Slow Recovery of Excitability and the Wenckebach Phenomenon in the Single Guinea Pig Ventricular Myocyte

Mario Delmar, Donald C. Michaels, and Jose Jalife

The cellular mechanisms of Wenckebach periodicity were investigated in single, enzymatically dissociated guinea pig ventricular myocytes, as well as in computer reconstructions of transmembrane potential of the ventricular cell. When depolarizing current pulses of the appropriate magnitude were delivered repetitively to a well-polarized myocyte, rate-dependent activation failure was observed. Such behavior accurately mimicked the Wenckebach phenomenon in cardiac activation and was the consequence of variations in cell excitability during the diastolic phase of the cardiac cycle. The recovery of cell excitability during diastole was studied through the application of single test pulses of fixed amplitude and duration at variable delays with respect to a basic train of normal action potentials. The results show that recovery of excitability is a slow process that can greatly outlast action potential duration (i.e., postrepolarization refractoriness). Two distinct types of subthreshold responses were recorded when activation failure occurred: one was tetrodotoxin- and cobalt-insensitive (type 1) and the other was sensitive to sodium-channel blockade (type 2). Type 1 responses, which were commonly associated with the typical structure of the Wenckebach phenomenon (Mobitz type 1 block), were found to be the result of the nonlinear conductance properties of the inward rectifier current, $I_K$. Type 2 sodium-channel-mediated responses were associated with the so-called “millisecond Wenckebach.” These responses may be implicated in the mechanism of Mobitz type 2 rate-dependent block. Single-cell voltage-clamp experiments suggest that variations in excitability during diastole are a consequence of the slow deactivation kinetics of the delayed rectifier, $I_K$. Computer simulations of the ventricular cell response to depolarizing current pulses reproduced very closely all the response patterns obtained in the experimental preparation. It is concluded that postrepolarization refractoriness and Wenckebach periodicity are properties of normal cardiac excitable cells and can be explained in terms of the voltage dependence and slow kinetics of potassium outward currents. The conditions for the occurrence of intermittent activation failure during diastole will depend on the frequency and magnitude of the driving stimulus. (Circulation Research 1989;65:761-774)
in terms of excitability recovery curves (e.g., AV node recovery curve). Yet, the phenomenon remains poorly defined from the cellular electrophysiological point of view, and, to date, its subcellular mechanisms are unknown.

In 1958, Rosenblueth analyzed the heart-rate dependence of propagation through the AV node and concluded that Wenckebach cycles cannot occur in a homogeneous conducting system but develop when an area of functional heterogeneity causes the impulse to stop momentarily and to resume its journey after a delay. Experiments in isolated tissue preparations support this view. In fact, previous studies from our laboratory using the Purkinje fiber-sucrose gap model show that the progressive prolongation and eventual failure of proximal to distal activation during the Wenckebach phenomenon can be explained in terms of delayed recovery of excitability of the distal element during diastole although that element may be comprised of normally polarized cells. As activation is progressively delayed, the proximal impulses find the distal cells less and less recovered until, finally, the electrotonically mediated depolarization across the inexitable zone (the sucrose gap) is unable to bring the distal cells to threshold and block occurs.

Thus, Wenckebach periodicity seems to be associated with postrepolarization refractoriness, which may occur in normally polarized cardiac cells whose response to a stimulus varies as a function of the stimulus timing during the diastolic interval. The purpose of this study was to investigate the cellular and subcellular mechanisms of periodic, rate-dependent activation failure in cardiac cells. We have used single, enzymatically dissociated guinea pig ventricular myocytes as well as computer simulations to demonstrate that Wenckebach periodicity is a characteristic of normally polarized cells. Moreover, our current- and voltage-clamp analyses provide compelling evidence that both postrepolarization refractoriness and the Wenckebach phenomenon in ventricular cells are direct consequences of the nonlinear properties of the inward rectifying current (I_K), as well as the slow deactivation kinetics of the time-dependent potassium current (IKs).

Some of these results have been presented in abstract form and in proceedings of a symposium.

Materials and Methods

**Dissociation Technique**

Single ventricular myocytes were obtained by enzymatic dissociation of Langendorff-perfused guinea pig hearts. Adult guinea pigs were anesthetized with sodium pentobarbital (35 mg/kg i.p.) immediately after administration of heparin (500 units i.p.). The hearts were quickly removed through a midline thoracotomy, and the aorta was cannulated for retrograde perfusion. After rinsing the excess blood by 1–3 minutes perfusion with normal oxygenated (100% oxygen) HEPES-Tyrode solution at 37°C, a low-calcium medium was perfused for 10 minutes. This was replaced by an enzyme-containing medium for 15 minutes and, finally, by an incubation (KB) medium for another 15 minutes. Thereafter, perfusion was stopped, and the ventricles were removed from the Langendorff apparatus and cut into small chunks that were kept in KB medium for another 30 minutes. The cells were released onto the stage of an inverted microscope (model IMT-2, Olympus, Cherry Hill, New Jersey) by gentle agitation of a small chunk into a superfusion and recording chamber. After 15 minutes in the chamber, the incubation medium was replaced with HEPES-Tyrode (normal Tyrode) solution, which was continuously superfused. Temperature was maintained at 32°C. The HEPES-Tyrode was not gassed to prevent bubble formation within the chamber. The composition of the solutions is detailed in Table 1.

