Evolution of Rhythms During Periodic Stimulation of Embryonic Chick Heart Cell Aggregates

Zeng Wanzhen, Leon Glass, and Alvin Shrier

During periodic stimulation of spontaneously beating chick heart cell aggregates, there is often an evolution of coupling patterns between the stimulator and the aggregate action potential. For example, at rapid stimulation frequencies, a rhythm that is initially 1:1 (stimulus frequency: aggregate frequency) can evolve to other rhythms such as 5:4 and 4:3. Time-dependent effects generated during periodic stimulation are characterized by three types of experiments to determine 1) the effect of periodic stimulation on the intrinsic cardiac beat rate (overdrive suppression), 2) the effect of periodic stimulation on the phase resetting properties of the aggregate, and 3) the time-dependent changes in the coupling patterns between the stimulator and the aggregate during periodic stimulation. The protocols involved variations in the duration and rate of periodic stimulation. A mathematical model is developed in the form of a two-dimensional finite difference equation based on the data from experiments 1 and 2. The model is used to predict the data generated by experiment 3. There is good correspondence with the experiments in that the theory reproduces complex transitions between various rhythms and displays irregular rhythms similar to those observed experimentally. These results have implications for the evolution of cardiac arrhythmias such as atrioventricular heart block and modulated parasystole. (Circulation Research 1991;69:1022–1033)

The sinoatrial node is the dominant pacemaker in mammalian hearts. In normal circumstances, all subsidiary pacemakers in the heart are entrained in a 1:1 fashion to the basic sinus rhythm. However, if the sinus rhythm is abolished, it is usual to observe the emergence of a subsidiary pacemaker after a period of quiescence. This suggests that the intrinsic rhythmicity of subsidiary pacemakers is normally suppressed. This inhibition of the intrinsic rhythmicity of spontaneous pacemakers is now called overdrive suppression.¹

Overdrive suppression plays an important role in normal mammalian hearts and in the pathophysiology of mammalian hearts. Many experimental findings support the interpretations that the atrioventricular node is a spontaneous pacemaker that is overdrive-suppressed by the normal sinus rhythm.² Overdrive suppression of the sinus node is a standard clinical test for the evaluation of sinus node function (the sinus node recovery test).³ ⁴ Finally, since parasystole is an arrhythmia in which the rhythm of an ectopic pacemaker can be modulated and entrained by the normal rhythm,⁵ ⁶ overdrive suppression may be important in interpreting complex parasystolic rhythms.

To date, there have been no attempts to develop quantitative theoretical models of overdrive suppression or to analyze the consequences of overdrive suppression on the evolution of complex rhythms in the heart. To carry out such a study, we consider a model system consisting of spontaneously beating aggregates of embryonic chick heart cells. Previous work has demonstrated overdrive suppression in this system⁷ and has documented complex evolution of rhythms during periodic stimulation,⁸–¹² but systematic studies have not been carried out. In the present study, we undertake a quantitative characterization of overdrive suppression in spontaneously beating embryonic heart cell aggregates and study the consequences of overdrive suppression on the phase-resetting properties of the aggregates and on the response of the aggregates to periodic stimulation of different

¹ From the Department of Physiology, McGill University, Montreal, Quebec, Canada.
² Previously presented by Z.W. in the Young Investigator Competition at the 11th Annual Scientific Section of the North American Society of Pacing and Electrophysiology in San Diego, Calif., June 1990.
³ Supported by grants from the Canadian Heart and Stroke Foundation, the Medical Research Council of Canada, and the Natural Sciences Engineering and Research Council of Canada.
⁴ Address for correspondence: Zeng Wanzhen, Department of Physiology, 3655 Drummond Street, McGill University, Montreal, Canada H3G 1Y6.
⁵ Received March 12, 1991; accepted May 15, 1991.
frequencies. We develop a simple quantitative model of the effects of stimulation history on the intrinsic rhythmicity of the aggregate that shows correspondence with a large body of experimental data.

