Aspects of Rate-Related Hyperpolarization in Feline Purkinje Fibers

DAVID J. BROWNING, JAMES S. TIEDEMAN, ADELBERT L. STAGG, DAVID G. BENDITT, MELVIN M. SCHEINMAN, AND HAROLD C. STRAUSS

SUMMARY We studied rate-related hyperpolarization (RRH) in feline Purkinje fibers by standard microelectrode techniques. Restrictions to ionic diffusion are shown to be less in this preparation than in canine preparations. The maximal hyperpolarization (V_H) and time constant (τ) for the exponentially decaying hyperpolarization following cessation of rapid drive were used as aggregate indices for the underlying rate of sodium pumping, the coupling ratio, and membrane resistance. Changes in V_H and τ in response to varying stimulation frequencies and durations of rapid drive, to cooling and addition of ouabain, and to changes in [K^+]_o and addition of cesium were used to assess the effects on RRH of sodium loading, pump inhibition, and short-circuiting potassium current, respectively. Steady state hyperpolarization was a linear function of stimulation frequency. Increased sodium loading led to an increase in V_H, but no change in τ. Onset and decay of RRH were symmetrical processes. The Qio's of τ and V_H were 1.57 and 2.00, respectively. For a time after exposure to ouabain, τ and V_H were unchanged; later, τ increased and V_H decreased, both linearly with time. Relative membrane resistance decreased with rapid drive and increased with an exponentially decaying time course following cessation of rapid drive. Cesium immediately increased V_H and decreased τ, and led to progressive deterioration of the fibers. From the steady state equation for RRH, we show that one need not invoke a change in coupling ratio to account for the observed steady state hyperpolarizations. From the nonsteady state equation, we show that the Qio for V_H is consistent with the Qio for Na^+, K^+-ATPase.

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The phenomenon of rate-related hyperpolarization (RRH), common to a variety of tissues, was first studied in the heart by Vassalle (1970). In sheep and canine Purkinje fiber preparations, this phenomenon consists of an initial depolarization (ID) at the onset of rapid stimulation, followed by a hyperpolarization, which is maintained as long as rapid stimulation is continued. When the stimulation frequency is abruptly reduced, a further terminal hyperpolarization (TH) ensues, and finally membrane potential decays back to the control level. In discussing RRH, Vassalle critically examined a variety of possible mechanisms and concluded that an electrogenic sodium pump accounted for his findings most satisfactorily. In this view, the ID seen at the onset of rapid stimulation in sheep and canine fibers arises from the Goldman diffusion potential as [K^+] in the extracellular space increases. Subsequently, an electrogenic pump is activated and hyperpolarization dominates, while extracellular [K^+] returns to resting levels (Vassalle, 1970; Kunze, 1977).

Implicit in this interpretation is the possibility that RRH may be used to study the electrogenic sodium pump in Purkinje fibers. Such an approach has previously been taken in many studies on nervous tissues, in which posttetanic hyperpolarization has been used to monitor electrogenic pumping, or in which the voltage clamp technique has allowed direct measurement of electrogenic current and estimation of the coupling ratio (Rang and Ritchie, 1968; den Hertog and Ritchie, 1969; Thomas, 1969; den Hertog, 1973; McDougal and Osborne, 1976).

In cardiac tissues, technical limitations have made the degree of control over experimental conditions that is obtainable in nervous tissue preparations only an ideal (Thomas, 1969; Beeler and Reuter, 1970; Johnson and Lieberman, 1971). However, answers to the analogous questions regarding the sodium pump in cardiac tissue are important, especially in view of the likelihood that pump rate will be a stronger function of [Na^+] than in tissues not regularly firing action potentials (Woodbury, 1963). As a first step in pursuing these questions in cardiac tissue, therefore, we sought a preparation in which membrane potential changes due to ionic depletion or accumulation in the extracellular space were minimized. On the basis of previous anatomical studies (Sommer and Johnson, 1968; Sommer and Johnson, in press) and preliminary experiments...
of our own, we chose feline Purkinje fibers as a preparation in which RRH reflects electrogenic pumping more specifically than in the canine and sheep preparations heretofore used.

For reasons discussed in Methods, we studied only the decaying limb of RRH after the termination of rapid drive. In early experiments, we verified that the decay of RRH is exponential in Purkinje fibers, in agreement with analogous studies in other excitable tissues (Rang and Ritchie, 1968; Thomas, 1969). We therefore felt justified in using the maximal value of hyperpolarization, $V_H$, and the time constant, $\tau$, for the decay of hyperpolarization as aggregate indices quantitatively characterizing the overall process underlying RRH. We have chosen the term "aggregate" in order not to imply specificity of $\tau$ or $V_H$ for any one component of RRH — pump rate, coupling ratio, or membrane resistance. Our first set of interventions was designed to vary the degree of sodium loading by varying either the basic frequency (BF) of stimulation, the high frequency (HF) of stimulation during rapid drive, or the duration of rapid drive. The next interventions, decreasing the temperature of the superfusate and applying ouabain, respectively, were designed to depress pump activity with sodium loading fixed. The last interventions were to vary the external ionic concentrations, $[K^+]_o$, and to apply cesium to change the potassium conductances that short circuit the electrogenic pump. All interventions were studied for their effects on $\tau$ and $V_H$. In other experiments, the time course of relative resistance following rapid stimulation was measured to define the time frame in which RRH may be considered proportional to electrogenic pumping.

From the information derived in these experiments, the relationship of electrogenic pumping to RRH is discussed, both for the steady state attained at the end of 3 or more minutes of rapid drive and for the nonsteady state conditions that exist until that time. The relations of the underlying determinants of RRH (pump rate, pump coupling ratio, ionic permeabilities, and intra- and extracellular ionic concentrations) to the aggregate indices $\tau$ and $V_H$ are examined.

**Methods**

A list of the symbols used in this study, along with their definitions, is found in the Glossary.

