J. Membrane Biol. 11, 331 – 352 (1973)

Some Effects of Prolonged Polarization on Membrane Currents in Bullfrog Atrial Muscle

David W. Maughan*

Department of Physiology and Biophysics,
University of Washington School of Medicine, Seattle, Washington 98195

Received 7 April 1972; revised 4 October 1972

Summary. Bullfrog atrial trabecula were voltage-clamped using a double-sucrose-gap method. Step depolarization produced a slowly changing outward current which was studied by analyzing the current "tail" produced by repolarization. The initial phase of the current tail (time constant 0.1 to 0.7 sec at −60 mV) had a reversal potential which depended upon the duration and magnitude of the preceding depolarization. Calculations based on trabecular geometry and the behavior of the currents in high external potassium suggest that part of the current tail reflects a restoration to a lower steady-state concentration of external potassium which had accumulated in narrow clefts between cells during the preceding depolarization. Step hyperpolarization produced a declining inward current (time constant 0.3 sec at −100 mV) which can be explained on the basis of a depletion of potassium from these intercellular clefts (about 0.5% of the trabecular volume).

Previous voltage clamp studies of cardiac muscle cells have indicated that slow changes in outward conductance occur in response to changes in transmembrane potential (Rougier, Vassort & Stämpfli, 1968; Brown & Noble, 1969*a*, *b*; Noble & Tsien, 1969*a*, *b*; Beeler & Reuter, 1970; de Hemptinne, 1971). These conductance changes appear to play a role in the delayed repolarization, repetitive firing, and frequency dependence of the action potential of sheep Purkinje and frog atrial bundles (Noble & Tsien, 1969*b*; Brown & Noble, 1969*b*; de Hemptinne, 1971). However, the interpretation of some of the voltage clamp data on which these studies have been based has recently been questioned (Johnson & Lieberman, 1971). Electron microscopy has disclosed the presence in these tissues of extracellular spaces of a relatively small volume (Sommer & Johnson, 1968; Baldwin, 1968, 1970) which may be sites of accumulation or depletion of

* *Present address:* Physiology Institute, University of Bern, Bühlplatz 5, 3012 Bern, Switzerland.

potassium during the prolonged polarizations used in the investigation of slow conductance changes. The resultant shift in driving force would show up on voltage clamp records as current changes. In frog atrium, the possibility of potassium accumulation during prolonged depolarization has already been suggested on the basis of voltage clamp data (Brown & Noble, 1969*a*). The possibility of K^+ accumulation during step depolarization, as well as K^+ depletion during step hyperpolarization, has been investigated in the present study. The results indicate that the potassium equilibrium potential shifts as a result of maintained current flow during step changes in membrane potential. It is concluded that the potassium equilibrium potential shift is negligible during the course of a typical half-second action potential, although the shifts may be appreciable during large step changes in potential employed in the study of slow changes of membrane conductance.

Materials and Methods

Preparation

Nonpacemaking muscle strands (trabecula) of diameter 0.10 to 0.15 mm and length 3.5 to 5.0 mm were dissected from atria of the bullfrog *Rana catesbiana*. The individual trabeculum is enveloped in an endocardial cell layer which is probably continuous throughout the atrium (Baldwin, 1968; Maughan, 1971). Trabecula contain 5 to 15 rather compact sub-units (bundles of tightly packed cells) divided from each other by connective tissue. It is estimated that connective tissue takes up 10 to 30% of the cross-sectional area (Baldwin, 1968; *personal observation*). The individual cells are usually 3 to 12 μ in diameter, the spacing between cells being about 200 Å (*cf.* Baldwin's (1968) electron-micrographs).

Experimental Apparatus

Trabecula were voltage-clamped using a double-sucrose-gap chamber and clamp circuit (Fig. 1, right) similar to that used by Rougier *et al.* (1968). A narrow segment of the trabeculum (200 to 300 μ) was electrically isolated from the two ends by cuffs of sucrose (300 to 400 μ). Vaseline-silicone grease, placed around the trabeculum at the partitions, formed a hydrophobic seal to prevent membrane hyperpolarization by a test solution-sucrose solution junction potential (Blaustein & Goldman, 1966; Rougier *et al.*, 1968). However, a contact site likely occurs in the connective tissue of the trabeculum where the otherwise protective cuff of vaseline-silicone cannot penetrate. Preliminary experiments indicated that such hyperpolarization occurs, probably contributing at least several millivolts to the transgap potential of the membrane (Maughan, 1971).

Perfusion Fluids

Normal Ringer's solution used was 117 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.08 mM Na_2HPO_4, 0.425 mM NaH_2PO_4, and 5 mM glucose, gassed with 100% O_2. High potassium Ringer's solutions were made by adding the appropriate amounts of

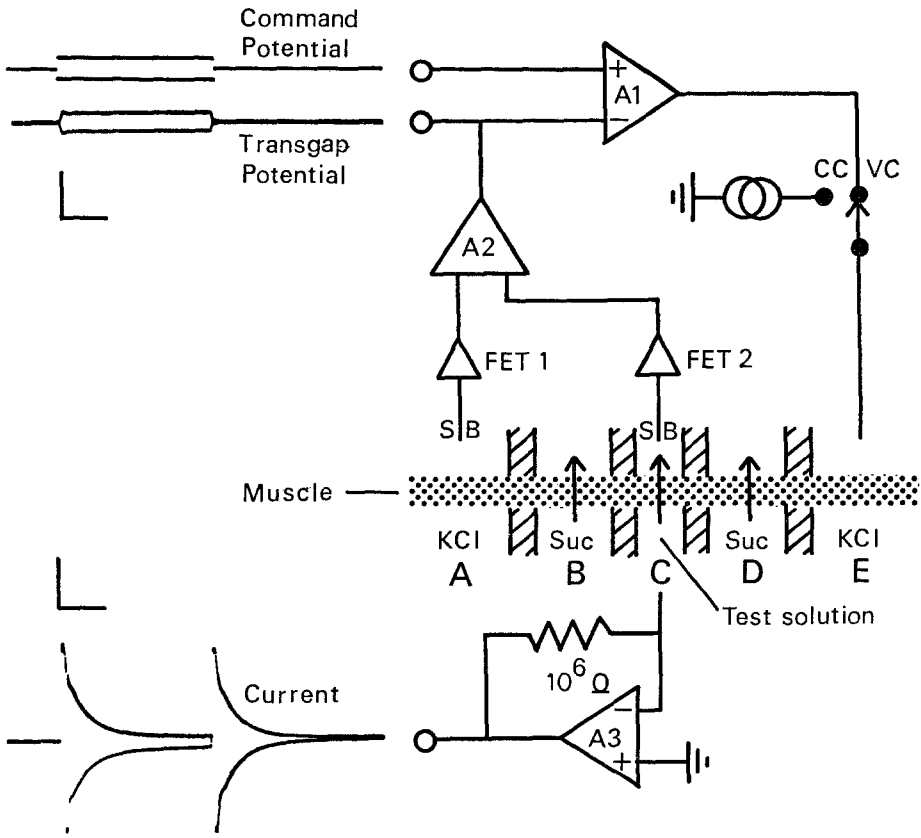

Fig. 1. *Left:* Membrane currents (lower record) associated with ± 10 mV rectangular polarizations of the test membrane (transgap potential, middle record; command potential, upper tracing). Holding potential -60 mV, holding current 0 namps. Voltage scale 50 mV, current 50 namps, time 1.0 msec. Trabeculum 6-20-70 (diameter 100 μ, test gap width 200 μ). In this and subsequent figures, upward deflections represent positive-going voltage changes and outward currents. *Right:* Schematic diagram of 5-compartment ($A-E$) experimental chamber and electronic circuit. An "average" (transgap) potential of cells in the test gap C is recorded across sucrose gap B, and the current to cells in the test gap is applied through the bundle across sucrose gap D. The difference between the transgap potential and a command potential appears amplified at the output A1 (SQ10, Philbrick-Nexus). Amplifier A2 (1517, Burr-Brown) and field transistors FET1 and FET2 (2N3819, Texas-Instruments) measure the transgap potential. The current amplifier A3 (1009, Philbrick-Nexus) has a field-effect transistor preamplifier. Command signals (including a d-c level adjustment) are supplied by conventional generators. For constant current (*CC*) stimulation, the circuit is switched over from the voltage clamp (*VC*) mode. Electrodes are platinized Ag–AgCl wires; voltage electrodes of A and C are embedded in 3 M KCl/2% agar salt bridges (*SB*). Transgap potential and current are recorded by a Hewlett-Packard 7702A paper-chart recorder with medium-gain Sanborn 8802A amplifiers. Fast transients are monitored by an oscilloscope. The gain of the feedback loop (0 to 5,000) is adjusted in individual experiments so that a 10-mV voltage step appears slightly underdamped (*left*)

KCl to normal Ringer's solution. Unless otherwise noted, normal Ringer's solution was used in all results presented in the figures.

