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# Potassium Movement during Hyperpolarization of Cardiac Muscle

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*Summary.* When a bundle of cardiac muscle cells is hyperpolarized, membrane current declines with time. Voltage clamp experiments on sheep and cat ventricular bundles showed that the magnitude of inward current depended on the external $K^+$ concentration. Following prolonged hyperpolarization, membrane current near the resting potential was generally outward. The half-time of decay of this outward current was approximately 2.5 sec at $-60$ mV. The potential measured in the absence of externally supplied current was generally more negative than it would have been without conditioning hyperpolarization.

The half-time of recovery of the current response following hyperpolarization was also approximately 2.5 sec at $-60$ mV, a factor of approximately 3.7 slower than the preceding decline of inward current. The rate of recovery has only a slight temperature dependence ($Q_{10} \cong 1.2$).

The experimental results are consistent with the idea that during hyperpolarization $K^+$ is depleted from approximately 3% of the total muscle volume, and that the replenishment of $K^+$ occurs primarily by $K^+$ diffusion from a much larger fraction of the extracellular space.

One little understood property of cardiac muscle is the valve-like regulation of $K^+$ movement across a polarized cell membrane (Noble, 1965). This property is called inward rectification because the membrane conductance to net inward current is greater than that to outward current. This property of inward rectification is an important feature of cardiac muscle since the small time-dependent currents modulating the plateau of the action potential are not swamped by what otherwise would be an large outward $K^+$ current (Trautwein, 1973).

A large component of the inward rectifying $K^+$ permeability in mammalian Purkinje and ventricular tissue appears to be time *independent* (McAllister & Noble, 1967; McGuigan, 1974). This static component (denoted $i_{K1}$ in Purkinje fibers) rectifies over a broad range of voltages, including the region normally associated with the plateau of

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the action potential. In Purkinje fibers, another component of $K^+$ permeability which is time *dependent* also shows marked inward rectification (Noble & Tsien, 1968). This time-dependent component (denoted $i_{K2}$), activated over a more limited range of voltages ($-90$ to $-60$ mV), is important in the control of pacemaker activity. In contrast, non-pacemaking ventricular tissue does not appear to posses a significant $i_{K2}$ system (McGuigan, 1974). Furthermore, other less specific time-dependent ionic channels through which $K^+$ can pass (such as the $i_{X1}$ and $i_{X2}$ systems in Purkinje fibers (Noble & Tsien, 1968)) are probably totally absent in ventricular muscle (McGuigan, 1974).

Although the membrane rectifying properties of both mammalian Purkinje and ventricular muscles are fairly well characterized in the depolarizing range of voltage, the rectifying properties in the hyperpolarizing range are not (Johnson & Lieberman, 1971). This lack of data is especially evident with regard to whether a significant time-dependent component of the $K^+$ rectifier operates over the hyperpolarizing range of voltages. In particular, there is some question as to whether, under voltage clamp conditions, the decline in inward membrane current which is known to occur reflects primarily a time-dependent change in membrane permeability (Ehara, 1971; Van der Walt & Carmeliet, 1971) or, alternatively, a reduced availability of extracellular $K^+$ in some restricted space (Maughan, McGuigan, Bassingthwaighte & Reuter, 1973).

This paper deals in greater detail with the behavior of the hyperpolarized membrane in mammalian ventricle. The experiments reported here strongly suggest that inward movement of $K^+$ during hyperpolarization can substantially reduce the extracellular $K^+$ concentration, and that the prominent decline in membrane current is largely due to a progressive reduction of extracellular $K^+$, rather than a time-dependent permeability change *per se*. Replenishment of $K^+$ appears to occur primarily by extracellular diffusion rather than $K^+$ movement across cell membranes.

## Materials and Methods

Thin ventricular trabeculae or papillary muscles (diameters between 0.3 and 0.7 mm) were excised from sheep and cat hearts. These preparations were current- and voltage clamped using the same sucrose gap chamber as that used by Beeler and Reuter (1970). The apparatus and general procedures, as well as crucial tests of the voltage-clamp technique, have been extensively described in previous papers (Beeler & Reuter, 1970; New & Trautwein, 1972).

