Frequency-dependent Excitability of "Membrane" Slow Responses of Rabbit Left Atrial Trabeculae in the Presence of Ba^{2+} and High K^+

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ABSTRACT Small trabeculae of rabbit left atrium immersed in TKBa solution (Tyrode with 10 mM K^+ and 1 mM Ba^{2+}) were used to study frequency dependence of "membrane" slow response excitability at long cycle lengths (>1 s). In TKBa, stimuli generate graded, low-amplitude (2–15 mV) subliminal responses of variable long duration (up to 450 ms). A full all-or-none slow response is generated when a subliminal response depolarizes the membrane to about −35 mV. Subliminal response amplitude and rate of rise augment with stimulus intensity-duration product. For a fixed stimulus, the subliminal response is larger and faster at higher frequencies. Sudden changes in stimulus frequency or time course induce changes in subliminal response that take four to eight cycles to attain steady state. For a fixed stimulus, slow response latency shortens progressively during the first few cycles after a sudden increase in frequency or when a rested preparation is excited (latency adaptation phenomenon, LAP). Slow response threshold stimulus requirements decrease during LAP (excitability hysteresis). The degree of excitability hysteresis is dependent on stimulation frequency and is more pronounced at higher frequencies. Frequency sensitivity of subliminal response (which causes frequency sensitivity of slow response excitability) is explained in terms of a transient state of enhancement set up by each stimulus. The enhanced state decays between stimuli with a half-time of ~4 s, thus allowing cumulative effects to become evident at rates above 0.1 Hz.

INTRODUCTION

It has long been known that two different and separable electrogenic mechanisms underlie action potentials of cardiac cells, namely a fast Na^+-dependent mechanism (Draper and Weidmann, 1951; Weidmann, 1955) and a slower mechanism dependent on Na^+ and Ca^{2+} (Wright and Ogata, 1961; Paes de Carvalho, 1966; Niedergerke and Orkand, 1964; Hagiwara and Nakajima, 1966; Paes de Carvalho et al., 1966, 1969; Beeler and Reuter, 1970; New and

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Trautwein, 1972; Reuter and Scholz, 1977). Although the slow response is normally observed only in sinoatrial and atrioventricular nodal cells (Paes de Carvalho et al., 1966, 1969), it can also be elicited artificially in other cardiac tissues under a number of experimental conditions, one of which is based on the effects of Ba^{2+} on cardiac cell membranes (cf. Cranefield, 1975).

It has been shown that Ba^{2+}-induced propagated slow responses elicited in large ribbons of rabbit left atrium exhibit excitability properties until then unsuspected in terms of heart muscle (Masuda, 1976; Paula Carvalho, 1977; Masuda and Paes de Carvalho, 1982). First, responses often seem to arise from a long (50–200 ms) subthreshold depolarization. Second, there is a marked dependence of excitability on the excitation history of the cell group involved. These facilitatory phenomena can be detected over times of 5–10 s, which longevity in the general realm of excitable systems is usually associated only with synaptic transmission (Kandel, 1977). The irregular time course of some of the recorded action potentials, however, suggests that strong electrotonic interactions may occur between adjacent, asynchronously firing regions. It is therefore difficult to determine whether some of these interesting time-dependent excitability characteristics are caused by slow response properties per se or by some sort of space- and/or time-dependent propagation failure.

It is our aim here to study time-dependent characteristics of slow response excitation in a situation where interpretation of the results is not complicated by the propagation disturbances and electrotonic interactions present in larger preparations.

METHODS

Preparation

Young rabbits were killed by a blow on the neck. Hearts were immediately removed and transferred to a petri dish containing normal Tyrode solution. The quiescent left atrium was isolated and its endocardial surface was exposed. Small atrial trabeculae (2–3 mm in length and 0.4–0.8 mm in diameter) were isolated and transferred to the tissue bath.

Experimental Set-Up

One end of the trabecula was pinned down to a Sylgard resin (Dow-184; Dow Chemical Co., Indianapolis, IN) bed. The other extremity was led to a glass suction pipette (tip diameter 600 μm) filled with normal or modified Tyrode solution. The pipette was connected through an Ag/AgCl interface to a DC isolated stimulator unit (2533; Devices Instruments Ltd., Hertfordshire, England). The stimulating circuit was closed by means of an Ag/AgCl electrode immersed in the bath. The total current injected through the system was measured as the voltage drop across a resistor of 10.77 kΩ.

Experimental recordings were started 45–60 min after the suction electrode was applied to the preparation.

Intracellular voltage was recorded by means of a 3 M KCl-filled microelectrode (DC resistance = 15–20 MΩ) led through an Ag/AgCl interface to a high impedance (2.10^{10} Ω) capacitance compensating preamplifier (WPI 750P W; Instruments, New Haven, CT), which measured voltage between the microelectrode and a distant

grounded reference electrode (Ag/AgCl) in the bath. A second microelectrode was used to record extracellular voltage.

First time derivatives of action potentials were obtained with the aid of an operational amplifier (type 0; Tektronix Inc., Beaverton, OR) previously calibrated by means of a known voltage ramp injected between the bath reference electrode and earth. The dV/dt signal was filtered to avoid undue high frequency noise. With a bandpass of 0–1 kHz, a 6–10-V/s ramp applied at the input resulted in a 2-ms rise time rectangular pulse.

Trabecular length constants (λ) were calculated both in normal Tyrode and test solutions by applying the short cable equation (Weidmann, 1952) to passive electrotonic potentials recorded along the preparation as a long (50 ms for normal Tyrode and 1 s for high K^+, Ba^{2+} Tyrode solution) polarizing pulse was passed through the

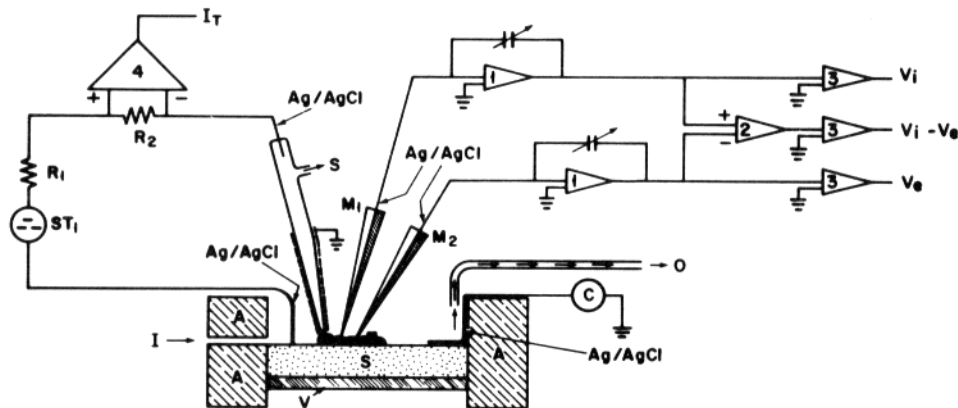

FIGURE 1. Experimental set-up: A, plexiglass cell. V, glass. S, Sylgard resin (Dow-184). M_1 and M_2, glass microelectrodes. ST_1, stimulator. R_1 and R_2, resistances of 20 kΩ and 10.77 kΩ, respectively. S, suction pipette; c, calibrator (CA5; Bioelectric Instruments Inc.). I and 0, inlet and outlet of the perfusing medium, respectively. The following amplifiers are shown: 1, high-impedance negative-capacity amplifiers (WPI-750); 2, high-gain differential amplifier (Tektronix 3A9); 3 and 4, type 3A74 Tektronix amplifier. I_T, total injected current. V_i and V_e, intracellular and extracellular voltage recordings, respectively.

suction pipette. Transmembrane electrotonic potentials were recorded by electronic subtraction of the intracellular microelectrode signal from the extracellular one. The extracellular microelectrode was positioned as close as possible to the intracellular one in such a way that no voltage deflection was observed when the intracellular electrode was withdrawn to the immediately neighboring extracellular space. This procedure seemed to avoid interference caused by external current flow and thus permitted genuine records of membrane voltage electrotonus. All records were photographed from the screen of 565 Tektronix oscilloscope (Tektronix Inc.) except that of Fig. 12, which was obtained from a Brush pen recorder (Gould Inc., Cleveland, OH).