Isotonic sucrose solution used was 200 mM sucrose (reagent grade) and 100 μM $CaCl_2$. The small concentration of calcium in the sucrose solution alleviated problems present in early experiments. With calcium, the closed loop gain of the feedback circuit during voltage clamps did not have to be increased as often to maintain the same potential control, and the muscle deterioration in the test gap (increase in baseline membrane potentials and conductances) was not as rapid. Both improvements can be explained by a reduction in (1) the loss of intracellular ions into the sucrose gaps and (2) the uncoupling of cells in the sucrose gaps. Only slight muscle deterioration was usually observed in the 2- to 3-hr experimental period. The temperature of all solutions was 22 ± 1 °C.

Simplified Electrical Analogue of a Trabeculum in the Muscle Chamber

A typical record showing the capacity charging transient following a step change in command potential is shown in Fig. 1 (left). The response to a ± 10-mV pulse is symmetrical. A two-patch electrical analogue of the trabecular membrane in the test gap, based on a simplified view of the morphology, can account for the two-phased capacitative transient. The initial phase of the capacity current can presumably be identified with the charging of the surface membrane capacity in series with the resistance of the bathing fluid. The second, slower phase of the capacity current can be attributed to the charging of the membrane capacity in series with a combination of cleft fluid resistance, r_C, and the resistance of the bathing fluid outside the cleft. Using the current record of Fig. 1, an estimate of r_C can be made by plotting current change during the rectangular depolarization relative to the steady level (at 3.4 msec) on a semilog scale. A straight line was drawn through the later points and extrapolated to the start of the pulse. Using Ohm's law, $r_C = 14$ KΩ, from the potential step and the value of the extrapolated current at zero time. This value of r_C is two orders of magnitude smaller than the resistance of the membrane in the test gap (r_m) of 2 MΩ for this preparation.

An "equivalent" depth of a typical cleft can be calculated using the lumped cleft resistance $r_C = 14$ KΩ. The number of cells in the exemplar trabeculum is estimated to be 160, assuming the trabecular diameter to be 100 μ, the fraction of connective tissue in the cross-section to be 0.2, and the average cell diameter to be 7 μ. The series resistance which an "average" cell faces can probably be taken as $r'_C = 14\ \text{K}\Omega \times 160 = 2.24\ \text{M}\Omega$. An equivalent cleft depth can thus be calculated: $r'_C\, Lw/\rho_R = 9$ μ, given nominal values of cell length $L = 180$ μ (Barr, Dewey & Berger, 1965) = gap width, cleft width $w = 200$ Å (Baldwin, 1968), and cleft fluid resistivity $\rho_R = 90$ Ω cm (resistivity of Ringer's solution). The fact that the equivalent cleft depth has the same order of magnitude as the average cell diameter agrees with the interpretation of r_C.

Measurement Error Relating to Distributed Elements

At rest, the series resistance of the fluid in the intercellular cleft will cause a transmembrane voltage differential such that the membrane potential at the center of the cleft will have a probable value which differs by less than 0.007 from a displacement of the membrane potential at the mouth of the cleft (r_C: $r_m = 0.007$, *see* previous section).

The transmembrane voltage differential along the axis of the cell bundle in the test gap is also small, being less than 2 mV during the flow of slowly changing current (Haas, Kern & Einwächter, 1970). My calculations agree with this observation of Haas *et al.*, giving less than a 5% decrement of voltage along the axis in the resting state (Cole, 1968, pp. 418–421). Specifically, the maximum relative change in transmembrane poten-

tial is $L^2 R_{myo}/R_m a = 0.03$, where L (gap width) = 200 μ, R_{myo} (specific myoplasm resistance) = 200 Ω cm (Woodbury & Gordon, 1965), R_m (specific membrane resistance) = 625 Ω cm² (*see* next section). Because the individual cells form a cablelike electrical syncitium (Woodbury & Gordon, 1965), a should be closer to the trabecular radius than the cell radius, thus a is taken as 50 μ. Since there is at most an order of magnitude drop in cardiac membrane resistance associated with the slowly changing membrane currents described in this study, underlying voltage processes will be subjected to a maximum voltage gradient of probably less than 30%.

Resting and Action Potentials; Resistance and Capacity

The resting and action potentials, as well as the resting membrane resistance, were used as guides to the general state of the preparation. Trabecula which yielded transgap potentials 25% less than the mean intracellular potential in intact atria (*see below*) were usually accompanied by inordinately low membrane resistances, and were discarded. The ratio of usable to unusable trabecula was about 1:4.

The procedure used to determine the (transgap) resting potential (E'_R) was as follows: At the start of the experiment, the test gap and end pools contained isotonic KCl. Normal Ringer's solution was then introduced into the test gap. The resulting repolarization took 1 to 2 min to reach a steady level. This potential change was denoted by E'_R, which in 18 trabecula was between −60 and −75 mV (−70.1 ± 8.3 mV, mean ± SD). In the same preparations, evoked action potentials were between 80 and 120 mV in amplitude (92.6 ± 7.6 mV) and between 150 and 1,000 msec in duration at 10% amplitude (367 ± 210 msec) at 22 °C.

The mean intracellular resting potential from intact (uncut) trabecula in two atria at 22 °C ($E_R = -81 \pm 4$ mV, 23 impalements) was approximately 10 mV larger than the above mean transgap resting potential. Supporting factors for E_R being greater than E'_R are: (1) The present method for obtaining the zero reference voltage is erroneous by a few millivolts since isotonic KCl in the bathing solution does not produce a transmembrane potential of zero (Burgen & Terroux, 1953). (2) The isolated trabeculum, which has cut ends, contains cells which have an average resting potential lower than those in the intact (uncut) trabeculum (Deck & Trautwein, 1964). (3) The transmembrane potential is an attenuated measure of the average resting potential of the cells in the test gap (Jirounek & Straub, 1971). The most direct measure of this short-circuiting factor is the ratio of the transgap potential to the intracellular potential. Since no accurate measures of the intracellular potential under the experimental conditions described in this study were obtainable because of technical difficulties, the relative contributions of factors (1) through (3) remain unknown.

The mean intracellular action potential amplitude from the two intact atria (92 ± 2 mV, 23 impalements) was not significantly different than the mean transgap action potential amplitude. The mean intracellular action potential duration from the intact atria (648 ± 50 msec, 23 impalements) was nearly twice as long as the mean transgap action potential duration. Since the action potential plateau is relatively sensitive to small changes in transmembrane current, the probable electrotonic interaction of the active segment in the center gap with the inactive segments in the sucrose gaps could account for the difference in mean durations.

The average resistance of the membrane at rest in the test gap (r_m) was 1.2 ± 0.3 MΩ (18 trabecula), obtained by dividing a transgap potential change of 10 mV by the applied current, measured at the end of a 2-sec rectangular current pulse. The average time constant of a (± 10 mV) voltage response to a rectangular current pulse was 7.2 ± 3.5 msec (5 trabecula). A calculation of specific membrane resistance (Ω cm²) and capacity

($\mu F/cm^2$) depends on a proper estimate of (1) the leakage current through the insulating cuffs of sucrose, and (2) the membrane area which faces extracellular space. Unfortunately, no proper measure of the leakage current is available in these experiments, although calculations indicate that only 10% of the recorded current may be leakage current from the sucrose gap for a 10-mV depolarization (J. A. S. McGuigan, *personal communication*). An estimate of the total area of individual cells can be obtained from electron-micrographs by Baldwin (1968), a factor of 15 larger than the surface area of the bundle (Maughan, 1971). Assuming that 90% of the recorded current flows through a total membrane area 15 times that of the trabecular area, the specific membrane resistance would be close to 12,000 $\Omega\,cm^2$, the capacity 0.7 $\mu F/cm^2$. These estimates, of course, are subject to the uncertainties in measuring leakage current and membrane area.