Cats weighing 1.5-3.5 kg were anesthetized with sodium pentobarbital, 30 mg/kg, ip, and the hearts were quickly removed and placed in a modified Tyrode's solution. Free-running Purkinje strands from either ventricle were dissected and secured under a flexible grid of fine plastic tubing in a Lucite bath with a volume of 14 ml. The preparation was superfused with a modified Tyrode's solution at 37°C ± 1°C (range) at a rate of 8-10 ml/min.

Fibers were stimulated with bipolar extracellular electrodes that were insulated except at the tips. Rectangular voltage pulses with amplitudes 1.5 times threshold and durations of 2-3 msec were generated by a Grass S-4 stimulator and delivered

<table>
<thead>
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<th>Glossary</th>
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<tr>
<td>BF = basic frequency of stimulation</td>
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<td>HF = high frequency of stimulation</td>
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<td>ID = initial depolarization of the maximal diastolic potential at the onset of rapid stimulation</td>
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<td>$J_D(t)$ = rate of sodium pumping as a function of time</td>
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<td>$\Delta J_D(t)$ = increase in rate of sodium pumping with rapid stimulation</td>
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<td>MDP = maximal diastolic potential</td>
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<td>$R_m(t)$ = membrane resistance as a function of time</td>
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<td>$R_m(t)$ = relative membrane resistance as a function of time</td>
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<tr>
<td>$r(t)$ = coupling ratio of the Na+, K+ pump as a function of time</td>
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<tr>
<td>RRH = rate-related hyperpolarization</td>
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<td>TH = terminal hyperpolarization of the maximal diastolic potential after the end of rapid stimulation</td>
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<td>$\tau$ = the time constant for decay of rate-related hyperpolarization after cessation of rapid drive</td>
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<tr>
<td>$V_H(t)$ = the hyperpolarization of the maximal diastolic potential as a function of time after cessation of rapid drive</td>
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<tr>
<td>$V_H$ = the maximal hyperpolarization of the maximal diastolic potential after cessation of rapid drive; in feline Purkinje fibers, which generally lack terminal hyperpolarization, $V_H$ is generally the same as $V_H(0)$</td>
</tr>
<tr>
<td>$V_H(\infty)$ = the hyperpolarization at steady state of the maximal diastolic potential at basic frequencies more rapid than 0.5 Hz; measured in mV positive to the hyperpolarization at 4 Hz</td>
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<tr>
<td>$V_o(t)$ = the hyperpolarizing displacement of the membrane potential by an anodal current pulse during measurements of relative membrane resistance as a function of time after cessation of rapid stimulation</td>
</tr>
<tr>
<td>$V_o(\infty)$ = the hyperpolarizing displacement of the membrane potential by an anodal current pulse during measurements of relative membrane resistance when recovery from rate-related hyperpolarization is complete</td>
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</table>
to the electrodes after passage through a Grass SIU 478A stimulus isolation unit. Pulse frequency was set by a custom-built programmable stimulator and monitored on a Hewlett Packard 5212A electronic counter. The basic frequency (BF) was 1 Hz and the high frequency (HF) was 4 Hz, except in those experiments in which these quantities were the ones varied. From 10 to 15 minutes separated each run, comprised of the stimulation sequence BF followed by HF followed by BF.

Transmembrane potentials were recorded with microelectrodes filled with 3 M KCl and having tip resistances of 10–20 MΩ. A short chloridized silver wire connected the microelectrode to the input of an emitter follower with input capacitance neutralization, the output of which was displayed on a Tektronix 565 oscilloscope.

Using a previously described (Tiedeman et al., 1975) circuit for measuring maximal diastolic potential (MDP), we attained much greater amplification than usual over the voltage range neighboring the MDP, without the artifacts inevitably resulting from high gain settings on oscilloscopes. A graphic presentation of the data was obtained by connecting this circuit to a Hewlett-Packard 7004 B X-Y plotter. This system allowed measurements of MDP to be made to a precision of 0.05 mV.

Solutions

The basic solution used was a modified Tyrode's solution of the following composition (in mM): NaCl, 135.4; KCl, 4.0; NaH₂PO₄, 1.8; CaCl₂, 2.7; MgCl₂, 0.5; NaHCO₃, 18; and dextrose, 5.5. The solution was gassed with a mixture of 95% O₂-5% CO₂. In one set of experiments, [K⁺] was varied to 2.7, 5.4, and 8.1 mM. For experiments in which ouabain was used, solutions were brought to 10⁻⁴ M from a 1 M stock solution of the drug (Sigma Chemical Co.). An analogous procedure was used in making solutions containing cesium (Sigma Chemical Co.).

Rapid Solution Changes

In one set of experiments, the superfusing solution had to be rapidly changed from one with a potassium concentration of 2.7 mM to one with 8.1 mM. For these experiments the bath was filled with paraffin except for a central channel of volume 0.5 ml. Solution changes were made by turning a stopcock on a manifold. The solutions were run through the channel at rates exceeding 10 ml/min, so that potassium concentration in the bath changed almost instantaneously. The change in the measured MDP therefore followed the time course for reequilibration of the extracellular space with the new solution, without bath reequilibration time affecting the result.

Resistance Measurements

Relative resistance measurements were made by positioning a current-passing electrode within approximately 100 μm of a recording electrode and passing anodal current pulses as previously described (Geduldig, 1968). After cessation of rapid stimulation, anodal current pulses 200 msec in duration were injected following every fifth action potential at a constant time during diastole.

The amplitude of the current pulse was selected to achieve hyperpolarizations of 3–4 mV at the recording electrode. The displacement in membrane potential at the recording electrode was recorded on the X-Y recorder and on 35-mm film with a Grass C4 Kymograph camera. The equation used to compute relative resistance is derived from the equation given by Geduldig (1968) by assuming constant internal longitudinal resistance, R:

$$R_{rel}(t) = \frac{[V_o(t)]^2}{[V_o(\infty)]^2},$$

where $V_o(t)$ = membrane potential displacement at time $t$ following cessation of rapid stimulation, and $V_o(\infty)$ = membrane potential displacement in the steady state at the BF.