Solutions and Drugs

The following solutions were used throughout the present study: (*a*) normal Tyrode solution: 137 mM NaCl; 2.7 mM KCl; 2.7 mM $CaCl_2$; 0.5 mM $MgCl_2$; 12 mM

$NaHCO_3$; 1.8 mM NaH_2PO_4; 6 mM dextrose; (*b*) high-K^+, Ba^{2+} Tyrode (TKBa) solution: 129.7 mM NaCl; 10 mM KCl; 1 mM $BaCl_2$; other constituents were unaltered. All solutions were continuously bubbled with a gas mixture (95% O_2 and CO_2) and were previously warmed to provide a bath temperature of 35–36°C. pH was 7.1–7.2.

The following drugs were tested: tetrodotoxin (Calbiochem-Behring Corp., San Diego, CA); acetylcholine chloride (Roche, Rio de Janeiro, Brazil); D-600 [α-isopropil-α(*N*-methyl-*N*-homoveratril-γ-aminopropyl-3,4,5-trimethoxiphenylacetonitryl)-HCl] was kindly supplied by Knoll SA (Rio de Janeiro, Brazil).

RESULTS

Electrophysiological Characteristics of Isolated Left Atrial Trabeculae in Normal Tyrode and TKBa Solution

Passive length constants (λ) were determined in trabeculae equilibrated in normal Tyrode and TKBa solutions. Fig. 2 shows results from typical experi-

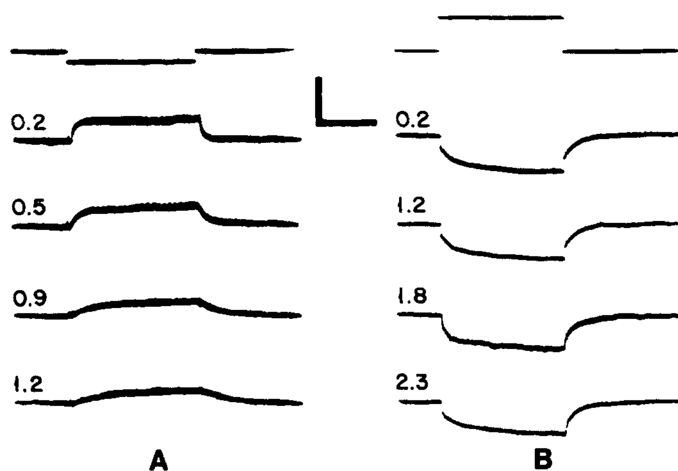

FIGURE 2. Passive transmembrane electronic polarizations recorded in normal Tyrode (A) and in high-K^+, Ba^{2+} Tyrode solution plus 15 μg/ml D-600 (B). Upper traces, constant current pulses. Other traces, recordings from different sites along the trabeculae. Numbers on the left of each trace are distances from the polarizing suction pipette measured in millimeters. Trabeculae lengths were 2.0 (A) and 2.5 mm (B). For these particular experiments λ was estimated as 1.06 (A) and 5.02 mm (B). Calibration: horizontal, 21 (A) and 420 ms (B); vertical, 77 μA and 7.7 mV (A); 30 μA and 15.7 mV (B).

ments. The upper trace in each panel is the total current pulse injected through the suction system. The other traces show purely passive electrotonic potentials at different distances from the suction electrode (located at $x = 0$) recorded differentially between an intracellular microelectrode and a nearby extracellular microelectrode. In normal Tyrode solution (Fig. 2A) the transmembrane voltage disturbance decreases in amplitude with distance while the time to reach a steady voltage value is increased. The trabecula used in Fig.

2A was 2.0 mm long and a sizable electrotonus could still be recorded at the far extremity of the preparation (not shown). Weidmann's (1952) expression for short sealed cables was used to calculate the passive length constant in nine different trabeculae, yielding a mean value of 1.10 mm (±0.06 mm SEM; range, 0.77–1.46 mm). Resting membrane time constants were estimated according to the equation that governs the temporal evolution of electrotonus in short sealed end cables (Eq. 4.12 from Jack et al., 1975). Applying this equation, a mean value of 5.9 ms (± 0.5 ms SEM; range, 3.5–7.5 ms) was obtained for the membrane time constant in normal Tyrode solution.

Strength-duration curves in normal Tyrode solution were determined in nine different trabeculae both for cathodal (pipette negative) and anodal pulses. Threshold was sharply defined and lower for cathodal pulses in all cases (rheobase, 3.0 μA; chronaxie, 1 ms).

Typical fast-rising atrial action potentials (104.2 ± 1.7 mV, amplitude; 28.1 ± 2.6 ms, duration at 50% amplitude, measured in 20 cells driven at 1 Hz) were observed in the isolated trabeculae under normal Tyrode superfusion. Conduction velocity of action potentials elicited by 1-ms-long threshold current pulses was determined in 14 trabeculae in normal Tyrode solution. Using multiple superficial impalements, velocities were measured from the slope of a linear distance-time plot and were found to range from 0.37 to 1.00 m/s with a mean ± SEM of 0.65 ± 0.05 m/s. Resting potential in normal Tyrode was −80.3 ± 1.1 mV (mean ± SEM of 20 cells).

Passive characteristics of trabeculae exposed to TKBa solution were studied with the same techniques used above, except that (*a*) cathodal pulses were used and (*b*) D-600 (15 μg/ml) was added to TKBa solution both in the bath and inside the pipette to ensure purely passive behavior. Fig. 2B shows that the steady voltage of electrotonus falls off <14% along the whole 2.50 mm of the trabecula. The same was observed in four other trabeculae so studied. Application of the equations for a short sealed cable yielded a space constant of 3.32 ± 0.50 mm (mean ± SEM). It is noteworthy that in five other trabeculae (lengths = 0.8–2.8 mm) electrotonic decay was too small to be measured.

Considering the above, spatial homogeneity of trabecular polarization in TKBa can be adopted as a close approximation. This conclusion is supported by the fact that the time course of the electrotonic potential change is nearly identical along the length of the preparation, although a small time difference (~10–20 ms) can be observed between the preparation extremities. In these conditions a time constant of 61.0 ± 3.4 ms (mean ± SEM) was estimated from the records taking as approximation the time needed for the electrotonic potential to reach 63% of its steady value. Considering this to be ~10-fold higher than the membrane time constant estimated in normal Tyrode, TKBa solution appears to cause a 10-fold increase in resting membrane resistance. This increase in membrane resistance accounts for the approximately threefold increase in λ. This effect is probably caused by the Ba^{2+}-induced decrease in membrane potassium conductance, g_K (Antoni and Oberdisse, 1965; Reid and Hecht, 1967; Sperelakis et al., 1967; Sperelakis and Lehmkuhl, 1966; Hermsmeyer and Sperelakis, 1970; Toda, 1970).

Rheobase and chronaxie were tested in rested preparations (quiescent for ≥30 s) both for cathodal and anodal stimuli. Rheobase was found to be about one order of magnitude higher in TKBa solution than in normal Tyrode and lower for anodal (pipette positive) than for cathodal stimuli. Chronaxie was also about one order of magnitude longer in TKBa than in normal Tyrode solution and about twice as long for cathodal as for anodal pulses. Also, it can be observed that in TKBa, contrary to normal Tyrode, the slow responses always originate in the free portion of the preparation. It seems that in TKBa, excitatory events that originate in the muscle within the suction pipette are unable to generate enough current to drive the free portion of the preparation.

