Analysis of cardiac pacemaker potential using a “voltage clamp” technique

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Diastolic depolarization was studied by means of a “voltage clamp” technique in short mammalian Purkinje fibers. Clamping the membrane potential at the maximal diastolic value resulted in an inward current rising with a time constant of 3 sec in 5.4 mM K, indicating that slow depolarization is essentially a time-dependent process. Membrane slope resistance during clamp increased as a function of time, suggesting a decrease in potassium conductance rather than an increase in sodium conductance. Clamping the membrane to the calculated potassium equilibrium potential resulted in a steady inward current; at more negative clamps the inward current decreased with time. The latter findings were obtained also in sodium-free solution, indicating that a fall in the activity of an electrogenic sodium pump does not play a major role in the diastolic depolarization process. Diastolic depolarization can be initiated at any time during the plateau by clamping to the maximal diastolic potential. The threshold for all-or-nothing repolarization was determined during the plateau and found to follow the expected time course.


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During diastole, the membrane potential of cardiac pacemaker cells slowly declines. Through this process, the threshold potential is attained and automaticity established. The slow diastolic depolarization is assumed to result from a net inward current and it has been proposed that one of the following mechanisms might account for it: 1) a gradual decline in potassium conductance; 2) a gradual increase in sodium conductance; and 3) a reduction of the activity of an electrogenic sodium “pump” (18). Measurements of membrane resistance indicate that the total conductance decreases during diastolic depolarization (15) and this has been taken to suggest that depolarization is associated with a fall in potassium conductance (6). In an attempt to separate time-dependent from voltage-dependent conductance changes, the potential of a Purkinje fiber was increased to the maximal potential value by current applied through a second microelectrode (13). The outcome of this experiment was suggestive of a time-dependent component of the conductance fall. However, the value of the experiment was limited since a given potential difference could only be produced at one point along the fiber. Recently a preparation has been developed in which cable complications are avoided (3). Making use of such preparation, the present investigation was aimed at studying the processes underlying pacemaker activity. Voltage was clamped at various levels while current and membrane conductance were recorded as a function of time. The results suggest, in confirmation of Trautwein and Kassebaum (13), that diastolic depolarization in cardiac pacemaker cells is caused by a time-dependent process, most probably by a progressive fall in potassium conductance.

METHODS

The voltage clamp method proposed by Deck, Kern, and Trautwein (3) consists in separating a small (1-2 mm) section of Purkinje fibers from the rest of the “false tendon” by means of ligatures. The injury caused by the ligatures to the membrane is allowed to “heal over.” The electrotonic potential distribution of a current pulse passed through an electrode placed at 1.5 mm from the cut end of a Purkinje fiber is uniform, due to the development of a new high resistance membrane (16). It is, therefore, possible to produce a spatially uniform potential displacement by means of a constant-current pulse in short fibers. The method involves damaging the fiber twice over a short distance and this resulted in some instances in irreversible depolarization. The preparations selected for this study showed a normal amplitude of the action potential: 119.6 mv ± 1.69 mv in 5.4 mm [K], which is the same as that of nonligated fibers (119.7 ± 1.9 mv, unpublished experiments). Details on the adequacy of the method are given in the paper of Deck, Kern, and Trautwein (3).
Purkinje fibers were isolated from sheep and calf hearts carried from the slaughterhouse to the laboratory in a cold (4°C) Tyrode solution. The strands were perfused with flowing Tyrode solution at 37°C in a small (15 x 5 x 2 mm) bath and held in place by two silk threads. The composition of the Tyrode solution, in millimoles per liter, was as follows: NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1.05, NaHCO₃ 11.9, NaH₂PO₄ 0.42, glucose 5.55. In some of the experiments, KCl was reduced to 2.7 mM. The fibers perfused in 5.4 mM K were not spontaneously active and were stimulated electrically at 30/min. A mixture of oxygen (95%) and carbon dioxide (5%) was bubbled through the solution. The rate of perfusion was about 60 drops/min. Sodium-free solution was obtained by omitting NaCl, NaHCO₃, and NaH₂PO₄, the osmotic pressure being maintained by choline chloride in proper amount. Atropine sulfate (5 mg/liter) was present in the choline solution. The ligatures were applied while bathing the fiber for a few minutes in a 9 mM calcium-Tyrode solution. The procedure was suggested by the faster healing of a cut end of heart fibers in high [Ca]ₐ (5). The second ligature was applied 15-60 min after the first one. The length of the ligated segment was measured through a micrometer eyepiece and averaged 1.9 mm. Stimulation of the ligated piece (to be referred to henceforth as the fiber) was accomplished by means of two silver wires placed in close proximity. About an hour after the second ligature, the tip of a Ling-Gerard electrode filled with 3 M KCl was inserted in the middle of the fiber and was used to pass current. The transmembrane potential was recorded between a microelectrode introduced about 300 μ from the first and a microelectrode in the perfusing fluid. The recording apparatus consisted of a push-pull cathode follower, a direct-coupled amplifier, and a cathode-ray oscilloscope (Tektronix model 502). The current was measured as a voltage drop across a resistor connecting bath to ground. A downward shift of the current trace indicates an inward-directed membrane current. The feedback apparatus for most experiments was of the type described by Weidmann (17). The voltage of one of the Y deflection plates of the Tektronix scope was connected to one end of a voltage divider, its other end being maintained at a fixed potential of -300 v with reference to ground. The voltage divider was set in such a way that its output was at earth potential when the membrane potential went through its maximal diastolic value. At a later stage of this work, on advice of Dr. W. Trautwein, Heidelberg, another feedback arrangement was employed which gave a better voltage control; its essential part was a Philbrick amplifier, type USA 4.