Results

Currents Following Step-Depolarization

Fig. 2 illustrates slow outward current changes which occur during and following 2-sec rectangular depolarizations of the membrane to five potential levels (-50 to -10 mV) from a holding potential of -60 mV (=resting potential). A transient inward current (Rougier *et al.*, 1968; Brown & Noble, 1969*a*; Haas *et al.*, 1970) precedes the slow current changes when the membrane is depolarized more than about 10 mV. The initial phase of the transient inward current, as well as the usual capacitative transients at the make and break of the voltage pulse, are too rapid to be recorded by the pen-recorder. The current record displays delayed outward current rectification (Rougier *et al.*, 1968; Brown & Noble, 1969*a*; Haas *et al.*, 1970). Following repolarization, an outward current tail declines to the resting level.

To determine the reversal potential of the current tail, the membrane was repolarized to different potential levels following a 2-sec 50-mV depolarization (Fig. 3). Repolarizations to -40, -50, -60, -70 and -80 mV show a monophasic tail which declines more quickly as the membrane is hyperpolarized, and which reverses direction when the membrane is repolarized to -90 mV. A 10-fold increase in extracellular K^+ (25 mM KCl-Ringer's solution) produced a corresponding decrease in this reversal potential from an estimated -85 mV to -50 mV (resting potential = -38 mV in the 25 mM KCl-Ringer's solution). From the Nernst equation, an estimate of the potassium equilibrium potential E_K is -88 mV with 2.5 mM $[K^+]_0$, and -30 mV with 25 mM $[K^+]_0$, taking the intracellular K^+ concentration as 89 mM (Haas, Glitsch & Kern, 1966). Although the reversal potential with 2.5 mM $[K^+]_0$ is near the presumed value of E_K, the shift in reversal potential with elevated K^+ concentration is only about 2/3 of that expected of a shift in E_K. The dependency of this portion of the current tail

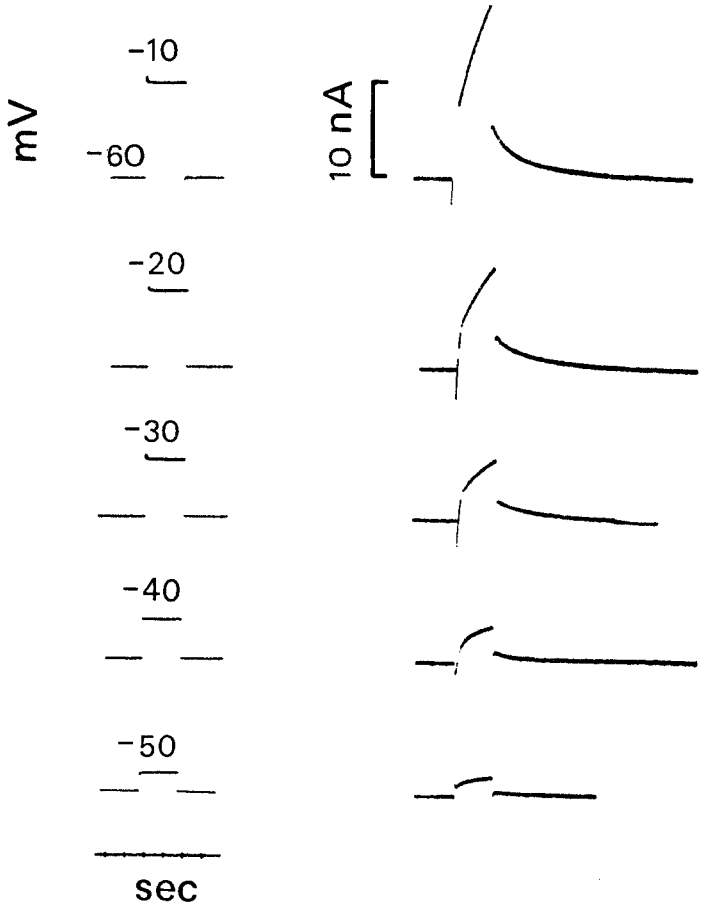

Fig. 2. Slowly changing currents (*right*) following step depolarization (*left*). The membrane was depolarized from −60 mV (holding potential = resting potential) to V_1, held for 2 sec, and then repolarized to −60 mV, where V_1 is given (in mV) at the right of the potential records. Records from exemplar trabeculum 10-8-70 (diameter 140 μ, test gap width 300 μ) were used to illustrate this and all subsequent figures

on extracellular K^+, however, supports the idea that potassium ions carry a substantial part, but not all, of the tail current. The corresponding reversal potential is denoted E'_K, to emphasize its dependency on potassium.

Thus, the outward current tails are consistent with the idea that the time course of the slowly changing currents during membrane depolarization and repolarization reflect activation and deactivation, respectively, of a time- and voltage-dependent current predominantly carried by potassium ions (de Hemptinne, 1971).

Surprisingly, there appear to be two other current reversals; between −20 and −30 mV, and between −90 and −100 mV. Repolarization to

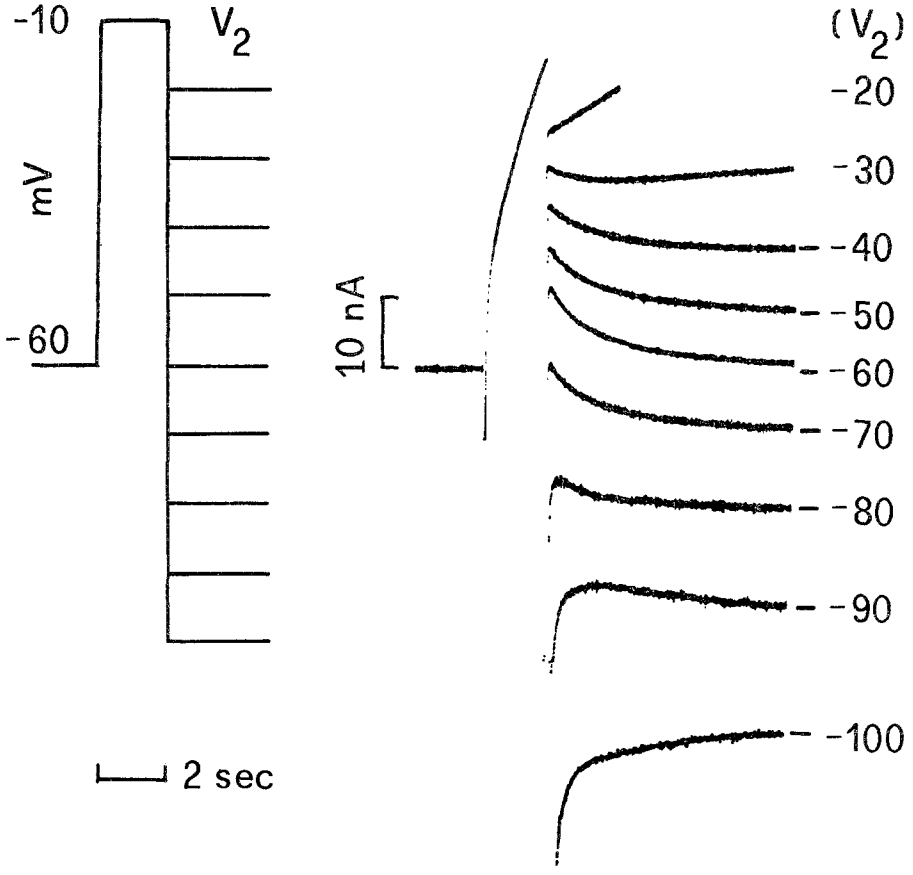

Fig. 3. Current responses to two-step voltage clamp from −60 mV. The membrane was depolarized to −10 mV, held for 2 sec, and then repolarized to V_2, where V_2 is given (in mV) at the right of the current records. Final current levels are indicated after a break in the records. Current records corresponding to the beginning of depolarization and $V_2 = -100$ mV have been retouched