Fig. 3A and B shows two pairs of simultaneous transmembrane recordings in two different preparations exposed to TKBa solution. Each panel shows that in spite of a wide separation of recording sites (see legend) there is almost perfect synchrony of slow responses. The continuous high-gain record of

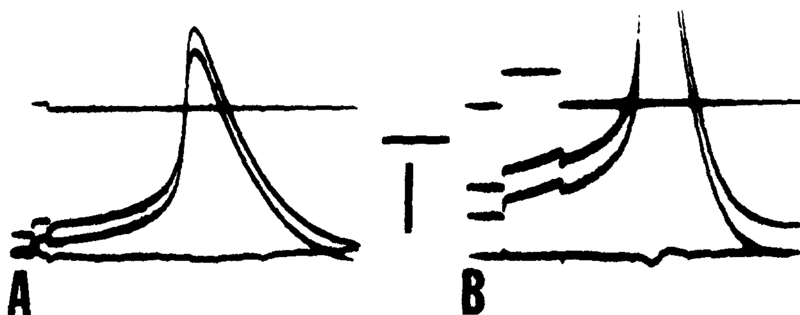

FIGURE 3. Simultaneous transmembrane and difference voltage recordings from pairs of cells in two different preparations in TKBa solution. Upper trace, current pulse. In A and B, the recording sites are separated by 2.0 and 1.2 mm, respectively. Calibration bars: horizontal, 50 (A) and 100 ms (B); vertical, 20 (A) and 7.7 mV (B). Difference record, 8.7 mV (A and B). Current strength, 90 (A) and 35 μA (B).

voltage difference between action potentials in each pair (bottom trace) shows clearly that voltage in one cell follows closely voltage in the other cell, discrepancies never rising beyond 1 or 2 mV at any given moment. This result is not surprising if one takes into account the prolongation of passive length constant shown above for the resting state. A simultaneous subthreshold rise of voltage in the whole preparation would obviously tend to provide synchronous firing. It can therefore be concluded that in short left atrial trabeculae exposed to TKBa the slow response behaves as a "membrane" action potential so that the rate of voltage change is directly proportional to net ionic current at any instant.

TKBa "membrane" slow responses are all-or-none action potentials (threshold voltage around −35 mV; see below) and exhibit properties thought to characterize slow responses obtained by different methods in several other species and preparations. In terms of mean ± SEM in 15 preparations, the total slow response amplitude is 59.6 ± 1.4 mV; the duration at 50% amplitude

is 83.7 ± 4.8 ms and the upstroke maximum dV/dt at long cycle length (see Fig. 6B) is 5.4 ± 0.1 V/s. Resting potentials are −55.0 mV ± 0.6 mV. TKBa slow responses are insensitive to tetrodotoxin (10 μg/ml, three preparations) but are abolished by D-600 (1–3 μg/ml, four preparations).

Onset of Slow Response Activity in Rested Trabeculae in TKBa Solution: the Latency Adaptation Phenomenon (LAP)

Fig. 4 shows superimposed transmembrane tracings of the first few cycles at the onset of slow response activity in two previously rested (for 60 s) preparations. The stimulation frequency was 1 Hz and the stimulus intensity was ~15% above threshold.

In Fig. 4A the first cycle corresponds to the longest latency (measured as the time interval between the onset of the current pulse and the slow response peak amplitude). Latency shortens progressively until a stable minimum is

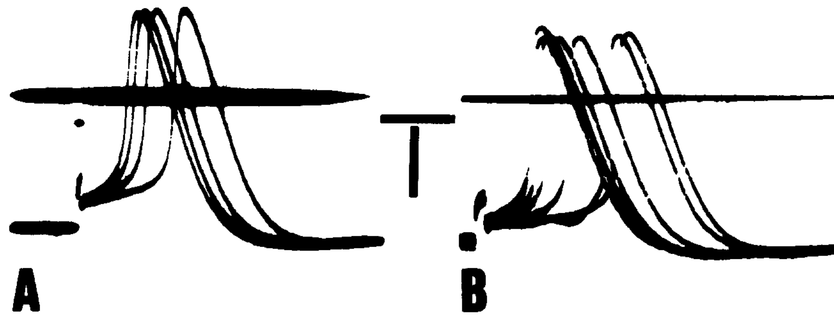

FIGURE 4. Superimposed transmembrane recordings during latency adaptation phenomenon in two different trabeculae (A and B). Both preparations had rested for 60 s before onset of stimulation (1 Hz). Panel A shows from right to left the first four consecutive slow responses. B shows from right to left the 3rd, 2nd, 1st, 4th, 5th, 6th, and 7th (stabilized) consecutive slow responses. Calibration bars: vertical, 20 mV and 200 μA (only for A); horizontal, 200 ms. The upper trace in B is not the zero potential.

obtained after four to eight repetitive cycles. We call this the latency adaptation phenomenon (LAP). This behavior is quite different from that observed in normal Tyrode solution where the same experiment yields constant latency in all cycles. It can be seen that a full all-or-none slow response arises in every case from a prolonged depolarization step that far outlasts the stimulus (see stimulus current record in upper trace, Fig. 4A). This depolarization step shows a positive slope that gently increases to form the upstroke of the slow response. Note that during LAP the initial slope of the depolarization step increases from beat to beat. As a result, slow responses appear progressively earlier in time. Also note that the four action potentials in Fig. 4A are qualitatively similar once the membrane potential has been depolarized beyond about −35 mV (see below for fine differences). This voltage is the threshold potential for the all-or-none slow response, whereas the initial depolarization step is an independent graded phenomenon that we shall call

the subliminal response. One reason for this distinction is that the subliminal response is potentiated from cycle to cycle after the onset of stimulation, whereas the slow response is not (see Fig. 5). Further reasons will become apparent as more data are presented.

Fig. 4B shows that the order of progression during the latency adaptation phenomenon may be altered in the first few cycles but the final stable latency always corresponds to the minimum latency for that preparation under a given set of stimulating conditions. In the particular instance shown, the first

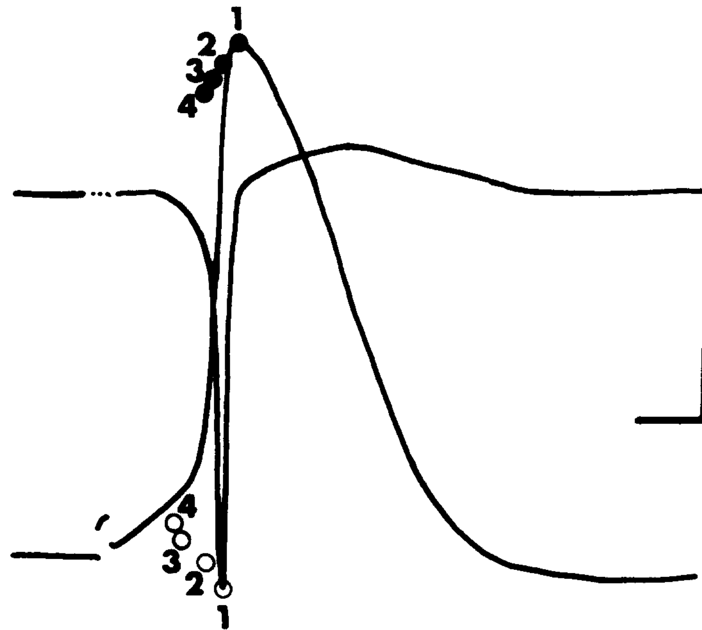

FIGURE 5. Slow response peak amplitude and maximum *dV/dt* during latency adaptation phenomenon (drawings from original records). Upper trace, slow response *dV/dt*. Lower trace, intracellular recording. Open and filled circles are, respectively, maximum *dV/dt* and slow response peak amplitude during the first four consecutive slow responses. Numbers correspond to cycle sequence (the 4th is the stabilized slow response). Stimulation frequency, 0.67 Hz. Calibration bars: vertical, 10 mV and 1.0 V/s; horizontal, 50 ms.

beat after rest was the third from right. Two beats at longer latencies followed. Then latencies shortened progressively to a stable minimum. This type of finding was rare and we have no explanation for it. LAP is reproducible and occurs as a progressive shortening during different runs in the same rested preparation except for small differences in latency during initial cycles. Final equilibrium latency is always more stable.

