action potential. In most aggregates, there is occasional "bursting" behavior, during which the beat rate of the aggregate transiently increases (17). During spontaneous activity, the interbeat interval of any cycle is highly correlated with that of the immediately preceding cycle (Fig. 1C).

**Response to stimulation with a single depolarizing current pulse.** Data were obtained at only one depolarizing amplitude in 10 aggregates and at two or more amplitudes in another 17 aggregates: these 27 aggregates were taken from 21 cultures. The mean diameters of these aggregates were in the range of 90–225  $\mu\text{m}$ , and the intrinsic interbeat intervals after impalement were in the range of 450–1,200 ms. Figure 2A shows the effect of injecting a single depolarizing current pulse of amplitude 10 nA and duration 20 ms into a spontaneously beating aggregate. The control interbeat interval ( $T_0$ ), the perturbed interval ( $T_1$ ), and the interbeat interval of the first poststimulus cycle ( $T_2$ ) are indicated. The coupling interval ( $t_c$ ) is defined to be the time from the zero crossing on the upstroke of the action potential immediately preceding the stimulus to the beginning of the stimulus. The phase  $\phi$  of the stimulus is  $\phi = t_c/T_0$  ( $0 \leq \phi < 1$ ). Activity in two widely separated cells in an aggregate is virtually synchronous, even after delivery of a stimulus (compare upper and lower traces in Fig. 2A). The time difference between zero crossings of all pairs of upstrokes recorded (Fig. 2) with electrodes  $\sim 120 \mu\text{m}$  apart is at most 50  $\mu\text{s}$ , which agrees with previous measurements in heart-cell aggregates during spontaneous activity (13).

Figure 2, B and C, shows the effect of delivering a depolarizing pulse of 20-ms duration at a coupling interval that is systematically incremented in 10-ms steps; in Fig. 2B the stimulus current amplitude is low (6.5 nA) and in Fig. 2C it is high (24 nA). At the high-stimulus amplitude graded action potentials are produced (Fig. 2C).

The normalized perturbed interbeat interval  $T_1/T_0$  is plotted as a function of  $\phi$  in Fig. 3 for various stimulus strengths. The progression in the data from Fig. 3A through Fig. 3F is typical of the changes in  $T_1/T_0$  seen in any one aggregate as the stimulus amplitude is increased. Since the stimulus artifact obscures the action potential upstroke when the stimulus is delivered late enough in the cycle to be a threshold stimulus,  $T_1$  can only be estimated to within one-half of the pulse duration in such instances. Also, when graded action potentials appear, one must arbitrarily decide on what is to be called an action potential. For example, we would call the event after the stimulus at  $t_c = 120$  ms in Fig. 2C an action potential but not the one after the stimulus at  $t_c = 110$  ms. Also note that beyond a certain point, increase of the stimulus amplitude produces no significant change in the data; the response appears to saturate (cf. Fig. 3E with Fig. 3F).