**Recording and Stimulation Techniques**

Recordings were attempted after at least 30 minutes of starting superfusion with the normal Tyrode solution. A healthy adult ventricular myocyte was identified by its rod-like, striated appearance and was either impaled with a 3M-KCl microelectrode (DC resistances, 20–40 megohms) or attached to a

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**Table 1. Composition of Solutions**

<table>
<thead>
<tr>
<th>Solution</th>
<th>HEPES-Tyrode</th>
<th>Low calcium medium</th>
<th>Enzyme medium</th>
<th>KB</th>
<th>IPS</th>
</tr>
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For cobalt-containing Tyrode, NaH₂PO₄ was reduced to 0.3 mM and no NaHCO₃ was added, to avoid precipitation. KB, incubation medium; IPS, internal pipette solution; BSA, bovine serum albumin; C, collagenase; B-OH-B, B-OH-butyric acid; PVP, polyvinylpyrrolidone; ATP, adenosine triphosphate; P-C, phosphocreatine. All concentrations are given in millimoles unless otherwise indicated.
suction pipette in the whole-cell recording configuration.\(^{16}\) The composition of the internal pipette solution (IPS) is also detailed in Table 1. Microelectrodes were coupled to a WPI amplifier (model MS-700, World Precision Instruments, New Haven, Connecticut) that allows single-electrode current-clamp procedures, whereas patch pipettes were connected to an Axoclamp 2A amplifier (Axon Instruments, Burlingame, California) in the "bridge" or "discontinuous single-electrode voltage-clamp" mode when the current clamp or voltage clamp protocols, respectively, were carried out (see below).

Power input was provided by one or two Frederick Haer \(p_{61}\) stimulation units. All signals were displayed on a storage oscilloscope (model 5113, Tektronix, Beaverton, Oregon) and photographed with a kymographic camera (model C4R, Grass Instruments, Quincy, Massachusetts).

Tip potentials in the suction pipette were measured as the voltage difference between a pipette filled with IPS and a 3M-KCl microelectrode (reference electrode) after the bath solution had been changed from normal HEPES-Tyrode to IPS.\(^ {17}\) The difference potential thus recorded when the internal and external solutions were identical was 7.88±0.68 mV (mean±SEM; \(n=11\)) more positive than when there was normal Tyrode in the bath. Hence, all voltage values obtained with the suction pipette method were corrected by 8 mV.

**Chemicals**

When manipulations were performed to abolish the active inward currents (see below), CoCl\(_2\) (2 mM; Sigma, St. Louis, Missouri) and tetrodotoxin (TTX, Sigma) were dissolved directly in a modified HEPES-Tyrode solution (see Table 1).

**Computer Simulation Techniques**

To reconstruct the electrical activity of a ventricular cell, we modified the equations of Beeler and Reuter\(^ {18,19}\) for the time- and voltage-dependent membrane currents. A cell was modeled as a 1-cm\(^2\) surface with a specific membrane capacitance of 1 \(\mu F/cm^2\) in parallel with four nonlinear conductances: sodium current \((I_{Na})\), calcium current \((I_{Ca})\), \(I_K\) (originally defined as \(i_{K}\)),\(^ {20}\) and \(I_{K1}\). The current-voltage relation of the time-independent, potassium outward current \(I_{K1}\) was modified to mimic more closely the activity of guinea pig ventricular myocytes\(^ {15,21-24}\) (see also our Figure 7). The computer programs were written in FORTRAN and run on a PDP 11/73 computer equipped with 2.5 megabytes of core memory and 56 megabytes of Winchester disk storage. Graphic outputs during the computer runs were displayed on either a VT125 graphics terminal (Digital Equipment, Maynard, Massachusetts) or a Tektronix 4010 graphics terminal. Hard copies were produced using an X-Y plotter (model 7470A, Hewlett-Packard, Palo Alto, California), a Tektronix hard copy unit (model 4631), or an Apple LaserWriter Plus printer (Apple Computer, Cupertino, California). Some simulations were run on an IBM PC/AT computer equipped with 1024 Kb of core memory, 40 Mb hard disk, and EGA graphics. This was connected remotely (via a 9,600 baud modem) to the Cornell National Supercomputer Facility at Cornell University, Ithaca, New York, and to the Center for Advanced Technology and Software Engineering at Syracuse University, Syracuse, New York.

**Current-Clamp Protocols**

Two types of current-clamp protocols were used in both single-cell experiments and computer simulations. In the first, repetitive depolarizing current pulses of fixed duration (5–100 msec) and critical amplitude were applied repetitively to study the various stimulus-response patterns that develop as a function of the driving cycle length. In all cases, stimulation was begun at a relatively long basic cycle length (e.g., 1,000 msec) at a fixed current pulse amplitude that was just barely threshold to activate the cell in a stable 1:1 manner. Thereafter, the cycle length was decreased in small steps (e.g., 10 msec), and the steady-state stimulus-response patterns were recorded at each step.

In the second type of current clamp protocol, trains of either brief suprathreshold stimuli (i.e., in those cases where all active currents were operative) or long (e.g., 300 msec) depolarizing current pulses (in the cases where \(I_{Na}\) and \(I_{Ca}\) had been blocked) were applied at constant but relatively long cycle lengths. Unless otherwise indicated, single test pulses of 100–200 msec were applied after every tenth basic stimulus at progressively earlier intervals.