It should be pointed out that small changes in the slow response time course can be detected during LAP. This is shown in Fig. 5, where the intracellular recording of the first slow response after rest is presented together with its first time derivative. Also shown (filled circles) is the position of the action potential

peak in the first four consecutive cycles, during which most of LAP takes place. Evolution of peak dV/dt during upstroke (open circles) is shown as well.

Both amplitude and upstroke velocity decrease during latency adaptation at this stimulation frequency. These data are representative of many similar experiments and demonstrate that LAP is not caused by enhancement of the slow response upstroke mechanism.

Frequency Dependence of LAP

Final equilibrium latency after LAP depends on the stimulation frequency. Fig. 6A shows that for a fixed-stimulus contour the relationship between cycle length and equilibrium latency is linear over an extended range, shorter cycle lengths causing shorter latencies. This effect is caused by a proportionally

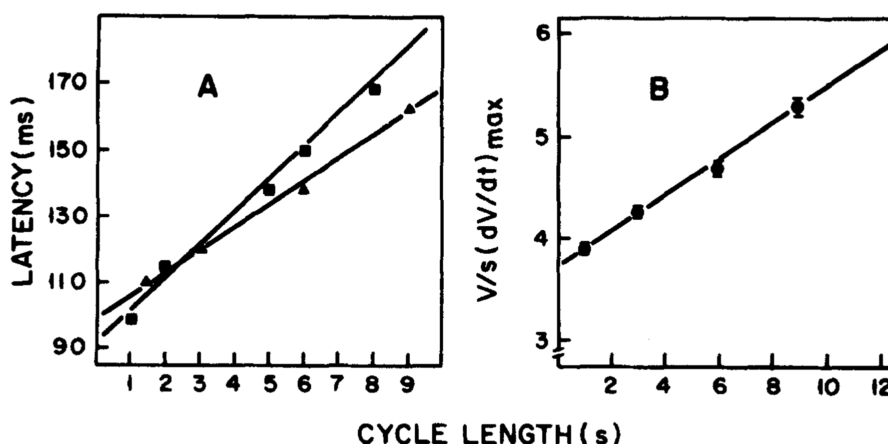

FIGURE 6. A: relationship between equilibrium latency (milliseconds) and cycle length (seconds). Different symbols correspond to different preparations. Lines are drawn according to equations: $y = 0.68x + 99$ (triangles, $r = 1.00$) and $y = 0.96x + 91.7$ (squares, $r = 0.99$). B: relationship between maximum dV/dt (volts per seconds) and cycle length (seconds). Line drawn according to equation $y = 0.17x + 3.77$ ($r = 1.00$). Circles and vertical bars are mean ± SEM of 20 measurements.

larger cycle-to-cycle growth of the subliminal response when LAP is obtained at shorter cycle lengths, causing the slow response threshold to be reached sooner.

Fig. 6B shows that the maximum dV/dt of the equilibrium slow response increases linearly with cycle length, which indicates that the data in Fig. 6A are not related to decreased efficiency of the slow response depolarizing mechanism at longer cycle lengths.

It should be pointed out that LAP occurs not only at the onset of activity in rested preparations but also upon sudden change in stimulation frequency. When cycle length is altered, a cycle-to-cycle change in latency is observed as the preparation moves between steady state points in the curve of Fig. 6A. In this context, LAP can be represented by either a shortening or lengthening of

slow response latency, depending on whether stimulation frequency is suddenly increased or decreased.

Based on evidence presented so far we suggest that amplitude and rate of rise of subliminal response are conditioned by a slowly decaying "enhanced state" created at each cycle and capable of accumulating from cycle to cycle to reach a final steady state essentially dependent on cycle length. The evolution of the enhanced state during LAP determines the progressive shortening of slow response latency as well as its final equilibrium value.

Slow Response Excitability Hysteresis during LAP

If stimulus effectiveness in generating subliminal response is enhanced during LAP, the minimum stimulus strength necessary to bring about the first slow response after a period of rest (I_{T_0}) should be higher than the minimum stimulus necessary to keep activity going after complete adaptation to a given rate (I_{T_∞}). Since latency adaptation depends on pacing rate (see Fig. 6A), I_{T_∞} should also be a function of stimulation frequency.

The following experimental protocol was adopted to test this hypothesis. First, we determined threshold current I_{T_0} by testing the rested preparation with pulses of increasing intensity applied at the very low frequency of 0.087 Hz (we found that shortening the resting period from 30 to 10–12 s does not alter I_{T_0}; see Fig. 8). The preparation was then allowed to rest for 90 s and stimulation was reinstated at a desired test frequency, using pulses of the same duration, and threshold intensity I_{T_0} as above. After a 30-s period of electrical stimulation, current strength was diminished in small steps. Time for latency adaptation was allowed between steps. Preparation behavior was recorded until the stimulus became ineffective in eliciting slow response activity. After a 90-s resting period, the procedure was repeated for a new test frequency. Fig. 7 shows data obtained during a single cell impalement in a representative experiment. Another four experiments yielded qualitatively identical results. The three vertical columns represent, from the left, test frequencies of 0.5, 0.25, and 0.17 Hz, respectively. In each column, row 1 shows rested responses to the first stimulus (I_{T_0}); row 2 shows adapted responses to I_{T_0} 30 s after test frequency was started; and row 3 shows adapted responses to a smaller stimulus current (~0.9 I_{T_0}). The bottom panels show subliminal responses to the second (or superimposed second and third) cycle after the stimulus was decreased by a small step to an intensity just below the minimum capable of eliciting slow responses consistently at that particular frequency (just below I_{T_∞} for the given frequency). Notice that the rested threshold stimulus (I_{T_0}) remained constant throughout the experimental procedure (row 1, columns A–C).

Rows 1 and 2 of Fig. 7 clearly confirm that latency shortening during LAP is less extensive at lower frequencies. Comparison of rows 2 and 3 shows that a decrease in stimulus strength prolongs latency while upstroke and amplitude of resulting slow responses remain unchanged. Such an increase in latency derives from the fact that weaker stimuli create smaller, slower rising subliminal responses. This is best seen by comparing panels C2 and C3. Latency

increases further as stimulus intensity approaches I_{T_∞}. Latencies as long as 350 ms have been seen on occasion.

The above comparison of rows 2 and 3 shows beyond doubt that, as expected, stimuli weaker than I_{T_0} can drive the preparation once it becomes adapted to a given frequency. The preparation stops responding only when driven by stimuli weaker than I_{T_∞}; once this has happened, simple restoration of stimulus intensity to I_{T_∞} will not cause immediate reappearance of slow response. In order to re-excite the preparation at first try, stimulus strength

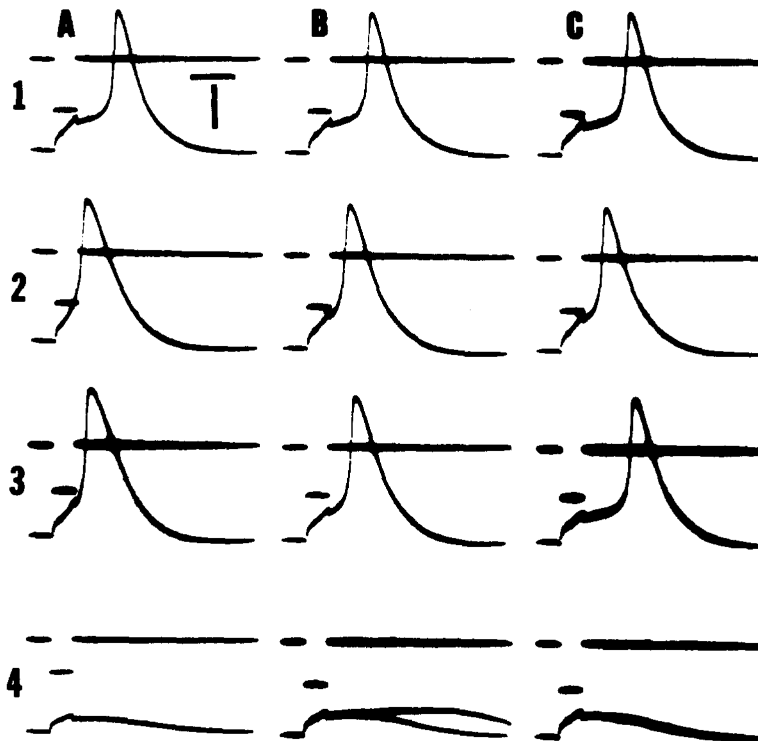

FIGURE 7. Relationship between slow response excitability hysteresis and stimulation frequency. Upper and lower traces are, respectively, current pulse and intracellular recording from a single impalement. See text for explanation. Calibration bars: vertical, 20 mV and 35 μA; horizontal, 100 ms.

must be restored back to I_{T_0}. We call this phenomenon excitability hysteresis of slow response and ascribe it to the same cumulative enhancement of subliminal responses proposed above to explain LAP.