−20 and −30 mV points to a phenomenon observed in all preparations which were depolarized by more than approximately 20 mV from the resting potential. That is, outward current continued to increase during pulses of up to 90 sec (maximum duration used). The increase in current was less than 0.2 namps/sec near the end of a prolonged depolarization. Such "creeps" in outward current have been observed under similar conditions in other bullfrog atrial trabecula (de Hemptinne, 1971; Haas *et al.*, 1970) and mammalian ventricle (J. A. S. McGuigan, *personal communication*). On the other hand, repolarization to potentials negative to −30 mV produced a current which reached a steady level within 10 sec or so. (Final current levels are indicated by a break in the records of Fig. 3.) A slower

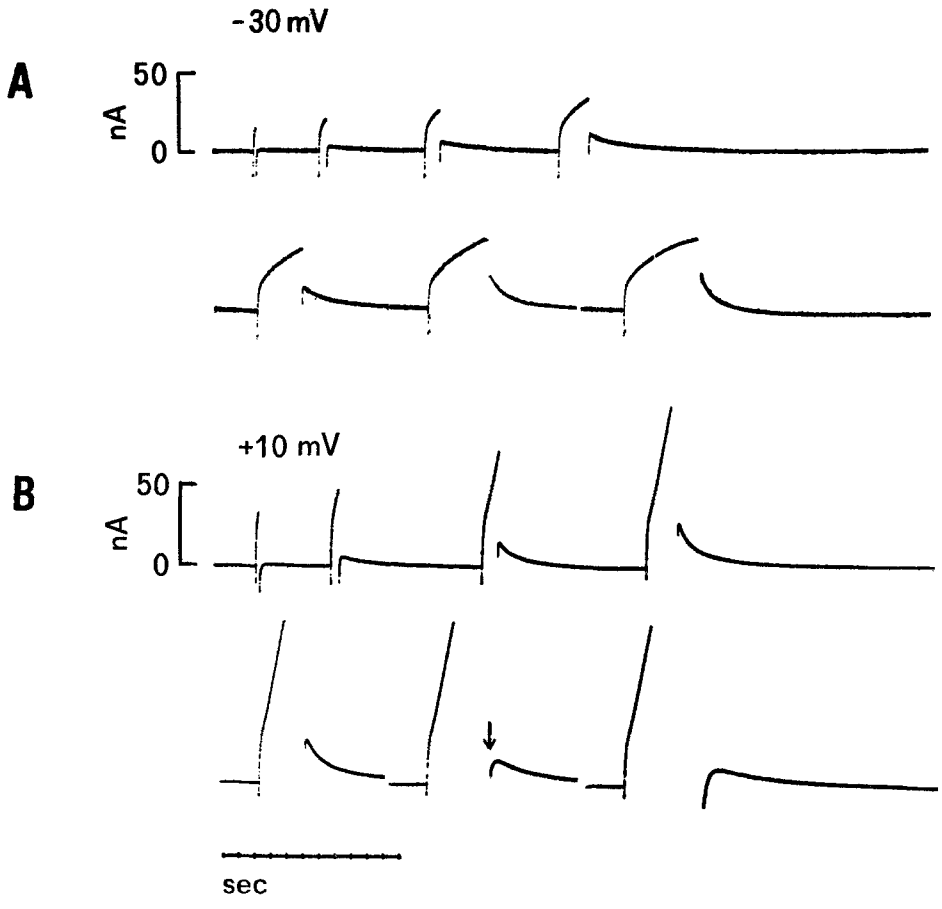

Fig. 4. Effect of preceding depolarization on current tails during repolarization. The membrane was depolarized from −60 mV to −30 mV (*A*) or +10 mV (*B*). Depolarization duration (left to right) 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 sec. Repolarization to −60 mV. Note the turn-over of the initial phase of the current tail (arrow) after prolonged depolarizations in *B* but not in *A*. Time marks 1.0-sec intervals

component of current appears, and reverses direction, between −90 and −100 mV. The time constant of this slower component is approximately 2.5 sec at −90 mV. While E_K' could be observed clearly in every case tested (12), the current reversal of this slower component could be clearly seen in only 6 trabecula. In the case illustrated in Fig. 3, the reversal potential for the slower component also showed a dependency on extracellular potassium, shifting approximately 36 mV with a 10-fold increase in $[K^+]_o$ from an estimated −92 mV in normal Ringer's solution.

Because potassium ions are likely to carry a substantial part of the outward current during membrane depolarization, it would be expected that maintained outward flow of K^+ would change the K^+ concentration in the narrow intercellular clefts. The corresponding changes in driving force for K^+ should be detected in the current tails as a shift in the tail reversal potential E'_K. Thus, the effect of depolarization amplitude and duration on E'_K was studied.

Fig. 4 illustrates current tails which follow depolarizations of 30 mV (*A*) and 70 mV (*B*) from a holding potential of −60 mV. The rectangular depolarizations were 0.2 to 5 sec in duration. The waveform of the current tail following the 5-sec 30-mV depolarization was monophasic, while the waveform following the 5-sec 70-mV depolarization was biphasic, with a negative initial component. A monophasic to biphasic waveform change is also observed when the duration of the preceding 70-mV depolarization is increased from 3 to 5 sec. The outward current tail at 3 sec in both *A* and *B* indicates that E'_K is negative to the holding (resting) potential, while the initial inward current tail following the 70-mV pulse (arrow) suggests that E'_K is positive to the resting potential during the first half-second after repolarization. Fig. 5*A* shows that E'_K lies between −50 and −60 mV at the moment of repolarization following the 5-sec 70-mV depolarization. Increasing the amplitude of the 5-sec depolarization by an additional 8 mV, from +10 to +18 mV, produced a corresponding positive shift in E'_K, from an estimated −58 mV to an estimated −49 mV (Fig. 5*B*), which is in the direction expected from an increased transfer of K^+ into the intercellular clefts. Decreasing the duration of the 78 mV by 1 sec, from 4.8 to 3.8 sec, shifted E'_K back to an estimated −55 mV (Fig. 5*C*), in the direction expected from a decreased transfer of K^+. Table 1 summarizes a more complete experiment.

Kinetics of Outward Current Tail

From the previous section, the general waveform of the current tail following depolarization depends not only on the level of repolarization (Fig. 3) but also on the duration and magnitude of the preceding depolarization (Fig. 4). The transient waveforms are impossible to explain solely on the basis of inactivation because the initial component of the current tail not only diminishes, but changes from a declining outward current to a declining inward current after prolonged depolarizations at a given level of repolarization. Thus, both positive and negative currents cannot flow through the same ionic channel if the driving force stays constant. It is possible that an additional current component with a reversal potential

