Phase Resetting Properties of Cardiac Pacemaker Cells

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ABSTRACT Aggregates of heart cells from chicken embryos beat spontaneously. We used intracellular microelectrodes to record the periodic behavior of the membrane potential that triggers the contractions. Every 5–12 beats, a short current pulse was applied at various points in the cycle to study the phase-dependent resetting of the rhythm. Pulses stronger than 2.5 nA caused the final rhythm to be reset to almost the same point in the cycle regardless of the phase at which the pulse was applied (type zero resetting). Pulses of ≤1 nA only caused a slight change of the phase. Increasing current intensities to between 1 and 2.5 nA gave rise to an increasing steepness in a small part of the phase-response curve. The observation of type zero resetting implies the existence of a critical stimulation that might annihilate the rhythm. Although we did find a phase at which more or less random responses occurred, the longest pause in the rhythm was 758 ms, 2.4 times the spontaneous interval. This suggests that the resting membrane potential was unstable, at least against the internal noise of the system. The conclusions are discussed in terms of the concepts of classical cardiac electrophysiology.

INTRODUCTION

The aim of the present study is to investigate whether the application of a short intracellular current pulse, a method that was classically used to study excitability, can also be helpful in the discrimination between different types of pacemaker cells in the heart.

Cranefield (1975, p. 264) describes "triggered activity" as the activity of "a focus that . . . will, if once arrested, remain quiescent until again triggered. In contrast, as far as we know, both normal Purkinje fibers and SA nodal fibers show true spontaneous activity; i.e., they will, if left alone and not excited, develop phase 4 depolarization and become self-exciting." In the actual discrimination between the two types, there is a problem, since "Once such a fiber has been triggered into activity, its action potentials are all but indistinguishable

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from those of a 'spontaneously' active fiber such as that of the SA node" (Cranefield, 1975, pp. 208–209).

An experimental proof of the triggered nature of a focus is that after a suitable stimulus it remains quiescent. A number of observations are available about the accidental termination of sustained rhythmic activity (e.g., Aronson and Cranefield, 1974; Cranefield and Aronson, 1974; Wit and Cranefield, 1976). Jalife and Antzelevitch (1979, 1980) demonstrated annihilation of sustained pacemaker activity in dog Purkinje fibers and strips of the sinoatrial (SA) node of the kitten by means of one single, critically timed stimulus pulse of proper magnitude and duration. They based their study on the work of Winfree (1970, 1972, 1973, 1977, 1980) on general properties of oscillators. This paper demonstrates how the excitable nature of cardiac pacemakers complicates the finding of such a suitable stimulus.

In a study on the model of Hodgkin and Huxley (1952) for the squid axon biased with constant current to exhibit triggered activity, Best (1979) computed the voltage shift one should impose on the membrane at certain points in the cycle to stop firing. His idea was to give a very short voltage clamp pulse. Because this is experimentally much more easily achieved by applying the right amount of charge with a short current pulse of proper magnitude, Rinzel and Miller (1980) repeated the calculations for current stimulation, and independently Chapman (1980) and Guttman et al. (1980) obtained the predicted experimental result: the activity disappeared.

In a recent review on the electrophysiology of the SA node, Brown (1982) states: "enough quantitative knowledge is now accumulating from voltage clamp experiments, to provide a basis for realistic models of SA node pacemaker activity." In our opinion, however, any model proposed should quantitatively predict the response to current pulses given at different points in the cycle. The Weidmann effect (depolarizing pulses early in the pacemaker cycle paradoxically delay the next discharge [Weidmann, 1951]), for example, has been used by Hauswirth as a performance test for the voltage response of the model of McAllister, Noble, and Tsien for Purkinje fibers (doctoral thesis; for reference see McAllister et al., 1975).

But apart from model discrimination, it would be very comforting to have a well-documented, negative answer to the question "can one truly stop any vital cardiac pacemaker by just one single pulse?" without reference to any specialized model. The close connection between these two aspects of cellular pacemakers is explained in the next section.

TRUE AND TRIGGERED PACEMAKERS

The type of model commonly used to describe cardiac electrical activity is a set of coupled differential equations. Generally, they are variations on a theme set by Hodgkin and Huxley (1952). They describe the membrane as an analog circuit, consisting of a capacitor in parallel with a number of nonconstant resistors with voltage-dependent kinetics, each representing the permeability of the membrane to a particular ionic species. The main discussion in the field is about the number and type of the contributing currents, the participation of active ionic

exchange mechanisms, and the form of the *I-V* relations involved. New insights lead to new terms in the original equations or to new, additional differential equations. For recent reviews, consult Bouman and Jongsma (1982), Brown (1982), and Irisawa (1978). Our purpose in this section is to stress the basic features that any model of cardiac activity should possess. They can be illustrated with the aid of a very simple set of differential equations:

$$dx/dt = -y + x\,(1 - x^2 - y^2);\; dy/dt = x + y\,(1 - x^2 - y^2),$$

or in polar coordinates:

$$dr/dt = r\,(1 - r^2);\; da/dt = 1,$$

where r is the radial distance from the origin, and a is the angle with the positive x axis. The equivalence is easily verified after differentiating

$$r^2 = x^2 + y^2$$

and

$$\tan a = y/x$$

on both sides with respect to time. On a particular radius from the origin (Fig. 1 *a*), we see that $dr/dt = 0$ for $r = 0$ or $r = 1$, while $dr/dt > 0$ for $0 < r < 1$ and $dr/dt < 0$ for $r > 1$. This means that a point with distance 1 from the origin will remain at that distance. Points further away will get nearer and points at a distance of <1 from the origin will move away from it. Because the angular velocity da/dt is constant over the whole plane, points on a certain radius stay in line as they turn counterclockwise around the origin, like ants on the arms of a windmill.