Data Analysis and Presentation of Results

The data consisted of curves representing MDP vs. time during and after rapid stimulation. Figure 1, which includes results of representative experiments on both cats and dogs, illustrates the reasons why only the decay limbs of these curves were analyzed. First, the baseline drift from pre- to post-HF amounts to 0.35 mV. This was a typical magnitude for such drifts, which were always less than 0.4 mV and were random with regard to whether they were in the hyperpolarizing or depolarizing direction. To minimize this inherent error of up to 0.4 mV relative to the magnitude of the measured quantity, $V_H$, we chose an HF higher than that of Vassalle (1970). The chosen HF of 4.0 Hz in many cases led to the phenomenon of two or more levels of repolarization during the period of rapid drive, as shown in Figure 1. This scatter in the onset limb of the curve precludes an accurate measurement of the time course. The decay limb corresponds to stimulation at the BF, for which action potential repolarization is complete. The magnitude of the discrepancy between incomplete repolarization at the HF and complete repolarization at the BF is given by the abrupt hyperpolarization seen for the first action potential after cessation of rapid drive. This should be distinguished from the slowly developing TH as shown in the record from the dog. Figure 1 displays several of the quantities referred to repeatedly in this paper.

For the purposes of curve fitting, the first 30 seconds of the decay curve were ignored as being the time interval during which the possibility of potassium depletion in the extracellular space was greatest (Kunze, 1977) and when membrane resistance deviated from its steady state value by the greatest amount (see Results). Because of the variability in absolute time constants and absolute maximal hyperpolarizations among Purkinje fibers during recovery from RRH, we have expressed val-
Values of $\tau$ and $V_H$ in all figures and tables as fractions of control values, unless otherwise noted. The control values of $\tau$ and $V_H$ were obtained as the means from several control runs (from two to five) performed prior to any intervention. Thus, following the method of McDougal and Osborne (1976), we were able to pool data from several fibers. So that rough conversions from normalized to absolute values of $\tau$ and $V_H$ might be made, the mean values $\pm$ SEM of $\tau$ and $V_H$ in 57 fibers (130 runs) were 59.5 $\pm$ 1.1 msec and 7.6 $\pm$ 0.1 mV, respectively.

Tests of statistical significance were performed by Student's $t$-test for equality of means with either equal or unequal variances as dictated by comparison of the variances of the pairs of points concerned (Snedecor and Cochran, 1967). 

Results

Choice of Preparation

A primary goal of our experiments was to have membrane potential changes reflect as specifically as possible the changes in electrogenic sodium pumping. Since changes in membrane potential during recovery from rapid stimulation can also reflect the effects of potassium depletion in the extracellular space, it is necessary to choose a preparation in which diffusion between extracellular space and superfusate is minimally restricted. On these grounds, anatomical data (Sommer and Johnson, 1968; Sommer and Johnson, in press) suggested the greater suitability of feline as opposed to canine Purkinje fibers. The experiments depicted in Figures 1 and 2 provide electrophysiological support for this suggestion.

Figure 1 shows patterns of RRH in the dog and the cat, drawn from two representative experiments. In agreement with Vassalle (1970), we found in the dog ID's at the beginning of rapid stimulation and TH's at the end. These were rarely seen in the cat and, when seen, were neither as large in amplitude nor as long in duration. In 12 experiments in dogs, the amplitudes of ID and TH averaged 1.46 $\pm$ 0.11 and 1.43 $\pm$ 0.15 mV, respectively, and the durations, as measured between onset and complete return from ID (or TH), averaged 47.9 $\pm$ 4.7 and 64.3 $\pm$ 9.3 sec, respectively. ID and TH values greater than 0.4 mV were seen in only 21% of cats ($n = 57$), and none was larger than 1 mV. The species variation in patterns of RRH can be explained on the basis of cleft size; the ID seen in the dog with the onset of rapid stimulation reflects the greater restriction to diffusion presented by its narrower clefts, leading to potassium accumulation.
Effects of Changes in BF, HF, and Duration of Stimulation

To determine the steady state characteristics of the mechanism underlying RRH, we performed experiments in which the frequency of stimulation was changed abruptly, in steps of varying magnitude. In one set of experiments, BF was kept constant at 0.5 Hz and runs were performed at HF values of 1, 2, and 4 Hz. In another set, HF was kept constant at 4 Hz and runs were performed at BF values of 0.50, 0.67, 1.00, and 1.33 Hz. In both sets of experiments, an interval of 3 minutes was allowed to elapse after changing to HF; this interval was sufficient for the MDP to reach steady state. Stimulus frequency was then abruptly returned to the basic level, and the decay of MDP was measured for \( \tau \) and \( V_H \).

The results of these sets of experiments are recorded in Table 1 and reveal two similarities. Neither changes in BF nor in HF affected \( \tau \) significantly. On the other hand, \( V_H \) depended in a linear manner on both BF and HF. As HF was increased, \( V_H \) increased, and as BF was decreased, \( V_H \) increased. These relations are illustrated in Figure 3, which includes a plot of \( V_H \) and \( V_H(\infty) \) vs. stimulation frequency, incorporating the data from both sets of experiments. The symbol \( V_H(\infty) \) refers to the hyperpolarization that persists even at steady state at any BF greater than 0.5 Hz; it is measured in millivolts positive to the MDP at 4 Hz. The justification for pooling the data as we have is that, in any given fiber, \( V_H \) (measured after stimulating at a higher frequency until a steady state MDP is reached) was observed to be a linear function of the increment in excitation frequency as well. For example, \( V_H \) measured after changing from a stimulation frequency of 1 Hz to one of 4 Hz was consistently observed to equal the sum of the \( V_H \) measured after changing the frequency from 1 to 2 Hz, plus the \( V_H \) measured after changing the frequency from 2 to 4 Hz, provided enough time was allowed to reach steady state at each frequency.