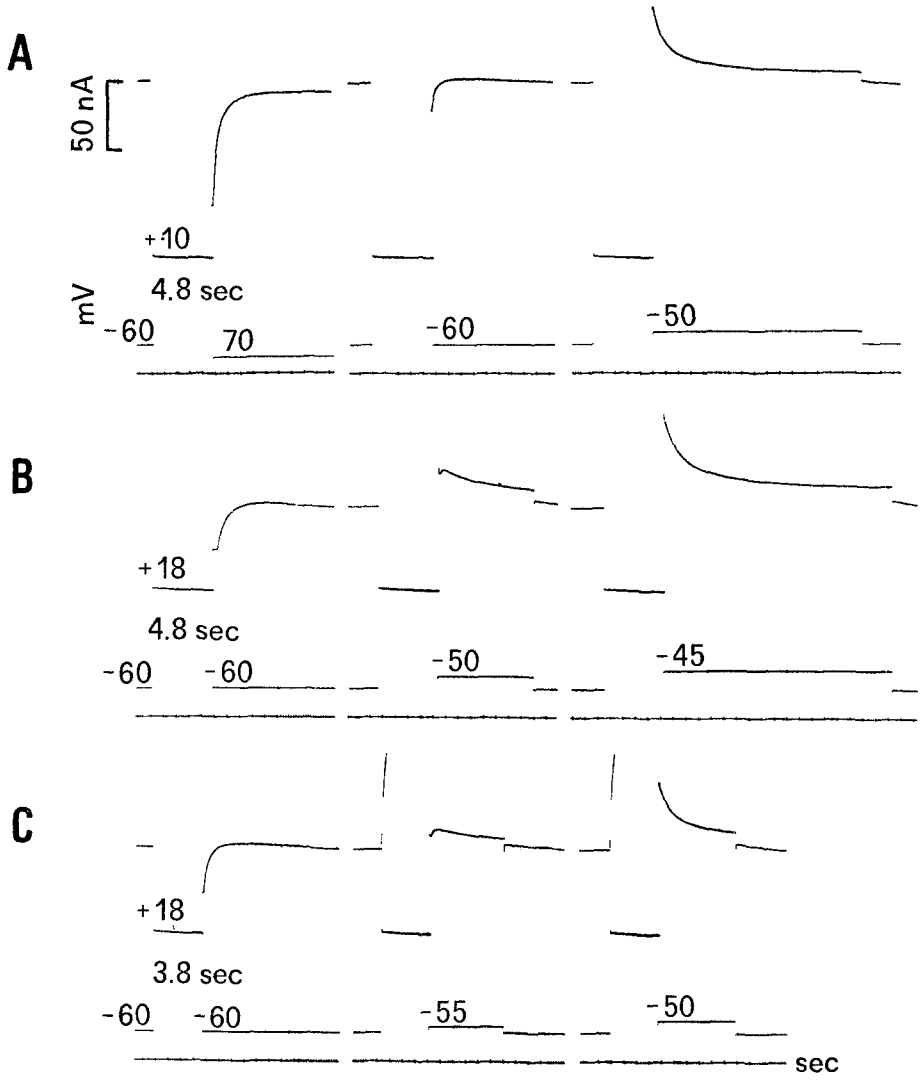

Fig. 5. Test of the hypothesis that an initial phase of the current tail after prolonged depolarization will have a reversal potential (E'_K) which increases as a function of the amplitude and duration of the preceding depolarization (*see* text). In *A* the membrane was depolarized from −60 mV to +10 mV, held for 4.8 sec, and then repolarized to −70, −60, or −50 mV (left to right). E'_K approximately −58 mV. In *B*, depolarization to +18 mV, held for 4.8 sec, and then repolarized to −60, −55, −50 mV (left to right). E'_K approximately −49 mV. In *C*, depolarization to +18 mV, held for 3.8 sec, and then repolarized to −60, −50, or −45 mV (left to right). E'_K approximately −55 mV. Compare the current records corresponding to −50 mV. Current traces upon depolarization are mostly off scale

Table 1. Reversal potential (E'_K) shift as a function of preceding depolarization

V_1 (mV)	V_1 duration (sec)	E'_K (mV)
+18	15.2	−39
	4.8	−49
	3.8	−55
	2.8	−60
	2.0	−78
+8	4.8	−58
	3.8	−60
−10	2.0	−85

This table is a more complete experiment of the type illustrated in Fig. 5. Holding potential, −60 mV; depolarization to V_1, repolarization to values around E'_K. The potential E'_K, at which the derivative of the initial portion of tail current reverses sign, is interpolated from the records. Measurement error approximately $\pm\frac{1}{2}$ mV. Trabeculum 10-8-70.

Table 2. Time constants of the current tail following repolarization

V_1 (mV)	t (sec)	V_2 (mV)	τ_{IP} (sec)	τ_{LP} (sec)
−10	2	−40	0.7	2.5
		−50	0.6	2.7
		−60	0.4	2.8
		−70	0.4	2.5
−30	2	−60	1.3	3.2
	10		0.6	4.3
	30		1.4	7.0
+10	2	−60	0.7	3.4
	5		0.3*	3.4
+20	2	−60	0.4	2.4

The membrane was initially depolarized from −60 mV to V_1, held for t sec, and then repolarized to V_2 (*cf.* Fig. 3). Current tails following repolarization are plotted relative to the final (steady) current level. Calculated rapid exponential component was obtained by subtracting slower exponential component from the experimental curve. All tail components are declining outward currents except those indicated by *, which denotes declining inward currents. Measurement uncertainty for time constants of initial phase τ_{IP} is 0.1 sec, for later phase τ_{LP}, 0.2 sec.

positive to the resting potential is activated very slowly over an appropriately positive potential range. Indeed, there is evidence that such a system, obeying first-order kinetics (time constant 2.5 sec at +10 mV) exists in *Rana rudibunda* (Brown & Noble, 1969*b*, system 2). As in the case of *Rana*

rudibunda, the linear summation of two exponential functions could often fit the present monophasic and biphasic current tails within experimental error, but unlike *Rana rudibunda*, the time constants depended on a complicated function of preceding depolarization. Table 2 lists some sample time constants for the initial phase τ_{IP} and latter phase τ_{LP} of the current tails for one trabeculum. In six experimental preparations, values of τ_{IP} were between 0.1 and 0.7 sec; τ_{LP} between 2 and >10 sec at -60 mV. The same two time constants do not fit all the tails in a given experiment at a given potential, but depend on the voltage and duration of the preceding depolarization. This is in contrast to what would be expected from a conductance system as described by Hodgkin and Huxley (1952) for the squid axon. According to the Hodgkin-Huxley analysis, the time constant of the decay of current tails should be independent of the previous depolarization, depending only on the membrane potential during repolarization.

Currents Following Step-Hyperpolarization

Fig. 6 illustrates slow inward current changes which occur during and following rectangular hyperpolarizations of the membrane to five potential levels (-65 to -90 mV) from the holding potential (-60 mV). During hyperpolarizations between -60 and -75 mV, a slowly increasing inward current occurs which is consistent with the idea that part of a time- and voltage-dependent potassium current system is activated at the resting potential, and that the time course on hyperpolarization reflects deactivation of the system. The current appears to reverse direction at approximately -75 mV, declining more rapidly as the membrane is hyperpolarized to -80 and -90 mV. Repolarization to the holding potential then reactivates the current system to the original resting condition. This reversal potential is 10 to 15 mV positive to the estimated value of E_K', shifting approximately $+35$ mV when the external K^+ concentration is increased 10-fold; both these facts again point to the probability that mixed charge carries the slowly changing currents. An estimate of the reversal potential of the current following repolarization from -100 mV is obtained from the two-step voltage clamp experiment illustrated in Fig. 7. A declining inward current, with an exponential time constant of approximately 0.3 sec, occurs during the 2-sec 40-mV hyperpolarization. Repolarization to different potential levels produces small current changes which are inward-going at -80 and -90 mV, and outward-going at -60 mV. The current appears to reverse direction at approximately -70 mV.

These current responses to membrane hyperpolarization are remarkably similar to those reported by Adrian, Chandler and Hodgkin (1970*b*) and,

