VOLTAGE CLAMP OF CARDIAC MUSCLE IN A DOUBLE SUCROSE GAP

A FEASIBILITY STUDY

LESLEY HARRINGTON *and* EDWARD A. JOHNSON

From the Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710. Dr. Harrington's present address is % Dr. J. Harrington, The Rockefeller University, New York 10021.

ABSTRACT A method of stabilizing the membrane potential of a small area of cardiac muscle membrane and the limitations of this method are described. Tiny bundles or strands, approximately 80 μm in diameter, of electrically interconnected fibers from the ventricles of rabbit hearts were used in a double sucrose gap. Current records associated with step changes in voltage were complicated by two capacitive surges of current of nodal and nonnodal origin and large "leakage" currents of nonnodal origin resulting mainly from the multifibered nature of the preparation and emphasized by the method. The transient, inward membrane currents in response to moderate depolarizing steps in command potential had the same duration as the upstroke of the action potential. In good runs, currents were smooth and free from notches. These initial currents behaved qualitatively like the initial sodium currents in squid axon and in other excitable membranes. A fraction of the initial sodium current persisted at least as long as 300 ms. The relationship between peak initial current and voltage was graded and linear in the positive direction. In the negative region the relationship was often very steep, indicating insufficient voltage control of all the membranes despite the squareness of the voltage record. Other indications of inadequacy of control could occur and thus even with this optimum preparation of cardiac muscle it was not feasible to analyze quantitatively either the initial or the prolonged sodium currents.

INTRODUCTION

Many attempts to voltage clamp heart muscle have been reported in the literature (see Johnson and Lieberman, 1971). The results of these studies have not been consistent but have varied from author to author and, perhaps not surprisingly, from preparation to preparation and from method to method. Furthermore, in most cases, voltage control in response to step changes in command potential has been slow, and resolution of currents within the first 10–100 ms at the beginning and the end of depolarizing steps in command potential could not be obtained. It has been suggested that these technical difficulties and the inconsistencies in the results arose from limitations imposed by (*a*) the preparation and (*b*) the method

(Sommer and Johnson, 1968; Johnson and Lieberman, 1971; Kootsey and Johnson, 1972). The present paper describes the application of the double sucrose gap voltage clamp technique to a naturally occurring preparation of cardiac muscle with the least undesirable morphology. In these experiments every effort was made to overcome the limitations imposed by the preparation and by the method; the results so obtained, noting all possible signs of shortcomings still present, are described.

SELECTION OF PREPARATION AND VOLTAGE CLAMP TECHNIQUE

Heart muscle is composed of bundles of fibers of different diameters in different animals, and these bundles and fibers are interconnected both morphologically and electrically, in various ways, so that, electrically, heart muscle is a syncytium. It is not surprising that such anatomical arrangements do not approximate the behavior of a long cylindrical cell, the behavior of which in turn can be approximated by one-dimensional cable theory. Although no preparations of heart muscle other than, perhaps, some grown in tissue culture have the ideal structure of a long cylindrical cell, or a bundle of such cells, some preparations approach the ideal more closely than others. Therefore a careful choice of preparations may remove some of the difficulties imposed by the anatomy of the preparation. Basically the preparation should behave as though little if any sarcolemma is in series with an appreciable external resistance. This criterion demands, morphologically, the absence of an extensive and narrow transverse tubule system and, also for a bundle of cells, that there be wide spacing between the cells, except where they are resistively connected (i.e., forming close cell appositions), and that the strand have a small overall diameter.

The small strands or *trabeculae carneae* which form a network on the endocardial surface of the ventricles of rabbit hearts were selected. This preparation morphologically was still not ideal; but, as a result of a comparative study of the hearts of frog, chicken, and several species of mammal (Sommer and Johnson, 1968; Sommer and Johnson, 1969), the strand preparation appeared to have the minimum number of complexities and to be the closest approach to the ideal of a long cylindrical cell. Its morphology has been described in detail elsewhere (Johnson and Sommer, 1967), but briefly is as follows. The strand can come in conveniently shaped bundles (approximately 3 mm in length and 30–80 μm in diameter), which can be dissected out intact, attached at each end to small lumps of the ventricular wall. It is composed of a number of muscle fibers (2–20), the adjacent membranes of which frequently become tightly apposed to form one of three kinds of cell apposition or junctional complex. One of these (the nexus, tight or gap junction, or *fascia occludens*) is presumably the locus of a resistive connection between adjacent fibers. The frequency and distribution of such junctional complexes in a 200 μm

length of strand from a rabbit heart have been determined, and they were represented by a quasi-electrical wire diagram (see Fig. 7 of Johnson and Sommer, 1967). The individual muscle fibers within a strand are approximately 10 μm in diameter. Fibers of larger diameter are found in strands from hearts of larger mammals such as the sheep or goat, but these strands are quite unsuitable in other respects (Sommer and Johnson, 1968). Except when they are forming junctional complexes, the individual muscle fibers in rabbit strands are relatively widely spaced (more than 1 μm apart), unlike the individual fibers in strands from the larger mammals, which are very closely packed. The muscle fibers, unlike ventricular muscle fibers, have no transverse tubules and therefore are classified as Purkinje fibers (Johnson and Sommer, 1967; Sommer and Johnson, 1968). Thus, none of the sarcolemma of the muscle fibers should be in series, externally, with any appreciable resistance (Sommer and Johnson, 1968).

The limitations imposed by the method are mainly dependent on the anatomy, but they also often involve the nature of the source of the controlling current. A theoretical analysis (Eisenberg and Johnson, 1970) of the electric field about a point source of current within cells of differing shapes indicated that an intracellular microelectrode should be avoided as a current source. If such an electrode is used, spatial and temporal variation in membrane potential about it must be anticipated. Use of the three-microelectrode method of voltage clamp (Adrian et al., 1970 *a*, *b*) is not possible because strands of heart muscle do not have naturally occurring ends. An artificial end made by cutting or tying the strand is, electrically, not sufficiently well defined, since the functional electrical end in all probability would be located not at the grossly visible end, but more likely at the irregular contour formed by the outline of the undamaged cells near the cut or tied end. The sucrose gap technique (Stampfli, 1954; Julian et al., 1962 *a*, *b*), although it presupposes that the preparation can be approximated by a one-dimensional cable, is preferable in one respect at least, in that the controlling current is injected along the cytoplasm of the fiber or fibers. In this case the current source looks like a disk in the plane of, and equal in area to, the cross section of the fiber. The double sucrose gap (Julian et al., 1962 *a*, *b*) theoretically makes possible the electrical isolation of a smaller segment of the above-described strands than could be obtained, for example, by tying or cutting (Deck et al., 1964; Fozzard, 1966; Vassale, 1966; McAllister and Noble, 1966), or by the use of a single sucrose gap (Mascher and Peper, 1969; Morad and Trautwein, 1968; Beeler and Reuter, 1970; Ochi, 1970). Hence this method was chosen.