Experiments were carried out in various Tyrode's solutions. The usual solution consisted of 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.42 mM $NaH_2PO_4$,

11.9 mM $NaHCO_3$, and 5 mM glucose, gassed with 95% $O_2$/5% $CO_2$. Modified $K^+$ Tyrode's solutions were made by adding (or subtracting) appropriate amounts of KCl. Slight variations in osmolarity were neglected.

The "isotonic" sucrose solution consisted of 200 mM sucrose and 100 μM $CaCl_2$. The small concentration of calcium in the sucrose retarded a progressive increase in core resistance of the tissue in the sucrose gap (Kleber, 1973), prolonging the period of adequate voltage control.

The temperature of all solutions was maintained at 36 °C by a thermostatically controlled temperature bath through which the perfusion tubes passed. For the experiments at room temperature (19–23 °C) the temperature bath was simply drained.

Muscle dimensions were measured by eye using a Zeiss binocular microscope with a calibrated gradicule. Corrections were made to compensate for asymmetrical refraction of the muscle image due to a meniscus in the bathing solution.

The potassium equilibrium potentials ($E_K$'s mentioned in the Results section) were estimated using the following method: At rest, the Goldman equation (Hodgkin & Katz, 1949), which relates the membrane potential ($E_R$) to the intra- and extracellular concentration of potassium ($[K^+]_i$, $[K^+]_o$) and the ratio of $Na^+$ to $K^+$ permeability $p$ can be written as (Woodbury, 1960):

$$\exp(F E_R/R T)=\frac{1}{[K^+]_i}\{[K^+]_o(1-p)+pM\}$$

where $R$ is the gas constant, $T$ is temperature, and $F$ is the Faraday; $M=[K^+]_o+[Na^+]_o$, and we assume $[Na^+]_i \ll [Na^+]_o$. Chloride permeability, which is relatively small at rest (Hutter & Noble, 1961; Fozzard & Hiraoka, 1973) is neglected here.

The use of the above equation depends on the assumption that $p$ is independent of $[K^+]_o$, which is found to be the case only when the membrane potential is close to or at $E_K$ in cardiac muscle exhibiting inward rectification, while the voltage dependence of $p$, or more precisely $P_K$, depends on $[K^+]_o$ (Noble, 1965). Unfortunately, at present the exact dependency of $P_K$ on $[K^+]_o$ is not known. Consequently, the above equation was used to give only rough estimates of $E_K$ as a function of $[K^+]_o$.

In four bundles (3 cat, 1 sheep), the best straight line fit to the above equation (eight values of $[K^+]_o$ between 1.27 and 129.6 mM; $M=142.6$ mM) gave, in the mean, $p-0.022$ and $[K^+]_i=165.1$ mM. Mean values of $E_R$ and $E_K$ (derived from the Nernst equation) were, respectively, $-78.8$ and $-90.6$ mV in 5.4 mM $K^+$ Tyrode, and $-65.9$ and $-72.2$ mV in 10.8 mM $K^+$ Tyrode.

## Results

### *1. Hyperpolarization and the Declining Inward Current Dependence on* $[K^+]$

The effect under investigation is a decline of inward current when cardiac muscle is hyperpolarized. This effect is seen in cat and sheep ventricular bundles (Fig. 1). In eight experiments, a second intracellular microelectrode, independent of one that controlled the voltage clamp, was used to see if the decline of current was an artifact arising from voltage nonuniformity in the preparation. When the two electrodes were at nearly the same position along the axis of the preparation (Fig. 1*a*),