Materials and Methods

Tissue Culture

Aggregates were prepared following the techniques of DeHaan and Fozzard as previously described. White Leghorn chick embryos were incubated for 7 days at a temperature of 37°C and a relative humidity of 85%. The embryos were decapitated, and the hearts were excised. The atria and apical portions of the ventricles were isolated separately, fragmented, and dissociated into single cells. Dissociation was carried out by a multiple cycle procedure in an enzyme-containing solution. The cell suspension was filtered through a membrane with a 12.0-μm-diameter pore size and centrifuged at 1,000 rpm for 15 minutes. The cells were resuspended and aliquoted into 25-ml Erlenmeyer flasks each containing 3 ml maintenance medium at a density of 5×10⁶ to 7×10⁶ cells/flask. The flasks were gassed with a mixture of 5% CO₂, 10% O₂, and 85% N₂, sealed with a silicone rubber stopper, and placed on a gyratory table (70 rpm) for 48–96 hours at 37°C to allow spheroidal aggregates to form. The dissociation medium consisted of 5.25×10⁻² g/ml crystalline lypoilized trypsin (245 units/mg, Worthington Biochemical Corp., Freehold, N.J.) and 5×10⁻⁶ g/ml deoxyribonuclease I (9.1×10⁶ units/mg, Worthington Biochemical) in a Ca²⁺-Mg²⁺-free, phosphate-buffered balanced salt solution with the following concentrations (mM): NaCl 116.0, KCl 5.4, NaH₂PO₄ 0.44, Na₂HPO₄ 0.95, and dextrose 5.6. The pH of the dissociation medium was adjusted to 7.3 with either 1N HCl or 1N NaOH. The maintenance medium consisted of 2% horse serum (Kansas City Biological), 4% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.), 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, CaCl₂ 1.8, MgSO₄ 0.8, NaH₂PO₄ 0.9, NaHCO₃ 20.0, and dextrose 5.5. The antibiotic gentamicin sulfate (10 mg/ml Garamycin, Schering Corp., Kenilworth, N.J.) was added to the medium to yield a final concentration of 5×10⁻⁵ 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 0.22-μm-diameter pore size.

Electrophysiology

After 2–4 days in culture, the reaggregates of cardiac cells were poured into a 35-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% CO₂, 10% O₂, and 85% N₂. The bicarbonate buffer in the medium maintained the pH at ~7.2–7.3. Temperature was maintained at 37±1°C. Under these conditions, more than 98% of the aggregates in a dish beat spontaneously.

Electrical activity was recorded intracellularly using microelectrodes filled with 3 M KCl (electrode resistance, 20–70 MΩ). Transmembrane potential was registered using an amplifier with negative capacitance compensation. The bathing medium was maintained at virtual ground via a current-to-voltage converter (10–100 mV/nA) through an agar salt bridge and a chlorided silver wire. Current pulses were injected into the aggregate through the same microelectrode used for recording the membrane voltage. The pulse amplitudes were measured from the current-to-voltage converter to the nearest nanampere. A programmable IBM PC–based stimulator and two programmable stimulators (pulsars 41 and 61, Frederic Haer) connected to external logic circuitry were used. Voltage and injected current waveforms were monitored on a digital oscilloscope (model 206, Nicolet Instrument Corp., Madison, Wis.) and recorded on an FM instrumentation recorder at a tape speed of 3 in./sec (3 dB frequency response at 3 in./sec, DC of 1,250 Hz, model 3964A, Hewlett-Packard Co., Palo Alto, Calif.) for later off-line analysis.

Experiments were carried out in 36 preparations. In these experiments, 15 focused on overdrive suppression; nine, on phase resetting; and 12, on periodic stimulation.

Three different types of experimental protocol were carried out.

Protocol 1: Overdrive suppression. The chick heart cell aggregates were stimulated using depolarizing stimuli at a rate faster than the intrinsic rate of the preparation. Frequencies and amplitudes of stimulation were chosen such that there was 1:1 entrainment between the stimulus and the aggregate action potential during the entire time of the stimulation. In general, the stimulus amplitude was between 1.5 and two times threshold, and the stimulus period was between 50% and 70% of the control cycle length. In each experiment, stimulation was carried out for various lengths of time but at a fixed frequency. After delivery of a train of stimuli, there was a rest period of ~30–60 seconds to allow the aggregates to return to control cycle length.

Protocol 2: Phase resetting. For control phase resetting, the heart cell aggregates were stimulated with an extra stimulus once every 10 spontaneous action potentials. The coupling interval from the last spontaneous beat to the stimulus was incremented in 5–10-msec steps to scan the spontaneous cycle. For phase resetting after overdrive, the aggregates were paced with a train of a given number of stimuli (eight to 20) at a basic frequency higher than the intrinsic rate such that there was 1:1 entrainment. Immediately after the last stimulus of the train, a premature stimulus was delivered at various coupling intervals.
The coupling interval was automatically incremented by 10–20-msec steps. After each premature stimulus, there was a rest period of 30 seconds before the next train of rapid stimulation.

Protocol 3: Periodic stimulation. The aggregates were stimulated periodically for several minutes at various frequencies with stimulus amplitudes corresponding to the levels used in protocols 1 and 2 above. The stimulation frequencies lay outside the range of 1:1 entrainment.