METHODS

Tissue Preparation

Rabbits were killed by a blow on the neck, and the heart was excised within 30 s, washed free of blood, and placed in a beaker of warmed Krebs-Henseleit (hereafter called K-H)

solution aerated with a gas mixture of 95% oxygen and 5% carbon dioxide. The right ventricular wall was removed by cutting along its septal border, and it was then transferred to a dissection chamber, the temperature of which was maintained at 37°C and which was irrigated with K-H solution aerated with the above gas mixture. Small *trabeculae carneae* or strands (30–80 μm in overall diameter) were removed from the endocardial surface of the right ventricular wall by excising a small portion of the wall to which each end of the strand was attached. After this dissection was completed, the unused portion of the right ventricular wall was left in the chamber for further dissection, and the freed strand was transferred to the sucrose gap chamber. The contractility of the strand could not be ascertained. Some strands had no muscle fibers. Others appeared to contract, but were being pulled mechanically by the rest of the ventricle. If an isolated strand contracted spontaneously it was discarded, since experience showed that such strands died very rapidly.

Sucrose Gap Chamber

The usual design and method of construction of the sucrose gap chamber requires the milling of channels and the drilling of precisely aligned holes in a block of Lucite. The architecture in the region where the sucrose and K-H solutions join with the tissue channel determines, in a largely empirical and hence somewhat unpredictable fashion, the size, form, and stability of the central nodal region of K-H solution which separates the sucrose streams. This necessitates many changes in the shape of this region before a satisfactory design can be developed for a given kind and size of preparation. We are indebted to Dr. T. W. Anderson for the following design and method of construction of the sucrose gap chamber. The novel feature of this design and method of construction was that the required channels were formed by first scratching grooves on the surface of a block of Lucite (Fig. 1 A) which, when covered by a plain sheet of Lucite, were converted into channels (Fig. 1 B). The narrow grooves shown in the figure were enlarged away from the central region of the chamber and were connected to large pools in which the current and voltage electrodes, together with their corresponding reference electrodes, were located.

After dissection, the strand was transferred to the central groove of the sucrose gap chamber. Excess fluid clinging to the strand was removed by gentle suction. The strand was adjusted so that the lumps of muscle at its ends were located in the lateral pools. To achieve this some strands had to be stretched up to a maximum of approximately 50% of rest length, in which case they were held at this length by catching the terminal lumps of muscle up against the edge of the walls of the lateral pools. The plain top cover in Fig. 1 A was dried and swabbed with silicone oil (dimethylsilicone fluid, General Electric Co., Chemical and Medical Div., Schenectady, N. Y., type SF99) and gently placed over and clamped securely against the grooved surface of the chamber block by tightening the holding screws. The flows of K-H solution into the current and voltage pools were turned on and were checked visually for absence of air bubbles. Electrical connections were then made to the electrodes and all DC levels checked. The sucrose flows were then turned on and the appearance of a resting potential was monitored. Because of the short expected lifespan of the preparation (usually 5 min or *less*, but rarely up to 20 min), the experiment was begun with high potassium in the voltage pool rather than by introducing it after an initial period in normal K-H solution which would have permitted us to follow the appearance of a resting potential. In experiments where K-H solution in the central pool was replaced by high potassium solutions, the holding currents increased by about the expected amount (see Results).

Differences in refractive index between sucrose and K-H solution could not be detected in this particular setup. Hence, the formation of the node could not be seen. Formation of

the gap and control of flow were empirically determined from electrical criteria, the magnitude of the resting potential and stability of the clamp currents. Nevertheless, even if the flows and node were visible, the formation of the node in and about the inner fibers could not have been seen.

The sucrose gap chamber was heated by radiation from an adjacent incandescent lamp. The temperature of the effluent fluids was approximately 34°C.

Electrodes

Electrical connections of the current pool, voltage pool, and the central nodal pool were made by glass-insulated, sintered silver-silver chloride electrodes (In Vivo Metric Systems, Redwood Valley, Calif., type GW-2). The sintered electrodes replaced earlier silver-silver chloride agar bridges with a corresponding reduction in resistance and in asymmetry voltages. The current and voltage electrodes were placed to the side of the corresponding pools shown in Fig. 1 B. The current reference electrode was placed upstream in the central K-H channel. The voltage reference electrode was placed as indicated in Fig. 1 A.

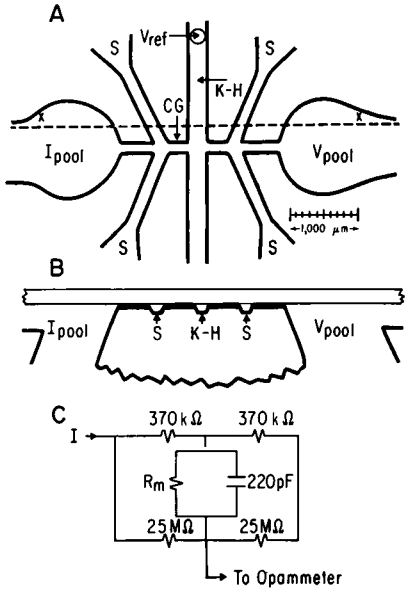

FIGURE 1

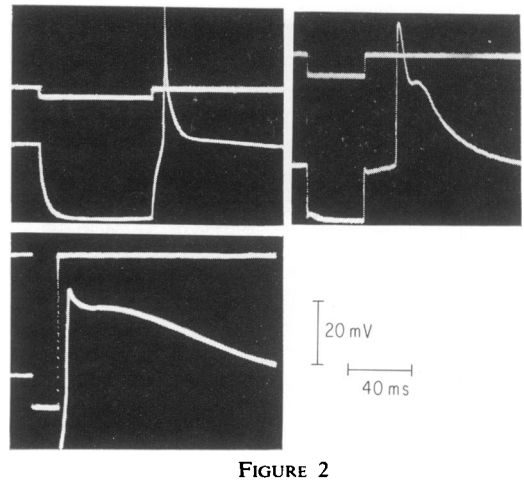

FIGURE 2

FIGURE 1 A and B: Scaled drawing of the main chamber of the sucrose gap apparatus: A, top view; B, section through *x-x* in A. K-H is the inlet and outlet of the Krebs-Henseleit channel; S, the inlet sucrose channels; I and V, the current and voltage pools (both containing isotonic K_2SO_4), respectively; CG, the central groove in which the strand lies; V_{ref}, the opening to the reference electrode inserted from below. Sucrose flows out through the I or V pool drain holes, or down the outlet channel of the K-H channel. C: Passive network used to test performance of control and recording electronics. R_m could vary between 5 MΩ and 50 kΩ. The opammeter is an operational amplifier in currentometric mode.
FIGURE 2 Typical action potentials elicited by anode break in the double sucrose gap chamber. All action potentials were attenuated. Upper trace, current; lower trace, voltage. In the upper right figure, capacitive coupling between current and voltage pools is the most likely cause of the distortion of the leading and trailing edges of the voltage response to the constant current pulse.

Electronics

The voltage clamp setup was basically the same as that described by Julian et al. (1962 *a*, *b*) and by Anderson (1969). With the real sucrose gap assembly replaced by a passive network (see Fig. 1 C) the rise time of the current amplifier which was purposely reduced to minimize noise was 25 μs, and that of the voltage amplifier 100 μs when R_m was 5 MΩ.