**Voltage-Clamp Protocol**

To study the time course of \(I_K\) deactivation, voltage-clamp experiments were performed using patch pipettes in the whole-cell configuration. Holding potential was maintained equal to the resting potential of the cell (i.e., between –80 and –90 mV). Conditioning depolarizing pulses of 500-msec duration to a membrane potential of 20–30 mV were applied every 10 seconds and were followed at various intervals by a single test pulse of 100-msec duration to 10–20 mV. The instantaneous current induced by each test pulse was an indication of the degree of \(I_K\) deactivation achieved at that particular interval.

**Results**

**Experimental Results**

Wenckebach periodicity in a single ventricular myocyte.\(^ {25}\) Rate-dependent activation failure can be shown to occur in single ventricular myocytes driven by current pulses of critical amplitude and appropriate cycle length. Figure 1 illustrates one example. In all panels, the top trace is a microelectrode recording and the bottom trace is a stimulus monitor. In panel A, the myocyte was driven at a cycle length of 2,000 msec by constant depolarizing cur-
Figure 1. Tracings of Wenckebach periodicity in a single guinea pig ventricular myocyte. Recordings were obtained with a conventional microelectrode filled with 3M KCl. In each panel, the top tracing represents the action potentials, and the bottom tracing represents the current applied through the recording microelectrode. The numbers at the right of each panel correspond to the basic cycle length (BCL). Current pulse amplitude (0.35 nA) was the same in all panels.

Current pulses whose amplitude (0.35 nA) and duration (20 msec) were programmed to be just barely threshold so as to activate the cell in a 1:1 manner (i.e., one response after every stimulus). In panel B, the cycle length was shortened to 910 msec, and a combined pattern of 5:4 and 4:3 Wenckebach periodicity was established. This pattern was stable and occurred repetitively for as long as the same stimulus parameters were maintained. In panel C, abbreviation of the stimulus cycle length (835 msec) led to a 2:1 activation pattern. Clearly, in spite of the fact that the electrophysiological characteristics of this cell were normal (e.g., its resting membrane potential was -82 mV), postrepolarization refractoriness and intermittent activation failure were easily demonstrable.

Additional examples of Wenckebach patterns are presented in Figure 2 to illustrate the different dynamic structures in the activation delays. The five superimposed traces in panel A were obtained from a myocyte whose resting potential was -80 mV. Depolarizing current pulses of 70-msec duration and 0.2-nA strength were applied at a constant cycle length of 800 msec. A 5:4 activation pattern was clearly manifest in which the stimulus-response latency, measured from the onset of the current pulse to 90% of the action potential upstroke, changed from an initial value of 66 msec to a maximum of 81 msec for the last successful stimulus. Note that the activation latency increased in decreasing increments, until failure occurred. This pattern corresponds with a typical sequence of Wenckebach periodicity, as described in the clinical literature.25,26

The traces in panel B were obtained 10 minutes later from the same cell. The resting membrane potential had depolarized slightly (-77 mV). In this case, depolarizing current pulses of 70 msec duration and 0.2-nA strength were used to pace the cell at a basic cycle length of 1,000 msec. The pattern changed to a 6:5 Wenckebach sequence in which the latency for the last two beats increased at increasing intervals. This is an example of so-called "atypical" Wenckebach structure27 and is commonly observed in the clinical setting. Finally, panel C shows a 4:3 Wenckebach cycle recorded in a cell that was being paced by brief (10-msec) current pulses of 0.6 nA. The typical structure of the Wenckebach pattern was observed. However, in this case, the range of activation delays at which the pattern was manifest was very brief (only 5 msec) and probably conforms to the phenomenon
known as "millisecond Wenckebach" encountered previously in isolated tissue preparations. 28

Diastolic changes in cell excitability. In all the examples of Wenckebach periodicity presented in the previous section, the stimulus cycle length was considerably longer than the action potential duration (see Figure 1). Thus, the current input always occurred after repolarization had been completed. This observation strongly suggests that some kind of slow recovery process is occurring during the diastolic interval, which eventually allows excitation of the myocyte if the stimulus occurs with sufficient delay after the last action potential. That this is indeed the case is illustrated in the example of Figure 3, which was taken from a cell whose resting membrane potential was stable at -79 mV. Action potentials were initiated periodically by depolarizing pulses (duration, 5 msec; amplitude, 1.65 nA), injected intracellularly through the recording microelectrode at a basic cycle length of 1,500 msec. To study diastolic changes in excitability, single 150-msec depolarizing pulses (S2) were applied after every tenth basic stimulus (S1) at progressively briefer S1-S2 intervals. The amplitude of S2 (0.35 nA) was such that it was above threshold at the longest S1-S2 interval of 820 msec (panel A). At this test interval, the cell was activated after a latency (S2-V2) of 115 msec (measured from the beginning of S2 to the midpoint of the action potential upstroke). When the S1-S2 interval was abbreviated to 630 msec (panel B), S2 also induced an active response, but the latency was longer (157.5 msec). Finally, at an S1-S2 interval of 560 msec (panel C), S2 failed to initiate an active response. Panel D shows graphically the complete input-output curve obtained from a similar experiment in a different cell. The curve shows an asymptotic S2-V2 value for S1-S2 intervals that were about 1,000 msec or longer. On the other hand, abbreviation of the coupling interval caused a larger prolongation of S2-V2 (the "ascending branch" of the curve) until refractoriness was observed at S1-S2=450 msec. This phenomenon, known as postrepolarization refractoriness, has been demonstrated in a variety of nonhomogeneous cardiac tissue preparations (see Reference 12), including the AV node and the ischemic nonhomogeneous myocardium. Our results demonstrate that postrepolarization refractoriness is a normal property of cardiac cells in response to critically timed stimuli of low amplitude.