The steady potential recorded during quiescence of a fiber will be referred to as resting potential.

RESULTS

Potential clamp in diastole. The experimental procedure consisted in clamping the membrane potential at the maximum diastolic value for a variable period of time. The results obtained in a spontaneously active fiber are illustrated in Fig. 1. At the left upper corner of the figure, two action potentials are shown, part of which is delimited by a rectangle. This part of the action potentials is reproduced at higher magnification in the rest of the figure, together with a record of the current. The voltage record begins with the late rapid repolarization and continues with diastolic depolarization. When the following action potential repolarized to its maximum diastolic value, the feedback circuit was closed: an inward current was recorded which progressively increased with time, whereas the membrane potential remained essentially constant. At the end of the clamp, the membrane abruptly depolarized and generated an action potential.

The same series of events was recorded in electrically driven fibers bathed in 5.4 mM K Tyrode (Fig. 2). In this and in the following pictures, the upper trace is a record of the current; again, only the lower part of the action potentials is shown. On the left side of Fig. 2, once the stimulation was interrupted, the membrane potential declined toward its resting value. On the right side of the figure, clamping the voltage to the maximum diastolic potential resulted in an inward current increasing with a time constant of 3.8 sec, a value not far from

![FIG. 1. Voltage clamp of diastolic depolarization of a spontaneously active fiber in 2.7 mM K. In this and in the following figures, the current calibration is the vertical bar in the upper left-hand corner of the illustration and it corresponds to 2 X 10⁻⁶ A.](image)

![FIG. 2. Voltage clamp in a driven fiber and time constant of the current rise. Left side of figure shows the control; right side illustrates rise time of the inward current as the voltage was clamped at the maximum diastolic potential for 0.5 sec. Stimulation of the fiber was stopped at the beginning of the clamp.](image)
the average 3.18 ± 0.24 sec. As usual, an action potential abruptly ensued at the end of the clamp (Fig. 2). After shorter clamp (0.5-1 sec), the membrane potential either returned to or, more often, temporarily overshot its normal course. When the membrane potential was clamped at the value of the resting potential during the phase of rapid repolarization, and thus prevented from attaining the maximum diastolic potential, an outward current was recorded which decreased slowly toward zero.

In 2.7 mM K the rate of diastolic depolarization was higher than in 5.4 (26.6 ± 0.53 mV/sec as against 2.9 ± 0.34 mV/sec). With respect to the current necessary to hold the level of maximum diastolic repolarization, the time constant of the current rise was lower in the lower potassium concentration: 1.6 in the experiment illustrated by Fig. 3, as against 3.8 for the same fiber in 5.4 mM K. The higher rate of diastolic depolarization and the quicker current rise may have a common basis, namely a quicker shift of the voltage-current curve for either potassium or sodium in 2.7 mM [K].