Row 4 of Fig. 7 shows that the longer the cycle length, the closer I_{T_∞} is to I_{T_0}, i.e., the less conspicuous excitability hysteresis is. The frequency dependence of excitability hysteresis is best shown in Fig. 8, where the relationship I_{T_∞}/I_{T_0} was plotted against stimulation frequency. As stimulation frequency decreases, I_{T_∞}/I_{T_0} approaches unity. At a frequency of 0.087 Hz (cycle length, 11.5 s), excitability hysteresis is no longer observed.

It is important to understand that I_{T_0} and I_{T_∞} can be defined for stimuli of a given fixed duration only. Changes in stimulus duration will create effects similar to changes in stimulus amplitude so that strength-duration curves can be constructed for both I_{T_0} and I_{T_∞}. The strength-duration curve for I_{T_0} is fixed by definition. However, as a result of frequency-dependent hysteresis, a plot of steady state threshold stimuli (I_{T_∞}-duration plot) yields a family of curves, one for each frequency, as shown in Fig. 9. The uppermost curve (filled circles) was obtained under a very low stimulation frequency (0.087 Hz). This curve is the same as the strength-duration curve for the rested preparation (I_{T_0}). The three other curves were determined at three different pacing

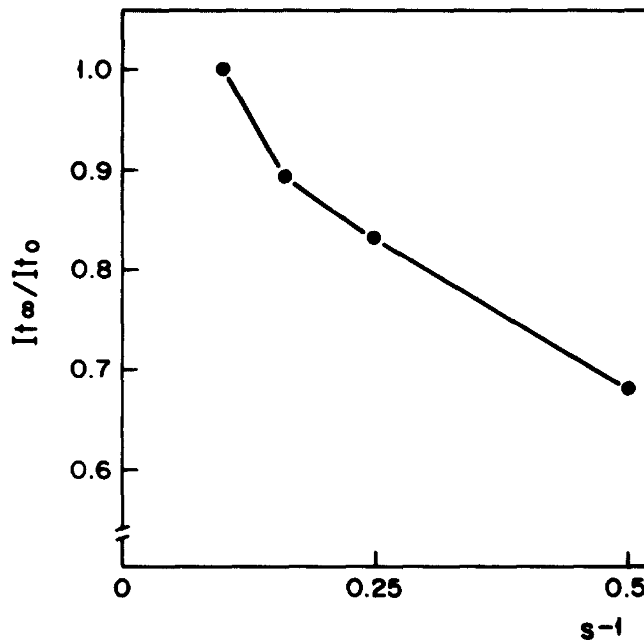

FIGURE 8. Relationship between $I_{T\infty}/I_{T_0}$ and stimulation frequency. Values obtained with same experimental protocol as Fig. 7 (see text for further explanation).

frequencies (open circles, 0.17 Hz; squares, 0.25 Hz; triangles, 0.5 Hz). These results demonstrate that as stimulation frequency increases, the steady state strength-duration curve is displaced downward. At higher rates, less stimulation charge is required to generate slow responses, i.e., slow response excitability increases with pacing rate.

Rate and Rhythm Dependence of Subliminal Response

It was pointed out above that latency depends not only on stimulation frequency but on stimulus strength and duration as well. In experiments such as the one shown in Fig. 7, it was consistently observed that upon sudden decrease of stimulus strength a few cycles of progressive latency lengthening

preceded final equilibrium. The concept of LAP can therefore be defined not only for a sudden change in rate but for a sudden change in stimulus characteristics as well. When stimulus strength is suddenly lowered just below I_{T_∞}, latency is seen to prolong for one or two beats before the slow response disappears. One such case is shown in Fig. 10. Panel A is the response to threshold stimuli (I_{T_∞}) delivered at a rate of 0.5 Hz. Panels B–D show, at the same rate, the first three cycles after stimulus intensity was slightly decreased. Notice that a greatly delayed (~300 ms) slow response is still present in B but disappears in succeeding cycles. Furthermore, the amplitude and duration of

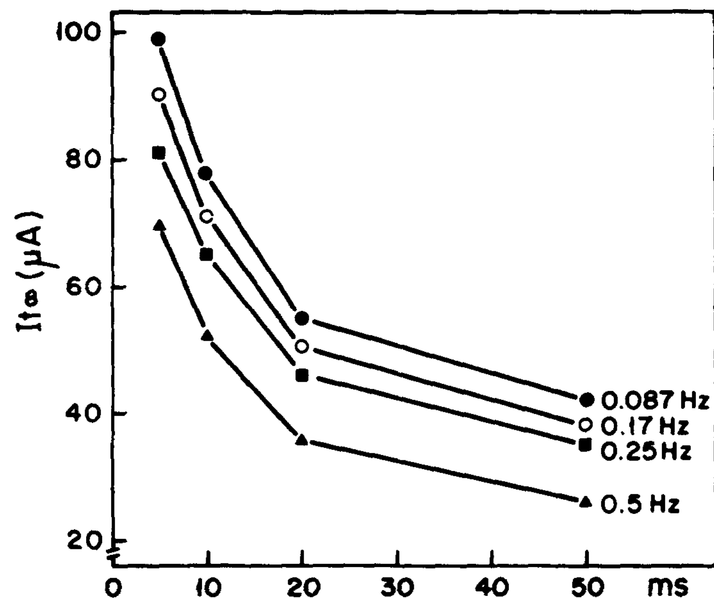

FIGURE 9. Family of steady state strength-duration curves obtained at different stimulation frequencies following same experimental protocol as Figs. 7 and 8.

the subliminal response decreases with each cycle from B to D (also see Fig. 7, panel B4). Such experiments reveal that changes in slow response per se are not directly responsible for generating time-dependent changes in excitability, because the slow response is present in B but not in C or D. If anything, because the slow response in B is 100 ms closer to the succeeding stimulus in C, any enhancement caused by the slow response itself should have potentiated the subliminal response in C. In fact, the amount of excitability enhancement obtained at each new cycle seems to be dependent on duration and amplitude of stimulus and/or subliminal response. The enhanced state created at each stimulation cycle decays slowly and, at the end of a cycle, can be accumulated with the enhancement generated by the following cycle. Upon a sudden change in rate or stimulus, therefore, the enhanced state at the end of a cycle can either accumulate or decrease progressively, thus conditioning a corresponding change in amplitude of succeeding subliminal responses. If this is

true, it must be possible to obtain temporal summation by means of the application of subthreshold stimuli to a rested preparation.