The main features of Fig. 3 are that 1) a stimulus of a

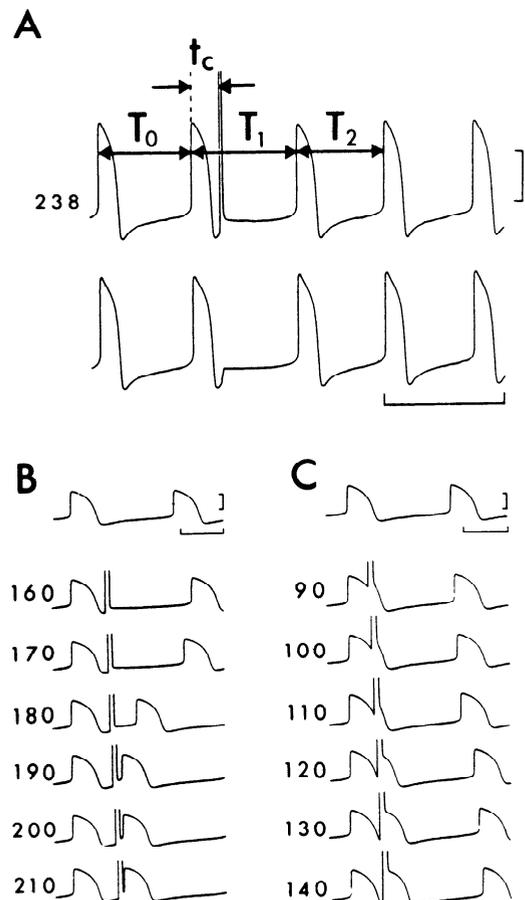


FIG. 2. Effect on the spontaneous rhythm of an aggregate produced by injecting depolarizing current pulses of 20-ms duration. A: response to a 10-nA current pulse. Perturbed cycle length ( $T_1$ ) is greater than the control cycle length ( $T_0$ ):  $T_0 = 768$  ms,  $t_c = 238$  ms,  $T_1 = 871$  ms,  $T_2 = 719$  ms. Stimulus artifact is off-scale vertical deflection in upper tracing and can be used as an approximate marker for time during which current is injected. Number to left of this and subsequent voltage traces is coupling interval ( $t_c$ ) in milliseconds. Lower tracing is from second microelectrode in same aggregate. Upstroke phase of action potentials in this and subsequent figures is slower than that shown in Fig. 1A. This artifact is due to relatively large sampling interval of the digital oscilloscope (1 ms). Horizontal calibration bar: 1 s. (Aggregate no. 27; diameter = 170  $\mu\text{m}$ .) B: phase resetting at a low current amplitude (6.5 nA). Uppermost trace shows spontaneous unperturbed activity. Coupling interval ( $t_c$ ) is incremented in 10-ms steps. Horizontal calibration bar: 200 ms. (Aggregate no. 1; diameter = 114  $\mu\text{m}$ .) C: phase resetting at a higher current amplitude (24 nA). Stimuli delivered earlier than  $\sim 100$  ms can now have an appreciable lengthening effect on action potential duration. Note also appearance of graded action potentials at, for example,  $t_c = 120$  or 130 ms. Coupling interval marking border between prolongation and abbreviation of cycle length is now at  $\sim t_c = 120$  ms. Horizontal calibration bar: 200 ms. (Aggregate no. 1; diameter = 114  $\mu\text{m}$ .)

given amplitude is capable of either delaying (i.e.,  $T_1/T_0 > 1$ ) or advancing (i.e.,  $T_1/T_0 < 1$ ) the time of occurrence of the next action potential depending on the phase in the cycle at which it is delivered; 2) the coupling interval at which the transition from delay to advance occurs moves to a smaller value as the stimulus amplitude is increased; 3) the transition from maximal prolongation (i.e.,  $T_1/T_0$  a maximum) to maximal abbreviation (i.e.,  $T_1/T_0$  a minimum) of cycle length takes place over an increasingly narrow range of the coupling interval as the stimulus amplitude is increased.