Solutions

The composition of the K-H solution in grams per liter was as follows: NaCl, 6.9; KCl, 0.35; $MgSO_4 \cdot 7H_2O$, 0.29; $CaCl_2$, 0.28; KH_2PO_4, 0.16; $NaHCO_3$, 2.1; glucose, 2.0. In high potassium solutions all the NaCl was replaced by KCl; sodium was still present as the bicarbonate. Choline chloride was substituted for NaCl in sodium deficient solutions with normal potassium. The sucrose was deionized by passing it through a deionizer (Crystalab, Inc., Hartford, Conn., model CL-5). Isotonic K_2SO_4 or KCl was used in both recording and current pools. The high potassium solution was necessary in the current pool because when hyperpolarizing currents were passed through the nodal membranes, depolarizing currents passed through the membranes in the current pool. These currents sometimes caused action potentials which were propagated to the central pool where they were recorded.

RESULTS

General Observations

Recording of the Resting Potential. Resting potentials of the expected magnitude of about 80 mV were never recorded. The attenuation of the true resting potential could be as great as 90% but more usually the attenuation was about 50%. Due to the closed arrangement of the gap apparatus the "true" resting potential could not be monitored with a microelectrode. Hence, how much of the attenuation was real and how much due to deterioration of the strand could not be known. The problem of attenuation and of leakage has been discussed by Dodge and Frankenhaeuser (1958), and specifically for the sucrose gap technique by Julian et al. (1962 *a*, *b*), where, with single axons, attenuation of the resting potential of approximately 5% was normal. With our multifibered preparation more attenuation was expected. Intuitively one would not expect that a narrow and well-defined central node could be formed around all the fibers by the stream of K-H solution separating the two sucrose streams. With a suitably designed chamber, the width of the central node formed by the K-H stream could be satisfactorily narrow at the endocardial surface of the strand. But, thereafter, the formation of the node about the inner muscle fibers must depend largely on diffusion inward of the sucrose and K-H solution. This inward diffusion from the endocardial surface of the strand must occur in both radial and axial directions between the fibers. The diffusive intermixing of the nodal K-H solution with the sucrose streams would enlarge the effective node towards the center of the strand and, consequently, dilute the ionic composition of the extracellular fluid. This must result in uncertainty about the width of the node and the composition of the extracellular fluid there, and for this reason we made no attempt to estimate the area of membrane from which we were

recording. In these regions, also, proper insulation by the sucrose could not be expected. Hence leakage paths for current must be present. It was noticed that the thicker preparations were often more stable and gave more normal resting potentials, but in these cases it seemed likely that the inside fibers had died.

Introduction of sucrose also resulted in apparent membrane hyperpolarizations (cf., Julian et al., 1962 *a*; Blaustein and Goldman, 1966), which in our case were inconsistent in amplitude and occurrence and often only became evident after many minutes. In the particular experiments shown in this paper, sucrose hyperpolarizations were negligible, but they were not uncommon in many of our experiments.

Recording of the Action Potential. Action potentials were also attenuated, as shown in Fig. 2. These action potentials were elicited by the breaking of a hyperpolarizing current pulse (anode break response). The plateaus of action potentials elicited in this way are generally depressed in amplitude and shorter in duration. The method of stimulation may therefore account for these characteristics in action potentials recorded in the sucrose gap. However, action potentials elicited by other methods (e.g., short depolarizing pulses) tended also to have a shorter duration (approximately 50–60 ms) compared with the duration of an action potential recorded by a microelectrode from such a strand *in situ* (approximately 120 ms).

Voltage Clamp Experiments. The currents associated with a 20 mV hyperpolarizing and a 30 mV depolarizing step change in command potential (from a holding potential of 80 mV, a holding current of 0.3 μA, and an original resting potential of 50 mV) are shown in Figs. 3 A and B. In A and B the upper trace is voltage, and the two lower traces are current on different time bases. Currents were routinely recorded on two time bases. In all experiments the corresponding hyperpolarizing step was alternated with each depolarizing step in voltage, except that hyperpolarizing pulses greater than 90 mV from the holding potential were not regularly applied. The holding current throughout the entire experiment was monitored on a chart recorder.

Capacitive Surges of Current. All current records began with a sudden transient surge of current about 75 μs in duration which inflected sharply with a more slowly decaying component. The first surge was, in general, at least an order of magnitude larger than the peak of the transient currents and was so brief that it did not show in photographic records and it was considered to result from charging of the membrane capacity. The second, relatively slowly decaying component, was symmetrical for step changes in potential in both the hyperpolarizing and the depolarizing directions, and its size and form did not depend on time, nor did its value alter during the course of an experiment. For larger depolarizing steps the major part of the slow decay coincided with the turn-on of the early transient currents, so obscuring their real time-course and amplitude. The decline of this second component was exponential with a time constant of about 500 μs in all experiments,

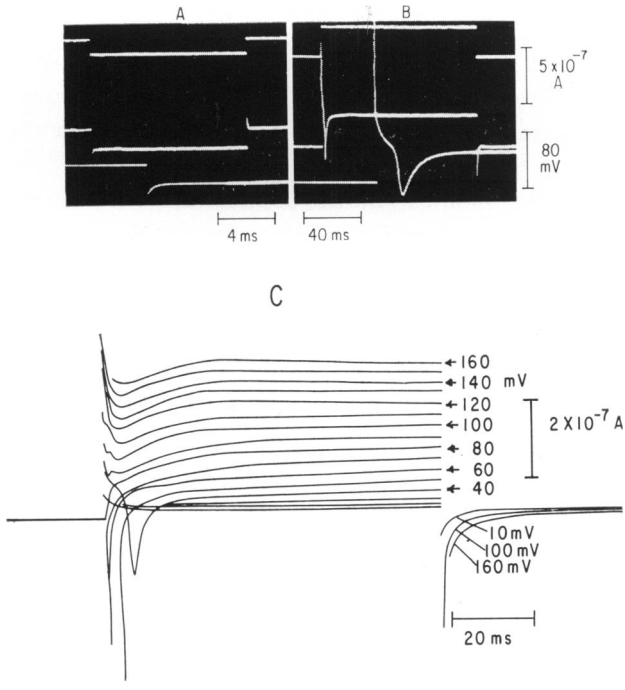

FIGURE 3 A: Response to a 20 mV hyperpolarizing step. B: Response to a 30 mV depolarizing step. Top trace, voltage; lower two traces, current, recorded on two time bases, upper of the two, slow and the lower, fast. The fast capacitive spikes have been retouched. The two time scales (4 and 40 ms) apply to both A and B. C: Tracings of a family of current records on slow time base from the same experiment as the records on a fast time base shown in Fig. 5.

regardless of the diameter of the strand or of the parameters of the sucrose gap system. The time constant, however, did appear to be smaller when very large depolarizing steps were applied. The form and time-course of these slow capacitive currents were very similar to those seen by Moore et al. (1967) and Narahashi and Haas (1968) in squid or lobster axons voltage clamped with a double sucrose gap technique, and those seen by Anderson (1969) in smooth (uterine) muscle, also voltage clamped with a double sucrose gap technique. The origin of such currents has been commented upon by these authors and by Dodge and Frankenhaeuser (1959), Takata et al. (1966), Hille (1967), and Moore et al. (1970).

In effect the membrane capacitance behaves as though part of it were in series with a resistance. This can be an inherent property of the membrane capacitance itself, as suggested by the above authors, a property variously and euphemistically described as being "lossy," as having "soakage" or being "frequency dependent," or as an imperfection of the type described by Cole (1968). On the other hand, the fiber membrane could be physically divided into two components, one of which is in series with a resistance as, for example, the membrane of skeletal muscle fibers

where part of it is in the form of the transverse tubular system. Although our preparation lacks a transverse tubular system, nevertheless, the membranes bathed by the sucrose gap on the current pool side of the central pool could represent such a membrane component: into which charge flows through the relatively high resistance of the sucrose to the central pool.