The degree of sodium loading was varied in yet a third way, by changing the duration of rapid stimulation. As shown in Table 1, \( \tau \) was independent of sodium loading by this method as well. With regard to \( V_H \), its measurement as a function of duration of rapid drive provides a means of unmasking the "true" MDP vs. time relationship for the hyperpolarizing limb of RRH, since \( V_H \) corresponds to a fully repolarized action potential after cessation of rapid drive. When \( V_H \) was plotted against duration of rapid drive for each run, the mean \( \tau \) for onset of hyperpolarization was 61 ± 4 seconds, whereas that for recovery was 60 ± 12 seconds. Therefore, onset and recovery from RRH are symmetrical processes for the higher values of HF used here, as they apparently were at the lower values of HF used by Vassalle (Vassalle, 1970; Vassalle and Carpentier, 1971; Carpentier and Vassalle, 1971).

Effects of Temperature

Since cooling is known to reduce pump activation (Page and Storm, 1965; Tamai and Kagiya, 1968;
Effects of Ouabain

Carpentier and Vassalle (1971) found that strophanthidin enhanced the initial depolarization and abolished the subsequent hyperpolarization in lamb Purkinje fibers. In the present experiments, we sought a more detailed understanding of the effects of cardiac glycosides on RRH, with emphasis on the time courses of these effects, since the effects of glycosides on the underlying Na⁺,K⁺-ATPase are known to be progressively time-dependent. After performing from two to five control runs, we changed the superfusate to Tyrode's solution containing 10⁻⁷ M ouabain. Starting at 5 minutes after application of the drug, runs were performed every 10 minutes until the fiber became either inexcitable or automatic to the point that further runs were impossible.

The first effect consistently noted was a gradual conversion of the RRH pattern of the cat into a pattern more resembling that in the dog or sheep, as illustrated by the representative experiment of Figure 4. Specifically, ID's and TH's begin to appear (panel B) and progressively grow in amplitude in successive runs (panel C). Concomitantly, the onset of exponential decline of the hyperpolarization is shifted to progressively later times, and in fact the fit one obtains using an exponential becomes poorer. Nonetheless we continued to impose the fit and to record an "apparent" time constant \( \tau \). Subsequently, \( \tau \) starts to increase and \( V_H \) to decrease, both effects being progressive with time. Eventually, hyperpolarization is abolished, and later runs show depolarizations during the period of rapid drive. Characteristically, these show a rapid first phase and a slower second phase (panel D). By the time this effect is noted, the MDP at the BF is no longer stable. Instead it shows a slow depolarizing trend, presumably as the reduced number of pumps increasingly fails to maintain the ionic gradients across the membrane.

The combined results from all fibers are shown in Figure 5. The progressive increase in \( \tau \) is apparent in panel A, as is the progressive decrease in \( V_H \) in panel B. The change in \( \tau \) achieves statistical significance earlier than does the change in \( V_H \), and the rate of change of \( \tau \) is greater than that of \( V_H \). Both

Hiraoka and Hecht, 1973), we performed experiments in which the effects on RRH of cooling from 37°C to 32°C and 27°C were examined. The results, recorded in Table 1, show that cooling led to an increase in \( \tau \) and a decrease in \( V_H \). The \( Q_{10} \) for \( \tau \) in this temperature range is calculated to be 1.57, whereas that for \( V_H \) is 2.00.

### Table 1 Effects on RRH Caused by Various Interventions

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Value of in-dependent variable</th>
<th>No. of fibers</th>
<th>[K⁺]o (mM)</th>
<th>( \tau )</th>
<th>SEM</th>
<th>( P &lt; )</th>
<th>( V_H )</th>
<th>SEM</th>
<th>( P &lt; )</th>
</tr>
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<tbody>
<tr>
<td>Changing BP</td>
<td>(Hz) 6.0</td>
<td>1.33</td>
<td>0.90</td>
<td>0.032</td>
<td>NS</td>
<td>0.79</td>
<td>0.041</td>
<td>0.005</td>
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<td></td>
<td></td>
<td>1.00</td>
<td>1.00</td>
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<td>1.00</td>
<td>0.010</td>
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<td></td>
<td>0.67</td>
<td>1.03</td>
<td>0.048</td>
<td>NS</td>
<td>1.25</td>
<td>0.029</td>
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<td>0.50</td>
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<td>NS</td>
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<td>0.067</td>
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<td>Changing [K⁺]o</td>
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<table>
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<th>( R_m ) at end of drive</th>
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Table 1. Normalised values of \( \tau \) and \( V_H \) for six different sets of experiments. In each set, the control values used for normalisation are those for which no \( P \) value is given. NS = not significant.
FIGURE 3 Linear relationship between steady state hyperpolarization and stimulation frequency. Pooled data from experiments in which the high frequency (HF) was held fixed and the basic frequency (BF) varied, and experiments in which BF was held fixed and HF varied. Left: Records of the decay limb of RRH from a representative experiment in which HF was held fixed at 4 Hz and BF varied to 0.50, 0.67, 1.00, or 1.33 Hz. The time constant for each curve is marked by a vertical bar, and, as shown, all lie within 8 seconds of each other. Right: Records of the decay limb of RRH from a representative experiment in which BF was held fixed at 0.5 Hz and HF varied to 4.00, 2.00, or 1.00 Hz. The time constants for each curve are marked and are independent of degree of sodium loading. Middle: Combined results from both types of experiments. The unfilled circle represents only experiments in which HF was varied. The unfilled triangles represent only experiments in which BF was varied. The filled triangles combine data from both types of experiments. The ordinate on the left refers to experiments in which HF was varied, as in the panel on the left. The ordinate on the right refers to experiments in which BF was varied, as in the panel on the right. The reference point for these experiments is the steady state value of MDP at 4 Hz (i.e., at the HF held constant throughout these experiments) and $V_H$ is measured in mV positive to this reference. The ordinate on the right refers to experiments in which HF was varied, as in the panel on the right. The reference point is the steady state value of MDP at 0.5 Hz (i.e., at the BF held constant throughout these experiments), and $V_H$ is measured in mV negative to this reference. Error bars represent ± SEM.