The subthreshold local response. An important element associated with postrepolarization refractoriness and rate-dependent block is the development of foot potentials that, in the case of complete block, are replaced by subthreshold local responses.11-29 As observed in our experiments (see Figures 1-3), when activation failure occurs, the shape of the subthreshold depolarization does not generally conform to the monophasic morphology of electrotonic potentials, which are related to the resistance-capacitance (RC) properties of the myocyte.15 In fact, the depolarizing responses seem to be distorted by what appears to be an active, yet subthreshold, event superimposed on the passive membrane-potential change.11

We have analyzed the ionic mechanisms responsible for the characteristic shapes of subthreshold responses. The recordings in Figures 4A and 4B were obtained from two different myocytes maintained in normal HEPES-Tyrode solution. Voltage recordings were obtained during the application of repetitive depolarizing current pulses (basic cycle length, 1,000 msec) through a suction pipette in the whole-cell current-clamp configuration. Two basic types of subthreshold responses were observed whose shapes depended on different transmembrane current systems. In the first type (panel A),

FIGURE 3. Tracings and plot showing slow recovery process during diastolic interval. Panels A-C: Single depolarizing pulses (S2 duration, 150 msec; amplitude, 0.35 nA) were applied after every tenth basic stimulus (S1) at progressively briefer S1-S2 intervals. Panel D: Input-output plot from a different cell. Postrepolarization refractoriness is apparent. S2-V2, latency measured from beginning of S2 to midpoint of action-potential upstroke.
depolarizing current pulses of constant amplitude and 100-msec duration induced a subthreshold response that consisted of a late depolarizing component, which developed monotonically during the course of the pulse. In the second type (panel B; pulse duration, 150 msec; notice the difference in time calibration), current pulses yielded responses consisting of an early transient component that was followed by a return to a stable, less depolarized level. Sodium-channel blockade eliminated the transient component and converted the response into one of the first type. This is illustrated in panel C, recorded from the same cell as in panel B 5 minutes after addition of 30 μM TTX to the superfusate. Although the resting membrane potential (−82 mV) did not change, the minimum amount of current needed to activate the cell increased from 0.85 to 1.1 nA. In addition, the shape of the response changed to that of the first type. These observations are consistent with those obtained by Rozanski et al11 in multicellular preparations where the subthreshold responses were either TTX- or verapamil-sensitive, depending on the level of membrane potential from which they were initiated. In the case of the single myocyte, however, the TTX-insensitive response was also insensitive to cobalt (see Figures 5 and 6), which eliminates the possibility that these responses could be mediated by the calcium current.

The role of I_{K1}. Recently, Tourneur23 demonstrated that "active" responses can be elicited from single guinea pig ventricular myocytes even while they are exposed to blocking doses of TTX and cobalt. We have confirmed these observations in 10 different myocytes superfused with both 2 mM Co and 30 μM TTX. The superimposed tracings in Figure 5 were obtained from one of these cells during the application of depolarizing current pulses at a very long cycle length (10,000 msec). Pulses were applied through the recording suction pipette; duration was 500 msec, and two slightly different current strengths (0.62 and 0.65 nA) were used. At the lower strength, the membrane potential change was clearly that of an RC response. However, a very slight increase in current amplitude during the subsequent pulse gave rise to a complex response with a foot potential that was followed by a fast upstroke and slow repolarization; these responses resembled an action potential. When the current pulse was turned off, the cell rapidly repolarized to its resting level. This type of response has been attributed to the nonlinear voltage dependence of I_{K1}, whose negative-slope characteristics allow the
appearance of a "threshold level" for further depolarization in the presence of a current input. However, as demonstrated by Tourneur,23 these outward current responses differ from action potentials in that they are not "all or nothing" and that the increased change in membrane potential results from a decrease, rather than an increase, in membrane conductance.

In addition to the aforementioned properties, our data demonstrate that these outward current-mediated responses are frequency dependent, and thus, their shape can change as a function of their timing in relation to a preceding response. The superimposed traces in Figure 6A were obtained from a single myocyte that was constantly superfused with 2 mM Co and 30 μM TTX. From a resting level of −81 mV, a long (500-msec) conditioning depolarizing pulse of constant amplitude (1.25 nA) applied once every 10 seconds changed the membrane potential to +86 mV, and thus, the upper portion of the response was off-scale. However, the resting membrane potential was restored immediately at the offset of the current pulse. At each oscilloscope sweep, a single test current pulse of 150-msec duration and 1.07-nA amplitude was applied at decreasing intervals after every conditioning response. Clearly, even in the absence of inward membrane currents, test pulses occurring at long intervals induced "active" action potential-like responses. Yet, as the test interval was decreased, the amplitude of the response also diminished, and its shape changed to that of the type 1 subthreshold response. Thus, the sodium channel determines the upstroke velocity and shape of the type 2 subthreshold response and probably plays a major role in so-called millisecond Wenckebach (see Figure 2C and "Discussion"). However, the recovery from inactivation of the sodium current is simply too fast to be able to account for the slow interval-dependent increase in the amplitude of the type 1 subthreshold response during diastole. Moreover, such a growth occurs even in the presence of very high concentrations of TTX, which invalidates the idea that sodium channel repriming kinetics are involved.