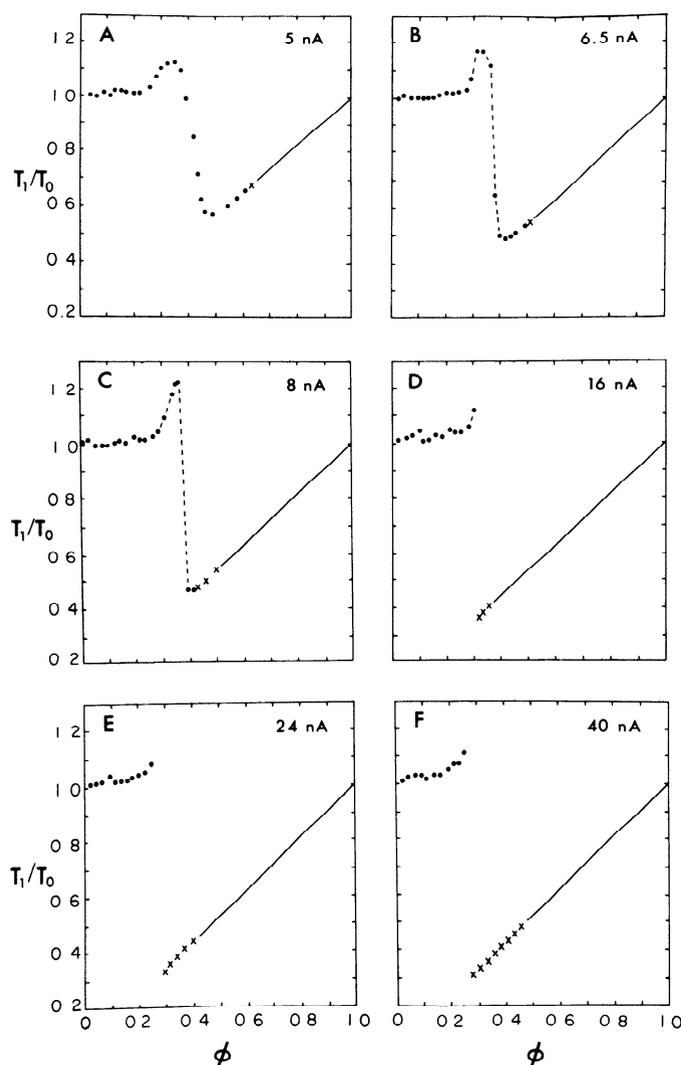


FIG. 3. Data points from phase-resetting runs carried out at 6 different stimulus amplitudes in one aggregate (same aggregate as in Fig. 2, B and C). Normalized perturbed cycle length ( $T_1/T_0$ ) is plotted vs. the normalized coupling interval ( $\phi = t_c/T_0$ ). Crosses are placed midway through stimulus artifact which obscures action potential upstroke. Solid lines are extrapolations based on results obtained in other aggregates and correspond to membrane reaching threshold during stimulus. Data points are found along dashed lines in A–C when phase-resetting run is repeated. (Aggregate no. 1: diameter = 114  $\mu\text{m}$ .)

In addition to the striking effect on the timing of the onset of the first action potential after a stimulus, there are other less marked effects on the durations of the poststimulus and subsequent cycles. A cycle that is shortened by a depolarizing stimulus falling late enough in its cycle tends to be followed by a poststimulus cycle that is longer than the control cycle, whereas a cycle that is prolonged by an early stimulus tends to be followed by a poststimulus cycle that is briefer than control (Fig. 2A). At lower stimulus amplitudes (producing responses such as those shown in Fig. 2B), for a prolongation of  $\sim 20\%$  (i.e.,  $T_1/T_0 = 1.2$ ) the poststimulus cycle is typically shortened by  $\sim 2\text{--}5\%$  (i.e.,  $T_2/T_0 \cong 0.95\text{--}0.98$ ), whereas for an abbreviation of  $\sim 50\%$  (i.e.,  $T_1/T_0 \cong 0.5$ ), the poststimulus cycle is typically lengthened to  $T_1/T_0 \cong 1.02\text{--}1.05$ .

*Discontinuity in the phase-resetting response:*

*all-or-none depolarization.* As stimulus amplitude is increased, the transition from prolongation of cycle length to abbreviation of cycle length occurs over an increasingly narrow range of the coupling interval. Indeed, in the aggregate of Fig. 3, at a stimulus amplitude of 8 nA, the transition from maximal prolongation to maximal abbreviation of cycle length occurs with a change in  $t_c$  of 10 ms. However, the transition is not discontinuous, since intermediate values of  $T_1/T_0$  were found on repeated stimulation with  $t_c$  in the transitional range of coupling intervals (i.e., with  $t_c = 160$  or 170 ms).

At a somewhat higher current than that necessary to obtain this continuous response, but at a current lower than that needed to obtain graded action potentials (Fig. 2C), the transition from maximal prolongation to abbreviation occurs much more abruptly. To probe this phenomenon in more detail, we investigated the effect of changing  $t_c$  in 1-ms increments in the range of stimulus amplitude and timing that produces the abrupt transition in another aggregate. The coupling interval at which this rapid transition occurs is always just a bit less than the action potential duration. Figure 4A shows one example, where a stimulus was delivered 11 times at a fixed coupling interval of 142 ms. Two types of responses occurred: one at a lengthened interbeat interval (3/11 trials) and