Data Analysis

Off-line analysis was carried out on a digital oscilloscope and by an automated PC–based computer system (Alembic Software). Magnetic tapes were played back and low-pass–filtered; the voltage waveform was sampled at 1 kHz by an IBM-compatible 386 computer interfaced with an analog-to-digital board (Omega). Interbeat intervals were determined from the digitized waveform by a pattern recognition program. The maximum diastolic potential (MDP) was the most negative potential during the cardiac cycle (Figure 2). The threshold potential (MDP + θ) was determined as the intersection of the slopes of the diastolic pacemaker potential and the action potential upstroke. The action potential duration (APD) was determined as the interval from threshold potential to the maximum diastolic potential. Figures of experimental tracings were obtained by reconverting the digitized record to an analog signal, which was sent to an analog x-y plotter (model 7015B, Hewlett-Packard). A nonlinear least-squares curve-fitting routine, the Leverberg-Marquardt method, was used to fit the data to nonlinear functions.

Theoretical Model and Terminology

The theoretical model is an extension of an earlier formulation that has been used to compute the effects of periodic stimulation on spontaneously beating cardiac tissue. Previous studies8,16–18 assumed that there is no change in the intrinsic cycle length, due to the stimulation history. Since the current experiments demonstrate that the intrinsic cycle length does vary as a consequence of stimulation, the previous theoretical model must be modified. The remainder of this section describes the theoretical model. Several subtle problems of a technical nature concerning the formulation of the theoretical model are addressed in the “Appendix.”

The basic idea of the model is that several quantities that have the units of time must be scaled not to the intrinsic cycle length of the unperturbed aggregate (T0) as in the previous studies8,16–18 but, rather, to the intrinsic instantaneous cycle length of the aggregate after periodic overdrive (T). After cessation of stimulation, T approaches T0 as the effects of stimulation wear off.

Protocol 1: Overdrive suppression. The change of the intrinsic cycle length due to overdrive suppression consists primarily of changes in the slope of phase 4 and, to a much lesser extent, changes in ADP (see Figure 2). Let MDP + θ be the threshold of the action potential and α the mean slope of phase 4, so that

\[ T = \frac{\text{MDP} + \theta}{\alpha} \]  

In the theoretical model, we assume that the change of T is due only to a change of α and that θ and ADP in Equation 1 remain constant. The physiological basis for this assumption is that the stimulation leads to changes in the currents that are important during phase 4, thus leading to a change in the slope of phase 4.

During overdrive stimulation there is a 1:1 entrainment between the stimuli and action potentials. Consequently, the perturbed cycle length is exactly equal to the stimulus period, t0. We assume that the ith stimulus gives rise to a change (Δi) in the slope

\[ \Delta_i = a \left( 1 - \frac{t_i}{T_i} \right) \]  

where a is a positive constant and T_i is the intrinsic cycle length after the ith stimulus. Therefore, if t_i < T_i, Δ_i is negative, and this will lead to an increase in T. Conversely, stimuli that lead to 1:1 entrainment but with a longer period than the intrinsic cycle length (underdrive) lead to a decrease in T.

Finally, we assume that the effects of many stimuli are additive (i.e., there is a linear superposition of the effects), but the effect of each stimulus decays exponentially with a time constant τ. Therefore, during periodic stimulation, the slope after i stimuli with stimulation period t_i is \( \alpha_0 + \Sigma_i \) where \( \alpha_0 \) is the slope in control and \( \Sigma_i \) is given by

\[ \Sigma_i = \Sigma_{i-1} \exp(-t_i/\tau) + \Delta_i \]  

After terminating the overdrive stimulation consisting of i stimuli, the slope exponentially returns to its control value:

\[ \alpha(t) = \alpha_0 + \Sigma_i \exp(-t/\tau) \]  

where t is the time interval after the termination of the stimulation.

The parameters \( \alpha_0, \tau, \) and a can be determined from experimental data. The slope in control (\( \alpha_0 \)) can be measured directly from the spontaneously beating preparations. We determined \( \tau \) and \( \Sigma_i \) by fitting experimental data in Figure 3b with Equation 4. To determine a, we apply Equation 3 to calculate the change of the slope due to the 1:1 stimulation with i stimuli. A computer program is used to calculate a by comparing the experimentally observed intrinsic cycle length after i stimuli, with the theoretically calculated T_i from Equations 1 and 4. If T_i is less than the observed value, a is increased by a step of 0.000002, and T_i is recomputed with a new value of a. If T_i is greater than the observed value, then a is decreased. This procedure can be repeated until T_i agrees with the observed value to a 1% accuracy. The procedure is repeated for several values of the stim-
ulation time and the average value of $a$ is reported (Table 1).