FIGURE 4 Effects of ouabain on RRH in feline Purkinje fibers. A: Control record before the application of ouabain. No ID or TH is seen. B and C: Records taken at 45 and 95 minutes, respectively, following the application of ouabain $10^{-7}$ M. The increase in $\tau$, decrease in $V_H$, and appearance of ID's and TH's are apparent. The decaying limbs of RRH in these records are not fit as well by exponential functions. D: Record taken at 105 minutes of exposure to ouabain. Hyperpolarization is abolished and a monotonic, biphasic depolarization during rapid stimulation is observed instead. Slow depolarization of the MDP is evident even at BF.
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### Table 2

<table>
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<th>Time after addition of ouabain (min)</th>
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### Figure 5

**Time course of effects of ouabain on RRH.**

A: Effects on TH. After a latent period, TH increases linearly with time. B: Effects on VH. After a longer latent period than that for TH, VH decreases linearly with time. Error bars represent ± SEM.

### Effects of [K+]o

For reasons discussed later, the experiments in which we varied [K+]o between 2.7 and 8.1 mM can be regarded primarily as manipulations of potassium, and hence membrane, conductance. The results of these experiments are found in Table 1.

Increasing [K+]o has no effect on TH. After a latent period, TH increases linearly with time. The progressive increase in the amplitude of the TH's with time after addition of ouabain is documented in Table 2.

### Relative Resistance Measurements

Changes in relative resistance (Rrel) following termination of rapid drive were measured by injecting constant current pulses during diastole. We found that at a [K+]o of 4.0 mM, Rrel was not constant, but rather increased in an exponential fashion from an initial low value to the control value measured at BF. In seven runs, the mean initial value following termination of rapid drive was 77 ± 7% of control, and the mean time constant for recovery was 27.7 ± 2.6 seconds. The results of a representative experiment are depicted in Figure 6, which shows a semilogarithmic plot of 1 - Rrel vs. time past cessation of rapid drive. The points are well fit by an exponentially decaying increase in Rrel.

### Effects of Cesium

Cesium, which has been proposed as a specific inhibitor of iK1 and iK2, and an activator of sodium pumping for [K+]o less than 5.4 mM (Isenberg, 1976), provided a particularly important intervention in confirming the electrogenic mechanism of RRH and in studying the magnitude of the short-circuiting potassium currents. To maintain comparability with our other experiments, [K+]o was kept at 4.0 mM, at which some small activation of sodium pumping by the addition of Cs+ is expected.
The first effect of Cs⁺ was an immediate depolarization of the membrane from 92.9 ± 2.2 to 66.5 ± 2.3 mV at 10 mM (n = 5). Both 10 and 20 mM Cs⁺ produced shortening of the action potential duration, loss of plateau, and decrease in MDF and phase 0 V_{max}. For short (less than 45-minute) periods in cesium-Tyrode’s solution, the effects were reversible; however, longer periods led to only partially reversible changes.

Cesium reduces the closeness with which one can fit recovery of RRH by an exponential, and therefore we measured an apparent time constant τ. As measured in Table 1 documents, 10 mM Cs⁺ led to a 30% decrease in τ and a 50% increase in V_H. Both effects were only slightly augmented with 20 mM Cs⁺. Relative resistance measured at the BF increased by 153 ± 3.0% (n = 2) after exposure to 10 mM Cs⁺ at [K+]o = 5.4 mM. In these two experiments, Cs⁺ eliminated the time dependence of R_{rel} following cessation of rapid drive.

The records from two experiments are shown in Figure 7. Panel A shows the recovery from RRH corresponding to the last control run preceding a solution change to 10 mM Cs⁺-Tyrode’s solution. Similarly, panel B shows recovery from RRH for the first run following a change to Cs⁺-Tyrode’s solution (10 minutes after the change). The decrease in τ, increase in V_H, and increase in area under the curve are apparent. In contrast, panel C shows the recovery from RRH corresponding to a run 45 minutes after changing to Cs⁺-Tyrode’s solution. Comparison with the run in panel B shows that τ is still reduced but that V_H has fallen, so that the area under the curve is less in C than in B.

Discussion

Minimizing Potential Changes Due to Diffusion

Previous studies focusing on the phenomenon of RRH in cardiac tissue have used sheep, lamb, and canine Purkinje fibers (Page and Storm, 1965; Vassalle, 1970; Carpentier and Vassalle, 1971). Useful qualitative data were thereby obtained; however, if the phenomenon is to yield fruitful data on the specific properties of the electrogenic sodium pump, the complicating membrane potential changes due to restricted diffusion of ions in the intercellular clefts should be minimized (Sommer and Johnson, 1968; Sommer and Johnson, in press). The anatomical study of Sommer and Johnson indicates that guinea pig, rabbit, and feline fibers are ideal in this respect, with canine fibers less so. From the first group of fibers we confined our attention to feline fibers and in preliminary reequilibration experiments compared them to canine fibers. The faster reequilibration of extracellular space and the different patterns of RRH are two functional correlates to the structural observation of clefts 1 μm wide in the cat as compared to 30 nm clefts in the dog (Sommer and Johnson, 1968).

Sodium Loading Experiments

No direct evidence has been obtained concerning whether or not [Na⁺]i, increases with an increasing HF-BF difference or longer duration of HF. A study by Brody and Akera (1977) suggests that no increase occurs, but a serious reservation is that the whole tissue sodium measurements used in this study are insufficiently sensitive to detect small changes in [Na⁺]. Most of the indirect evidence available indicates that [Na⁺] should increase under these circumstances to balance the documented loss of potassium (Langer, 1967, 1968; Langer and Brady, 1974), and we favor this hypothesis.