Another feature of the initial current was sometimes seen (see arrow Fig. 8 B). This was an increase in current immediately after the initial surge and preceding the slow secondary component. When it occurred it got larger as the experiment progressed. We have attributed it to capacitive coupling between the current and voltage electrodes. Normally it was insignificant, but as the resistance of the pathway from the current pool to the central node increased, presumably as a result of ions being leached out of the fibers bathed by the sucrose, this capacitive coupling became noticeable. It occurred more often when recording from strands of very small diameter. The effect of this capacitive coupling in an unclamped preparation is seen in the top right-hand picture of Fig. 2.

Transient Currents. In Fig. 3 A the current in the hyperpolarizing direction, once the capacitive components had declined, remained constant in the inward direction. In Fig. 3 B, where a depolarizing step in voltage was applied, the current went inward before the slow capacitive component had declined fully. The peak of the inward current was reached at about 2 ms after the beginning of the pulse.

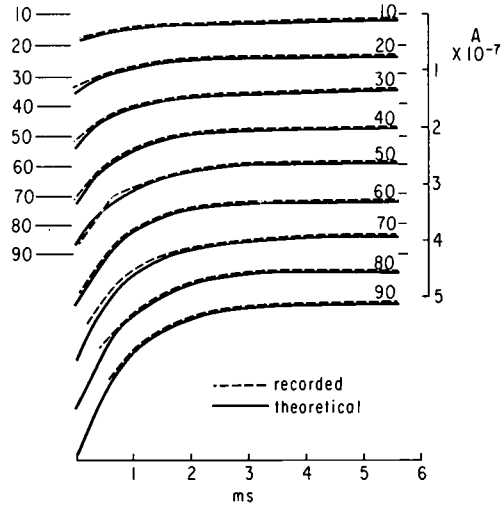

FIGURE 4 Tracings of current records (dotted lines) for different hyperpolarizing steps in command potential applied to the same strand as in Fig. 5. The solid lines are a set of exponentials of identical time constant. The initial and final values were determined as described in the text. The numerals refer to the value, in millivolts, of the step in command potential from the holding potential, indicated by the horizontal bar at beginning and end of a trace.

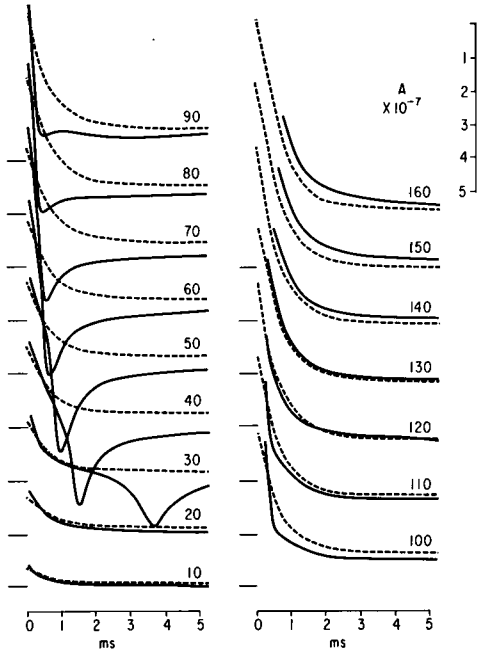

FIGURE 5

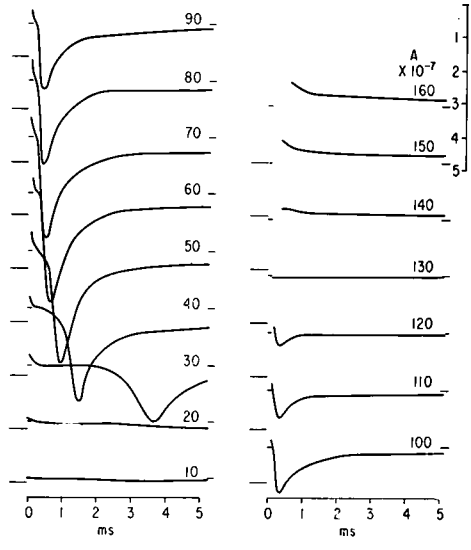

FIGURE 6

FIGURE 5 Tracings of current records (solid lines) for different depolarizing steps in command potential. The dotted lines are the same set of exponentials, changed in sign, as in Fig. 4. The numerals at the end of each trace are the values (millivolts) of the step in command potential with reference to the holding potential.

FIGURE 6 Family of current traces for different depolarizing steps in command potential, from Fig. 5, corrected for the slow capacitive component. The horizontal bar at the beginning and end of each trace is the steady-state value of the theoretical curves of Fig. 5, which has the same value (changed in sign) of the current in response to the corresponding hyperpolarizing step in command potential. The numerals on the horizontal bar at the end of the trace are the values (millivolts) of the step in command potential with reference to the holding potential.

The current then declined, at first rather rapidly and thereafter more slowly, and apparently became positive. At the longer time of about 10 ms, as seen in the records of Fig. 3 C, the current started to increase further, taking approximately 30 ms to reach an apparent steady-state value.

Fig. 4 shows a set of tracings of a family of current records associated with hyperpolarizing steps in command potential, and Fig. 5 shows the corresponding family of currents for the depolarizing direction. As noted above, the slow secondary capacitive component obscured the time-course of the initial transient currents. Hence the slow capacitive components were removed by hand. As stated above, the slow capacitive decline was exponential. Hence, currents recorded for very small depolarizing steps (that is, before ionic events coincided with this decay), and for

all hyperpolarizing steps, could be fitted according to $I = I_\infty - (I_\infty - I_0) \exp(-t/\tau)$, where I_0 was the current value at the point of inflection and I_∞ the final steady-state current value. Both I_0 and I_∞ varied linearly with the size of a hyperpolarizing step change in command potential. Using these experimentally determined values of I_0 and I_∞, and the same time constant, $\tau = 600$ μs, chosen to give the best fit, a set of exponential curves was fitted to the currents recorded in the hyperpolarizing direction, where the fit was good for all steps. The same set of exponentials, changed in sign, was fitted to the currents recorded in the depolarizing direction (Fig. 5). The fit was still good for the smaller steps (up to 80 mV) but not for the bigger depolarizing steps where exponentials with a smaller time constant would have fitted better. Removal of the slow capacitive component from the current records in the hyperpolarizing direction resulted in step changes of current. Removal of the capacitive components in the depolarizing direction resulted in the set of curves shown in Fig. 6.