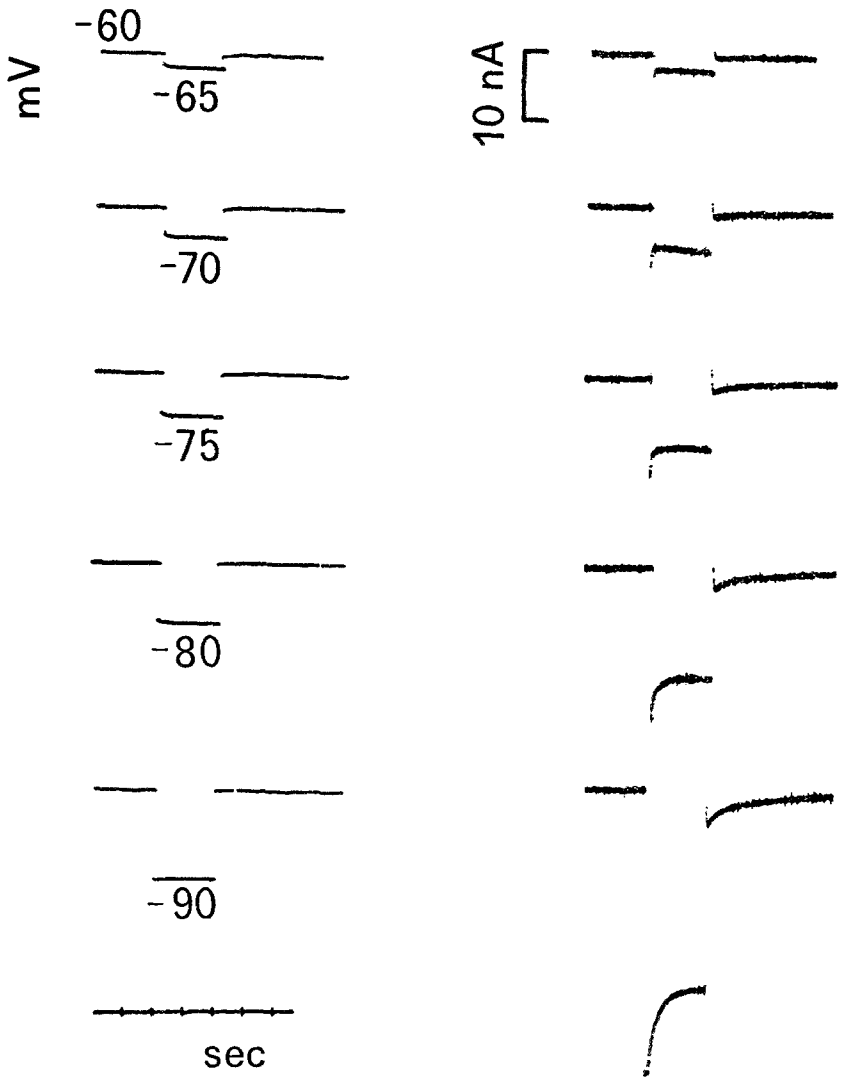

Fig. 6. Slowly changing currents (*right*) following step hyperpolarization (*left*). The membrane was repolarized from −60 mV to V_1, held for 2 sec, and then repolarized to −60 mV, where V_1 is given (in mV) below each potential record

more recently, by Almers (1972) for frog sartorius muscle. Some features of the skeletal muscle currents could be explained by the Adrian-Freygang hypothesis of transverse-tubular potassium depletion (Adrian & Freygang, 1962). The confined space of the extensive tubular system is similar to that of the intercellular clefts in frog atrial trabecula. If depletion of potassium in the clefts is responsible for the decrease in inward current following

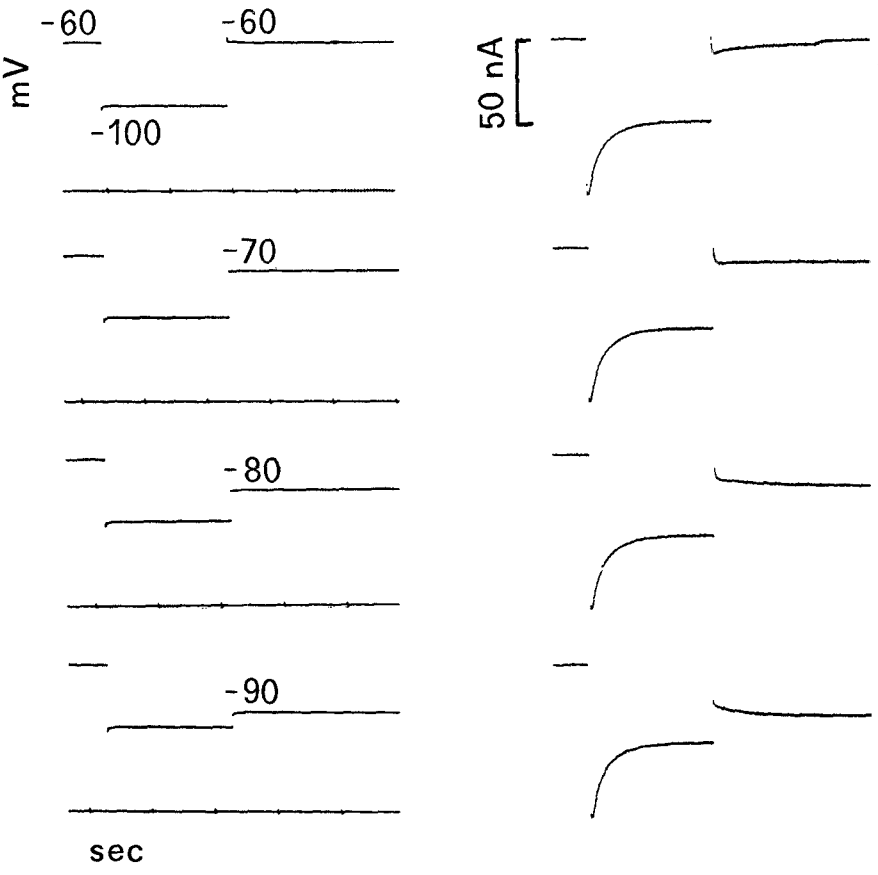

Fig. 7. Current responses to two-step hyperpolarization from −60 mV. The membrane was hyperpolarized to −100 mV, held for 2 sec, and then repolarized to −60, −70, −80, −90 mV (top to bottom)

hyperpolarization, one would expect the total quantity of current which flows in the transient to be less than the total charge Q on the cleft potassium ions. Following Adrian *et al.* (1970*b*), the depletion hypothesis predicts that $Q < F[K^+]_{oc}\,\rho$, where F is the Faraday, $[K^+]_{oc}$ is the initial cleft potassium concentration, and ρ is the fraction of the muscle volume occupied by the clefts. Given $[K^+]_{oc}$ equal to the external potassium concentration $[K^+]_o$, and assuming that Q represents nearly all of the cleft potassium, the inequality can be replaced by $Q \simeq F[K^+]_o\,\rho$.

Table 3 gives estimates of ρ calculated at different levels of hyperpolarization and preceding depolarization for $[K^+]_o$'s of 2.5 and 25 mM. If ρ is in fact an estimate of the fraction of the muscle volume occupied by

Table 3. Test of the cleft-K^+ depletion hypothesis

$[K^+]_0$ (mM)	V_h (mV)	V_1 (mV)	t (sec)	V_2 (mV)	$\tau_2(V_2)$ (sec)	$\Delta I(V_2)$ (namps)	ρ
2.5	−60			−90	0.30	23	0.008
2.5	−60			−100	0.25	50	0.015
2.5	−60	−2	2.0	−90	0.13	40	0.006
2.5	−60	−10	2.0	−90	0.26	20	0.006
2.5	−60	−10	2.0	−100	0.15	32	0.006
25.0	−40			−50	0.63	40	0.003
25.0	−40			−60	0.54	68	0.004
25.0	−40	+18	2.0	−50	0.35	74	0.003

Potassium concentration of bathing medium $[K^+]_0$, holding potential V_h, exponential time constant of the declining inward current during membrane hyperpolarization $\tau_2(V_2)$. The initial value of the current $\Delta I(V_2)$ is relative to its final (steady) level at V_2. In some experiments, the membrane was first depolarized to V_1 for t seconds, then hyperpolarized to V_2. It follows from the text that the fraction of muscle volume occupied by the clefts, ρ, is approximated by the relationship

$$\rho = \tau_2(V_2)\,\Delta I(V_2)/vF[K^+]_0$$

where F is the Faraday and v is the muscle volume in the test gap. For trabeculum 10-8-70, $v = 3.5 \times 10^{-6}$ cm^3. Subtracting the connective tissue space from the trabecular volume gives an estimate of ρ 10 to 30% larger.

the intercellular clefts, one can calculate a value for the diameter (width) of the cell. Taking the cross-section as x microns square, the intercellular spacing as 200 Å (Baldwin, 1970) and the average $\rho = 0.005$, the solution to the equation $(x+0.01)^2 - x^2 = 0.005\,x^2$ gives a cellular width of 5 μ, which is within the 3- to 12-μ range of cell diameters measured by Baldwin (1970).

Discussion

Activation of Outward Current and Reversal Potential Shift

Brown and Noble (1969) and de Hemptinne (1971) have analyzed some time- and voltage-dependent features of the outward current in bullfrog atrium. Brown and Noble's current system 1 (de Hemptinne's current system I_K) had a range of activation and a reversal potential which were both positive to the resting potential in normal Ringer's solution. The present results indicate that the range of activation and the reversal potential (E_K') can extend to potentials more negative than the resting potential. E_K' is dependent on $[K^+]_0$, although the K^+-dependent shift is only about 2/3 of the shift in E_K calculated from the Nernst equation.