The lack of dependence of τ on HF-BF difference and on duration of HF differs from observations on rate constants (reciprocal time constants) for recovery from posttetanic hyperpolarization in some nerve preparations (McDougal and Osborne, 1976). On the other hand, our finding is consistent with the evidence in which Thomas (1969) showed the pump rate constant to be independent of [Na⁺]i in snail neurons as measured directly with a sodium-sensitive microelectrode.

The observation that a steady state V_H depends linearly on frequency of stimulation has implications regarding the coupling ratio of the sodium pump. Langer has reported an approximately linear relationship between net sodium efflux (and influx) and stimulation frequencies for frequencies up to 35 beats/min at steady state (Langer, 1967; Langer and Brady, 1974). Since sodium influx occurs mainly during phase 0, which is little affected by high frequencies of stimulation, it is likely that the linear relationship also obtains at higher frequen-

![Figure 7](https://circres.ahajournals.org/doi/figure/10.1161/01.RES.44.5.620)
cies. On the other hand, potassium efflux at steady state is known to be independent of stimulation frequency at frequencies up to 80 beats/min (Langer and Brady, 1966, 1974). At higher frequencies it is likely that potassium efflux increases as well, although not as rapidly as sodium efflux (Langer and Brady, 1974).

To explain the imbalance between sodium influx and potassium efflux, Langer (1968) has hypothesized that the increased sodium influx is discharged by an increased electroneutral exchange with calcium. Our observation that \( V_H \) increases linearly with increasing frequency of stimulation raises the possibility that not all the resulting sodium efflux is coupled to calcium influx. It is possible that an increasing part of the sodium efflux is uncoupled to account for this, thereby increasing the coupling ratio.

Although the experimental data that would enable us to answer this question directly are not available, one can show on theoretical grounds that it is not necessary to invoke an increase in coupling ratio to account for the observed potential changes at steady state during rapid stimulation. One can calculate from (1) Thomas' (1972) equation for the electrogenic component of the membrane potential, (2) estimates for the change in \([K^+]\), with stimulation frequency changes measured by Langer and Brady (1966, 1974), (3) the assumption that the change in \([Na^+]\), would be equal in magnitude and opposite in sign to the change in \([K^+]\), (Langer and Brady, 1966, 1974), and (4) the assumption that \( r = 3:2 \) (Thomas, 1972), that \( P_K \) must increase by a factor of 1.61 to yield the observed 9.57-mV hyperpolarization in changing stimulation frequency from 0.5 to 4 Hz (see Fig. 3) without invoking a change in \( r \). From independently obtained data relating membrane conductance to potential (Carmeliet, 1961, (Fig. 27)) and the relations between \( P_K \), \([K^+]\), \([K^+]_o\), and \( G_K \) predicted from constant-field theory, one can calculate that \( P_K \) increases by a factor of 1.66 for the 9.57-mV hyperpolarization. Thus, although this rough calculation does not provide actual estimates for the changes in \( P_K \) and \( r \), it makes the point that the expected change in \( P_K \) is great enough to account for the steady state \( V_H \) values we observed, without the necessity of invoking a change in \( r \). This argument, however, does not apply to the nonsteady state situations obtaining during onset of and recovery from RRH, when net ionic flux occurs, and to which the steady state equations do not apply (see below).

**Pump Inhibition Experiments**

The experiments in which Purkinje fibers were cooled before rapid stimulation yielded \( Q_{io} \) values for \( \tau \) and \( V_H \) of 1.57 and 2.00, respectively. These values must be interpreted with caution because, for example, the \( Q_{io} \) of \( \tau \) is a composite quantity in that it is the product of \( Q_{io} \) values for the time constants of pump current and membrane resistance (Bayliss, 1959). Similarly, the \( Q_{io} \) of \( V_H \) is the product of the \( Q_{io} \) values for the magnitudes of pump current, membrane resistance, and coupling ratio. These statements follow from the fact that \( V_H \) during nonsteady state is given not by Thomas' equation (1972), which applies to the steady state, but rather by a form of Ohm's law: \( V_H (t) = \text{change in electrogenic current} \times \text{membrane resistance} \). In this equation, any change in passive currents due to internal ionic concentration changes has been neglected as being small relative to pump current. If we let \( J_p (t) \) equal the rate of sodium pumping in \( m/\text{cm}^2 \cdot \text{sec} \), \( r(t) \) be the coupling ratio, and \( R_m(t) \) be the membrane resistance in \( \Omega \text{cm}^2 \), all as functions of time, then from the fact that:

\[
\text{change in electrogenic current} = (\Delta \text{sodium pumped} - \Delta \text{potassium pumped}) \cdot F
\]

\[
= [\Delta J_p (t) - \frac{1}{r(t)} \Delta J_p (t)] \cdot F,
\]

it follows directly that:

\[
V_H(t) = \frac{r(t) - 1}{r(t)} \cdot F \cdot \Delta J_p (t) \cdot R_m (t).
\]

In erythrocytes and nerve membranes, the term \( \Delta J_p (t) \) includes a sigmoid dependence both on \([Na^+]\), and on \([K^+]_o\), (Garay and Garrahan, 1973; McDougal and Osborne, 1976). In addition, it has been suggested that \( \Delta J_p (t) \) depends on stimulation frequency itself (Prasad et al, 1978).

From the \( Q_{io} \) of 2.00 for \( V_H \) and the mean \( Q_{io} \) of 0.67 measured for \( R_m \) by Coraboeuf and Weidmann (1954) in sheep Purkinje fibers, we can estimate a \( Q_{io} \) of 3.00 for the product of \( Q_{io} \) values for the maximal values of \( \Delta J_p \) and \( r \). If we assume the coupling ratio to be unchanged between 27 and 37°C, this \( Q_{io} \) applies to \( \Delta J_p \) and is close to the \( Q_{io} \) of 3.4 for \( Na^+,K^+\)-ATPase, calculated from the activation energy reported by Charnock et al. (1971). Thus, it seems likely that the stoichiometry of the pump is unchanged over the range of temperatures we considered.