The family of corrected currents shown in Fig. 6 resembles more closely the early membrane currents recorded in squid axon or in frog node of Ranvier. At all hyperpolarizing steps (not shown) the currents were inward. At small depolarizing steps the currents were outward. With depolarizing steps of more than 20 mV (i.e., to below about -70 mV in Fig. 7), transient and inward currents appeared. As the depolarizing steps were increased, this transient current reached its peak more quickly and decreased in amplitude, and also reversed direction when the voltage step was above about 130 mV (i.e., to $+30$ mV absolute potential). Indications of this reversal potential were clear in the corrected records, but virtually undetectable in the original traces. The transient currents decayed from their peak value, at first very rapidly, but then more slowly. They remained less than the steady-state value of the corresponding hyperpolarizing current (shown by the horizontal bar at the end of each trace) for at least 5 ms after the beginning of the pulse, even for depolarizations up to $+30$ mV absolute potential. For larger voltage steps their value was always greater. Fig. 7 shows a plot of the peak transient currents (closed circles) against voltage for the set of corrected traces shown in Fig. 6. Also plotted are the currents in response to the hyperpolarizing steps (closed circles). These points lie on a straight line which was extrapolated in the depolarizing direction (dashed line). An unknown proportion of these currents in the hyperpolarizing direction, and presumably also in the depolarizing direction, must be of nonnodal membrane origin. That is, the current which resulted in unclamped preparations in attenuation of the measured transmembrane potential and of the action potential is being measured. A large proportion of the current which is recorded apparently flows through the extracellular space of that part of the strand bathed by sucrose on the current pool side, a pathway which is in parallel with the intracellular pathway taken by what is considered to be membrane current. This most likely accounts for the high slope conductance (reciprocal 450 kΩ) in the hyperpolarizing region of the current-voltage relationship in Fig. 7. In other experiments the slope conductance

was often higher. But whatever the origin of these large hyperpolarizing currents, provided that the transient currents in response to the depolarizing steps are caused solely by a change in permeability of one ion, then the potential at which this extrapolated line crosses the peak initial current curve is considered as the equilibrium potential for the transient current (cf. Dodge and Frankenhaeuser, 1958; Anderson, 1969). In Fig. 7, when the straight line drawn through the hyperpolarizing currents was extrapolated in the depolarizing direction, it intersected the peak initial current curve at +30 mV, the potential in Fig. 6 where the corrected current records showed no initial transient current either inward or outward. The initial current reversed direction in the range +30 to +50 mV in nearly all experiments.

In general, the relationship between peak current and voltage was graded, reaching a maximum inward value at about −50 mV (absolute potential). The relationship in the positive direction was always linear, at least to the reversal potential. This linearity was not unexpected since it is what is observed in squid axon or frog node of Ranvier, but it has not been reported in previous studies of cardiac muscle (Rougier et al., 1968; Beeler and Reuter, 1970; summarized by Johnson and Lieberman, 1971). Nevertheless, although such nonlinearity may reflect inhomogeneity

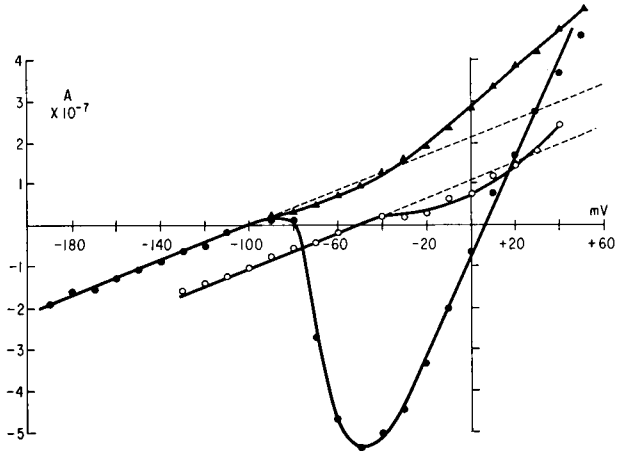

FIGURE 7

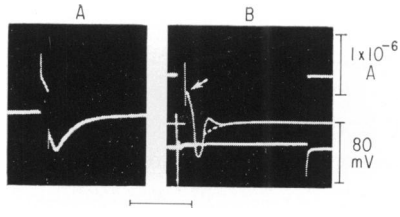

FIGURE 8

FIGURE 7 Current-voltage relationship in a strand. ●, hyperpolarizing currents (from Fig. 4) and the peak transient currents in the depolarizing direction (from Fig. 6); ▲, currents measured at 100 ms. (from Fig. 3 C); ○, currents measured at 5 ms in hyperpolarizing and depolarizing directions from an experiment done on the same strand, held at −50 mV. The slope in the hyperpolarizing direction is the same in both cases. The reversal potential (see text) also is the same.

FIGURE 8 A: Current record obtained when the membrane was depolarized by 30 mV and repolarized to the holding potential before the inward current had reached its peak value. The current continued inward. Time cal. bar: 5 ms. B: Composite of responses to two consecutive 40 mV depolarizing steps. The first response was notched. The second response (dashed line, put in by hand) was not. The currents were recorded on two time bases. Time cal. bar: 5 and 50 ms. The significance of the arrow is mentioned in the text on p. 638.

of membrane potential control as postulated by Johnson and Lierberman (1971), its absence cannot, as was implied by these authors, be taken as evidence of adequate voltage control. On the contrary, the analysis by Kootsey and Johnson (1972) showed that the characteristics of the current-voltage relationship are more or less determined by the magnitude of the conductance of extracellular space as well as that of the membrane.

In most experiments the relationship between peak current and voltage in the negative region was very steep, and in some cases steep enough to approximate a threshold. This indicated insufficient voltage control of all the membranes, despite the squareness of the voltage records (p. 410, Cole, 1968; Kootsey and Johnson, 1972). Also in voltage steps to potentials around the beginning of this negative slope region, the transient currents sometimes turned on after long (up to 10 ms) and fluctuating delays. Observations like those in Fig. 8 also demonstrate the kind of behavior that must be attributed, in the absence of evidence to the contrary, to inhomogeneity of membrane potential control, even though in these cases again the voltage record appeared satisfactory. There were many preparations also in which the voltage record obviously was not satisfactory. In Fig. 8 A the membrane was repolarized to the holding potential before the peak of the inward current had been reached. The recorded current, however, did not decline back to the resting level but continued inward. Another sign of imperfect control was the presence of notches ("abominable notches," p. 326, Cole, 1968) on the falling phase of the fast initial transient current (Taylor et al., 1960). These were often incipient in our experiments as seen in Fig. 8 B, a composite of two consecutive photographic records. The notch occurred in the first record. The dotted line (put in by hand) was the current course in the following photograph where the same potential step was applied. In this case the current rose smoothly without notching. Transient oscillations in current often occurred, particularly in the region of peak inward current flow, but sometimes throughout an entire run. These could sometimes be removed by reducing the flow of K-H solution into the central groove, but usually, once the gap had been established, changes in flow rate short of turning it off had little or no effect. All of these instabilities were more apparent when tinier strands of smaller diameter were used. Certainly, a more stable and more predictable preparation would be needed in order to undertake an analysis of the kinetics of the early transient current. Despite the difficulties in obtaining any results, we will now list our consistent observations, apart from those listed above concerning the "shape" of the currents.