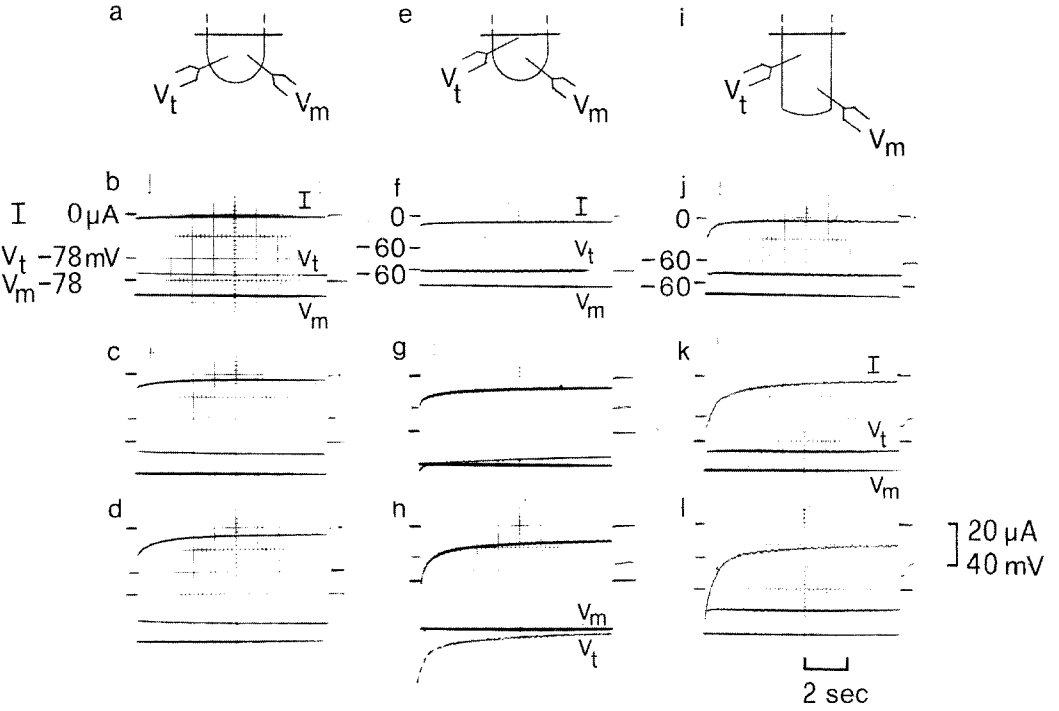


Fig. 1. Declining inward current during hyperpolarization. Voltage recording by two microelectrodes (lower two traces in each frame) near the end of a cat papillary muscle (*b–d*, *f–h*) and a sheep ventricular muscle (*j–l*), and the corresponding current (top trace). In this and subsequent figures, downward deflections represent negative-going voltage changes and inward currents. Frames *b–d* taken from bundle bathed in 5.4 mM $K^+$ Tyrode; *f–h* and *j–l* in 10.8 mM $K^+$ Tyrode. Positions of the two voltage electrodes corresponding to frames *b–d* are given in *a*; to frames *f–h*, in *e*; *j–l*, in *i*. Membrane potential $V_m$ is used to control the voltage clamp; $V_t$ is recorded by a second electrode, independent of the one that records $V_m$. In *a* and *e*, bundle diameter at rubber membrane (horizontal line) is 0.60 mm; in *i*, 0.51 mm. The drawings are approximately to scale. Several voltage traces are retouched to darken line. Current and voltage calibration at far right. Holding potential: *b–d*, −78 mV; *g–h* and *j–l*, −60 mV. Temperature, 36 °C. Bundle 12.11.3, *a–h*; 6.11.3, *i–l*

little difference between the voltage signals could be seen (Fig. 1 *b* − *d*). This indicates little or no circumferential nonuniformity but since both electrodes were probably impaled in superficial cells, the test does not rule out the possibility of radial nonuniformity. However, since current is injected into cells throughout the entire cross-section of the bundle at one end, and not from a point within the bundle, appreciable radial nonuniformity is unlikely (*cf.* Morad & Trautwein, 1968; *see*, however, Jakobsson, Barr & Conner, 1975, for a theoretical refutation of this). Uniformity along the axis of the bundle was tested in eight preparations

by placing the second microelectrode at different points along the axis (Fig. 1*e* and *i*). In six of these, the voltage recorded by the independent electrode varied from that at the site of the control electrode by as much as 2.9× the amplitude of the hyperpolarizing pulse, and the decline of voltage roughly parallelled the decline of the inward current (Fig. 1*f*–*h*). In two of the eight preparations, however, control was excellent at the site of the independent electrode and the declining inward current was still recorded (Fig. 1*j*–*l*). It seems very unlikely, therefore, that the declining inward current results from poor voltage control. But because of the uncertainty of adequate spatial voltage control along the muscle axis, no detailed quantitative analysis of the current kinetics during hyperpolarization was carried out. The only analysis of the kinetics of this current were estimates of the half-time decline.