In the experiments in which \( 10^{-7} \text{ M ouabain} \) was applied to the Purkinje fibers, the observed effects were descriptively complex. In interpreting these effects, the fact that ouabain noncompetitively inhibits the underlying \( Na^+,K^+\)-ATPase [Gibson, 1973 (Fig. 3)] seems to cast the most light on the results.

The most likely explanation for the ouabain-induced conversion of the feline RRH pattern to the canine pattern (Figs. 1 and 4) involves the reduced rate of ionic pumping in the face of undiminished \( K^+ \) efflux per action potential, with the resulting likelihood that \( K^+ \) accumulates in the clefts. As a noncompetitive inhibitor of \( Na^+,K^+\)-ATPase, ouabain reduces the number of pumps without, however, changing the dependence of the remaining pumps on \([Na^+]\). (Gibson, 1973). Thus, fewer pumps must turn over faster to discharge the \( Na^+ \) load.

* The detailed calculation may be obtained from the authors upon request.
imposed by HF, which in turn requires [Na\(^+\)]
 to increase to higher levels the greater the number of
muscle was less than for early runs, when more pumps
were available to balance the loss. The particular
balance between cleft size and the ability to dissi-
pate K\(^+\) efflux, which in the control fiber is mani-
fested as no or minimal ID's or TH's, is therefore
disrupted progressively. As Table 2 documents, for
later runs, accumulation and depletion of potas-
sium, measured as larger ID and TH values, become
greater at the beginning and after the end of rapid
drive, respectively.

Those sodium pumps that are still active are
driven further up the sigmoid-shaped pump activa-
tion curve (Garay and Garrahan, 1973; McDougal
and Osborne, 1976) to discharge the sodium load.
When they reach the saturation plateau of this
curve, they are unable to balance sodium influx at
rapid drive, and this is reflected in a monotonic but
biphasic decline in MDP throughout the period of
rapid stimulation (Fig. 4D). A simple test of this
hypothesis was to reduce HF. With the smaller K\(^+\)
efflux thereby produced, one would expect the
smaller number of pumps to be able to recover the
deficit and hence return the pattern of RRH back
to that observed in the control state. When we did
this experiment, the expected result was observed.

Interventions Changing Membrane
Resistance

Considering now the results of experiments in
which [K\(^+\)]\(_o\) was varied between 2.7 and 8.1 mm,
the observed good agreement between the changes in
R_{rej} and V_H supports the view that the decrease in
V_H at higher [K\(^+\)] values was caused by a reduc-
tion in R_{rej}. Such an interpretation is consonant with
the known increase in potassium conductance with
increasing [K\(^+\)]\(_o\) (Carmeliet, 1961). The lack of ef-
flect on τ of changing [K\(^+\)], over the range we did
contrasts with the results of similar experiments in
rabbit cervical vagus, in which increasing [K\(^+\)], led
to a decrease in the time constant for decay of
posttetcnic hyperpolarization (Rang and Ritchie,
1968). Our result is further evidence that the sodium
pump in cardiac tissue has a weaker dependence on
[K\(^+\)] than have sodium pumps in other excitable
tissues (Bosteels and Carmeliet, 1972; Isenberg,
1976).

The correct interpretation of the decreased rela-
tive resistance immediately after cessation of rapid
stimulation, which then increases toward control
value with an exponentially declining time course,
is undoubtedly complex. The exponentially declining
increase can be interpreted in terms of two
changes associated with the recovery limb of RRH.
First, in analogy to frog sartorius muscle, a gradual
decline in pump rate after sodium loading, with
possible internal ionic concentration changes, might
be associated with increased membrane resistance
(Harris and Ochs, 1966; Geduldig, 1968). Second,
membrane resistance is known to increase with
membrane depolarization (Carmeliet, 1961; Hellam
and Studt, 1974). However, since both factors de-
depend directly on the time course of pump rate, one
would expect the time constants for recovery of R_{rej}
and from RRH to be similar. The observed discrep-
ancy of 32 seconds demonstrates, therefore, that
the issue remains unresolved.

In the experiments in which we added cesium, 10
mm, we observed V_H to increase by 150%. Part of
this increase can be attributable to an increased
membrane resistance amounting to 153% in our
measurements of R_{rej}. Moreover, the increased
membrane resistance can be attributed to blockade
of potassium channels in Purkinje fibers and not
simply to depolarization into a voltage range in
which anomalous rectification increases R_{rej}. Figures
3 and 4 in Isenberg's paper (1976) show that Cs
abolishes such anomalous rectification. Despite the
relative agreement between the increases in V_H and
R_{rej}, however, it is unlikely that an increased resist-
ance accounts for the total effect of Cs\(^+\) on V_H, in
view of the pump stimulation that Cs\(^+\) is known to
cause at a [K\(^+\)]\(_o\) of 4.0 mm (Isenberg, 1976).

The stimulus of Cs\(^+\) to pump activity probably
accounts for the decrease of τ by 30%. The possi-
bility cannot be ruled out, however, that a Cs\(^+\)-
induced change in r(t) contributes to the fall in τ
for V_H(t). In the two fibers in which we followed
recovery of R_{rej} after cessation of rapid stimulation,
R_{rej} remained constant, so that it seems unlikely
that a change in R_{rej} (t) contributed to the fall in τ
with addition of Cs\(^+\).

As a blocking agent of i_{Na} and i_{K}, Cs\(^+\) would be
predicted to increase the average apparent coupling
ratio given by:

\[
\text{Average apparent coupling ratio} = \frac{\text{(net extruded Na})}{\text{(net extruded Na}^+ - \text{electrogenically extruded Na})}.
\]

The average apparent coupling ratio reflects not
only r(t) but also the degree of short circuiting by
potassium. Since Cs\(^+\) blocks short-circuiting potas-
sium ions, the average apparent coupling ratio
should increase in the presence of Cs\(^+\) and approach
the time-averaged value of r(t). Comparison of the
area under the curves in Figure 7, A and B, will
demonstrate that these relations do obtain.