Experimental Results

Effect of the Holding Potential. The transient currents were larger the more negative the holding potential. The currents at a less negative holding potential were a constant fraction of those at a more negative potential. The peak of the

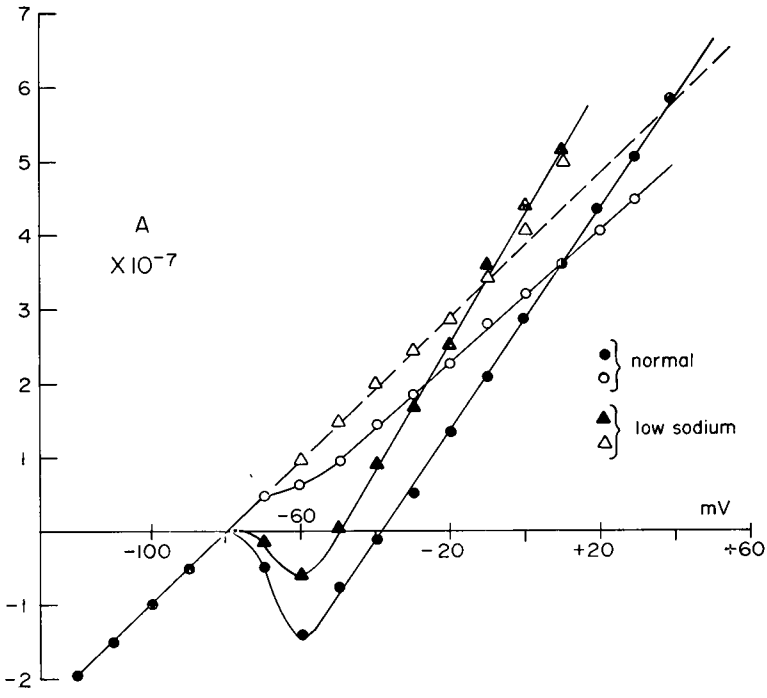

FIGURE 9 Current-voltage relationships from a strand in normal and sodium poor solutions. ●, peak currents, and ○, currents at 100 ms for normal solutions; ▲, peak currents, and △, currents at 100 ms in low (20%) sodium solution. The currents in the hyperpolarizing direction were the same for both runs.

transient current for a given depolarizing step was an S-shaped function of the holding potential like the inactivation curves of squid axon (Hodgkin and Huxley, 1952 *b*), of frog node of Ranvier (Dodge and Frankenhaeuser, 1958), and for sheep and goat Purkinje strands (Weidmann, 1955).

Effect of Low Sodium. In sodium-poor solutions, the transient currents were reduced and the reversal potential decreased by about the predicted amount for a sodium concentration cell. Fig. 9 shows the result of an experiment in 20% normal external sodium. The reversal potential decreased by 48 mV, compared with the expected theoretical shift of 43 mV at 34°C. After sodium was replaced by choline chloride, recovery on return to normal solution was incomplete. Sodium was not replaced by Tris because of its quinidine-like actions (Cline et al., 1968), which might have interfered with the above experiment. It was impossible to use sucrose. When potassium replaced sodium in the external solution, the reversal potential for the inward current decreased by the expected amount. The inward currents recovered completely when the potassium was removed and sodium returned to the perfusing fluid. The effects of low sodium and the subsequent recovery are also shown in Fig. 12, where sodium was replaced by potassium.

Effect of TTX. Tetrodotoxin (TTX) (2×10^{-5} g/ml) reversibly reduced or abolished the peak initial currents without affecting the reversal potential. This dose was not at the top of the dose-response curve and was not necessarily enough to block the transient currents in all preparations. The dose is high but has been found necessary in mammalian ventricular tissue (Dudel et al., 1967; Coraboeuf and Vassort, 1967). Fig. 10 shows a family of currents corrected for the slow capacitive component discussed above. These curves were obtained 100 s after the perfusing solution had been switched to one containing TTX. The strand used was the same one from which the tracings shown in Figs. 4 and 5 were obtained and the same set of exponential curves was used to remove the slow capacitive component as used to obtain Fig. 6. The currents in the hyperpolarizing direction, although not shown in Fig. 10, were unchanged. In this particular experiment TTX considerably reduced but did not abolish the inward currents. Complete suppression of the inward currents was often seen; but this incomplete block allowed determination of a reversal potential which in this case was unchanged from +30 mV, the reversal potential before TTX was introduced. The peak current-voltage plot of Fig. 11 also shows that the reversal potential remained the same when the inward currents had started to recover 50 s after removal of the TTX containing K-H solution. The hyperpolarizing currents are not shown in Fig. 11, but they were the same as in Fig. 4.

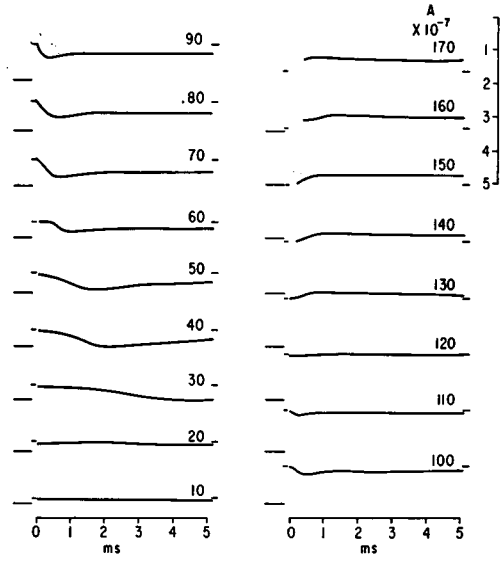

FIGURE 10 Family of current traces (corrected for the slow capacitive component as described in the text) from the same strand as the data of Fig. 6, obtained 100 s after exposure to TTX (2×10^{-5} g/ml) showing partial block of the transient currents. The horizontal bars at beginning and end of each trace are the same as in Fig. 6. The numerals are the values (millivolts) of the step in command potential with reference to the holding potential.

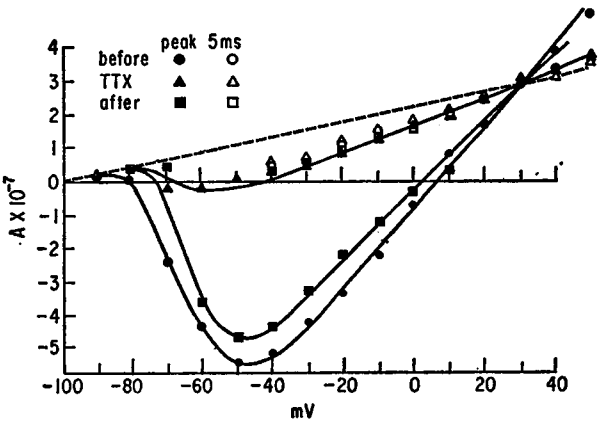

FIGURE 11 Current-voltage relationships from the same strand before (Fig. 5), during (Fig. 9) and after TTX was introduced. ●, ▲, and ■, peak currents before, during, and after TTX, respectively; ○, △, and □, currents recorded at 5 ms before, during, and after TTX action, respectively. The hyperpolarizing currents are not shown, but were the same in each experiment, and the same as in Fig. 4. The dashed line is the line drawn through these currents extrapolated in the depolarizing direction. The reversal potential is the same for all six sets of current records.

These observations in the presence of low sodium and of TTX indicate, as expected, that the initial ionic current is a sodium current which behaves, at least qualitatively, like the sodium current in squid axon.

Currents at Longer Times. (*a*) *Currents at 5 ms.* In Fig. 6 the currents occurring at 5 ms are all less than the corresponding hyperpolarizing currents, for voltage steps to less than +30 mV absolute potential. The same observations hold for later times, provided that the delayed changes (see below) in outward currents had not started to appear. These currents occurring at 5 ms are plotted in Fig. 11 (open circles). A line through these points crosses the extrapolated hyperpolarizing line at the same potential as the line drawn through the peak currents. A line drawn through the currents occurring at 5 ms when the preparation was exposed to an incomplete blocking dose of TTX (open triangles in Fig. 11) and through the currents measured at 5 ms (open squares) when the TTX had been removed also crossed the extrapolated hyperpolarizing line at the same potential. These results indicated that at 5 ms at least, and in other experiments at longer times, some current was being carried by sodium ions.