Membrane conductance during clamp. The results thus far illustrated can be interpreted to suggest that diastolic depolarization is essentially a time-dependent process, but they do not provide information on the ionic species for which the membrane undergoes conductance changes. In this connection, mainly Na and K ions appear of importance for Cl ions contribute but little to the total membrane conductance (1, 8). A time-dependent decrease of potassium conductance ($g_K$) could conceivably account for the inward current crossing the membrane when the potential is clamped to the maximum diastolic level. However, a time-dependent increase in sodium conductance ($g_Na$) would equally well explain the findings. It was then of interest to measure the membrane slope conductance during clamp of diastolic depolarization. This could not be done in the usual manner (15) and a technique suggested by Dr. S. Weidmann was adopted, which involved feeding square pulses of constant voltage from a pulse generator into the amplifier: corresponding changes of the membrane potential were thus simulated. With the feedback circuit switched off, the square pulses would appear superimposed on the action potential. With the feedback loop in operation, the amplifier would pass not only the current needed to hold the maximum diastolic potential, but also extra pulses corresponding to the injected square waves and resulting in periodic changes of the membrane potential. Superimposed on the usual current time course, the record showed pulses, the height of which was a measure of the slope conductance. It was found that the amplitude of the current pulses during the clamp decreased with time: the average diminution of the pulse amplitude was 23.8 ± 3.4% in five experiments.

This finding is consistent with a decrease of membrane...
Clamping the resting potential at the same voltage required less current in the lower potassium solution. At the end of the clamps an action potential ensued. Clamping the resting potential at the maximum diastolic level (in 5.4 mm K) or at a value in between (in 2.7 mm K) resulted in the usual time-dependent increase of the current. A time-dependent increase of \( g_{\text{Na}} \) would have required an opposite finding.

Most Purkinje fiber preparations become spontaneously active in 2.7 mm K and it has been suggested that at this \([K]_p\), potassium permeability falls (14). In Fig. 5, the resting potential in 5.4 and 2.7 mm K was about the same, as is usual in these solutions. When the potential was clamped to the same value, the current required in lower potassium was smaller (Fig. 5), suggesting that the chord conductance for this ion had decreased.

The experiments illustrated in Fig. 5 allowed the study of the current time course in the resting potential region in absence of activity. The current associated with the clamp at maximum diastolic potential decreased rapidly after the initial surge, as more clearly seen in records taken at higher speed, described an initial downward concavity, and then slowly increased again (Fig. 5). The slow increase of the current was similar to that observed during clamp in diastole. The time course of the current can be accounted for by a delayed voltage-dependent increase followed by a time-dependent decrease of potassium conductance.

"Voltage clamp" in sodium-free solution. The question arises as to whether the inward current resulting from a changing potassium conductance would also be apparent in Na-free solution. For this to occur, the sodium substitute must have a conductance of the same order of magnitude as that of the sodium ion; if the conductance were to be less, the resulting hyperpolarization would bring the resting potential closer to the potassium equilibrium potential (\( E_K \)). But as \( E_K \) is approached, the potassium current fall due to the time-dependent decrease of \( g_{\text{K}} \), becomes less and at \( E_K \), the current will show no slow component. In quiescent fibers, perfused in 5.4 mm K and later studied in choline solution, the resting potential was 75.6 ± 1.96 mv and the maximum diastolic potential 80.4 ± 1.71 mv; the difference between these two values (6.8 ± 0.75 mv) is similar to that of nonligated Purkinje fibers (6 ± 0.47 mv (14)). The results obtained in sodium free solution are illustrated in Fig. 6. In control Tyrode solution, holding the potential at the value of maximal negativity resulted in the described current change. In Na-free choline solution, the resting potential increased by 7 mv (Fig. 6), whereas the average increase for all preparations was from 75.6 to 81.4 mv. Thus, hyperpolarization brought the resting potential fairly close to the value of the maximum diastolic potential previously recorded in Tyrode solution. When the resting potential was held 6 mv higher, the time course of the current was essentially flat (Fig. 6). A steady current was recorded at a potential of 86.4 mv.
± 1.53 mV (5 exps.), a value not far from the potassium equilibrium potential (89 mV), calculated on the assumption that $[K]_i = 151$ mEq/liter as in heart muscle (11). Clamping beyond the K equilibrium potential should result in a gradual decrease of the current, if $g_K$ falls as a function of time: the lower part of Fig. 6 shows such current diminution. That this change was due to a fall in membrane conductance was suggested by the increase of the slope resistance as a function of time. The gradual fall of the current was similar to that recorded in the presence of sodium under the same clamping conditions.