Thus, when we let the movement take place, we see all radii moving with the same constant speed. At the same time, along each particular radius the points creep toward a limit point, say L, at a distance 1 from the origin on that radius. Over time, all points L on the different radii mill around a circle, forming periodic solutions of the differential equation. All the other points spiral towards this circle (Fig. 1 *b*).

Because this closed curve attracts neighboring points, we call it an attracting limit cycle. The set of points that tends ultimately to coincide with a particular point L in its movement along the limit cycle is called the isochron of L; in this case it is the straight radius through L. The only point that does not approach the limit cycle is the one initially at the origin. In fact, it does not move at all. Because it does not move toward the limit cycle, we say it is not in the cycle's basin (or domain) of attraction. Because it does not move at all, we call it a fixed point or rest point of the differential equation. Because all neighboring points leave it, we call it unstable. If a real-life version of this system happens to be in a state corresponding to the origin, it will be excited toward periodic activity by the slightest noise perturbing it. A pacemaker cell with this structure—one limit cycle attracting almost every point in space, and all rest points unstable—will be called a true pacemaker, or truly automatic.

A slightly more complicated picture arises if we consider

$$dr/dt = r\,(1 - r^2)\,(r^2 - 1/4);\ da/dt = 1.$$

Now we have two cycles, one extra for $r^2 = 1/4$. For $r^2 < 1/4$ we have $dr/dt < 0$ and for $1/4 < r^2 < 1$ we have $dr/dt > 0$. So all points inside the smaller circle will now move toward the origin and only the points outside it will spiral toward the (outer) limit cycle as before (Fig. 1 *c*). Hence the origin is now a stable rest

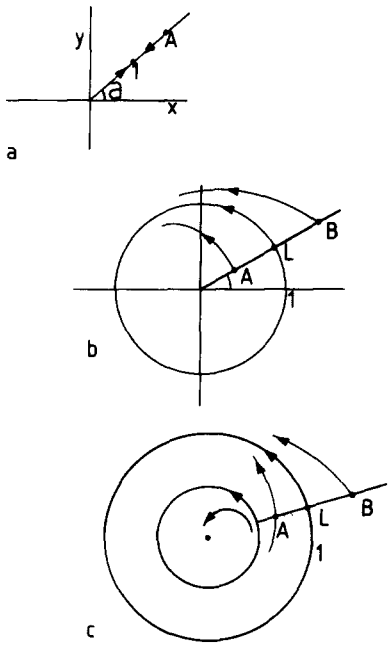

FIGURE 1. (*a*) The position of a typical point *A* can be given either by its Cartesian coordinates (*x*, *y*), or in polar coordinates (*r*, *a*), with *r* the distance to the origin and *a* the angle with the positive *x* axis. See Introduction for further explanation. (*b*) In time, the points *A* and *B* will spiral toward the limit cycle and will ultimately coincide with the point *L* on the same radius. (*c*) Two periodic solutions: the outer one is approached by all points outside the inner circle. The origin "attracts" all points inside the smaller circle. The set of points that ultimately coincides with *L* in its motion around the circle is that part of the radius through *L*, which lies outside the smaller circle.

point and the circle with $r^2 = 1/4$ is an unstable cycle, forming the boundary between the basins of attraction of the origin and the limit cycle with $r^2 = 1$, respectively. Also, the isochrons stop at this boundary. A pacemaker with such a structure will be called a triggered pacemaker. It can either be quiescent or it can, if swept out of the inner circle, show endless, repetitive, stable activity.

Now, if we can model the pacemaking activity of a cardiac cell by a system of differential equations with a stable periodic solution, i.e., one we will be able to observe, then at least two types of pacemaker cells should be distinguished. A

pacemaker that can, if left alone, exhibit only one mode of periodic behavior is truly automatic. If, besides this, other (quiescent) stable modes exist, one would speak of triggered activity. For a more thorough discussion of the mathematics involved, see Winfree (1980) and the references given therein, Guckenheimer (1975), Hirsch and Smale (1974), and Kawato (1981). Discussions of the more complicated properties of spatially distributed pacemakers that exist in the heart can be found in Bouman and Jongsma (1982), Cranefield (1975, 1977), or Wit and Cranefield (1976, 1977).

EXPERIMENTAL DISCRIMINATION BETWEEN PACEMAKER TYPES

In principle, it is possible to identify the nature of a pacemaker experimentally either with current or voltage clamp pulses. We used current pulses and will explain the theoretical basis given by Winfree (1980) for that case. Consider the net current through the membrane:

$$I_{\text{membrane}} = CdV/dt + \text{sum ionic currents},$$

where V is the membrane potential and C is the membrane capacity.