Fig. 11 shows that, indeed, temporal summation of subthreshold stimuli can occur during a subliminal response. In this experiment, we applied a subliminal conditioning pulse (0.78 I_{T_0}) followed at different intervals by an even weaker test pulse (0.50 I_{T_0}), and repeated the first conditioning pulse at a rate of 0.125 Hz. Panel A shows that, alone, the first stimulus generated only a subliminal response. In panel B, the addition of the test pulse 50 ms after the conditioning pulse elicited a slow response. Panel C shows that

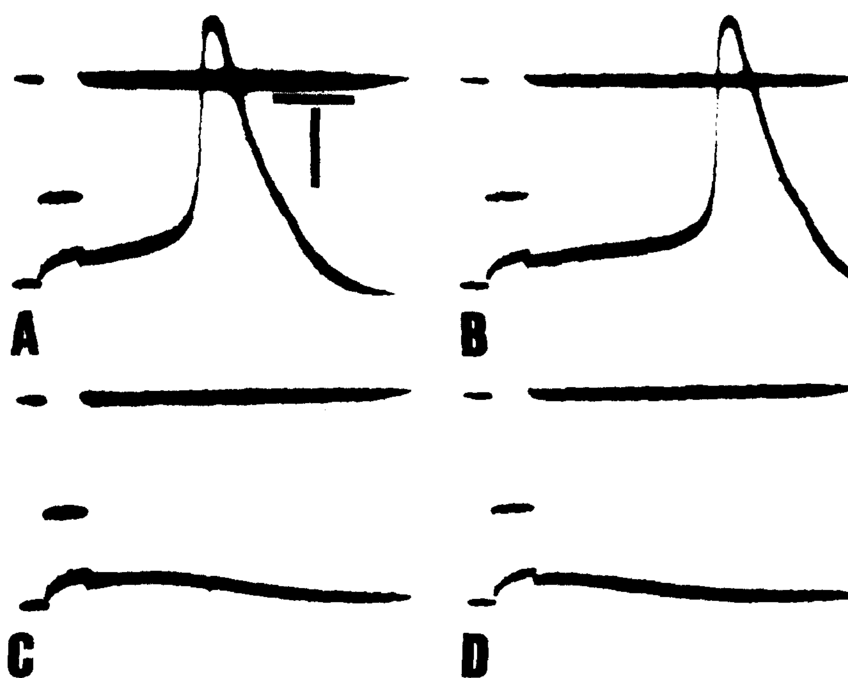

FIGURE 10. Progressive loss of slow response upon sudden lowering of stimulus intensity just below threshold. A: slow response elicited by threshold stimulus ($I_{T\infty}$). B–D: successive cycles immediately after decreasing stimulus intensity. Stimulation frequency, 0.5 Hz. Calibration bars: vertical, 20 mV and 45 μA; horizontal, 100 ms.

excitation is still achieved by the pulses at a longer coupling interval (125 ms). However, the subliminal response generated by the second stimulus is smaller, so that slow response latency is increased. At a stimulus interval of 140 ms (D), the test pulse fails to elicit a slow response. A small increase in strength of the first and/or second pulse, however, would restore the slow response (data not shown).

Experiments such as this illustrate that the subliminal response is indeed a graded phenomenon capable of temporal summation throughout its duration. It should be emphasized, however, that cumulative enhancement of excitability can be demonstrated at times well beyond the duration of the

subliminal response, which itself lasts no more than 450 ms. This has already been made evident in this paper by the wide range of frequencies over which accumulation can occur. We now establish this more directly by showing that the enhanced state caused by subthreshold stimuli can show cycle-to-cycle accumulation even when repeated at rates of 1 Hz or lower. This is illustrated in Fig. 12, where subliminal excitation is established at three different frequencies (0.33, 0.5, and 1 Hz). In each panel, the upper trace is the stimulus current and the lower trace is membrane potential recorded with a pen recorder at low paper speed. The preparation was allowed to rest for 90 s after

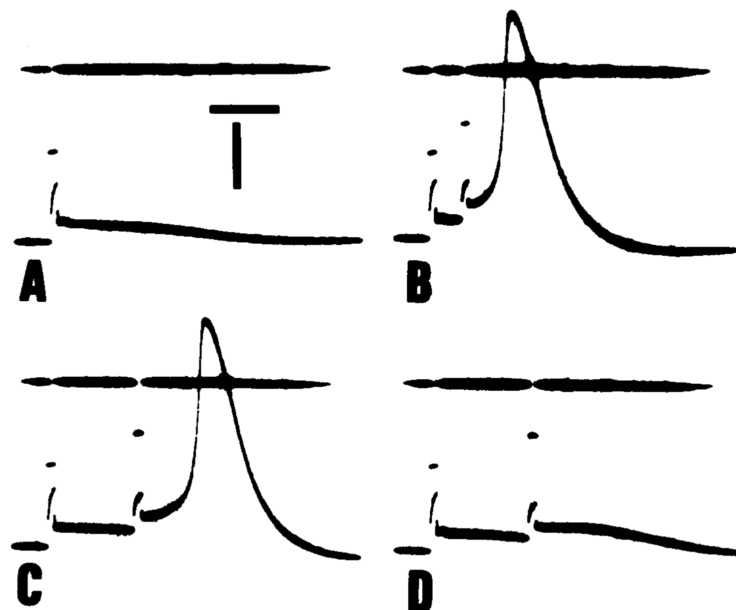

Figure 11. Slow-response paired-pulse stimulation. In each panel, upper trace is current pulse and lower trace is intracellular recording. A: subliminal response to a single subthreshold (0.78 I_{T_0}) pulse applied at 0.125 Hz. B–D: responses to a second subthreshold stimulus (0.50 I_{T_0}) applied at progressively larger delays after the conditioning pulse. Note absence of slow response in D (delay, 140 ms). Calibration bars: vertical, 20 mV and 185 μA; horizontal, 100 ms.

each test. In row 1, the experiment was carried out with a stimulus strength equal to 0.80 I_{T_0}. At a rate of 0.33 Hz (panel A), a long train containing 40 stimuli was unable to elicit a slow response, although oscillations of the subliminal response amplitude can be seen. Both at 0.5 Hz (panel B1) and at 1.0 Hz (panel C1), the same stimulus strength promoted activity on the 16th cycle. In row 2, records were obtained with a larger subthreshold stimulus (0.85 I_{T_0}). Slow responses were obtained on the 24th cycle at 0.33 Hz (A2), on the 16th cycle at 0.5 Hz (B2), and on the 17th cycle at 1.0 Hz (C2). It is thus clear that excitability enhancement can be accumulated from cycle to cycle even if stimuli generate only a subliminal response. This statement is supported

by the progressive increase in subliminal response amplitude that precedes the onset of activity in every successful train. For a constant stimulus, the chance of obtaining slow responses increases with stimulation frequency. Because the stimulation frequencies used in Fig. 12 allowed enough time for complete recovery of resting potential between successive subliminal responses, one is led to conclude that although the amplitude and time course of these responses are modulated during excitability enhancement, enhancement effects are not mediated simply by summation of subliminal depolarizations.