**Protocol 2: Phase response curve.** The phase resetting behavior is determined in control and after overdrive suppression. In both circumstances, we measure the perturbed cycle length, $T(\delta)$, due to a single stimulus delivered at a delay ($\delta$) after the upstroke of an action potential (see Figure 6). During the control, the phase response curve (PRC) is given as

$$
PRC = \frac{T(\delta)}{T_0} = f\left(\frac{\delta}{T_0}\right)
$$

where $f$ is a nonlinear function. After overdrive stimulation, the perturbed cycle length is normalized to $\bar{T}$ rather than $T_0$. The resulting function that we call the normalized PRC (designated PRC) is given by

$$
PRC = \frac{T(\delta)}{\bar{T}} = \bar{F}\left(\frac{\delta}{\bar{T}}\right)
$$

In this equation, the function $f$ is not necessarily equal to $\bar{F}$. However, the experimental studies in the text show that these two functions are approximately equal, especially at high amplitudes at which most of the experimental studies are carried out. Therefore, in this manuscript we assume the functions $f$ and $\bar{F}$ are equal. The phase ($\phi$) of the stimulus is defined as $\delta/T_0$ for control and as $\delta/\bar{T}$ during overdrive.

**Protocol 3: Periodic stimulation.** The theoretical computation of the effects of periodic stimuli on a nonlinear oscillator are described in detail elsewhere for the situation in which the period of the oscillator does not depend on the prior stimulation.\textsuperscript{18,19} In this situation, the effects of periodic stimulation are given by

$$
T_0\phi_{i+1} = T_0\phi_i + t_i + T_0 - T_0 f(\phi_i)
$$

where $t_i$ is the period of the stimulation, $\phi_i$ is the phase of the $i$th stimulus, and $f$ is the PRC curve defined in Equation 5.

In the current case, the period of the oscillator is changed due to the stimulation. One way to account for these effects is to determine the phase of each stimulus based on the intrinsic control cycle length after the $i$th stimulus, rather than on $T_0$. In this case, we obtain

$$
\bar{T}_i\phi_{i+1} = \bar{T}_i\phi_i + t_i + \bar{T}_i - \bar{T}_i f(\phi_i)
$$

To compute the dependence of $\bar{T}_i$ on the stimulation history, we apply Equations 1–3 to obtain

$$
\Sigma_{i+1} = \Sigma_i \exp\left(-\frac{t_i}{\tau}\right) - a[1 - f(\phi_0)]
$$

$$
T_{i+1} = \text{APD} + \frac{\theta}{\alpha_0 + \Sigma_{i+1}}
$$

By substituting $\Sigma_{i+1}$ from Equation 9 into Equation 10, we obtain a two-dimensional finite difference equation (Equations 8 and 10) that is used to carry out the computation of the dynamics during periodic stimulation. In carrying out the simulations with periodic stimulation, we add a small amount ($\pm 1\%$) of random noise to help account for small changes in the intrinsic cycle length that are experimentally observed.\textsuperscript{14} The addition of noise does not alter the evolution of rhythms during periodic stimulation but simply gives rise to fluctuations of the data that more closely resemble the experimental results.

**Results**

**Experimental Results**

**Protocol 1: Overdrive suppression.** Figure 1 shows tracings from a 7-day ventricular aggregate with intrinsic cycle length of 520 msec that was stimulated with a stimulus period of 300 msec for 10 stimuli (top panel), 100 stimuli (middle panel), and 400 stimuli (bottom panel). After 3, 30, and 120 seconds of stimulation, the first cycle after cessation of stimulation was prolonged over control by 40%, 120%, and 270%, respectively.

The change of the intrinsic cycle length due to overdrive suppression arises primarily because of a decrease in the slope of the diastolic pacemaker
depolarization, which prolongs the duration of phase 4. Changes in the APD contribute very little to the cycle length change during overdrive (Figure 2). To characterize the overdrive effect, we determined the MDP and threshold (see “Materials and Methods”). The slope of the depolarization between the MDP and threshold, which represents the mean slope of phase 4, was calculated by dividing the voltage difference by the time difference.

The systematic changes of the slope immediately after cessation of different periods of overdrive, ranging from 6 to 180 seconds, are shown in Figure 3a. The intrinsic cycle length is 950 msec, and the stimulation period is 600 msec. The longer the period of overdrive, the smaller the slope and, therefore, the greater the intrinsic cycle length.

After overdrive, the slope gradually recovers to the control value with a monotonic time course that is well described by Equation 4, with a time constant in the range of 6–35 seconds. Figure 3b shows an example of the recovery time course for the slope after 180 seconds of overdrive stimulation shown in Figure 3a. The solid line in Figure 3b is a fit of the data to Equation 4 using $\alpha_0=0.036 \text{ mV/msec}$, $a=0.00077 \text{ mV/msec}$, and $\tau=26$ seconds. Thereafter, these parameters are used to calculate the change of the slope as a function of stimulation duration (solid line in Figure 3a).