When no stimulus is applied, I_{membrane} is zero; hence:

$$-Cdv/dt = \text{sum ionic currents}.$$

This differential equation for the membrane potential gets an extra term during stimulation of a pacemaker cell with depolarizing current. Hence, the directions in which the points tend to move are changed. This means that a point initially on the limit cycle will no longer follow it. As long as the stimulus lasts, the point will move in a different direction (Fig. 2*a*). After we stop the stimulus, the previous laws of motion are restored. Now our stimulated point will either be outside the basin of attraction of the limit cycle or it will be inside the basin and will then lie on a particular isochron.

In the first case, we will observe how the resulting state is influenced by noise. We will demonstrate the triggered nature of its former activity if the system remains quiescent. However, if the noise is large enough to push the system out of a relatively small stable set, we have an identification problem, which might be settled by looking at the statistical properties of the responses in repeated trials.

In the second case, we need to do more. If we start the same stimulus (current pulse) at a slightly different point in the cycle, the system will be in the neighborhood of the first result at the end of the stimulus. If we scan the whole cycle in this way, this continuity will cause the resulting points to lie on a closed curve, say R in Fig. 2*b*. If rhythmic activity always returns, the whole of R lies in the basin of the limit cycle. Now two things can happen. Either R intersects all isochrons or it does not.[1] The last event is important. It means that the endpoint of some isochrons is not inside R. Because isochrons end at the border of the

[1] We restrict the discussion to the simplest possible case. To keep the treatment general, the distinction should be: "R intersects all isochrons an odd number of times or it does not," with some modifications of the part of the discussion hereafter (see Winfree, 1980).

limit cycle's basin of attraction, some points must have crossed that boundary during the stimulus. A shorter stimulus would have left that point in the other region.

A slightly different reasoning proves Winfree's assertion that a weaker stimulus could do the same with perhaps a different point in the cycle. If we scan a

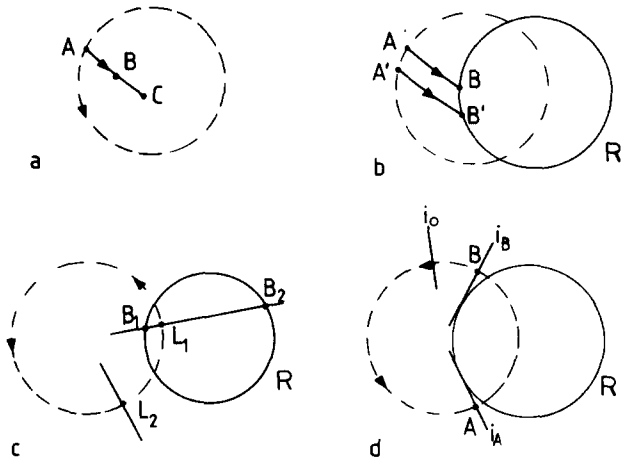

FIGURE 2. (*a*) Schematic picture of the events during stimulation. The dashed circle is the limit cycle under unperturbed conditions. When a stimulus starts, a different rule governs the motion of points. If the system is in state *A*, for example, at the beginning of stimulation, the stimulus will cause it to deviate from the limit cycle it normally follows. The strength of the stimulus determines the direction. One stimulus duration will bring it, for example, into *B*; the same strength and a longer duration could bring it into *C*. (*b*) The dashed curve is the spontaneous limit cycle. A stimulus of fixed strength and duration carries point *A* to *B* and a neighboring point *A'* to *B'*. The whole spontaneous cycle is mapped to a closed curve of points, *R*, drawn with a continuous line. (*c*) After termination of the stimulus, the perturbed points will again conform to the free run differential equation. If they are in the domain of attraction of the limit cycle, they will return to it. The isochrons on which they are landed determine the points on the limit cycle with which they will ultimately coincide. For example, points B_1 and B_2 will finally merge with L_1. In this example, no point will finally approach L_2 because its isochron does not have a point in common with *R*. (*d*) The two isochrons just tangent to *R* are i_A and i_B. After a sufficient number of spontaneous revolutions, all stimulated points will be near the part *A*–*B* of the spontaneous cycle. The latencies with which they follow an unperturbed control vary by the amount of time to travel from *A* to *B*, which is only a fraction of the whole period.

pacemaker cycle with a certain stimulus and observe which isochrons our points remain on after the stimulus, we can conclude whether our stimulus is strong enough to test the stability of our limit cycle. We simply have to ascertain whether only a limited number of isochrons are intersected.

In practice, this event is detected without knowledge of the form of the isochrons. Consider Fig. 2*d*. When we wait until a sufficient number of sponta-

neous cycles have occurred after the end of the stimulus, all stimulated points will have reached the part of the limit cycle between *A* and *B* along their respective isochrons. Thus, the responses will show a range of phase differences corresponding to the time to travel from *A* to *B*, i.e., less than one spontaneous interval.