Increasing the amplitudes of the voltage clamps to more negative potentials produced larger more rapidly declining currents. For example, for the bundle in 5.4 mM $K^+$ Tyrode (Fig. 1*b*–*d*), the initial current amplitude at −106 mV was −6 μA and the half-decline time, $t^{\frac{1}{2}}$, was 0.80 sec; at −120 mV, the initial amplitude was −12 μA and $t^{\frac{1}{2}}$ was 0.45 sec. These currents were sensitive to $K^+$ since, at a given potential, the magnitude of the inward current of a bundle bathed in 10.8 mM $K^+$ Tyrode was approximately twice that of the bundle in 5.4 mM $K^+$ Tyrode (compare traces *d* and *h*).

Fig. 2 depicts current-voltage relations obtained from the cat ventricular bundle of Fig. 1, illustrating the inward current dependency on $[K^+]$, as well as the inward rectifying characteristics of the muscle. The current-voltage relations from sheep ventricular bundles were similar. Over a voltage range negative to the presumed potassium equilibrium potential $E_K$ the initial current levels (measured within 50 msec following the switch in potential) from a bundle in 10.8 mM $K^+$ were approximately twice those in 5.4 mM $K^+$ Tyrode (Fig. 2*A*). The steady state current-voltage relation (Fig. 2*B*), determined approximately 9 sec later, shows a similar $K^+$ dependency. In this and five other experiments of the same kind, the farther removed from the presumed values of $E_K$ (arrows, estimated at rest—*see* Materials and Methods), the more nearly the initial currents were proportional to the external $K^+$ concentration (over at least a range of 10.8 to 2.7 mM). This dependency of $K^+$ suggests that for sufficiently negative potentials the primary charge carrier for inward current is $K^+$.

An analogous dependence of the magnitude of inward current on $K^+$ is found in skeletal muscle, where the decline in current over a similar