Figure 4 gives a global picture of the overdrive effect, combining the results of different stimulation runs for five preparations. The abscissa represents the period of stimulation and time of relaxation in seconds, and the ordinate represents $T$ after the cessation of the overdrive, divided by $T_0$, where $T_0$ is computed from the last five beats before each stimulation train. The extent of overdrive and the time needed to return to the control frequency (when $T/T_0=1$) depend on the rate and duration of stimulation. There is also considerable variability in the degree of prolongation of the intrinsic cycle length in the different preparations, due to the variable sizes of the preparations and the different stimulation frequencies. However, in all cases there is a slow development of the overdrive effect. The decay of this effect after overdrive consists of an initial rapid decay followed by a slower decay. The experimentally measured parameters for five aggregates are summarized in Table 1.

Figure 5 shows a plot of the theoretically computed curves (solid lines) superimposed with Figure 4b using the parameters $\alpha_0=0.036 \text{ mV/msec}$, $a=0.00077 \text{ mV/msec}$, and $\tau=26$ seconds. Note that the asymmetrical buildup and decay of the overdrive effect is reproduced by the theoretical model. However, the initial decay occurs more rapidly in the experimental data than in the theoretical model.

**Protocol 2: Phase response curve.** The response of biological oscillators to an isolated stimulus depends on the phase of the oscillation at which the stimulus is delivered and the magnitude of the stimulus. The unperturbed cycle length is denoted as $T_0$. The upstroke of the action potential is defined as zero phase. A stimulus delivered at some coupling interval $\delta$ after the up-
stroke of the action potential induces a perturbed cycle length T. Figure 6a shows the prolongation (upper tracing) and shortening (lower tracing) of T with coupling intervals of 410 and 440 msec, respectively, in a 6-day ventricular aggregate with T₀=1,000 msec.

Experiments were then carried out to determine the PRC after overdrive stimulation in the same preparation. Figure 6b shows the corresponding protocol for this approach. Periodic trains of eight stimuli with a stimulus period of 600 msec were given, followed by a premature stimulus and then a rest period of 30 seconds before the next train of stimuli. This rest period was adequate for full recovery of phase 4 depolarization to the control level. The effects on the spontaneous electrical activity of stimulation with coupling intervals of 510 and 530 msec are shown in the upper and lower tracings, respectively. The transition point from prolongation to shortening after overdrive is between the coupling intervals shown in Figure 6b, which occurs in a range greater than that of the control shown in Figure 6a.

Figure 7 summarizes a typical experiment dealing with phase-resetting behavior carried out on a 7-day atrial aggregate with T₀=820 msec. The normalized perturbed cycle length (T/T₀) during control stimulation (open circles) is shown as a function of (δT/T₀) for a low stimulus amplitude (Figure 7a, 12.7 nA) and a high stimulus amplitude (Figure 7c, 22.8 nA). These data are typical of PRC curves found in other experiments.14 After overdrive stimulation with 10 stimuli with a stimulus period of 550 msec for the same stimulus amplitudes, the measured values of

---

**Table 1. Experimental Parameters During Overdrive Stimulation**

<table>
<thead>
<tr>
<th>Curve</th>
<th>T₀ (msec)</th>
<th>tᵢ (msec)</th>
<th>a₀ (mV/msec)</th>
<th>APD (msec)</th>
<th>θ (mV)</th>
<th>a mV/msec</th>
<th>τ (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>520</td>
<td>300</td>
<td>0.130</td>
<td>180</td>
<td>44.2</td>
<td>0.00380</td>
<td>6.92±3.95</td>
</tr>
<tr>
<td>b</td>
<td>950</td>
<td>600</td>
<td>0.036</td>
<td>160</td>
<td>30.4</td>
<td>0.00077</td>
<td>26.08±2.94</td>
</tr>
<tr>
<td>c</td>
<td>1,000</td>
<td>500</td>
<td>0.033</td>
<td>200</td>
<td>26.4</td>
<td>0.00062</td>
<td>20.20±14.00</td>
</tr>
<tr>
<td>d</td>
<td>720</td>
<td>400</td>
<td>0.074</td>
<td>228</td>
<td>34.4</td>
<td>0.00115</td>
<td>22.26±9.06</td>
</tr>
<tr>
<td>e</td>
<td>660</td>
<td>400</td>
<td>0.074</td>
<td>164</td>
<td>36.7</td>
<td>0.00200</td>
<td>15.25±5.04</td>
</tr>
</tbody>
</table>

Curves a–c correspond to the curves shown in Figure 4. T₀, control cycle length; tᵢ, stimulus period; a₀, mean slope of phase 4; APD, action potential duration; θ, threshold of action potential; a, fraction of change of the slope due to single stimulus; τ, time constant of the change of the slope of phase 4 (mean±SEM).
T/T₀ show systematic shifts as a function of δ/T₀ (filled triangles in Figures 7a and 7c).