METHODS

Aggregates of heart cells from embryonic chicken were formed using the method of DeHaan (1967) with slight modifications. Hearts were removed from 7-d-old chicken embryos as aseptically as possible, and after trimming of vessels they were cut into small cubes. The pieces were dissociated with collagenase (type 1; Worthington Biochemical Corp., Freehold, NJ) in Puck's saline A (Marcus et al., 1956) for 15 min. Then the suspension was mechanically agitated by passing it through a large-bore pipette and was again dissociated for 15 min. The resulting cell suspensions were cooled, centrifuged at 200 *g* for 10 min, washed in growth medium, and finally resuspended in growth medium. A 25-ml Erlenmeyer flask with 3 ml of suspension containing 1.5×10^6 cells was placed on a gyratory shaker at 37°C for 24–48 h. The resulting aggregates ranged from 50 to 350 μm in diameter. The dissociating medium consisted of saline A (pH 7.2–7.4) to which was added 450 U/ml of collagenase (CLS fraction type I; Worthington Biochemical Corp.), 0.001% DNAase (DP fraction 1,400 U/g; Worthington Biochemical Corp.), 10 μM $CaCl_2$, and 60 μM $MgCl_2$. The growth medium consisted of 5 ml serum (1:1 horse and bovine serum), 20 ml Medium 199, 10 ml chicken embryo extract (Flow Laboratories, Inc., McLean, VA), and 65 ml Dulbecco's salt solution. The ionic composition of the growth medium (in mM) was 116.4 NaCl, 0.8 $MgSO_4$, 1.03 NaH_2PO, 1.8 $CaCl_2$, 25.1 $NaHCO_3$, and 2.5 KCl. No antibiotics were added.

Before experimentation, the aggregates were poured onto a plastic petri dish and incubated in a pH- and humidity-controlled incubator. During the experiments the dishes were placed on the heated stage of an inverted microscope. A thin layer of mineral oil (Klearol) was layered on top of the medium to prevent evaporation. A continuous flow of heated air/5% CO_2 mixture was passed over the surface to keep the pH at 7.2–7.4. Temperature was kept constant between 36 and 36.5°C.

Transmembrane potentials were measured with conventional intracellular microelectrodes with tip resistances of 10–40 MΩ. Current pulses of 20–200 ms duration and amplitudes between 0.1 and 30 nA were applied through the recording electrode by means of a balanced bridge circuit. In order to obtain a reliable detection of the beginning of the action potential, the signal was low-pass-filtered, usually 2 or 4 kHz, and electronically differentiated to avoid the effects of improper balance of the bridge circuit. The remaining artifacts in the derived signal consisted of on and off transients of the current pulse. These were adequately suppressed by switching the derived signal to ground during these transients with an electronic switch. A switching time of ~5 ms was usually sufficient. The remaining signal was free of artifacts of the stimulus and was fed into a level trigger.

Pulses were given with 5–12 action potentials in between, to ensure that the aggregate was in steady state at the application of each pulse. Both the intervals between successive pulses and the intervals between successive action potentials were measured on-line by a PDP 11/10 computer (Digital Equipment Corp., Marlboro, MA) using a Laboratory Peripheral System (LPS 11; Digital Equipment Corp.) with 1 ms accuracy.

After a series of pulses had been given, the intervals could be displayed in the format of Fig. 3, or as an "oscilloscope" display like that in Fig. 4, to judge the regularity of the spontaneous interval and the resolution of the "scope" display. The data could then be

stored on a disk for later processing. If necessary, an extra series of pulses could be given at critical phases to obtain an unambiguous plot.

RESULTS

To be of any use as a model of a cardiac pacemaker, an aggregate of embryonic heart cells should be capable of generating a constant spontaneous rhythm with not too many irregular beats. Although the whole embryonic heart was used in the culturing of the aggregates, most of the batches consisted of spontaneously and regularly active aggregates. When irregular aggregates were found, it appeared that the whole batch, cultured from 7–10 eggs, was irregular. In successful experiments stable, uninterrupted penetrations lasting up to 5 h were possible, during which time the basic interval of the aggregate did not vary more than 10% (range). In some experiments the aggregates were incubated overnight with 1 μM cytochalasin B to reduce the contractions in the hope of obtaining a

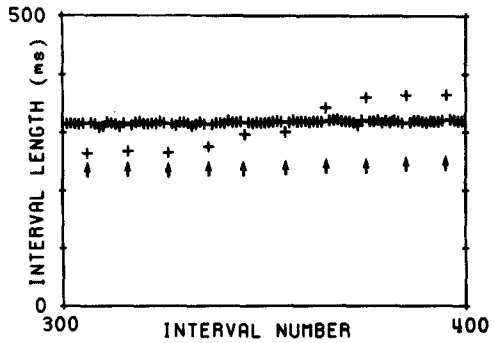

FIGURE 3. A typical sequence of experimental intervals. The length of the interval between two subsequent action potentials is plotted vertically against the interval number. After every 10 action potentials a depolarizing current pulse was given of 50 ms duration and 1 nA. Arrows mark the stimulated intervals.

more stable penetration. In this concentration cytochalasin B does not interfere with the electric phenomena in pacemaking (Sachs et al., 1974).

Fig. 3 shows the sequence of intervals in a typical experiment. In this case, after every 10 action potentials a depolarizing pulse of 50 ms, 1 nA was given at various points in the cycle. Clearly, the main effect was to advance or delay the following action potential with only a minor influence on subsequent intervals.

To obtain a systematic impression of the effects of the pulse, depending upon the point in the cycle, or phase, where it started, the data of Fig. 3 are plotted in Fig. 4. The horizontal axis represents time, like the time basis of an oscilloscope. Each symbol in a horizontal row marks the occurrence of an action potential, the sweep being triggered on the last action potential before a stimulus was given. Along the vertical axis, sweeps are positioned according to the stage in the cycle (phase) at which the pulse was given. For a pulse starting just after the triggering action potential, the times of subsequent action potentials are plotted on the lowest row; for a pulse applied 100 ms after the triggering spike,

the responses are plotted at height 100 ms, etc. As an example, in Fig. 4 the membrane potential trace in which a pulse was given at 160 ms is redrawn at the corresponding height. For identification of the stages in the cycle, the time course of the intracellular membrane potential is drawn along the vertical axis.