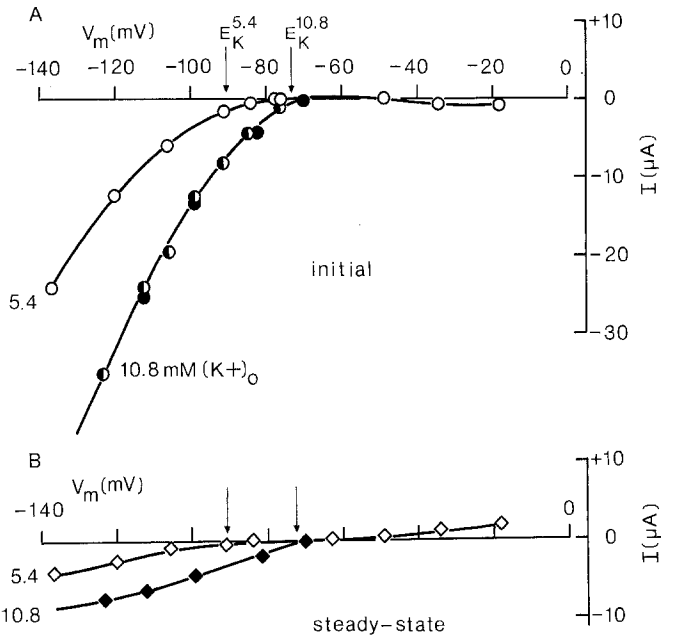


Fig. 2. Current dependency on $[K^+]$. (*A*) Initial current-voltage relation taken immediately after onset of hyperpolarization, or slightly after onset of depolarization; (*B*) Steady-state current-voltage relation taken at 9 sec after the onset of hyper- or depolarization, when the current had reached an approximately steady level. Open symbols, bundle in 5.4 mM $K^+$ Tyrode; solid symbols, bundle in 10.8 mM $K^+$ Tyrode. Half open symbols in (*A*) represent a different, later, impalement. The initial currents in the hyperpolarizing range in (*A*) were measured by taking the value extrapolated to the beginning of the pulse; in the depolarizing range, by taking the value at 50 msec. The slow oscilloscope traces could not record either the fast capacitative transients at the beginning or end of the pulse (*see* Beeler & Reuter, 1970) or the rapid transient inward current during depolarization (New & Trautwein, 1972). Arrows: estimated $E_K$ for bundle in 5.4 and 10.8 mM $K^+$ Tyrode, respectively, calculated on the basis of mean measurements of the resting potential dependency on $[K^+]$ and the Goldman equation (*cf.* Materials and Methods). Resting potentials, $-76$ mV in 5.4, $-60$ mV in 10.8 mM $K^+$ Tyrode; $T=36$ °C. Bundle 12.11.3

voltage range was found to be due primarily to $K^+$ depletion from the lumen of the diffusion-limited transverse tubules (Almers, 1972*a*; Barry & Adrian, 1973). In cardiac muscle, potassium depletion may also occur in analogous restricted diffusion-limited spaces under similar voltage-clamp conditions (Johnson & Lieberman, 1971; Maughan, 1973). Thus, one simple explanation for the decline in inward current in Figs. 1 and 2 is that a progressive reduction in availability of $K^+$ occurs during hyperpolarization, because the replenishment of $K^+$ diffusing in from less restricted extracellular spaces is slower than the influx of $K^+$ into the cells. The following experiments were designed to test this idea.

### 2. *Hyperpolarization Shifts the Zero-current Potential*

If hyperpolarization diminishes the $K^+$ concentration in some restricted extracellular space, a negative shift in the potassium equilibrium potential should occur. However, as Almers (1972*a*) points out, any change in the equilibrium potential would be partly shunted by any membrane where the extracellular space is not diffusion limited and where hyperpolarization should consequently cause little or no depletion. Furthermore, any shift in the transmembrane potential $V_o$, measured in the absence of externally supplied current (which at rest is equivalent to the membrane resting potential, $E_R$) will be less than any corresponding shift in $K^+$ equilibrium potential, because the membrane is not *exclusively* permeable to $K^+$.

In some bundles, no shifts in $V_o$ could be detected; in others, the shifts were small (less than 10 mV), but always in the expected negative direction. In still others, prominent negative shifts occurred (which might be due to a diminished ability of the membrane to rectify currents). Fig. 3 illustrates one example.

The membrane potential was first switched from −60 (the holding potential) to −97 (Fig. 3*A*) or −129 mV (3*B*) for the duration shown above