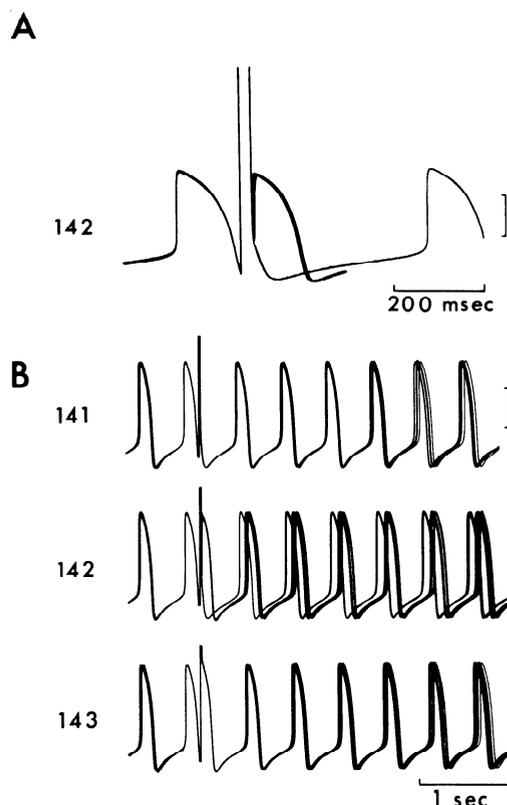


FIG. 4. A: superimposed tracings from 11 repeated trials with a 27-nA amplitude, 20-ms duration current pulse at a fixed coupling interval ( $t_c$ ) of 142 ms. Eight of these trials produced an advance in time of occurrence of next beat, whereas other 3 produced a delay. Unperturbed interbeat interval of this aggregate was  $\sim 462$  ms. Thus discontinuity in the response  $T_1$  is  $\sim 0.83$  of spontaneous cycle length. B: 5/5 trials attempted at  $t_c = 141$  ms produced only delays ( $T_1 \cong 565$  ms), whereas 7/7 at  $t_c = 143$  ms produced only advances ( $T_1 = 153$  ms). 3/11 trials at  $t_c = 142$  ms produced advances, the other 8 produced delays. (Aggregate no. 10: diameter = 149  $\mu\text{m}$ .)

the other at a shortened interbeat interval (8/11 trials). Responses with intermediate values of  $T_1/T_0$  were not observed. In addition, the variability in  $T_1$  at fixed  $t_c$  seen at slightly lower stimulus amplitudes did not occur. At  $t_c = 141$  ms only prolongation of the cycle length was observed (5 trials), whereas at  $t_c = 143$  ms only shortening was observed (7 trials) (Fig. 4B). In experiments on other aggregates in which as many as 50 trials at a fixed coupling interval were carried out, a similar dichotomy in the response was observed. This threshold-like behavior may be called all-or-none depolarization.

The abrupt transition shown in Fig. 4 was seen in all aggregates in which  $t_c$  was changed in 1-ms increments. The current amplitude at which it is seen varies from aggregate to aggregate, since the electrophysiological properties of aggregates can differ, even if they are of comparable size (9, 13). However, in all aggregates where both all-or-none depolarization and graded action potentials were seen, all-or-none depolarization occurred at a level just below that at which graded action potentials made their appearance.

**Skipped beats.** In 24 aggregates, all of which had intrinsic periods of  $<1$  s, the largest prolongation observed was one with  $T_1/T_0 = 1.41$ . However, in two other aggregates, both of which had spontaneous cycle lengths of  $>1$  s (only 3 preparations studied had such long interbeat intervals), much larger maximal prolongations could be obtained. These long delays were usually associated with subthreshold oscillatory activity in the pacemaker range of potentials (Fig. 5) and were only produced within a narrow range of coupling intervals; increase or decrease in  $t_c$  by as little as 10 ms usually destroyed the effect. The response is variable in that the long prolongation is often of a different length and might be seen at a slightly different coupling interval when the phase-resetting run is repeated keeping the stimulus amplitude and duration fixed. Indeed, there can be significant fluctuation in the response when repeated trials at a fixed coupling interval are carried out; threshold is attained at one or other of the peaks of the subthreshold oscillation (Fig. 5, A and B). The longest prolongation observed in this aggregate was  $T_1/T_0 = 5.3$  (Fig. 5B:  $t_c = 620$  ms).

Careful attempts to annihilate spontaneous activity in the aggregate using a single 20-ms duration depolarizing pulse were made by systematically varying the stimulus timing and amplitude as described above. In no case was it possible to annihilate spontaneous activity.