It can be seen that pulses occurring during the plateau phase of the action potential did not change the interval. When the pulse was given at progressively later phases after the triggering action potential, the disturbed cycle was first prolonged to a maximum of 366 ms, elicited by a pulse given at 141 ms. Again increasing the phase of the pulse caused the delay to diminish gradually, until the effect of the pulse on the cycle length was nil at ~160 ms (see the voltage trace in Fig. 4). By positioning the pulse at still later stages in the cycle, the disturbed interval became shorter than normal, until a maximal shortening to

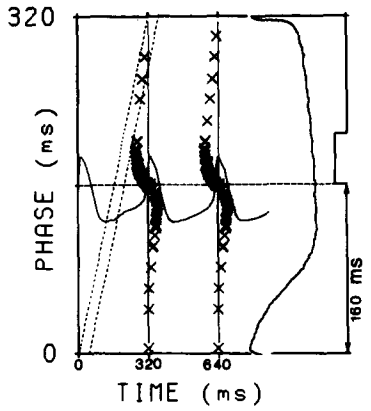

FIGURE 4. Response to a stimulus of 1 nA, 50 ms. Each symbol represents one action potential. Vertical axis: milliseconds after triggering action potential at which the pulse started. Horizontal axis: time of occurrence of action potentials after the stimulus. The two vertical lines are drawn at intervals equal to the spontaneous period, 320 ms. The two dashed lines mark the beginning and end of the stimulus. The symbols on the dashed horizontal line correspond to the drawn membrane potential in which a pulse was applied at 160 ms after the first action potential in the trace. See text for further explanation.

264 ms was reached at a phase of 192 ms. Thus, the action potential occurred at a latency of 72 ms, i.e., 22 ms after the termination of the pulse. So in these stages the pulse was still subthreshold in the classical sense, i.e., it did not cause a sustained depolarization resulting in an action potential. Pulses in later phases did not advance the action potential that much. At still later stages, the action potentials started during the pulse, as can be seen in Fig. 4: the two dashed parallel lines, 50 ms apart, mark the beginning and the end of the stimulus. Here, first an action potential was missed by the triggering circuitry because it followed the end of the pulse within the closed period of the electronic switch, at phase 221 ms, and in the uppermost row, at phase 302 ms near the end of the cycle, because it came shortly after the onset of the pulse.