E'_K was found to shift to more positive values when the amplitude or duration of the preceding depolarization was increased. Since the reversal potential measurements by Brown and Noble (1969) and de Hemptinne (1971) were both taken after large depolarizations, similar shifts may account for the reversal potential being positive to the resting potential under the conditions of their experiments.

Under similar conditions, other tissues with analogous diffusion-limited spaces in close apposition to the membrane show a shift in E_K, and thus a shift in the (K^+-dependent) membrane potential. For example, Frankenhaeuser and Hodgkin (1956) attributed the positive shift in E_K which occurred in squid axons as a function of the preceding depolarization to potassium accumulation in a (approx. 300 Å) space between Schwann cell and axolemma. In analogous experiments, Adrian, Chandler and Hodgkin (1970*a*) attributed a shift in E_K in frog sartorius muscle to potassium accumulation in the (200 to 1,000 Å diameter) transverse tubular system. McAllister and Noble (1966) showed in sheep Purkinje tissue that large depolarizations may temporarily displace the resting membrane potential by about 10 mV, which they attributed to potassium accumulation in a confined space immediately outside the membrane. This space, as shown by Sommer and Johnson (1968) could be the intercellular clefts in the Purkinje fibers.

Summary of Cleft K^+ Accumulation-Depletion Hypothesis

The possible influence of intercellular clefts in bullfrog atria on membrane currents is summarized in Fig. 8. A line drawing of part of the cross-section of a trabeculum, based on an electron-micrograph by Baldwin (1970), is given in Fig. 8 (left). Narrow clefts between cells stain with ruthenium red, indicating aqueous contact with the space outside the mouth of the cleft (Baldwin, 1970).

Fig. 8 (right) depicts one such cleft. When the membrane is depolarized from the resting potential (upper inset) much of the outward current is carried by K^+ entering the clefts from adjacent cells. It is likely that the fraction of charge carried by K^+ across the membrane (transport number t_K) is greater than the fraction of charge carried by K^+ into the extracellular fluid outside the cleft (t'_K). This results in an increase in the potassium concentration in the cleft. When the membrane potential is restored, e.g., to a level negative to E'_K at the moment of repolarization (middle inset), the concentration of cleft K^+ declines because K^+ moves back into the cells (Table 3), reflected by the declining inward current in Fig. 5.

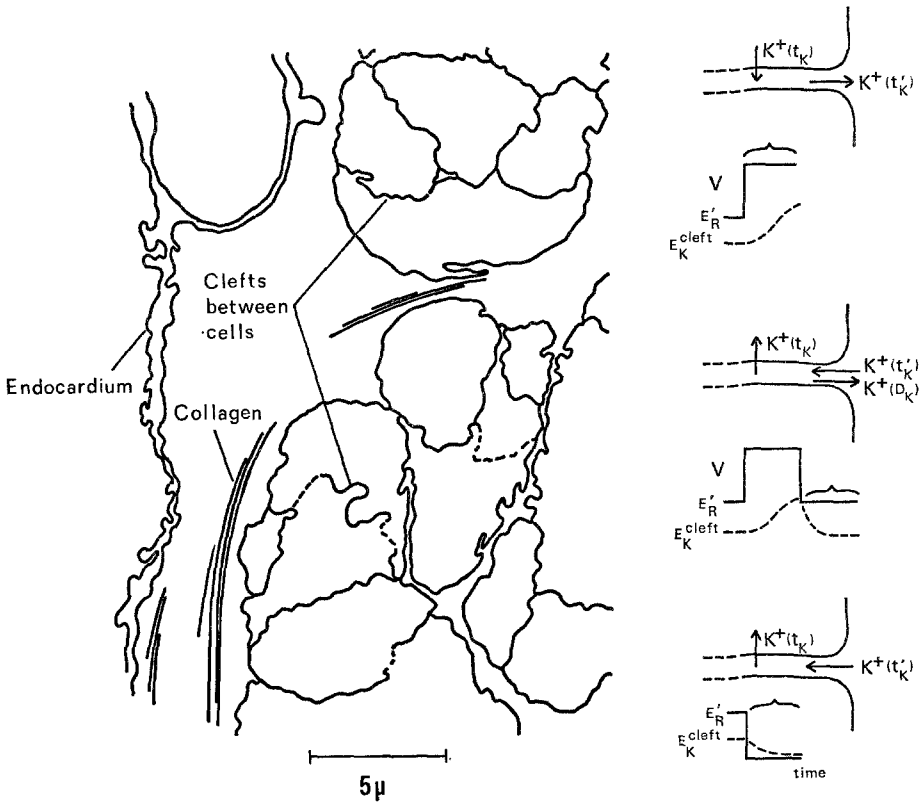

Fig. 8. Summary of cleft K^+ accumulation-depletion hypothesis. *Left:* Line drawing of part of a cross-section of bullfrog atrial trabeculum (Baldwin, 1970, based on Fig. 3), showing clefts between cells. Dashed line represents indistinct morphology. *Right:* Detail of intercellular cleft near its opening. When the membrane is first pulsed with a prolonged depolarization (*top*), potassium ion accumulation occurs in the cleft (indicated by the rise in K^+ equilibrium potential across the cleft membrane) because the fraction of charge carried by K^+ across the cell membrane (t_K) is greater than the fraction of charge carried by K^+ in the extracellular fluid outside the cleft (t'_K). When the membrane is repolarized (*middle*), the cleft K^+ concentration falls to the resting level or some other steady level depending on the level of repolarization (indicated by the decline in E_K^{cleft}) resulting in a declining inward-going current. Part of the decrease in K^+ is due to diffusion out of the cleft (diffusion constant D_K). When the membrane is hyperpolarized (*bottom*), K^+ depletion occurs in the intercellular cleft (indicated by the fall in E_K^{cleft}) because $t_K > t'_K$. The result is a declining inward membrane current. For simplicity, the transfer of K^+ across noncleft membrane is not illustrated

During prolonged depolarizations, the increase in outward current and persistent "creep" could be explained by very slow changes in membrane permeability. Another explanation could be that (1) the membrane conductance (largely g_K) increases, and (2) the driving force for K^+ movement decreases as a consequence of extracellular K^+ accumulation during pro-

longed depolarization. While the change in driving force would decrease rather than increase outward current, (3) the influence of K^+ accumulation on membrane conductance may be sufficient to increase the current (Noble, 1966). Carmeliet (1961) and Hall, Hutter and Noble (1963) calculated the expected conductance change at E_R as a function of $[K^+]_0$, assuming constant field equations applied in the case of a potassium-selective membrane. They found that the calculated values fit the observed values in Purkinje fibers over a range in $[K^+]_0$ of 4 to 24 mM and 2.7 to 148 mM, respectively, although the observed relations displayed inward rectification rather than the calculated outward rectification for $V=E_R$ (<0 mV). Assuming that the constant field equations are applicable in the present case, the calculated change in conductance at E_K' was found to be insufficient (by about 2/3) to account for the increase in membrane conductance coincident with a positive shift in E_K'. For example, Fig. 5 shows a 30% increase in membrane conductance associated with a 6-mV shift in E_K'. In contrast, the constant field equation predicts a 10% increase (on the basis of a shift of E_K' from -55 to -49 mV, and assuming that the membrane is exclusively permeable to potassium at the time of the measurement). An increase of less than 10% is predicted if the selectivity of the membrane to other ions is considered. Presumably the greater part of the increase in membrane conductance during prolonged depolarization is due to activation of the time-dependent conductance and, possibly, an increase in potassium-dependent conductance which exceeds the constant field prediction. The present results cannot specify the contributions of these two factors.

After repolarization, in addition to the uptake of K^+ across the cleft membrane into the cell myoplasm, diffusion of K^+ out of the cleft into the wider extracellular space probably contributes to the decrease in cleft K^+. The half time of freely diffusing potassium from a volume corresponding to that of a trabeculum of 150 μ in diameter (trabeculum 10-8-70) is approximately 0.6 sec.[1] This is the same order of magnitude as the time constant of the rapid phase (τ_{IP}) of the current tail (Table 2). Thus, it appears that the rapid change in driving force due to passive diffusion out of the clefts may influence the waveform of the initial phase of the current tail.