Time course of voltage response increase; the role of I_K. The nonlinear properties of I_K with respect to membrane potential can accurately explain the shape and time course of the type 1 voltage response to depolarization during a long current pulse. However, I_K is time independent and, hence, cannot determine the dynamic changes observed in the amplitude of the response at high driving frequencies or brief test intervals (see Figures 2, 3, and 6). Thus, additional current systems must be considered to account fully for the dynamic behavior of the type 1 and type 2 subthreshold response associated with rate-dependent failure and postrepolarization refractoriness. As demonstrated above, the sodium channel determines the upstroke velocity and shape of the type 2 subthreshold response and probably plays a major role in so-called millisecond Wenckebach (see Figure 2C and "Discussion"). However, the recovery from inactivation of the sodium current is simply too fast to be able to account for the slow interval-dependent increase in the amplitude of the type 1 subthreshold response during diastole. Moreover, such a growth occurs even in the presence of very high concentrations of TTX, which invalidates the idea that sodium channel repriming kinetics are involved.
Another important current that should be considered is the delayed outward rectifier, \( I_K \). Indeed, since \( I_K \) activation continues throughout the action potential, its slow deactivation during the diastolic interval is an excellent candidate for controlling the frequency-dependent changes in the amplitude and time course of the subthreshold local response.\(^{19}\) To determine this possibility, we have analyzed qualitatively the kinetics of \( I_K \) deactivation. The superimposed tracings presented in Figure 7 were obtained from a voltage-clamp experiment in a guinea pig ventricular myocyte. After breaking the patch seal, the membrane potential was held at the resting level (−85 mV) and conditioning-clamp depolarizing pulses of 500-msec duration to +20 mV were applied every 10 seconds to induce a partial activation of the \( I_K \) current. Each conditioning depolarization was followed at varying intervals by a single test pulse of 100-msec duration to +15 mV. It is clear that both the amplitude and time course of the resulting current gradually changed as functions of the clamp interval. Consequently, since the instantaneous current induced by a given pulse is an indication of the amplitude of \( I_K \) at that particular interval, we determined the time course of \( I_K \) deactivation by measuring the amplitude of the current during the initial 5 msec after the onset of each test pulse and found it to be very similar to the time course of growth of the subthreshold response.\(^{19}\) These data provide strong support to the hypothesis that the rate of deactivation of \( I_K \) during diastole determines the dynamic changes in the amplitude of foot potentials and subthreshold events associated with postrepolarization in the ventricular myocyte.

**Computer Simulations**

The model. To provide additional support to our hypotheses concerning the determinants of postrepolarization refractoriness and Wenckebach periodicity, we have carried out numerical studies using a modified version of the Beeler and Reuter model.\(^{18}\) For these simulations, we changed the current-voltage (I-V) relation of the time-independent \( I_{K1} \) current to approximate more closely the activity of guinea pig ventricular myocytes.\(^{19,21-23}\) Figure 8 shows an action potential obtained with the original Beeler and Reuter model (panel A) and one plotted after the I-V relation had been modified (panel B) to mimic the negative slope conductance region in the guinea pig heart. The only major difference between the two is a slight prolongation of action potential duration in the guinea pig response, in accordance with experimental results in that species.\(^{15}\)

**Wenckebach periodicity and post-repolarization refractoriness.** We have demonstrated that delayed recovery of excitability and rate-dependent activation failure can be shown to occur in single guinea pig ventricular myocytes.\(^{19,21-23}\) Figure 9 illustrates the frequency-dependence of activation when an input of the appropriate magnitude (−1.4 μA/cm², 100 msec) was used to trigger the simulated action potentials. Panel A shows a 1:1 stimulus-response pattern when the basic cycle length was held constant at 860 msec. Shortening the pacing cycle length to 840 msec and 820 msec (as shown respectively in panels B and C) caused the appearance of Wenckebach-like patterns, whose stimulus-response ratios varied as a function of cycle length.
Figure 9. Tracings showing Wenckebach periodicity in the modified Beeler and Reuter\cite{18} model. A depolarizing current pulse (-1.4 \mu A/cm^2 in strength; 100-msec duration; not shown in the figure) was applied repetitively at various basic cycle lengths (BCL).

In panel D, a further decrease in cycle length (810 msec) led to a 2:1 activation pattern.