Clearly, at every point in the cycle the response was unambiguous. Also,

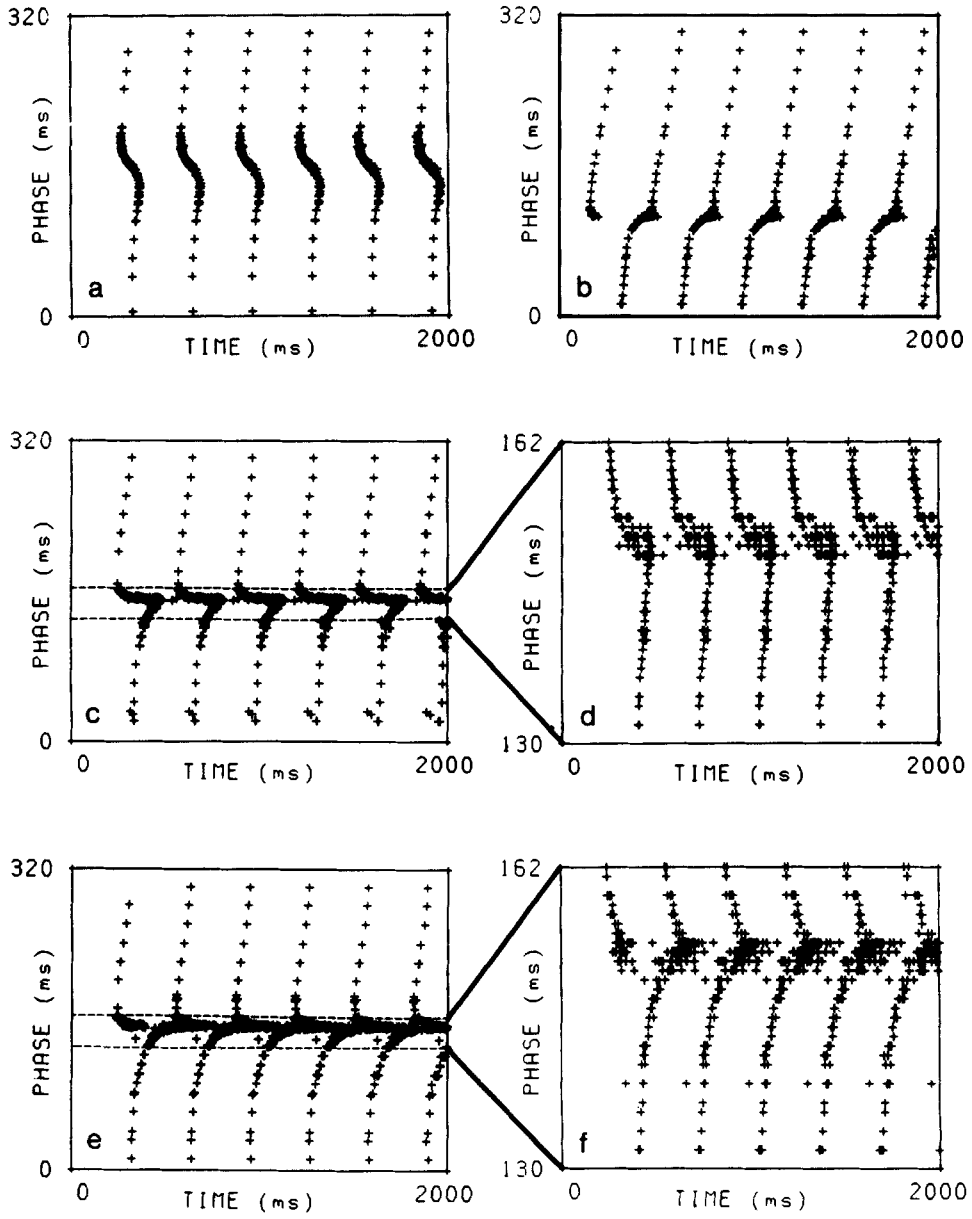

FIGURE 5. (*a*) Same data as in Fig. 4, but illustrating the long-lasting effect of the phase shift. Pulse of 1 nA, 50 ms. 82 pulses. (*b*) Response to pulses of 6 nA. 105 pulses. (*c*) Response to pulses of 1.5 nA. 146 pulses. (*d*) Same data as in *c*. Phases are between 130 and 162 ms in the enlarged view. (*e*) Response to pulses of 1.7 nA. 156 pulses. (*f*) Same data as in *e*. Phases are between 130 and 162 ms in the enlarged view. There were 10 action potentials between subsequent pulses. All responses are from one penetration. For further explanation, see text.

because the second column is a nearly identical copy of the first, the pacemaker seems to be back near its limit cycle almost completely within the first beat.

The main observation shown in Fig. 4 is the gradual change of the response as the stimulus scans the cycle. In Fig. 5*a*, the data from Figs. 3 and 4 are plotted again, showing that the phase shifts remained very clearly recognizable for a long time interval. Winfree (1980) christened this type of response "type 1 resetting," because in a different plot (time from stimulus to actual next beat against expected time of next beat [see Winfree in Bouman and Jongsma, 1982]), the measured responses would lie on parallel curves, each rising with an average slope of one.

A completely different picture was observed for a much higher stimulus intensity. Fig. 5*b* (upper right panel) shows the "oscilloscope" plot for a pulse of 6 nA and 50 ms duration. Two differences between Figs. 5*a* and *b* should be noted. First, the delaying effects in the beginning of the cycle appear earlier and are stronger; second, at approximately one-third of the cycle, an "all or nothing" phenomenon occurred, as can be seen from the abrupt end to the first column of plus signs in Fig. 5*b*. In this phase, pulses brought the membrane near threshold. As a result, the pacemaker either generated an action potential, after which it returned to maximal diastolic potential, continuing with a more or less normal diastolic depolarization, or it failed to reach threshold but used about the duration of an action potential to return to maximal diastolic potential, continuing as if it had fired (Fig. 6*a*). As can be seen in Fig. 6*a*, the whole process starts after the termination of the stimulus. This particular behavior was only observed in a very small part of the cycle, at phases between 103 and 107 ms after the triggering action potential. In all other stages the responses were strikingly constant: Fig. 6*b* shows 20 superimposed responses to the same pulse at 97 ms.

For a stronger pulse, 8 nA, the threshold was earlier in the cycle, at phase 90 ms (Fig. 6*c*). Also, it seemed that the scatter in occurrence of action potentials after this pulse was smaller than with a pulse of 6 nA. In both cases, the responses were graded if judged according to the membrane potential immediately after the stimulus. The position of the discontinuity in Fig. 5*b* was apparently determined by the way our triggering circuitry "defined" the difference between an action potential and a "passive response." However, the timing of subsequent action potentials was clearly all or nothing: in 27 trials with 6-nA pulses and 39 trials with 8-nA pulses in the critical range of phases, there was a distinct zone in which no action potentials fell. In four additional aggregates we found the same result, with comparable numbers of trials per stimulus strength. Current strengths ranged from 4 to 30 nA. The position of the threshold in the cycle varied not only with the stimulus strength but also with the cycle length. For example, an aggregate with spontaneous interval 265 ms had a threshold between 205 and 210 ms for a pulse of 6 nA, 50 ms (its diameter was 150 μm).