Fig. 7 shows a plot against voltage of the currents occurring at 5 ms (open circles), recorded from a strand, the same one in Fig. 6 and in Fig. 10, held at −50 mV, a potential at which no discernible transient currents occurred. Note the nonlinearity in the voltage range −40 to +40 mV. The slope of the current-voltage relationship in the hyperpolarizing direction was the same as when the holding

potential was −100 mV. The line drawn through these currents intersected the extrapolated hyperpolarizing line at the same potential, i.e. +30 mV.

(*b*) *Currents at 100 ms.* Currents occurring at 100 ms are plotted against voltage in Fig. 7 (closed triangles) for the experiment from which the tracings of Figs. 4 and 5 were obtained. The situation in this case was not straightforward because currents other than sodium (presumably potassium, as discussed later) contributed to the recorded currents at these later times. Hence, the current-voltage curve did not cross the extrapolated hyperpolarizing curve at the same potential as did the peak current curve. This late current curve is nonlinear, the slope being reduced in the voltage range −80 to −30 mV. When the strand was exposed to TTX the nonlinearity in this voltage range diminished, and in many cases it completely disappeared. That is, the currents occurring at long times were changed in value, quite unlike the situation when squid or frog node of Ranvier is exposed to TTX. The nonlinearity reappeared after the TTX was washed out. The currents in the hyperpolarizing direction did not change during these procedures. The nonlinear relationship occurring at long times was also reduced or abolished when low external sodium solutions were perfused, as seen in Fig. 9.

Unless a potassium or some other current increased in sodium poor solutions, or in the presence of TTX, or with a different holding potential, and these possibilities were not indicated when the currents were measured at 5–10 ms, then it appears that even as late as 100–200 ms some sodium current was still flowing. The nonlinearity of the current-voltage relationship even at very long times would seem to be due to the effect of a prolonged component of a sodium current. One would not, on the basis of the present observations, ascribe the nonlinearity to anomalous or inward reactification of potassium or to the presence of any other ionic currents.

Time-Course of the Sodium Current. Repolarization of the membrane during peak inward current flow was associated with a large tail of current, presumably carried by sodium ions. Unlike the tail in squid axon (Hodgkin and Huxley, 1952 *b*) it did not decline rapidly. The decline appeared to be biphasic, the initial tail being somewhat faster than the second. These tails, although much reduced in amplitude, could be obtained as late as 300 ms after the beginning of the pulse which is what would be expected from the preceding discussion. This procedure of measuring tails of current upon repolarization was not pursued because the slow capacitive component obscured the tails and made analysis difficult.

Delayed Outward Currents. Large outward currents of the sort carried by potassium ions in squid axon or frog node of Ranvier were not seen, and indeed, were not expected. Smaller and very delayed changes in outward, or outward going, currents were usually seen (Fig. 3 C). These small, delayed currents increased in amplitude and approached their maximum value more rapidly as the depolarizing steps were increased. Sometimes these delayed currents would disappear during an

experiment or would not be present at all. We ascribe this to damage to the fiber (Dodge and Frankhenhaeuser, 1958); though it is odd that the delayed currents were affected before the early transient currents. A plot of these currents in Fig. 3 C occurring at 100 ms, as a function of voltage, is given in Fig. 7. At the larger depolarizing steps to above −30 mV absolute potential the currents are greater than the extrapolated hyperpolarizing currents, indicating a "delayed potassium rectification." Often the absolute value of these late currents was smaller than the corresponding hyperpolarized values, although the slope of a line drawn through them was steeper than the extrapolated hyperpolarizing line indicating some rectification. However, sometimes it appeared that there was less outward current at the sodium reversal potential at 100 ms, or later, than at earlier times. This is seen in Fig. 9, in the experiment where low sodium was introduced. In these cases there was no detectable or obvious increase in the delayed current, and indeed a slow decline in current would occur after about 20 ms. This finding could be interpreted as evidence for a new current component with an equilibrium potential very much higher than E_{Na}. We, however, prefer to attribute it to inhomogeneity of membrane voltage control; more membranes being recruited as the magnitude of the depolarizing step was increased. We suggest this interpretation because such behavior was inconsistent and did not occur in the majority of preparations. Also, occasionally such an effect was seen in the earlier currents, giving a curved peak current-voltage relationship (Rougier et al., 1968; Beeler and Reuter, 1970). There is also a possibility, which is untestable, that contractions of muscle fibers could increase the area of membrane in the gap, resulting in slow inward currents. Such currents would not interfere with measurements of transient currents, since the delay between excitation and contraction is 25–60 ms (Johnson and Sommer, 1967), but they could conceivably interfere with the recording of later currents. Such anomalies, whatever their cause, are further reason for skepticism in interpreting the observations.

Although the delayed currents were small, or maybe even absent, some attempt was made to investigate their nature. A quantitative study could not be considered since the voltage control had already been shown to be inadequate to study instantaneous currents occurring upon repolarization of the membrane to different voltage levels, a procedure which we consider to be the most appropriate for studying currents occurring at long times. Moreover, that type of experiment would be further complicated by the presence of the slow capacitive currents and by the tails of inward sodium current (see above). Therefore we decided that the most revealing experiment would be to see what happened to the currents occurring at long times and to the current tails occurring upon repolarization to the holding potential when the normal K-H solution was replaced by a high potassium, low sodium solution (i.e., all NaCl replaced by KCl, some sodium remaining as $NaHCO_3$). In this case, if the late currents were being carried even only partly by potassium ions, one would expect that with large potential steps the late currents would be relatively unchanged, and with the smaller depolarizing steps, that the late currents would be inward (see

Fig. 1, Frankenhaeuser, 1962 *a*), or at least reduced in amplitude. In normal K-H solution, step repolarizations to the holding potential are associated with small, slowly decaying, inward current tails, which the above experiments have shown were most likely carried by sodium ions. Both this sodium current and the inward going slow capacitive component would obscure any component of potassium since at the holding potentials that we used, any current carried by potassium should be outward and, because of the closeness of the potential to the potassium equilibrium potential, very likely small. However, removal of sodium ions should remove the component of current carried by sodium ions, and if the potassium conductance does increase upon depolarization, then, in the high external potassium solution, on repolarization to the original holding potential, inward currents carried by potassium ions should be present (Frankenhaeuser, 1962 *b*), albeit in the present case somewhat obscured by the slow capacitive component.

The results of such an experiment are shown in Figs. 12 and 13. The effects of the new solution were always quick and complete recovery nearly always occurred upon return to normal K-H solution. Fig. 12 shows the effects upon (*a*) the hyperpolarizing currents, whose value more than doubled (the increase in the holding currents is indicated in the caption); (*b*) the peak transient currents which were, as expected, reduced and eventually abolished, together with a decrease in the reversal potential; and (*c*) the currents occurring at 120 ms, which were increased in amplitude, and more so than the increase in the corresponding hyperpolarizing currents.