Voltage clamp during the plateau. The relationship between the process of diastolic depolarization and events occurring during the plateau were explored by clamping the fiber to the value of maximum diastolic potential at various intervals after the action potential beginning. The current required to clamp an activated membrane back to its maximal diastolic level presented the following characteristics (Fig. 7): 1) the initial surge of current decreased toward the zero value and the rate of fall was slower the earlier the clamp; 2) as the clamp was applied later during activity, the minimum of the current gradually approached zero. In five experiments of this kind, the minimum of the current strength in the course of the earliest clamp varied between 6 X 10^-8 to 21 X 10^-8 A; 3) a current rise with a long time constant (probably underlying diastolic depolarization) was observed after the current had gone through its minimum. The rise occurred irrespective of the moment when the action potential was interrupted and proceeded at essentially the same rate. As is suggested by the voltage tracing, the feedback system did not allow an "instantaneous" clamp. A more rigorous voltage control would have resulted in a larger initial surge of current. Also, it is clear from photographs obtained at a higher speed that membrane current was still falling when membrane potential had reached a steady level.

Threshold for repolarization. Repolarization during the plateau can be initiated by means of a pulse of anodal current of appropriate strength (2, 15). The preparation employed appeared suited for the determination of the threshold for repolarization at different times during the plateau. The membrane potential was displaced for a short time (20–30 msec) to levels progressively closer to the maximum diastolic potential. When, at the end of the clamp, the membrane potential failed to return to the plateau, the voltage at which this all-or-nothing repolarization had occurred was taken as threshold potential. This procedure was repeated at different moments during the plateau. In Fig. 8 are shown the tracings obtained, superimposed on the upstrokes of the action potential. Repolarization thresholds were -65 mV when the 20-msec pulse was applied 60 msec after the onset of activity; -60 mV at 120 msec; -51 mV at 180 msec; -45 mV at 240 msec; and -39 mV at 300 msec. Comparable values were found in two more experiments. A similar change of the “abolition threshold” in the course of the action potential has been reported for the squid giant axon after treatment with tetraethylammonium chloride (19) and has been computed for a uniform polarization of Purkinje fibers (10).

DISCUSSION

The results obtained in this study show that diastolic depolarization of Purkinje fibers is: 1) a time-dependent process; and 2) due to a fall in potassium conductance. The arguments in favor of these statements are as follows: 1) a progressively rising inward current is recorded when the membrane is clamped at its maximum diastolic value; 2) the total membrane conductance decreases...
during clamp of the pacemaker potential; 3) the current necessary to hold the membrane near the calculated K equilibrium potential follows a step function; 4) the inward current initially is large and slowly approaches a lower value when the membrane is clamped beyond the potassium equilibrium potential, and the slope conductance at the same time decreases.

These findings are all consistent with a fall of potassium conductance over a long time course when the fiber is repolarized to and above the maximum diastolic potential.

While diastolic depolarization can be explained to sufficient extent by a decrease of $g_K$, some of the findings tend to exclude other mechanisms. To have a direct effect on the pacemaker potential, an electrogenic sodium pump is required to slow its activity on repolarization as a function of time. However, it would be hard to believe that the pumping rate could increase as a function of time when polarizing the fiber beyond $-95$ mV in $5.4 \text{ mM} K$. The inversion of the current’s slow component is more readily accounted for as an indication of K equilibrium potential. Furthermore, the same finding is observed in Na-free Tyrode, at the time when $[\text{Na}]_i$ must have been negligible and the contribution of an electrogenic pump accordingly minimal.

The question of a time-dependent increase in $g_{Na}$ is answered, to a large extent, by the finding of a decreasing slope conductance; for, if the sodium conductance were to increase as a function of time, the slope conductance would also increase. The possibility might still be suggested that, in addition to an increase in $g_{Na}$, the voltage-current relationship for sodium becomes regenerative (or more regenerative) as a function of time. However, the fall in slope conductance in sodium-free solution, when the membrane is clamped beyond the potassium equilibrium potential, does not support such a suggestion.