A pulse with little or no effect would cause all action potentials to occur along more or less vertical lines, regardless of which phase the pulse was given at, as in Fig. 5*a*. However, if at every phase a pulse elicited an action potential, the rhythm would look as if it were started anew by the pulse: perturbation to

(approximately) the same isochron is equivalent with all action potentials appearing at a fixed time (plus an integer number of cycles) after the pulse (cf. Figs. 5*b* and 4). In Fig. 5*b* the responses clearly do not ascend along a vertical line, but increase one period to the right, as the stimulus runs through the cycle. Hence, we conclude that for these pulse strengths, the response is of a different type compared with pulses of 1 nA. Again, in Winfree's terminology, if plotted as

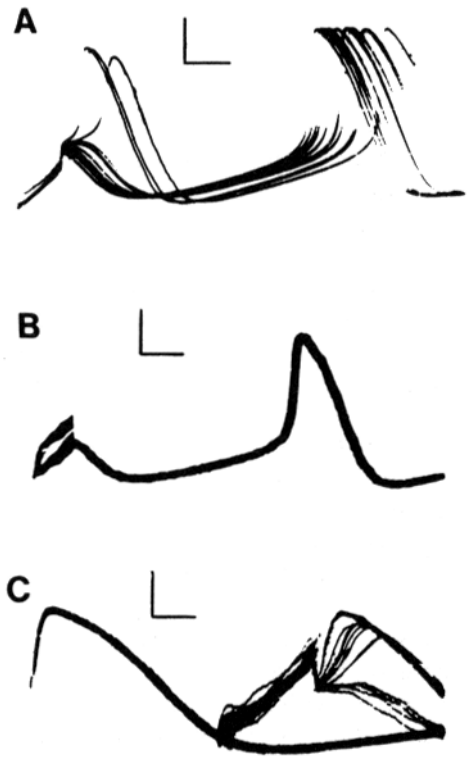

FIGURE 6. (*A*) All or nothing response to a pulse of 6 nA, 50 ms, applied 105 ms after preceding action potential. (*B*) 20 superimposed tracings of the response to the same pulse as in *a*, but at 97 ms after the preceding action potential. (*C*) Threshold behavior in response to a pulse of 8 nA, 50 ms, applied 90 ms after the preceding action potential. All pulses were with depolarizing current. Calibrations: (*A*, *B*) 20 mV, 50 ms; (*C*) 20 mV, 25 ms.

time from stimulus to actual next beat against time to expected next beat, the data would lie along horizontal curves, hence his term "type zero resetting."

The basic conclusion is that this stimulus has carried all parts of the limit cycle to only a limited number of isochrons. Hence, as explained in the Introduction, both a smaller and a shorter pulse can be found, either of which is able to carry at least one point of the limit cycle into the singular point to assess its stability.

Figs. 5*c* and *e* give the responses to stimuli of 1.5 and 1.7 nA, respectively. For both these current strengths, the transition from delay to advance took place

in a very short range of phases, ~5 ms wide. The large scatter in this part of the phase-response curve is consistent with its steepness. A slight variation in timing of the pulse gave rise to a large change in the response. An enlarged portion of the plots is shown in the two corresponding panels on the right, *d* and *f*, respectively. (Our triggering and recording circuitry allowed for a resolution in discrete steps of 1 ms.) Although here the scatter is greater than in the upper two plots, the action potentials still seem to cluster around more or less preferred places. Five intervals were longer than 700 ms, and the longest was 758 ms, corresponding to a delay of 604 ms; all were caused by a pulse of 1.7 nA at phases between 150 and 160 ms. A test against random distribution around the circle (Rayleigh's test for a circular average; see Batschelet, 1981, or Mardia, 1972) always gave a significant response for the average of repeated trials at a particular phase. Because at every point the response was unambiguous, again the plots are of different types: type one for 1.5 nA, type zero for 1.7 nA.

Fig. 7 shows the grand total of all responses of the same penetration, in the same aggregate as used in Figs. 3–6; it is a three-dimensional plot, with a helicoidal surface in a plot of time (vertical axis) against phase and current intensity. Although some scatter is introduced through slight variations in spontaneous period, all pulse strengths greater than 1.7 nA did cause a type zero resetting. In other experiments we observed type zero resetting for pulses from 4 to 30 nA onward for comparable sizes of aggregate and pulse durations.

DISCUSSION

The purpose of this study was to gain insight into the relation between the excitability of a cardiac pacemaker and its phase resetting properties. We now give a possible interpretation of our results.

In the classical electrophysiology of resting nerve and muscle fibers, a suprathreshold stimulus is one that gives rise to an action potential. In a spontaneously beating preparation, even a stimulus of zero intensity is always suprathreshold when applied for (at most) one spontaneous interval. If you think of a threshold as something you cannot cross without external help, then an unperturbed pacemaker does not meet a threshold in its spontaneous cycle. A possible way out of this semantic puzzle can be found with the aid of the theoretical work of FitzHugh (1961). However, see also Game (1982). FitzHugh illustrates the general form of his argument with a two-variable model, to which it is in no way restricted. In his example, the pacemaker cell is simplified to the interaction of only two variables, the membrane potential and something like the potassium conductance. This permits a graphic analysis.