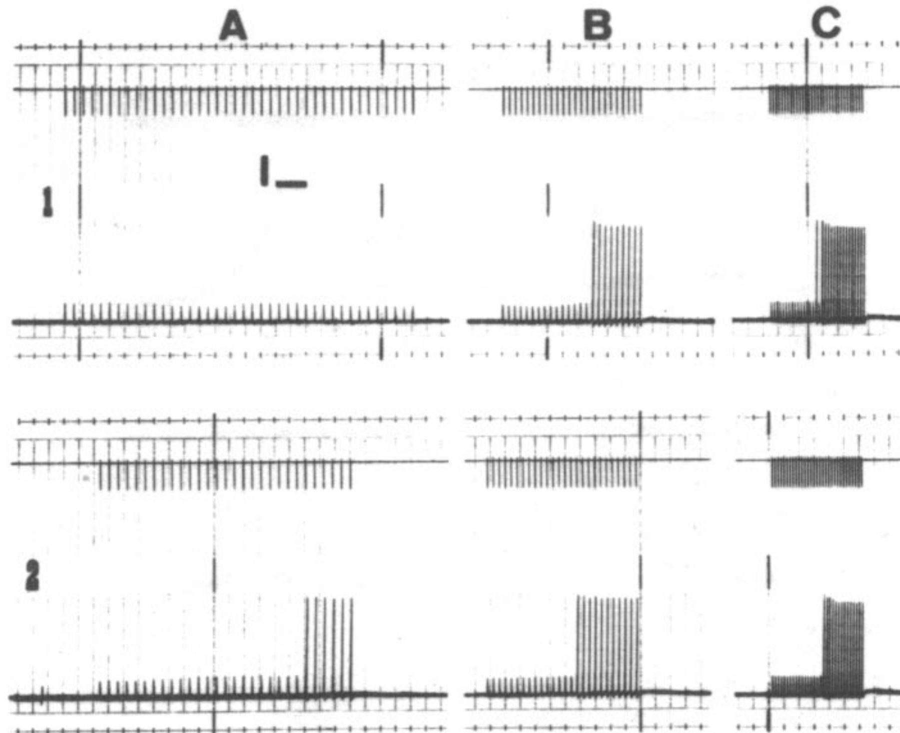

FIGURE 12. Cumulative excitability enhancement. In each row, the upper trace is the current pulse and the lower trace is the intracellular recording (continuous pen recordings from the same cell). Stimulus current pulse: 0.80 I_{T_0} (row 1) and 0.85 I_{T_0} (row 2). Stimulation frequency: 0.33 (A), 0.5 (B), and 1 Hz (C). Notice peak amplitude oscillations in subliminal responses. Calibration bars: vertical, 15 mV and 120 μA; horizontal, 10 s.

DISCUSSION

Slow Response Stimulation through a Suction Electrode

Relatively large suction electrodes have been used previously to polarize cardiac tissues (Bonke, 1973; de Mello, 1977). These authors assumed, as we do, that current enters cells within the suction pipette and leaves them in the free portion. We assume that our trabeculae are composed of roughly parallel interconnecting fiber bundles (Sommer and Johnson, 1979). Current entering

fibers at one extremity should therefore run along fiber bundles and leave cells approximately according to the laws governing current flow in a cable (Hodgkin and Rushton, 1946; Jack et al., 1975).

The isolated left atrial trabecula in normal Tyrode solution seems to behave as a linear short cable during weak polarization pulses. The measured length constant (1.10 ± 0.06 mm, SEM) is in reasonable agreement with the values found in dog pectinate muscle (Sakamoto and Goto, 1970), papillary muscle (Kamiyama and Matsuda, 1966; Sakamoto, 1969; Sakamoto and Goto, 1970), sheep and cow ventricular trabeculae (Weidmann, 1970), and rabbit *crista terminalis* (Bonke, 1973). The shorter value found by Bonke (1973) for rabbit atrial trabeculae may be the result of technical differences.

TKBa solution causes an increase of at least three times in trabecular length constant, which seems to contribute to the occurrence of spatially uniform slow responses ("membrane" action potentials). The absence of detectable extracellular voltage changes (not shown) and the close resemblance in time course of slow responses recorded at different points along the trabecula strongly suggest that a "membrane" slow response occurs.

As pointed out by Bonke (1973), the tissues inside and outside the suction electrode are oppositely polarized during the current pulse, since the same current that crosses the cell membrane within the pipette must do so in the reverse direction in the much larger free segment of the preparation. Also, that difference in available membrane surface area causes current density to be greater at the suctioned extremity. In TKBa, however, the preparation tends to behave as a space-clamped cell, and any regenerative activity appearing inside the pipette should be shunted by the extensive membrane area offered for current flow in the free segment. In our experiments, although depolarization during cathodal (pipette negative) pulses may have elicited slow responses in the suctioned segment, such responses neither propagate nor influence appreciably the passive hyperpolarization caused by current outflow at the free segment. When slow responses appear at the free segment in these occasions, they are clearly anodal break excitations originated in the free segment itself (not illustrated). For this reason, slow response rheobase is lower for anodal (pipette positive) pulses, which cause depolarization of the free segment. All excitability tests were conducted with anodal pulses.

The increase in chronaxie observed in TKBa solution is clearly related to the increase in membrane time constant. On the other hand, the large increase in rheobase appears to be at least in part technique dependent, as the higher membrane resistance in TKBa increases the input resistance of each fiber and diverts a larger proportion of the applied current via extracellular space.

TKBa Slow Response

The bulk of the experimental evidence available to date strongly suggests that cardiac muscle slow response (of which SA and AV nodal action potentials and TKBa slow responses are examples) is a predominantly Ca^{2+}-dependent response (Reuter, 1973; Cranefield, 1975). Ca^{2+} spikes with similar electrophysiologic and pharmacologic characteristics have been reported in a large

number of muscle, nerve, and other cells throughout the animal kingdom (Hagiwara and Byerly, 1981).

Ba^{2+} has long been known to induce spontaneous activity characterized by a slow action potential configuration (Antoni and Oberdisse, 1964), which was later identified as slow response (see Cranefield, 1975). The effect of Ba^{2+} seems to be mediated both by a loss of resting potential caused by a decrease in resting g_K (Sperelakis and Lehmkuhl, 1966) and by a direct role of Ba^{2+} as a charge carrier via the slow channel (Reuter, 1973). Simultaneous increase of extracellular K^+ to 10 mM has been shown in rabbit left atrium, to abolish the spontaneous activity induced by 1 mM Ba^{2+}, while maintaining a resting potential low enough to ensure complete inactivation of the fast response (Cukierman, 1978). Left atrial preparations immersed in TKBa solution could still be excited electrically and yielded slow responses with characteristically low rates of rise (3.5–6 V/s), which were found to be tetrodotoxin insensitive and could be abolished by D-600, acetylcholine, and Mn^{2+} (Masuda, 1976; Cukierman, 1978; Masuda and Paes de Carvalho, 1982).

It can be seen from different records in this paper that the "membrane" slow response in TKBa is a stereotypical all-or-nothing action potential. Its threshold voltage is about −35 mV, which is in general agreement with voltage-clamp data for the slow inward current (New and Trautwein, 1972; Reuter, 1973). Provided the repetition rate is kept at ≤1 Hz, upstroke velocity and amplitude show a modest but clear direct linear relationship with cycle length, slow response depression increasing with rate (Fig. 6B). This may be the same phenomenon described at higher rates as "fatigue" (loss of amplitude and upstroke velocity). Fatigue at high rates (above 1 Hz) is observed in our preparations (not shown) as well as in normal AV nodal slow response (Paes de Carvalho, 1964) and in artificial slow responses (Cranefield, 1975).

Under steady state conditions and at any given constant stimulation frequency, upstroke velocity was found to be independent of latency or stimulus pulse parameters (not shown). As cycle length seems to be the sole determinant of fatigue, its mechanism must obviously be thought of in terms of a time-dependent depression process set up at each cycle and vanishing with a very long time constant. This idea is borne out by the observation that upstroke velocity and amplitude decrease slightly from cycle to cycle at the onset of activity in a rested preparation (Fig. 5). We have no data that might otherwise contribute to elucidate the mechanism of slow response fatigue.

It is interesting to note that, based on the observed constancy of upstroke velocity, the depolarizing current underlying the slow response does not appear to be inactivated by the preceding depolarization of several hundred milliseconds at a voltage close to the threshold, during the subliminal response. We are therefore led to suggest that either inactivation is not present at these near-threshold voltages or it proceeds with a time constant so large that latencies can exceed 300 ms with no change of upstroke velocity. New and Trautwein (1972) showed that, in mammalian ventricle, the slow inward current inactivation time constant changes with voltage, showing a minimum value of ~70 ms from −25 to +10 mV and increasing towards 180 ms at −35 to −40 mV at the negative side and +40 mV at the positive side.

Graded Subliminal Response

The occurrence of prepotentials preceding spiking is a well-known phenomenon in nerve and smooth muscle preparations (Gulrajani and Roberge, 1978; Connor et al., 1974) and has been ascribed to different mechanisms in different tissues (Connor et al., 1974; Connor, 1978; Gulrajani and Roberge, 1978; Prosser, 1978).