**Response to stimulation with a single hyperpolarizing current pulse.** The response to a hyperpolarizing pulse has also been studied in nine aggregates. We summarize the results (further details can be found in Ref. 17): 1) the response to a hyperpolarizing current pulse is the reverse of that produced by a depolarizing pulse, in that a stimulus falling early in the cycle produces an abbreviation of cycle length, whereas one falling later produces a prolongation of cycle length; 2) the transition from maximal prolongation of cycle length to maximal abbreviation of cycle length becomes more abrupt as the stimulus amplitude is increased, until it eventually becomes effectively discontinuous. An apparent discontinuity occurs at a small value of  $t_c$ , when the stimulus

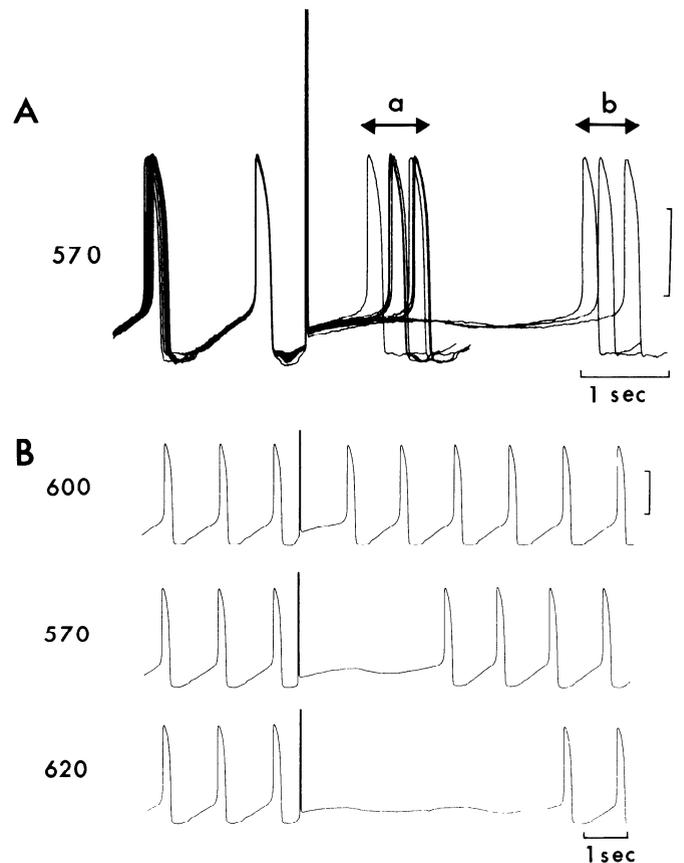


FIG. 5. A: 10 repeated trials at fixed coupling interval ( $t_c$ ) of 570 ms. Pulse amplitude was 9 nA and pulse duration 20 ms. Threshold is attained either at first crest of subthreshold oscillatory activity (group of 7 action potentials labeled a) or at the second crest (group of 3 action potentials labeled b). B: 3 trials from a phase-resetting run in which the coupling interval was changed in 10-ms steps. Pulse amplitude was 6.5 nA, pulse duration 20 ms. Action potential after delivery of stimulus fires on first ( $t_c = 600$  ms), second ( $t_c = 570$  ms), or third ( $t_c = 260$  ms) crest of subthreshold oscillation in membrane voltage. (Aggregate no. 8; diameter = 170  $\mu\text{m}$ .)

falls during the plateau of the action potential (all-or-none repolarization); and 3) a high-amplitude stimulus can produce graded action potentials, sometimes via an anodal-break effect.

**Pulse-induced rapid repetitive activity.** Rapid repetitive activity in response to a high-amplitude depolarizing current pulse was a rare phenomenon seen in only two aggregates from two different cultures. Figure 6A shows the induction of repetitive activity at a rate considerably faster than the spontaneous beat rate after injection of a single 20-ms duration current pulse. The increase in rate is mediated by an increase in the slope of diastolic depolarization, which gradually declines back down to normal. The exact form of the response varied from trial to trial carried out at fixed  $t_c$ . The effect was not dependent on the coupling interval of the stimulus, in that it could be elicited at phases scattered throughout the spontaneous cycle. It is unlikely that this effect is due to dislodging of the electrode and a subsequent speeding up of the rate due to the resultant "leakage" current, since maximum diastolic potential (MDP) remains relatively unaltered from beat to beat. In some trials, there was a slight decrease in the absolute value of MDP. The two