The present position may be summarized as follows: 1) a time-dependent decrease of $g_K$ appears to be the primary mechanism underlying diastolic depolarization; 2) a voltage-dependent drop of $g_K$ and, toward the threshold, a voltage-dependent rise of $g_{Na}$ speed up depolarization; and 3) there is no evidence indicating that a time-dependent increase of sodium conductance or a time-dependent decrease of sodium extrusion are factors of a major importance underlying the pacemaker potential.

The discussion of some of the findings may be facilitated by referring to Fig. 9: this is a plot of a hypothetical voltage-current relationship in the region of $E_K$ (range of diastolic depolarization). The dashed trace represents the sodium current; traces 1 and 2 represent the potassium current in $5.4$ and $2.7 \text{ mM} K$, respectively. The $K$ current in the two solutions increases from zero at the potassium equilibrium potential to the same value at the resting potential (point A); at the latter potential value, the $Na$ and $K$ currents, flowing in opposite directions, have the same magnitude. The equality of the $K$ currents at the resting potential in $5.4$ and $2.7 \text{ mM} K$ is obtained through a fall in $K$ conductance in the lower potassium solution (Fig. 5). As a result, the difference between the
resting and the equilibrium potential increases in lower [K]. The maximum diastolic potential of an activated cell is higher than the resting potential, supposedly as a result of an increase of $g_K$. This has been reproduced by rotating the potassium curve clockwise (curve 3). The maximum diastolic potential corresponds to point B. With the fall of $g_K$ as a function of time, holding the potential at the maximum diastolic value requires a progressively increasing current, since the $K$ current decreases from B toward C (Fig. 1). During the phase of rapid repolarization, potential clamp at the resting level will cause an outward current to flow (A-D in the graph), which will then decrease back to A as a function of time. The time dependent fall in $g_K$ on clamping the resting potential at the maximum diastolic level, without a previous depolarization, is of interest; for voltage- and time-dependent changes in $g_K$ in this potential region may be relevant to the mechanism of subthreshold oscillations leading to the onset of automaticity in low [K]. The beginning of spontaneous activity in low [K], should require $g_K$ to fall to the extent that the $K$ curve no longer intersects the $Na$ curve. When the resting potential is made to coincide with $E_K$, the current is not affected by a time-dependent change of $g_K$. For clamps beyond $E_K$, the large inward current (point $E$ in Fig. 9) decreases slowly toward point $F$, as the potassium conductance decreases.

If it seems reasonable to assume an increase of the potassium conductance above its resting value at the maximum diastolic potential, it is not clear whether such an increase is voltage dependent (9) or time dependent (9). In Fig. 7, the time course of the steadily increasing currents is the same in successive clamps indicating that on repolarization $g_K$ decreases as function of time, independently of the timing of the clamp during the plateau. However, a time-dependent increase in $g_K$ during the plateau is suggested by the following findings: 1) when the clamp was applied earlier, the minimum of the current was higher and the potential fell immediately after the end of the clamp was larger (Fig. 7); 2) with the earlier abolition of the action potential, the maximum potential value was about the same as the potential immediately preceding the upstroke and increased gradually with successive repolarizations (Fig. 8). If reference is made to Fig. 9, the potential at which the upstroke begins would be located at the intersection of the sodium curve and a potassium curve intermediate between curves 2 and 3; for not enough time had elapsed between action potentials for the resting potential to be attained. With the earliest induced repolarization, the voltage returns to the same point as before excitation (Fig. 8) and then decreases as the potassium curve shifts toward curve 2 (Fig. 9). With later repolarizations, as $g_K$ increases with time during the plateau, the maximum diastolic potential is gradually approached. When the potential is clamped at the maximum diastolic potential early during the plateau (Fig. 7), the minimum current results from the failure of $g_K$ to increase to curve 3. These conclusions do not exclude a slow, time-dependent fall of $g_{Na}$ during the plateau as described by Deck and Trautwein (4). In fact, the large initial current (Fig. 7) might be due to the sudden increase of driving force for Na influx in combination with a relatively high value of $g_{Na}$. Such a mechanism has been demonstrated in the nerve, where a large sodium current flows when repolarization is initiated during the period of high sodium conductance (7). The decline of the inward current might then signify a time-dependent fall of $g_{Na}$ toward its resting value. This current drop would be assisted if, as a consequence of repolarization, the low $g_K$ characteristic for the plateau were to increase with a time lag.

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