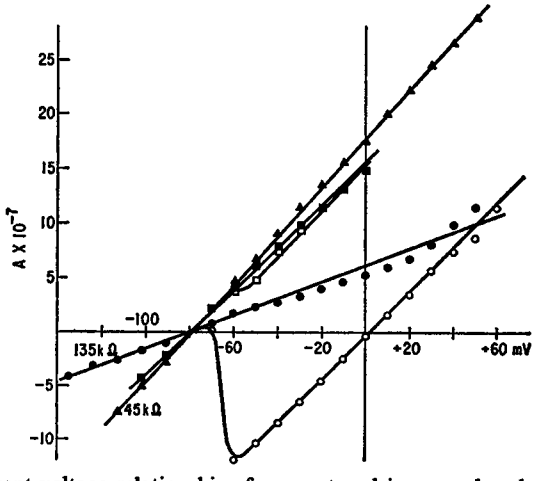

FIGURE 12 Current-voltage relationships from a strand in normal and in high potassium (131.4 mM), low sodium (25 mM) bathing solution. ○, peak currents and ●, currents occurring at 120 ms in normal K-H solution; □, peak currents, and ■, currents at 120 ms recorded 2.25 min after introduction of high potassium, low sodium solution; and ▲, currents at 120 ms, recorded 5 min later. The holding current at −80 mV (the holding potential) in the normal K-H solution was 2.3×10^{-7} A, for the currents occurring at 2.25 min, 8×10^{-7} A, and for the currents 5 min later, 1×10^{-6} A.

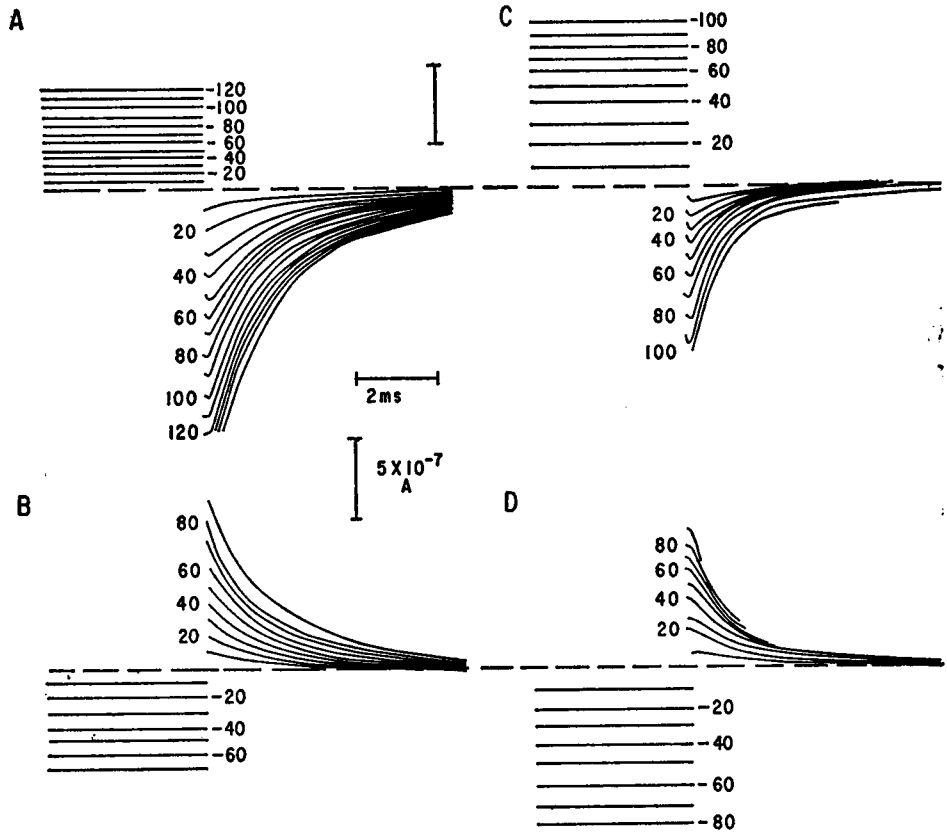

FIGURE 13 Membrane currents associated with the end of depolarizing (A and C) and hyperpolarizing (B and D) steps in potential, and with repolarization (A and C) and depolarization (B and D) from these steps to the holding potential of 100 mV. Amplitude of steps is marked. A and B in normal K-H solution, holding current 2.25×10^{-7} A; C and D in high potassium, low sodium bathing solution, holding current 8.0×10^{-7} A.

The nonlinearity in the -50 to $+40$ mV range was reduced and eventually abolished. Fig. 13 shows in a different strand the effects on the tails of current that occurred when the membrane was repolarized to the holding potential of -100 mV. In the normal K-H solution the tails declined more slowly than the corresponding currents for a step change in command potential of equal magnitude but of opposite sign. These current tails for step returns to the holding potential from a hyperpolarized potential can be considered as purely slow capacitive current. In the high potassium solution the time constant of such tails of capacitive current apparently decreased as did the time constant and magnitude of the tails of current for a return to the holding potential from a depolarized potential. Certainly no evidence was obtained for a declining potassium current as a component of the tails of current. The experiment does show, however, that the major membrane conductance at the holding

potential is a potassium conductance, since the holding currents and the currents in the hyperpolarizing direction and, presumably, in the depolarizing direction, increased by large amounts when the potassium concentration was increased. Any voltage- or time-dependent increase in this potassium conductance would therefore appear to be nonexistent or extremely small, and apparently must play only a small role in the generation of the cardiac action potential.

DISCUSSION

A voltage clamp method with some reliability has been developed for cardiac muscle. We have been able to observe a transient inward current of about the same duration as the upstroke of an action potential. This initial current behaved qualitatively like the sodium currents of the squid giant axon (Hodgkin and Huxley, 1952 *a*) and frog node of Ranvier (Dodge and Frankenhaeuser, 1958). This initial sodium current was followed by two other currents, an apparently slowly inactivating sodium current and an unusually delayed and very small potassium current.

The records of these currents were complicated by two factors: (*a*) a slow capacitive component and (*b*) large attenuating currents, resulting from the multifibered nature of the preparation and accentuated by the method. These artifacts made analysis of the records difficult, but did not invalidate the results. What was much more disappointing was that as our method improved, it became more apparent that the spatial and temporal control of the membrane potential was not adequate. Thus it was not feasible to make a quantitative, kinetic analysis of the data. In fact, it was found that the most stable preparations were those which theoretically should be bad, for example, the very thick strands, where insulation should be worse and where membrane area would be expected to be larger.

The slow sodium currents were novel and not of a sort that was well documented for other preparations. Hence, we had no precedents for exploring their behavior under our inadequate experimental conditions. The majority of successful strands lived for about 5 min or so, and only on extremely rare occasions did a functional preparation last for 20 or 30 min. Our main reason, however, for not exploring these slow currents further in this paper was our concern that they may have arisen from some inhomogeneity of the preparation or deficiency in the method, of which we were not aware and which made us hesitate to take them at their face value (see Johnson and Lieberman, 1971; Kootsey and Johnson, 1972).

Despite the negative nature of these conclusions, we think it worthwhile to report them, if only as an indication of the difficulties involved in clamping cardiac muscle, and, in particular, in interpreting the results, especially since we used a preparation and a method which should have minimized these complexities.

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