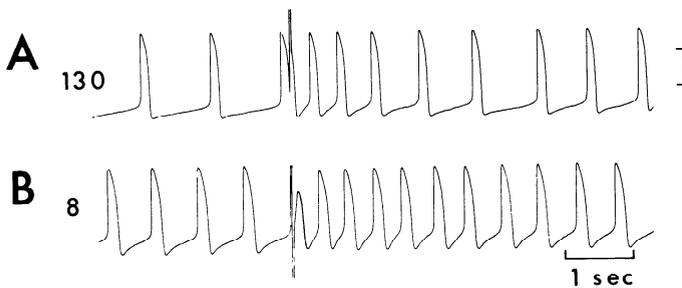


FIG. 6. Rapid repetitive activity induced by a single 20-ms duration current pulse. *A*: depolarizing pulse of amplitude greater than 100 nA, with coupling interval ( $t_c$ ) = 130 ms. Exact current is not known due to saturation of current measurement circuitry. Rapid activity was not seen at a current amplitude estimated to be about two-thirds of this amplitude; only a single extrasystole occurred. (Aggregate no. 13; diameter = 190  $\mu\text{m}$ .) *B*: hyperpolarizing pulse of amplitude 37 nA, with  $t_c$  = 8 ms. Action potential upstroke and stimulus artifact retouched. (Aggregate no. 11; diameter = 132  $\mu\text{m}$ .)

aggregates in which this phenomenon was seen came from cultures in which aggregates appeared to be contracting normally under the microscope and which had the usual high percentage of spontaneously beating aggregates. Furthermore, the action potential parameters measured from the aggregates in which the rapid repetitive activity was seen were within the usual range.

In contrast to the case of a depolarizing stimulus, rapid repetitive activity was often seen in response to a high-amplitude hyperpolarizing stimulus (e.g., Fig. 6*B*). Also, in contrast to the case of a depolarizing stimulus, the effect was strongly phase dependent, in that rapid repetitive activity could only be provoked by a stimulus falling very early in the cycle (e.g., Fig. 6*B*). In addition, there were marked decreases in the absolute values of the maximum diastolic potential and of the overshoot potential for several beats after injection of the stimulus (Fig. 6*B*).

## DISCUSSION

A large number of studies have demonstrated a biphasic response of spontaneously beating cardiac tissue to a single stimulus. This includes experiments on heart cell aggregates (8, 13, 15, 17, 19, 28, 29) and isolated cardiac tissue (14, 20–23, 27, 30) as well as clinical data from patients with parasystole (4). A similar response has been found theoretically in ionic models (2, 3, 8), in electronic models (26), and in simple two-dimensional limit-cycle oscillations (18, 28, 31).

Over an intermediate range of stimulus amplitudes, in response to a depolarizing current pulse, there is an abrupt transition from lengthening to shortening of the perturbed cycle length (Fig. 4). There are fundamental problems in ascertaining whether or not the normalized perturbed cycle length  $T_1/T_0$  is truly a discontinuous function of the normalized coupling interval  $t_c/T_0$  (16, 17). In Fig. 4*A* an abrupt transition of the cycle length can be seen without any evidence, even after many trials, of intermediate responses.

Investigation of the response of an ionic model of the aggregate to perturbation with a depolarizing current pulse suggests that, at lower stimulus amplitudes,  $T_1$  is fundamentally a continuous function of the coupling