The open circles in Figure 7a and 7c are the control PRC. In the present study, we propose that an analogous quantity, the normalized phase response curve PRC, can be obtained by dividing the measured time intervals by T (see Equations 5 and 6). The results are shown by filled squares in Figures 7b and 7d.

If the scaling accounted completely for the differences in phase-resetting behavior after overdrive, then there would be a perfect superposition of the PRC and PRC curves in Figures 7b and 7d. However, there is a discrepancy around the transition point from cycle lengthening to cycle shortening at the low stimulus intensity (Figure 7b). In contrast, the data are quite closely superimposed at the higher stimulus amplitudes (Figure 7d). Since we are more interested in the higher stimulation amplitudes, which were used during periodic stimulation in most of these experiments, we will focus on the PRC at high stimulation amplitudes.

Protocol 3: Periodic stimulation. We now consider the changes in the rhythms set up between the stimulator and the aggregate during periodic stimulation of 2–5 minutes. Figure 8a shows a typical evolution of rhythms recorded from a 7-day atrial aggregate when stimulated with a stimulus period (tₛ=470 msec) that is less than the intrinsic cycle time (T₀=730 msec). The rhythm is initially 1:1 but then undergoes transitions: 1:1→10:9→8:7→7:6→6:5. One way to characterize such recordings is to determine the time interval between each stimulus and the preceding action potential. The time interval between a given (ith) stimulus and the preceding action potential is δᵢ. Since we do not measure Tᵢ directly during the periodic stimulation, in this experiment we consider δᵢ rather than φᵢ (see Equation 8). Figure 8b plots δᵢ as a function of the time during periodic stimulation from the same recording shown in Figure 8a. For the stimulus period less than the control cycle length of the aggregate, there is a 1:1 rhythm at the onset of the stimulation. Thereafter, there is a progressive shortening of the interval from the stimulus to the subsequent action potential for the first 29 stimulated action potentials, which culminated in a dropped action potential. This is represented by a downward shift of the first 29 data points in Figure 8b, leading to a dropped beat. After the dropped beat, the next stimulus induces an immediate action potential corresponding to a value of δ=0. Thereafter, during periodic stimulation we observe the repetition of this type of pattern, which undergoes changes with the evolution of the rhythms. Figure 8c shows a plot of δₙ₊₁ versus the immediately preceding values of δₙ, called the Poincaré map, from the data in Figure 8b. Figure 9a shows a continuous recording from the same preparation during periodic stimulation with a period (tₛ=860 msec) greater than the intrinsic cycle length. In this case there is a 1:1 rhythm at the onset of the stimulation period, fol-
followed by an evolution to a complex rhythm. This is found in the range of stimulation conditions where we would expect to find chaotic dynamics based on previous results.\textsuperscript{9,10,17} Figures 9b and 9c show, respectively, the evolution of $\delta_t$ as a function of time during periodic stimulation and the Poincaré map for the same data.

We now consider the theoretical computation of the entrainment rhythms. For the preparation shown in Figures 8 and 9, the parameters were estimated to be $\alpha_0 = 0.04 \text{ mV/msec}$, $\alpha = 0.0006 \text{ mV/msec}$, and $\tau = 25$ seconds. The theoretical calculations using Equations 8 and 10 are shown in Figure 10a for $t_1 = 470$ msec and in Figure 11a for $t_1 = 860$ msec (compare with Figures 8 and 9).

During stimulation at rates faster than the intrinsic rate (overdrive), there is a progressive buildup of overdrive suppression. The effect of this is to prolong the time-dependent intrinsic cycle length so that 1:1 entrainment is no longer possible. The evolution of the rhythms computed from the model and observed experimentally both showed this effect (compare Figures 8a and 10a).

An opposite effect is seen with stimulation slower than the intrinsic rate (underdrive). Now the effect is to decrease the time-dependent intrinsic cycle length, once again destabilizing the 1:1 entrainment, producing irregular rhythms. However, the effect of stimulation history is smaller than during overdrive stimulation.

Finally, we plot the Poincaré map showing $\delta_{R_1}$ as a function of $\delta_t$. Figures 10b and 11b give the Poincaré maps from the simulations in Figures 10a and 11a, respectively. Although the points approximately fall along a curve, the slight "thickness" of the curve reflects the time-dependent effects and the added noise that have been included in this calculation but were not included in our previous work.\textsuperscript{9,10,17}

**Discussion**

In the present study we have characterized the effects of the stimulation history on the time-depen-
dent intrinsic cycle length of chick heart cell aggregates. The time-dependent variation of the intrinsic cycle length influences the phase-resetting properties of the aggregates as well as the response of the aggregates to periodic stimuli. This work has implications in basic physiology, clinical medicine, and the theory of nonlinear systems.