Slow recovery of excitability and postrepolarization refractoriness can be demonstrated also in the mathematical model. Figure 10 shows the results. All inward and outward currents were maintained in these simulations. An S2 current input of the same characteristics as those used in the simulation illustrated in Figure 9 (i.e., -1.4 \mu A/cm^2 in strength, 100-msec duration) was applied at variable delays with respect to the last of a train of 10 action potentials induced by a suprathreshold S1 stimulus. As in the experimental preparation, abbreviation of the S1-S2 interval (1,200 msec in panel A; 743.5 msec in panel B) caused prolongation of the activation latency (S2-V2 interval) until failure occurred at the S1-S2 of 743 msec (panel C). Panel C shows two superimposed traces to illustrate that, as in the experimental situation (see Figure 3), the shape and amplitude of the subthreshold response is also a function of the coupling interval. In fact, at brief delays (560 msec) the subthreshold depolarizations conform to the normal RC properties of the cell membrane, whereas at longer intervals (743 msec) their amplitude increases progressively and their shape becomes that of the type 1 response. Panel D shows the input-output plot in which the changes in S2-V2 interval are plotted as a function of S1-S2. These simulations reproduce very accurately all the characteristics associated with postrepolarization refractoriness in the isolated ventricular myocyte.

The subthreshold response. We have also used the mathematical model to confirm the existence of time- and voltage-dependent regenerative responses that are mediated by the inward rectifier current I_K1. The results of these simulations are presented in Figure 11. Several superimposed voltage traces are shown in each panel under the conditions in which both I_Na and I_Ca had been deleted. In panel A, only the time-independent I_K1 current was operative (i.e., I_K=0). After a large (-3 \mu A/cm^2) depolarizing conditioning pulse of 300 msec, a smaller pulse of constant amplitude (-2.5 \mu A/cm^2) and duration (150 msec) was applied at various test intervals that were different for each run. The voltage changes showed the characteristic shape of subthreshold local responses, but their amplitude remained constant at all coupling intervals. In panel B, when the...
FIGURE 11. Superimposed tracings from a current-clamp simulation. Inward membrane sodium and calcium currents were omitted. The horizontal line in all panels represents zero potential. Panel A: Only the time-independent inward rectifying potassium current was operative; a single depolarizing pulse of constant magnitude (not shown) was applied at various test intervals after a conditioning pulse; no interval-dependent changes in the amplitude or shape of the response are observed. Panel B: Both time-dependent potassium current ($I_K$) and time-independent potassium current were operative; the subthreshold response increases progressively in amplitude and becomes distorted at progressively later intervals. Panel C: Hyperpolarizing pulses applied under similar conditions as in panel B do not reveal any time-dependent changes in the electrotonic response.

The maximum value of $I_K$ was increased to normal, there was a time-dependent increase in the amplitude of the individual responses, with those occurring earlier conforming to the RC properties of the system and those initiated later gradually taking the shape of subthreshold local responses. Also note that, in the absence of $I_K$ (panel A), the membrane potential reaches a maximum steady level during the conditioning pulse, whereas the existence of a slow repolarizing phase in panels B and C indicates the activation of the time-dependent potassium current $I_K$. These results mimic very accurately those encountered in multicellular and single cell preparations (see Figure 6). Finally, panel C of the same figure shows that, also consistent with experimental results, when hyperpolarizing pulses are applied using the same current parameters (i.e., $-2.5 \mu A/cm^2$ strength, 150-msec duration), no time-dependent changes are seen, and the voltage responses are those expected from an RC circuit. These occurrences reflect the linearity of the $I_K$-V relation at hyperpolarized levels of membrane potential (see Figure 8).

Discussion

Advantages and Limitations of the Technique

The isolated ventricular myocyte is the simplest and most direct preparation in which to study the role of slow recovery of excitability in the development of rate-dependent activation failure. However, one can never be certain that, after enzyme treatment and dispersion, the physiological properties of the single cell are the same as those of the intact tissue. The same uncertainty holds for the effects of intracellular dialysis by the pipette filling solution. Moreover, of particular importance is that, to prolong the viability of the isolated myocytes during recording, we had to maintain the temperature at 32° C, which probably slowed the kinetics of the currents involved. Hence, low temperature might have prolonged the time course of the excitability recovery process, but it would do so concomitantly with a prolongation of the action potential duration. Since both processes are intimately related, we would anticipate that postrepolarization refractoriness would be demonstrable in the myocyte even at 32° C. Indeed, previous experiments in isolated tissue preparations as well as our computer simulation results (Figure 10) support our contention that the functional refractory period of well-polarized ventricular myocytes may outlast the action potential duration when stimuli of critical magnitude are applied.

In an attempt to test our hypotheses more rigorously, we simulated the experimental results using a mathematical model of ventricular cells modified from Beeler and Reuter. We demonstrated a striking correlation between the data from the biological preparation and the results generated by the computerized model. It should be noted, however, that experimental evidence has been accumulated that suggests that the Beeler and Reuter model is an oversimplified description of the actual ionic flow across the sarcolemma of a ventricular myocyte. In fact, a major weakness of this model is its inaccurate rate representation of the time course of the sodium inward current. The possible implications that these shortcomings may have in the interpretation of our results are discussed below (see "Ionic Mechanisms: Role of the Inward Currents").