To describe then the qualitative behavior of the experimental results, an extra ingredient is needed in the phase portrait of the differential equation besides a limit cycle and a (possibly stable) rest point. FitzHugh argues that a threshold could be thought of as a boundary in the state plane, separating the region of passive responses from the regenerative region. In two dimensions, this role could be played by a single trajectory, which is locally unstable (see Fig. 8): points besides the part of the trajectory from *A* to *B* move rather sharply away from it to either the excited (action potential) part of the cycle, or to the diastolic depolarization. During stimulation, the trajectories are changed, so some point

in the diastolic depolarization can be brought to the separatrix *AB* if the stimulus is strong enough and lasts for a sufficiently long time. For example, suppose a pulse of 4 nA 50 ms is such that the point *C* is transported to *C'*. Because after stimulation the point *C'* finds itself on the trajectory *AB*, it faces a very unpredictable future. The slightest amount of noise will put it after a random time interval to either a trajectory on the left of *AB* or on the right, so it will either

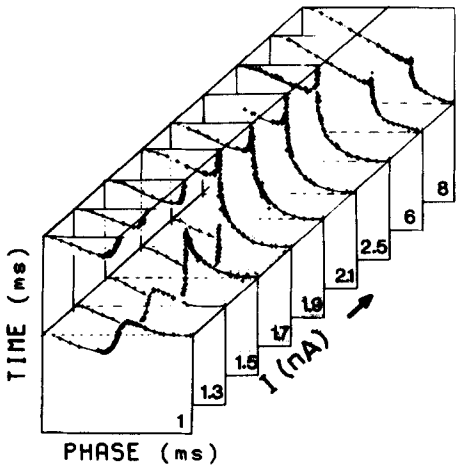

FIGURE 7. Three-dimensional view of the first part of the responses for different stimulus strengths. All results are from one penetration, the same as for Figs. 4–6. The data lie on a helicoidal surface. The phase advance region in the left of each panel has about the same form in the first row of action potentials, which is partially behind the surface, as in the second (upper left row), which is shown. To help in depicting a three-dimensional helix, action potentials behind the surface are masked. To present a look to the inside, the upper right part has been cut away. Connecting lines between the measured ponts are drawn by hand. The graph shows the responses to 972 pulses, with 10 action potentials between successive pulses. A small vehicle, riding through the valley of maximally advanced first action potentials from high to low stimulus intensities, could reach smoothly the front panel of 1 nA from a stimulus of 8 nA in the back of the figure. In the front panel a smooth transition to the right, the delay region, is possible. But from here, along the edge of the figure, one can travel smoothly back to higher stimulus intensities, where, at 8 nA for example, a smooth hill of action potentials can be climbed. Looking downward, the driver would see, one period deeper, the action potentials at which he started. Horizontal plane: phase and stimulus strength; vertical axis: time of occurrence of action potentials.

repolarize or generate an action potential. A subthreshold stimulus would be one that does not bring any point from the spontaneous cycle to the unstable part of the separatrix, whereas a suprathreshold one would pose the dilemma for at least one point of the spontaneous cycle. We used only depolarizing stimuli and always found one threshold phenomenon in the case of type zero resetting and no threshold in the case of type one. This would be consistent with the idea that separatrix leaves an unstable rest point monotonically, rather than in an

oscillatory way. Further experiments would be necessary to clarify this point. Because of the noise and the fact that the isochrons near the boundary of the domain of attraction of the limit cycle are close together (Guckenheimer 1975), a statistical treatment will be inevitable.

At the moment we are not able to improve the experimental resolution reliably, because preferably both stimulus intensity and duration should be reproducibly varied and measured digitally. Therefore, the question of whether a stable singular point (stable resting potential) exists, which would imply that the activity was indeed triggered, is, strictly speaking, not answered completely. However, the window through which it should have been reached with a pulse of 50 ms is restricted to within a range of 5 ms (<2% of the cycle) and current strengths between 1.5 and 1.7 nA for the aggregate we described in detail above.

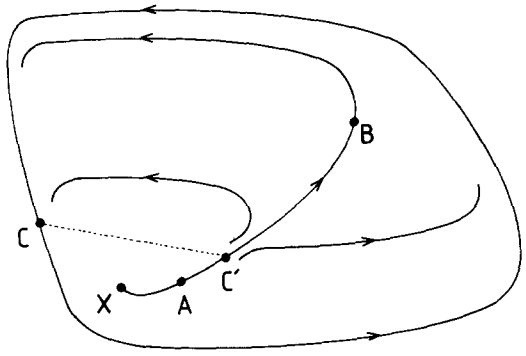

FIGURE 8. Fitzhugh's state plane, redrawn. Horizontal variable: membrane potential, vertical potassium conductance. *C* is on the stable limit cycle. It is perturbed to *C'* on the separatrix, a trajectory that is unstable between *A* and *B*, to model the all or nothing response. The purpose of the study was to determine whether the set *X* consisted of one single unstable rest point, in case of a true pacemaker, or consisted of a stable rest point surrounded by a certain domain of attraction within an unstable cycle, the simplest possible portrait of a triggered pacemaker. It depends on the relative positions and forms of the post-stimulus locus and the separatrix, and whether intersection of both is equivalent with type zero resetting. See Discussion for further explanations.

The crucial observation of type zero resetting in heart tissue has been reported earlier (e.g., Jalife, et al., 1980; Jalife and Moe, 1976; Jalife and Antzelevitch, 1979, 1980), but has not always been recognized as such. Winfree (1980, pp. 111, 172, 318) discusses the interpretation of reports of type zero resetting in excitable tissues.

Several writers (e.g., Wit and Cranefield, 1976; Jalife and Antzelevitch, 1979; Chapman, 1980; Guttman et al., 1980) have demonstrated the triggered nature of different pacemakers by the annihilation of activity with one single stimulus. It is well known that a constant bias current or treatment with drugs can modify or even stop pacemaker activity (see, for example, Cranefield, 1975; Jalife and Antzelevitch, 1980). The present study shows that it can be rather difficult to stop pacemaker activity without exerting a continuous influence.

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