The area under the curve in Figure 7B, after
correction for the increased resistance in the
presence of Cs\(^+\), is still 1.90 times the area under
the curve in Figure 7A. This corrected area corre-
sponds to the net electronegatively extruded sodium. In view
of the fact that net extrusion of sodium will be less in
the presence of Cs\(^+\) (since the sodium load in-
curred by rapid stimulation is less at the depolarized
membrane potentials seen with 10 mm Cs\(^+\)), this
implies that the average apparent coupling ratio
must be larger. A result this unequivocal was not
seen in every experiment, since in the 11 experi-
ments performed, the mean increase in area was
only 60 ± 43% after correction for increased mem-
brane resistance. Consequently, in these cases we
cannot draw any conclusions about change in the
coupling ratio.
In contrast, the area under the curve in Figure
8C, in which the fiber had been exposed to 10 mM
Cs\(^+\) for 45 minutes, is less than the area under the
curve in Figure 7A. We cannot make any definite
comparison between the average apparent coupling
ratio in the presence and absence of Cs\(^+\) for this
case, because the terms defining the ratio are chang-
ing in different directions, producing opposite ef-
fects on the ratio, unlike the case in Figure 7, A and
B. The point this figure makes, therefore, is that
Cs\(^+\) is not an ideal blocking agent for \(i_h\) and \(i_n\),
because the fiber tends to deteriorate in the pres-
ence of Cs\(^+\), rendering the effects of the ion time-
dependent.

To summarize the present study, we have exam-
ined the steady state relation of hyperpolarization
to stimulation frequency as well as the nonsteady
state process of recovery from RRH in terms of the
aggregate quantities \(\tau\) and \(V_H\). Although our exper-
imental design does not permit us to determine the
functional relationships of the more fundamental
quantities (change in rate of sodium pumping \(\Delta J_\text{Na}(t)\), coupling ratio \([r(t)]\), ionic conductances
\([R_m(t)]\), and intra- and extracellular ionic concen-
trations) with time, we have been able to make a
semiquantitative assessment of these functions and
indicate the tendencies of the steady state values of
\(J_\text{Na}\), \(r\), and \(R_m\) at different frequencies.

We have found that the steady state hyperpolar-
ization is a nonlinear function of stimulation frequen-
cy, and that no increase in coupling ratio need be
invoked to account for the observed hyperpolar-
izations at steady state. The time constant for re-
covery from RRH was observed to be independent
of interventions designed to change the degree of
sodium loading. Membrane resistance, as reflected
in \(R_m\), was found to increase in an exponentially
decaying manner on abrupt return to lower fre-
quencies. Finally, the short circuiting effects of po-
tassium ions were observed to be substantial in
experiments in which Cs\(^+\) was applied.

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Mechanism of Augmented Premature Responses in Canine Ventricular Muscle

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SUMMARY In an effort to determine the mechanism inducing paradoxical augmentation of the very premature action potential (AP), we performed the following experiments. Premature stimuli (S1) were applied to isolated canine ventricular muscle at variable intervals after every 10th to 15th driving stimulus (S0) at 0.5–1 Hz. Action potentials (R1 and R2) elicited by S1 and S2 of equal strength and duration were recorded by conventional microelectrode methods. Measurements were made of the amplitude (Amp), duration (APD), plateau area (PA), and maximal rising velocity (Vmax) of each AP and the proximity (P), the interval from 90% repolarization point of R1 to S0. In normal K+ solution, R1 had a greater plateau area (PA1) than R2 at P less than 300 msec. Moreover, PA1 increased progressively with decreasing P at [Ca++] of 0.4, 1.6, and 6.4 mM and reached its maximum at P of 40–90 msec. The maximum AP [(PA1 - PA1)/PA1 x 100] was estimated at 15 ± 9, 28 ± 6, and 61 ± 11% at each [Ca++]+, which showed that AP1 increased significantly (P < 0.01) as the [Ca++]+ increased. Verapamil (10-6 M) suppressed completely this augmentation in the plateau of the premature AP. In addition, experiments in high K+ (21 mM)-high Ca++ (6.4–10 mM) solutions revealed that the Amp, APD, and Vmax of R2 were greater than those of R1. They progressively increased with a reduction of the S1-S0 interval, just as did the plateau of the premature AP at normal [K+]. These findings suggest that augmented Ca++-influx may be triggered by a very premature depolarization in canine ventricular muscle, in contrast to the prevailing concept that recovery from inactivation of the Ca++ current is delayed in mammalian heart muscle. Circ Res 44: 624-632, 1979

IT IS well known that durations of action potentials in cardiac muscle are dependent on preceding diastolic intervals and heart rate. However, when the cycle length of stimulation is abruptly reduced, paradoxical prolongation of action potentials (AP's) and plateau durations has been noticed in the ventricular muscle of dogs (Hoffman and Suckling, 1954; Edmands et al., 1966; Miller et al., 1971; Inuma and Kato, 1978b), rabbits (Gibbs and Johnson, 1961), cats (Bass, 1975), and sheep (Cohen et al., 1976). Although some authors reported that changes in inward Ca++ current might occur in such premature responses (Bass, 1975; Hiraoka and Sano, 1976), the underlying mechanism of this paradoxical prolongation still remained to be elucidated.

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Recently, we studied the effect of the extracellular Ca++ concentration ([Ca++]o) and verapamil on the premature AP and obtained findings that suggested enhanced Ca++-influx in very premature depolarizations. In addition, a study has been made of the responses to premature stimulation of the Ca++-dependent AP (Trithart et al., 1973). The purpose of this report is to present the results of these studies and to discuss the possible contribution of increased Ca++-influx to the paradoxical augmentation of the premature AP.

Methods
The Effect of [Ca++]o, and Verapamil on the Premature AP

A small portion (8 × 2 × 1 mm) of trabecular muscle was dissected from the right ventricle of healthy dogs as described before (Inuma and Kato, 1978a). The preparation was mounted in a plaxi-
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