The Development of Wenckebach Periodicity

As classically defined, typical Wenckebach cycles consist of a succession of beats in which the PR interval increases progressively by decreasing increments until failure occurs. Our data support the hypothesis that Wenckebach periodicity can be established when heart cells are stimulated at frequencies that do not allow complete recovery of excitability. Because of partial recovery after a given action potential, the stimulus-response interval is prolonged. This induces an abbreviation of the diastolic interval in the following cycle, as well as an additional encroachment upon the recovery...
phase, and thus further prolongation of the subsequent stimulus-response interval. When the stimulus finally falls within the refractory period, failure occurs, allowing for prolongation of the diastolic interval and recovery of excitability for the next beat. Accordingly, each stimulus-response interval in the Wenckebach cycle must correspond to the intersection between the instantaneous cycle length and the recovery of excitability (input-output) curve. 19

Wenckebach patterns, typical and/or atypical, were observed in the ventricular myocytes. This was true regardless of the duration of the stimulating current pulse. Yet, when brief duration pulses (<20 msec) were applied (see Figure 2C), the range of variation in the stimulus-response interval was usually less than 5 msec. In such cases, the structure of the repetitive activation cycles is equivalent to what El-Sherif et al. 28 designated as “millisecond Wenckebach.” When this occurs, the progressive beat-to-beat delays would be difficult to identify in the routine electrocardiogram, and the diagnosis of second-degree block of the Mobitz type 2 would be established. 31

The Shape of the Subthreshold Response

Our results are quite consistent with previous experiments in nonhomogeneous ventricular muscle preparations 11 in that the shape of the subthreshold depolarization does not conform exclusively to the resistance-capacitance properties of the membrane. In fact, the amplitude of such a response increases progressively, and its shape becomes distorted as it approaches the threshold for regenerative discharge.

Rozanski et al. 11 were unable to identify “active” subthreshold responses in their sucrose-gap preparations when the resting potential was more negative than −80 mV. On the other hand, both our experimental and computer results show that such responses are demonstrable in well-polarized ventricular cells, that is, with resting membrane potentials more negative than −79 mV. This disagreement may be the result of differences in the techniques used to apply intracellular current and elicit the subthreshold response. Indeed, it may not be possible in the sucrose gap preparation to fine-tune the amount of current entering the cell from which records are taken, which would make it difficult to find the ideal value of current input necessary for finding active responses before threshold is reached. On the other hand, it is also possible that because of the low temperature in our experiments, a significant shift in sodium channel activation threshold might have occurred without major changes in resting potential, which would have improved our chances of demonstrating postrepolarization refractoriness and rate-dependent activation failure in well-polarized cells.

In the experiments of Rozanski et al., 11 the amplitude and shape of the subthreshold responses changed in a characteristic manner that depended on the coupling interval and on the resting membrane potential. Also, depending on the resting potential, subthreshold responses were sensitive to either TTX or to verapamil. These authors therefore concluded that the different shapes of such responses were the result of slow time-dependent recovery from inactivation of inward current channels (either INa or ICa). Our results confirm the existence of the type 1 and type 2 responses described by Rozanski et al. 11 In the type 2 responses, a rapid transient depolarization was followed by a decay (Figure 4B). These responses are sensitive to TTX and are, therefore, most probably related to partial sodium-channel activation. In the type 1 responses, membrane depolarization increases monotonically until the end of the pulse. In our experiments, these responses were insensitive to TTX (Figure 4C), as well as to 2 mM Co (see also Figures 5 and 6). Thus, it is very unlikely that the type 1 responses are calcium-channel dependent. Moreover, in the experiments of Rozanski et al., 11 it is possible that the use of verapamil as a calcium-channel blocking agent might have affected the outward current components as well, particularly at the depolarized levels of membrane potential. 32,33

Our experiments are consistent with the hypothesis that the TTX-insensitive (i.e., type 1) responses are the consequence of the presence of a negative slope region in the current-voltage relation of the inward rectifier current IK1. It is well known from experiments in multicellular preparations that the current-voltage relation of IK1 shows inward-going rectification that, in the case of guinea pig ventricular muscle, produces a region of negative slope conductance. 21,34 Whole-cell voltage-clamp experiments from the same species 22 have confirmed this observation. Our results confirm and expand the experiments of Tourneur, 23 who studied the mechanism of outward current-mediated responses in membrane potentials of isolated guinea pig ventricular myocytes. Under conditions in which all inward currents had been abolished and only outward currents were operative, relatively long (150-msec) depolarizing current pulses induced membrane potential changes whose shape resembled that of the subthreshold response. The mechanism of such responses is related to the existence of a negative slope conductance in the IK1 I-V relation; this finding has been confirmed by computer simulations in Tourneur’s study 22 as well as ours (see Figure 11).

Ionic Mechanisms: Role of the Inward (INa and ICa) Currents

Previous studies 35-37 have demonstrated that there are at least two exponentials (one fast, one slow) in the repriming process of the sodium current of the ventricular myocyte. Brown et al. 35 reported average time constant values of 2.1 msec and 40.8 msec (fast and slow, respectively) obtained from rat ventricular myocyte held at a membrane potential of −80 mV and maintained at room temperature. Sim-
ilar results were obtained by Fozzard et al. in
dialyzed Purkinje cells. More recent experiments have shown that the slow reactivation of the sodium current has an average time constant of 90 msec. Accordingly, after an action potential, the vast majority of sodium channels must be reactivated within the initial 100 msec after the repolarizing phase. In two of eight of our experiments, the ascending branch of the recovery of excitability curve was very steep, and the duration of postrepolarization refractoriness was relatively brief. In these cases, the type 2 (TTX-sensitive) response was demonstrable. Thus, the sodium channels were available very rapidly after repolarization, which was crucial in determining the fast time course in the recovery. However, in most of the experiments, the recovery of excitability was much slower, and the active responses were of the first type (TTX-insensitive) and could only be elicited when initiated several hundred milliseconds after the membrane potential had repolarized. Consequently, even though slow repriming of $I_{Na}$ can be a key limiting factor for reexcitation immediately after an action potential, it cannot account for the development of postpolarization refractoriness and rate-dependent activation failure at slow frequencies or long coupling intervals.