1 The diffusion half time is the time taken for the concentration of potassium at the axis of a cylinder to fall to one-half its initial concentration, assuming a homogeneous initial distribution of ions in an unstirred volume. Given a diffusion coefficient for potassium in Ringer's solution of 1.75×10^{-5} cm^2/sec (calculated at 25 °C from the Nernst limiting law by Kushmerick and Podolsky, 1969) the diffusion time is 0.56 sec (Crank, 1956, p. 67). The entire intratrabeculuar space is most likely unstirred since both the endocardium enveloping the bundle and the narrow interspaces offer an effective block to fluid convection.

When the membrane is hyperpolarized from the resting potential to a value below E_K (lower inset, Fig. 8), the decay of the inward current is also likely due to the depletion of K^+ from the clefts (Table 3). Current changes due to the deactivation of the outward current system are detected when the membrane is hyperpolarized approximately 10 mV or less (Fig. 6). The apparent reversal of this current, at -75 mV, appears to be approximately 10 mV positive to E_K' measured after a 2-sec 50-mV rectangular depolarization (Fig. 3). One possible explanation of this difference in estimates of E_K' is that the selectivity of the outward current system to potassium decreases as a function of hyperpolarization. Further hyperpolarization, e.g., to -100 mV (Fig. 7), would be expected to shift E_K' even further in the positive direction; this effect is observed where the reversal potential of the current measured after the 40-mV conditioning hyperpolarization is approximately 5 mV positive to the reversal potential measured in the absence of any conditioning polarization.

The shifts in the K^+ equilibrium potential are probably small during the course of a typical half-second action potential since other experiments indicate that relatively small outward currents pass during the plateau period (de Hemptinne, 1971). Even when the potential is prolonged for an extended period of 2 sec near the plateau potential (Fig. 3), the shift in E_K is likely to be less than 3 mV, since the reversal potential of the mixed outward current (E_K') is within an estimated 3 mV of E_K. Thus, it appears that potassium accumulation in intercellular clefts does not appreciably affect the time course of the normal action potential. Where equilibrium potential shifts become a matter of concern is in the study of slow changes in membrane conductance, where the membrane may be strongly polarized for extended periods of time.

This paper is based on part of a Ph. D. Thesis submitted to the Department of Physiology and Biophysics of the University of Washington. I thank Professor J. W. Woodbury for his advice, helpful discussions and for detailed editing of the thesis and manuscript; Professors S. Weidmann, G. Mainwood and H. Reuter for helpful comments; Dr. B. Hille for help with the design of the chamber; and Mr. R. Ward for constructing the final clamp circuit.

This research was supported by Training Grant No. GM 00739 from the National Institutes of Health.

References

Adrian, R. H., Chandler, W. K., Hodgkin, A. L. 1970*a*. Voltage clamp experiments in striated muscle fibres. *J. Physiol.* **208**:607.

Adrian, R. H., Chandler, W. K., Hodgkin, A. L. 1970*b*. Slow changes in potassium permeability in skeletal muscle. *J. Physiol.* **208**:645.

Adrian, R. H., Freygang, W. H. 1962. The potassium and chloride conductance of frog muscle membrane. *J. Physiol.* **163**:61.

Almers, W. 1972. Potassium conductance changes in skeletal muscle and the potassium concentration in the transverse tubules. *J. Physiol.* **225**:33.

Baldwin, K. M. 1968. The fine structure and electrophysiology of heart muscle cell injury. Ph. D. Thesis. University of Washington, Seattle, Wash.

Baldwin, K. M. 1970. The fine structure and electrophysiology of heart muscle cell injury. *J. Cell Biol.* **46**:455.

Barr, L., Dewey, M. M., Berger, W. 1965. Propogation of action potentials and the structure of the nexus in cardiac muscle. *J. Gen. Physiol.* **48**:797.

Beeler, G., Reuter, H. 1970. Voltage clamp experiments on ventricular myocardial fibres. *J. Physiol.* **207**:165.

Blaustein, M. P., Goldman, D. E. 1966. Origin of axon membrane hyperpolarization under sucrose gap. *Biophys. J.* **6**:453.

Brown, H. F., Noble, S. J. 1969*a*. Membrane currents underlying delayed rectification in frog atrium. *J. Physiol.* **204**:717.

Brown, H. F., Noble, S. J. 1969*b*. A quantitative analysis of the slow component of delayed rectification in frog atrium. *J. Physiol.* **204**:737.

Burgen, A. S. V., Terroux, K. G. 1953. On the negative inotropic effect in the cat's auricle. *J. Physiol.* **120**:449.

Carmeliet, E. E. 1961. The potassium and chloride permeability in Purkinje fibers. *In:* The Specialized Tissues of the Heart. Elsevier Publishing Co., Amsterdam.

Cole, K. S. 1968. Membranes, Ions and Impulses. University of California Press, Berkeley, Calif.

Crank, J. 1956. The Mathematics of Diffusion. Oxford University Press, Oxford.

Deck, K. A., Trautwein, W. 1964. Ionic currents in cardiac excitation. *Pflüg. Arch. Ges. Physiol.* **280**:63.

de Hemptinne, A. 1971. Properties of the outward currents in frog atrial muscle. *Pflüg. Arch. Ges. Physiol.* **329**:332.

Frankenhaeuser, B., Hodgkin, A. L. 1956. The after-effects of impulses in the giant fibres of *Loligo*. *J. Physiol.* **131**:341.

Haas, H. G., Glitsch, H. G., Kern, R. 1966. Kalium-Fluxe und Membranpotential am Froschvorhof in Abhängigkeit von der Kalium-Außenkonzentration. *Pflüg. Arch. Ges. Physiol.* **288**:43.

Haas, H. G., Kern, R., Einwächter, H. M. 1970. Electrical activity and metabolism in cardiac tissue: An experimental and theoretical study. *J. Membrane Biol.* **3**:180.

Hall, A. E., Hutter, O. F., Noble, D. 1963. Current-voltage relations of Purkinje fibres in sodium-deficient solutions. *J. Physiol.* **166**:225.

Hodgkin, A. L., Huxley, A. F. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117**:500.

Jirounek, P., Straub, R. W. 1971. The potential distribution and the short-circuiting factor in the sucrose gap. *Biophys. J.* **11**:1.

Johnson, E. A., Lieberman, M. 1971. Heart: excitation and contraction. *Annu. Rev. Physiol.* **33**:479.

Kushmerick, M. J., Podolsky, R. J. 1969. Ionic mobility in muscle cells. *Science* **166**:1297.

Maughan, D. W. 1971. Voltage clamp analysis of slowly changing membrane currents in *Rana catesbiana* atrial trabecula. Ph. D. Thesis. University of Washington, Seattle Wash.

McAllister, R. E., Noble, D. 1966. The time and voltage dependence of the slow outward current in cardiac Purkinje fibres. *J. Physiol.* **186**:632.

Noble, D. 1966. Electrical properties of cardiac muscle attributable to inward going (anomalous) rectification. *J. Cell. Comp. Physiol.* **66**:127.

Noble, D., Tsien, R. W. 1968. The kinetics and rectifier properties of the slow potassium current in cardiac Purkinje fibres. *J. Physiol.* **195**:185.

Noble, D., Tsien, R. W. 1969*a*. Outward membrane currents activated in the plateau range of potentials in cardiac Purkinje fibres. *J. Physiol.* **200**:205.

Noble, D., Tsien, R. W. 1969*b*. Reconstruction of the repolarization process in cardiac Purkinje fibres based on voltage clamp measurements of membrane current. *J. Physiol.* **200**:233.

Rougier, O., Vassort, G., Stämpfli, R. 1968. Voltage clamp experiments of frog atrial heart muscle fibres with the sucrose gap technique. *Pflüg. Arch. Ges. Physiol.* **301**:91.

Sommer, J. R., Johnson, E. A. 1968. Cardiac muscle, a comparative study of Purkinje fibers and ventricular fibers. *J. Cell Biol.* **36**:497.

Woodbury, J. W., Gordon, A. M. 1965. The electrical equivalent circuit of heart muscle. *J. Cell. Comp. Physiol.* **66**:35.