Our present study of the slow response shows that it always arises from a low depolarizing step, the duration and amplitude of which depend on stimulus parameters (intensity, duration, and rate or rhythm). We have suggested that this graded depolarization is indeed a subliminal response and that, at least under our experimental conditions, slow response excitation is a direct consequence of the stimulus efficiency in creating an effective subliminal response. The subliminal response is not some spurious reflection of anodal excitation within the suction pipette but a real independent entity. There are several reasons for our believing this is the case. First, the subliminal response in Fig. 7 (panel 4B) is far too long (nearly 350 ms) to be considered a shunted slow response (or a shunted action potential plateau), as total slow response duration lies around 220 ms. Second, "membrane" slow response duration in TKBa seems to be fairly independent of rate and rhythm (see Figs. 4 and 7) so that a slow response arising within the pipette should not exhibit the time-dependent variations shown for the subliminal response in this paper. Third, records in Fig. 10C and D, where longer anodal (pipette-positive) pulses were used, show unequivocally that in the free segment the subliminal response starts during the pulse and lasts undisturbed for a long time beyond pulse break. Fourth, control experiments were performed in which D-600 (20 μg/ml) and tetrodotoxin (10 μg/ml) were added to the suction pipette fluid so that both fast and slow responses were presumably blocked within the suction pipette. In these conditions the unaffected free segment was still capable of yielding subliminal responses.

It is impossible to ascertain from our data whether subliminal and slow responses are mediated by independent sets of ionic channels. Preliminary data on D-600 depression of slow response in conditions where the subliminal response was clearly visible were always accompanied by depression of the subliminal response itself (Cukierman, 1978). However, one must point out that the frequency dependence of subliminal response is opposite to that observed for the upstroke velocity and amplitude of the slow response.

Subliminal Response Enhancement and the Effect of Cycle Length on Slow Response Excitability

Masuda (1976) and Masuda and Paes de Carvalho (1982) showed, for left atrial strips immersed in TKBa, that under a given stimulus configuration there is a minimum frequency at which propagated slow responses can be elicited. They also demonstrated excitability hysteresis and temporal summations, but were unable to rule out entirely the possible interference of conduction asynchronies and electrotonic interactions in interpreting their data. In the present study, these effects are examined under conditions of spatially uniform membrane voltage. We have extended their findings and

are now in a position to state some characteristics of the time dependence of slow response excitability. (*a*) The strength-duration curve for slow responses depends on the rate of drive; in other words, recent experience of the preparation alters its excitability as if some enhancing factor were generated by each stimulus and then slowly disappeared. (*b*) Slow response latency is entirely determined by the time the subliminal response takes to reach slow response threshold. (*c*) Excitability enhancement resulting from repetitive identical stimuli is caused by subliminal response enhancement; the enhanced state is evident as a potentiation of the subliminal response, both in terms of its final amplitude and its rate of depolarization towards slow response threshold. (*d*) At frequencies above 0.1 Hz, enhancement after a resting period is cumulative from cycle to cycle; half-time of disappearance of the enhanced state is on the order of 4 s. (*e*) Full slow response firing is not necessary for enhancement to occur; repetition of ineffective subthreshold stimuli (0.8 I_{T_0}) can eventually lead to slow response firing. (*f*) The enhanced state cannot be equated with any detectable change in the resting level of membrane potential; on the other hand, the voltage time course of the subliminal response is a good indicator of the existing steady state level of enhancement.

At this moment, we can but wonder about the possible mechanism involved in the extremely long-term "memory" underlying cumulative enhancement of subliminal response. Enhancement does not seem to be secondary to catecholamine release during stimulation. Thus enhancement effects can be easily observed in large atrial preparations in TKBa when slow response excitation is elicited by distant electrical stimulation or even by propagation of normal action potentials originating in a Tyrode-exposed segregated extremity of the tissue (Masuda, 1976; Masuda and Paes de Carvalho, 1982). Furthermore, LAP-related phenomena have been observed in propagated slow responses elicited in high-K^+/isoproterenol (Pappano, 1970) and in high-K^+/epinephrine (Masuda, 1976). LAP can also be observed in our preparation when slow response is excited in high K^+/epinephrine, in spite of the fact that spatial homogeneity is lost in this solution. In such cases, catecholamine excess in the bath would be expected to swamp any additional effect of catecholamine release by stimulation. Enhancement does not seem to be caused by interstitial accumulation of K^+ during activity either. Even though some progressive increase in interstitial K^+ may occur in cardiac muscle with repetitive driving (Cleeman and Morad, 1978), this presumptive increase could not be construed as a possible explanation. Not only is K_o^+ already fairly high (10 mM in our preparation; 10–25 mM in high-K^+/epinephrine experiments), but its increase would presumably tend to augment g_K (Weidmann, 1956; Noble, 1965) and shunt both subliminal and slow responses. In fact, slow response excitability enhancement is attenuated by increasing K_o^+ up to 25 mM: in 25 mM K_o^+/1 mM Ba^{2+} Tyrode solution, both LAP and hysteresis phenomena disappear. Also, stronger stimuli, as compared with 10 mM K^+/Ba^{2+} Tyrode solution are necessary to induce subliminal responses capable of triggering slow response (S. Cukierman, manuscript in preparation).

Prepotential or subliminal response enhancement such as reported in this paper finds no counterpart in single cell electrogenic phenomena in different

tissues. Potentiation and facilitation with similar or longer time courses are well known only in relation to synaptic transmission, where internal calcium accumulation in the presynaptic terminals has been implicated as its possible mechanism (Kandel, 1977). Our present data do not allow us to address the intrinsic mechanism of subliminal response enhancement. Although the basic property may reside at the membrane itself, it is clearly impossible to rule out some kind of cumulative alteration in the cell environment.

Relationship between Subliminal Response and Other Oscillatory Behaviors of Cell Membranes

Digitalis-induced after-depolarizations (Ferrier et al., 1973; Kass et al., 1978), oscillatory after-potentials (Hiraoka et al., 1979), and the after-depolarization that leads to triggered activity in simian mitral valve and canine coronary sinus (Cranefield, 1975) may well share a basic common mechanism with the subliminal response described here. In digitalis-induced after-depolarizations, amplitude increases with the number of preceding beats (Ferrier et al., 1973). Rate of stimulation exerts an important effect on the amplitude of delayed after-depolarizations in low resting potential myocardium islets in the mitral valve or in coronary sinus (Cranefield, 1975). As to oscillatory after-potentials (OAP), Hiraoka et al. (1979) observed that (*a*) as the stimulating cycle length decreases, the OAP time to peak decreases; (*b*) as the number of stimulated beats increases the amplitude of the following OAP increases; (*c*) solutions containing K^+ tend to abolish OAP. This last observation is paralleled by the K^+ depression of automatic activity generated by Ba^{2+} (Antoni and Oberdisse, 1964; Cukierman, 1978). As mentioned above, we have also found that increasing K_0 beyond 15 mM substantially depresses the subliminal response.

It may be tempting to relate LAP to the cumulative enhancement of a delayed after-depolarization. This phenomenon might just add to the subliminal response and cause its progressive increment in successive cycle at the onset of activity in a rested preparation. This seemed to be indeed the case in some of our preparations in which "triggered" activity could be induced. However, in several others LAP could be observed in the absence of noticeable delayed after-depolarizations (see also Masuda and Paes de Carvalho, 1982). If this phenomenon is related to LAP, it certainly cannot explain cumulative cycle-to-cycle enhancement of isolated subliminal responses to pulses smaller than I_{T_0}. This observation leads us to suggest that subliminal response enhancement is the basic phenomenon and that delayed after-depolarizations may represent enhanced subliminal responses that are revealed after recovery from a "positive" (hyperpolarizing) after-potential at the end of an action potential (or slow response).

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