It is important to note, however, that in those six cells in which we observed type 1 responses and slow recovery time, the resting membrane was also well polarized (−83.5±1.9 mV) and the shape of the action potential was similar to what has been described for a normal guinea pig ventricular myocyte. Hence, although it can be argued that the sodium channels might have been somehow "damaged" by the dissociation procedure, it is also possible that a process of "accommodation" was taking place. In other words, because of the long duration of the current pulse, sodium channels were partially inactivated during the slowly rising phase of the electrotonic potential. Another possibility is that the cells with type 2 responses were in fact Purkinje cells, whose behavior might have been different from that of the ventricular myocyte. Such a possibility seems unlikely, however, given the disparity in numbers of the two types of cells.

Reactivation of the calcium current seems to be a very complex process that depends not only on the interpulse interval but also on the duration of the conditioning pulse. Analysis of the data published by Mitchell et al. shows that at least 80% of the repriming of the calcium current is completed within 150 msec after the end of a conditioning pulse. Similar values are reported by Shimoni et al. In all cases, holding potentials between −45 and −50 mV were reported. Those results suggest that, under conditions of sodium-channel inactivation (i.e., action-potential threshold less negative than −40 mV), reactivation of the calcium channel might also be of significance in determining the diastolic recovery of excitability of the ventricular myocytes. However, this slow calcium current is probably not very relevant when the subthreshold responses occur in the range of potentials between −80 and −55 mV. On the other hand, a different population of calcium channels ($I_{Ca}$) has been recently identified in heart cells. Although this channel is active at more hyperpolarized levels of membrane potential (more negative than −50 mV), its conductance is known to be very small, and its reactivation kinetics appear to be too fast (a time constant of about 160 msec in single Purkinje cells) to account for the phenomenon of postrepolarization refractoriness that we have described in this paper.

Role of the Outward Currents

As indicated above, in the majority of cases in which input-output plots were constructed, the curve decayed slowly as the S1-S2 interval was prolonged (see Figure 3D for example). This kind of input-output curve was best fitted by logarithmic functions with time constants of about 200 msec and was related to the occurrence of the type 1 responses observed during failure in the Wenckebach cycle (Figures 2A and B). Our data strongly suggest that in these cases the outward currents $I_{K}$ and $I_{Ki}$ are the major determinants of the interval-dependence in the amplitude and shape of the subthreshold response (Figure 6). Since the negative slope region (20–30 mV positive to the resting potential) of the $I_{K}$-I-V relation corresponds also to the threshold for the action potential, the inward rectifier must also contribute to this threshold and be a factor in the development of rate-dependent block.

The time-dependent outward current $I_{K}$ plays a major role in the growth of the subthreshold response during diastole. The study of the kinetics of $I_{K}$ has been complicated by potassium accumulation and depletion in the clefts separating the cell membranes and by the existence of a slow current component ($I_{K}$). Nevertheless, voltage-clamp studies have recorded an activation time constant of the $I_{K}$ component of about 370 msec and deactivation of 260–290 msec, whereas the time constant of this $I_{K}$ is estimated to be 1–2 seconds. Recently, Matsuura et al. reported that, in the ventricular myocyte at 37°C and holding potentials negative to −50 mV, the time course for current deactivation is best described by a monoexponential function with a time constant of about 200 msec, which corresponds quite closely with the slow recovery of excitability in our experiments. Other results in isolated guinea pig ventricular myocytes show that $I_{K}$ has an average time constant of activation of 535 msec when measured at 37°C. Moreover, in another study performed at room temperature, $I_{K}$ relaxation was best fitted by two exponentials whose average time constants were 730 msec and 2.92 seconds. These time- and voltage-dependent characteristics of $I_{K}$ strongly suggest that this current system does not completely deactivate upon repo-
larization from an action potential and that part of this deactivation process takes place during the diastolic interval. Such a deactivation should have a major influence on the amplitude of a closely coupled subthreshold response. Hence, even in the absence of any inward currents, and as a result of slow \( I_K \) deactivation, relatively long depolarizing pulses of constant amplitude, which are applied at progressively longer diastolic intervals, should lead to responses of progressively greater amplitude. Furthermore, since the negative-slope region of the \( I_K \) conductance is reached at the longer pulse intervals, the response should deviate more and more from its characteristic RC shape and give rise to what appears to be a local "active" response.

**Conclusions**

Our results demonstrate that rate-dependent activation failure is a property of normal ventricular cells and can be adequately explained by the fact that recovery of excitability after an action potential can follow a very slow time course and greatly outlasts the action potential duration. Such a slow recovery is a consequence of the slow deactivation kinetics of the repolarizing current \( I_K \). Our data further suggest that this current and \( I_K \) are the major determinants of the time course and shape of the subthreshold local response associated with postrepolarization refractoriness and Wenckebach periodicity.

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