interval  $t_c$  (8). Although the response is continuous, the transition from prolongation to abbreviation of the cycle length can be very steep. For example, in the ionic model of a 150- $\mu\text{m}$ -diameter aggregate, for a current pulse amplitude of 16.9 nA, an increment in the coupling interval of as little as 10  $\mu\text{s}$  must be used to demonstrate the underlying continuity (Ref. 8, Fig. 3*B*). At double this amplitude, even increments in  $t_c$  as small as 1  $\mu\text{s}$  are not sufficient to determine continuity (Ref. 17, Figs. 3–9): an increase in  $t_c$  of 1  $\mu\text{s}$  suffices to convert prolongation into abbreviation of cycle length. In fact, comparison of the computed voltage traces in these two instances reveals that the membrane potential at the end of the pulse is 7  $\mu\text{V}$  more positive when abbreviation rather than prolongation of cycle length is produced. Since the amplitude of membrane voltage noise in the aggregate is many times this value (12), it is not surprising that both prolongation and abbreviation of cycle length can be seen at a fixed value of  $t_c$  in the experiments (Fig. 4*A*). In fact, in a 150- $\mu\text{m}$ -diameter aggregate, the opening or closing of only six ionic channels during the current pulse may suffice to produce a change in the membrane potential of 7  $\mu\text{V}$  and thus interconvert the two responses shown in Fig. 4*A* (17). The situation in the model of the aggregate is probably similar to that which occurs in the Hodgkin-Huxley model of the quiescent giant axon of the squid, where increments in the take-off potential of  $10^{-12}$  mV must be used to demonstrate that a discontinuous all-or-none threshold phenomenon does not exist in the model (6). In that case the continuous “quasi-threshold phenomenon” of FitzHugh occurs (8). Due to the presence of membrane noise, further evaluation of all-or-none phenomena in the aggregate would necessitate the formulation of an inherently stochastic model representing a population of single channels.

Winfree (31) has collected an impressive array of examples showing that the phase-resetting behavior of many biological oscillators can be classified into two main types: type 1 phase resetting is seen at a sufficiently low stimulus strength, whereas type 0 phase resetting is seen at a sufficiently high stimulus strength. In type 1 phase resetting  $T_1/T_0$  is a continuous function of  $\phi$  (Fig. 3, *A–C*). In type 0 phase resetting  $T_1/T_0$  is a discontinuous function of  $\phi$  with a jump of  $\sim 1$  cycle length (Fig. 3, *E and F*; see Refs. 16, 17, 31 for further discussion and more precise definitions).

Apart from our study, there appears to have been only one other study in which the transition from type 1 to type 0 resetting in a cardiac oscillator was systematically investigated (29). In that study, as the stimulus amplitude was increased, there was a direct transition from type 1 to type 0 phase resetting. This is in contrast to the present findings, where we demonstrate type 1 phase resetting at low amplitudes, type 0 phase resetting at high amplitudes (Fig. 2*C*), and discontinuous, all-or-none phase resetting (i.e., neither type 1 nor type 0) at intermediate amplitudes (Fig. 4).<sup>1</sup> One possible reason for this

<sup>1</sup> The critical factor here is that after several cycles there is still a phase shift between the action potentials after prolonged and abbreviated interbeat intervals (Fig. 4*B*:  $t_c$  = 142 ms). Thus the behavior in Fig. 4 cannot be classified as either type 1 or type 0 phase resetting (16, 17). As discussed above, if  $T_1/T_0$  is a continuous, very steeply decreasing function in the neighborhood of  $t_c$  = 141–143 ms, then the phase resetting would be type 1 but there is no way for us to resolve this experimentally.

difference in the findings of the two studies, which were both carried out using embryonic chick heart-cell aggregates, is the absence or reduction of the fast inward sodium current in the aggregates studied by Van Meerwijk et al. (29). This absence is probably due to the fact that collagenase was used to disperse the cells, not trypsin (9). Modeling indicates that the presence of the fast sodium current is crucial in producing an (apparent) discontinuity (8).

A related all-or-none phenomenon can be seen during subthreshold oscillatory behavior (Fig. 5) and has also been described by Antzelevich and Moe (1) in Purkinje fiber. The grouping of the action potentials after the stimulus produces jump discontinuities when  $T_1/T_0$  is plotted vs.  $t_c/T_0$ . A simple two-dimensional model incorporating a threshold element shows similar behavior (16).

Despite strenuous attempts to do so, we have not found it possible to abolish spontaneous activity in the aggregate with a single 20-ms duration depolarizing current pulse. A similar result was found by Van Meerwijk et al. (29). On the other hand, a single subthreshold stimulus of the right size delivered within a narrow range of coupling intervals can abolish spontaneous activity in strips of kitten atrium containing sinus nodal tissue (20) and in depolarized Purkinje fiber (21). More recently Gilmour et al. (14) have shown that spontaneous activity in a piece of human ventricular myocardium can be annihilated by a critically timed stimulus.