There is an extensive literature demonstrating overdrive suppression in man and in animals. Many different factors have been implicated in overdrive suppression including release of neuromediators, change of the activity of the Na⁺/K⁺ pump due to buildup of intracellular sodium, the accumulation of potassium outside the cells and an increase in the influx of calcium. Pelleg et al. have demonstrated the importance of the Na⁺/K⁺ pump in overdrive suppression of chick heart cell aggregates by showing that ouabain, which blocks the Na⁺/K⁺ pump, reduces overdrive suppression. Further experiments are needed to clarify the mechanism underlying overdrive suppression in this preparation.

One of the standard protocols for studying biological oscillators is to determine the PRC of the oscillator using some appropriate input (see Reference 20 for an extensive review). The results we have obtained here show that after stimulation the PRC may change. Although the changes can be partially accounted for using the normalization of the stimulus phase and response to the value of the intrinsic cycle
Theoretical computation of the rhythms during periodic stimulation. Panel a: The time intervals from stimuli to preceding action potentials ($\delta$) plotted as a function of stimulation duration ($t_s=470$ msec). The simulation is carried out for the same set of conditions as in Figure 8 using mean slope $a_0=0.04$ mV/msec, positive constant $a=0.0006$, time constant $\tau=25$ seconds. The phase response function $f(\phi)$ was adopted from a previous study and is given by $Y_1=4\sin(\phi)$, $Y_2=Y_1^{0.57}((1+k-1)\phi^{3/2}+(c_1\phi^{3/2})$, and $f(\phi)=I+Y_1+Y_2$ where $\phi=\delta T_0$, where $T_0$ is control cycle length. The parameters were obtained by fitting $f(\phi)$ with the experimental PRC to obtain $c_1=0.42$, $c_2=0.43$, $n_1=7$, $n_2=80$, and $k=0.6$. Panel b: Poincaré map showing $\delta_1$ as a function of $\delta$ for the time series shown in panel a (compare with Figure 8c).

Figure 11. Theoretical computation of the rhythms during periodic stimulation. Panel a: The time intervals from stimuli to preceding action potentials ($\delta$) plotted as a function of stimulus duration ($t_s=860$ msec). Panel b: Poincaré map for the time series shown in panel a (compare with Figure 9c).

length (now modified due to stimulation), the effects can be more subtle than this (see Figure 7b). This change in the shape of the phase-resetting curve from a smooth continuous function in control to a discontinuous one after overdrive stimulation is a novel observation. Further detailed analysis and experiments are needed to determine the nature and time course of this observation.

In recent years, a simple theoretical model has been developed for periodically stimulated biological oscillators. Biological oscillations have been associated with stable limit cycle oscillations in nonlinear differential equations. Previous reports from many groups assumed that, after a perturbation, a stable limit cycle is reestablished with the same period and amplitude as before but that the phase of the oscillation is reset; theoretical predictions based on this model concerning the various rhythms set up between the stimulator and the biological oscillator as stimulation frequency and amplitude vary show close agreement with experiment. The present study shows one type of experimental protocol in which these previous assumptions break down. When the aggregate is driven at a higher frequency than the intrinsic rate for a long time interval, the effect of overdrive suppression cannot be ignored. This shows that the theory for the periodic stimulation of biological oscillators must be extended to take into account time-dependent effects, and we have given one simplified theoretical model in which this may be carried out.

The construction of the theoretical model is not unique. We have tried different models to characterize the overdrive effect, as well as the rhythms during periodic stimulation. The model we present here is simple (a two-dimensional map), it gives reasonable agreement with the data, and it has a clear physiological picture. However, there are several limitations of the theoretical model. These are discussed in the "Appendix."

This work has implications for complex rhythms observed in intact hearts and other systems. During periodic stimulation at rapid rates, there are time-dependent changes (e.g., "fatigue") in the atrioventricular nodal and cardiac Purkinje fiber conduction properties. One consequence of this is that conduction properties can change during the course of lengthy stimulation in a fashion analogous to that
shown in Figure 8. Complex evolution of rhythms can also be observed during periodic stimulation of the respiratory rhythm and other cardiac rhythms, indicating that analogous phenomena to those observed here may be of significance in the study of other oscillating systems.

Overdrive suppression would also be expected to play a role during modulated parasystole of a ventricular ectopic focus. Since the ectopic focus usually has a cycle length longer than the sinus cycle length, the persistent stimulation of the ectopic focus during modulated parasystole would be expected to change the time-dependent intrinsic cycle length and hence the PRC. Clinical data and theoretical models of modulated parasystole will have to be reexamined to see if such effects are playing an important role.

Finally, the sinus node can also be overdriven. Such effects are sometimes observed during a supraventricular tachycardia. In the sinus node recovery test, the effects of overdriving the sinus node are determined. An unusually long sinus node recovery time is often associated with sick sinus syndrome. In the clinical setting, neurohumoral factors can play a role in determining the sinus node recovery time. The current in vitro experiment eliminates these neurohumoral factors. A notable difference between overdrive suppression in our experiments and in the clinical context is that the duration of pacing does not have a marked effect on the sinus node recovery time in normal individuals, whereas here it does affect the extent of the elevation of the intrinsic cycle length (Figure 1). Further clinical studies are needed to clarify the time course of buildup and decay of the overdrive suppression.

This work has demonstrated time-dependent effects of stimulation on intrinsic automaticity and phase resetting in spontaneously beating chick heart cell aggregates. A quantitative theoretical model incorporating these effects is able to account for the evolution of complex rhythms observed during periodic stimulation. This provides a conceptual and theoretical framework for understanding analogous effects in the intact heart.

**Appendix**

The theoretical model contains a number of assumptions. These assumptions have been motivated by a desire to maintain a comparatively simple mathematical structure consistent with a plausible physiological mechanism and also in reasonable accord with experimental observations. In developing this theoretical model we have also tried a number of alternative formulations. This "Appendix" provides further amplification on the assumptions of the current model and is directed toward mathematically inclined readers who might wish to extend the current model.

The time dependence of the buildup and decay of the overdrive suppression presents striking features that are not easy to account for using various plausible mechanisms. In general, the buildup of the intrinsic cycle length during stimulation is comparatively gradual, whereas the decay of the intrinsic cycle length after cessation of stimulation is initially quite rapid for the first few beats and is then followed by a slower time course.

The qualitative appearance of the decay of the intrinsic cycle length suggests that the dynamics might be well fit by a sum of two exponentials. When the data is fit to a sum of two exponentials, one finds a range of fast time constants from 1 to 4 seconds and a range of slower time constants from 15 to 40 seconds. A compartmental model with two compartments could provide a good fit to this exponential decay. For example, such a model could correspond to the accumulation of potassium in two compartments, with transport between the compartments and decay from one or both of the compartments. When we tried to develop this sort of model, we found that the buildup of the overdrive effects was symmetrical to the decay in that it consisted of a rapid buildup followed by a slower decay; this is not in agreement with what is observed (see Figure 3). The present model better reproduces the asymmetry in the buildup and decay processes. The rapid initial decay arises because of the possibility of having a small value for $\alpha$ in Equation 1. Thus, even though $\alpha$ decays exponentially, the decay of the period of the oscillation is not a simple exponential function. The slow buildup is partially accounted for by having the $\Delta t$ in Equation 2 depend on $1 - t_i/T_i$. The current model does not account for the comparatively rapid decay of the cycle time after stimulation for short durations.

In the theoretical model we have assumed that a simple linear scaling of both the coupling interval and the perturbed cycle length accounts for the effects of overdrive stimulation on the PRC (Equations 5 and 6). The data presented in this paper show that the situation is much more subtle than what we have assumed. As demonstrated in Figure 7b, we sometimes observed striking differences in the form of the PRC after overdrive stimulation. The current theoretical model in which we assume simple scaling offers no way to account for the apparent discontinuities that are observed in Figure 7b. However, the discrepancies observed only occur over comparatively narrow ranges of delay; therefore, the discontinuity does not markedly affect the computations during the periodic stimulation protocol. Another difficulty in the scaling of the PRC results from the comparatively small changes in the APD even after prolonged stimulation. The consequence of this is that the simple linear scaling that we have applied here breaks down in situations of large overdrive effect, since stimuli delivered during the peak of the action potential will still have negligible effect on the perturbed cycle length, even though a strong effect is predicted for such stimuli using the current formulation after long duration overdrive.
It is difficult to account for these subtle effects on phase resetting during conditions of overdrive. The experiments are probing the effects of perturbations during transient behaviors. A deeper physiological understanding of the mechanisms leading to the overdrive may be needed before a more detailed theoretical model can be developed. In particular, theoretical models posed as differential equations in which there is a stable limit cycle oscillation corresponding to the cardiac cycle will probably be necessary to account for the transient responses.

Acknowledgments

We thank Adam Sherman (Alembic Software) for excellent computer programs used in the stimulation and analysis aspects of this work.

References


Key Words • overdrive suppression • phase response curve • phase locking • mathematical modeling
Evolution of rhythms during periodic stimulation of embryonic chick heart cell aggregates.
W Z Zeng, L Glass and A Shrier

doi: 10.1161/01.RES.69.4.1022

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/69/4/1022

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/