The exact nature of the conditions necessary for allowing spontaneous activity in a cardiac oscillator to be annihilated by a simple brief stimulus are unknown. However, a necessary requirement is that the stimulus brings the state point of the system sufficiently close to a stable equilibrium point (2). Modeling work leads us to believe that in our faster-beating aggregate preparations ( $T_0 < 1$  s) there is normally only one equilibrium point (Fig. 7A), which lies in the plateau range of potentials (8). The current-voltage curve in the ionic model of the slower-beating aggregates is of the form shown in Fig. 7B, and the two equilibrium points lying at the more negative potentials are unstable (8). This means that spontaneous activity cannot be annihilated by a single brief small-amplitude depolarizing stimulus; instead, the existence of subthreshold oscillatory activity similar to that experimentally found (Fig. 5) is possible. We associate this activity with the presence of an unstable equilibrium point in a deterministic model (17). Such activity might also be seen if there existed only one equilibrium point lying in the pacemaker range of potentials (Fig. 7C). The ionic model does not include the slow inward current and therefore cannot be used to predict the stability of the equilibrium point lying in the plateau range of potentials (Fig. 7, A and B). DeHaan and DeFelice (12) observed subthreshold oscillatory activity that they propose is due to stochastic fluctuations about a stable equilibrium point, and they discussed the role of these oscillations in excitability.

Investigation of an ionic model of Purkinje fiber demonstrates that spontaneous activity cannot be annihilated by a brief stimulus, unless modifications are made to the equations (5). The results of the numerical simu-

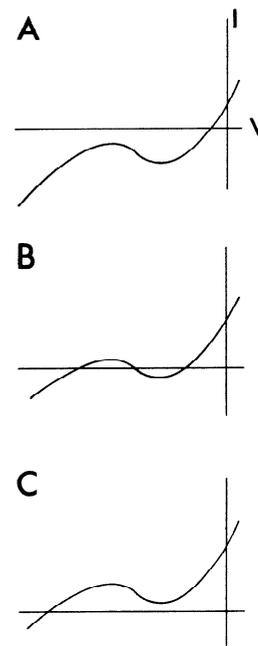


FIG. 7. Schematic steady-state current-voltage ( $IV$ ) curves for 3 different aggregates. Curves are shifted in a hyperpolarizing direction as one moves from A to C, corresponding to a decrease in beat rate. Intersection of the  $IV$  curve with the horizontal axis  $I = 0$  gives equilibrium point(s) of system. Middle equilibrium point in B is saddle point. Perturbation of state point of system into a neighborhood of equilibrium point C would lead to subthreshold oscillatory activity in pacemaker range of potentials if that point had complex eigenvalues. Same holds true for most negative equilibrium point in B.

lation are basically consistent with the experimental work on Purkinje fiber (21). It remains to be seen in the aggregate, however, whether it is possible to reproducibly convert an unstable equilibrium point into a stable equilibrium point, thus allowing annihilation of spontaneous activity with a single pulse.

Pulse-induced repetitive activity was observed in preparations that were  $<200 \mu\text{m}$  in diameter (Fig. 6). In response to a depolarizing pulse, the activity could be elicited at all phases of the cycle by a stimulus amplitude many times the threshold level: there was no vulnerable period. Similar observations have been previously made in quiescent and in spontaneously active cultured heart cells (25). We believe that rapid repetitive activity was only infrequently observed in response to a depolarizing stimulus because of saturation in the current carrying capacity of the electrodes. With a hyperpolarizing stimulus, however, much larger currents could usually be passed, and rapid repetitive activity could be routinely seen. The phase dependence of the effect of a hyperpolarizing stimulus remains unexplained. The mechanism underlying the behavior shown in Fig. 6 cannot be classical reentry, since the size of the preparation is too small to support a long-loop reentrant pathway.

In this paper we characterized the effects of single stimuli delivered at various phases of the cardiac cycle. Such data are of direct relevance in understanding the mechanism of modulated parasystole since the phase-resetting curves shown above are similar to those derived in humans from clinical data (4). These experiments also demonstrate that a single pulse can cause a transient increase in beat rate, even under conditions in which a

macroreentrant mechanism is not possible. Finally, knowledge of the phase-resetting properties of the aggregates can be used to predict the effects of periodic stimulation (15, 17, 19). In this way the origin of extremely varied, complex, and irregular rhythms observed during periodic stimulation of the aggregate and other cardiac oscillators can be understood from the phase-resetting response to a single isolated stimulus.

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