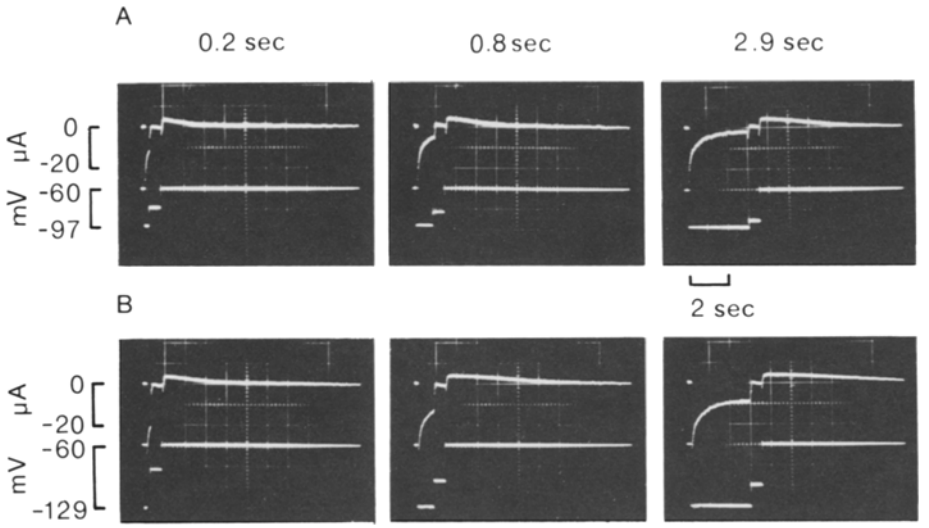


Fig. 3. Increasing the magnitude and duration of preceding hyperpolarization shifts the zero current potential. Conditioning pulse to −97 mV (*A*) or −129 mV (*B*), for duration shown above each trace. The test pulse after each conditioning pulse is, in this case, the zero-current potential taken when the initial portion of the current is at the original (resting) level. *See text* for further explanation. $[K^+]_o = 10.8$ mM; $T = 36$ °C; Bundle 6.12.3*B*

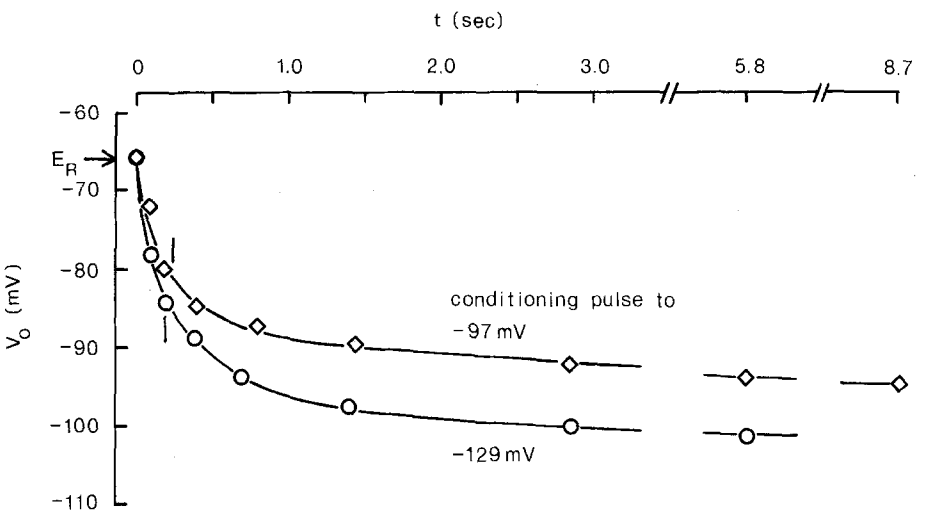


Fig. 4. More complete experiment of Fig. 3, showing a negative shift in the zero-current potential ($V_o$). Initial $V_o$ level at $E_R = -67$ mV. Half-decline times for $V_o$ (vertical lines) are 0.25 and 0.20 sec, corresponding to hyperpolarizations to $-97$ and $-129$ mV, respectively

each trace. The resting potential in each case was $-66$ mV. The potential was then switched to some intermediate level at which the current was "initially" zero; *i.e.*, the zero-current potential, $V_o$. $V_o$, determined by systematic trial and error, became progressively more negative upon increasing the duration of hyperpolarization.

Fig. 4 illustrates more complete tests from the same bundle. In both cases the negative shift in $V_o$ was virtually complete in 6 sec. For the $-37$ and $-69$ mV hyperpolarizations, the maximum shifts from a resting potential of $-66$ mV were $-28$ and $-35$ mV. The respective half-decline times were 0.25 and 0.20 sec, compared with half-decline times of 0.23 and 0.20 sec for the corresponding current changes during hyperpolarization. This similarity of half-decline times for $V_o$ and currents is consistent with $K^+$ depletion.

### 3. *Recovery Following Hyperpolarization*

Following rectangular hyperpolarization of the cardiac membrane, reapplication of a similar pulse within approximately 30 sec produced a diminished current response; *i.e.*, some time was needed between identical conditioning and test pulses in order to re-establish the original current response. Although significant axial voltage gradients usually existed

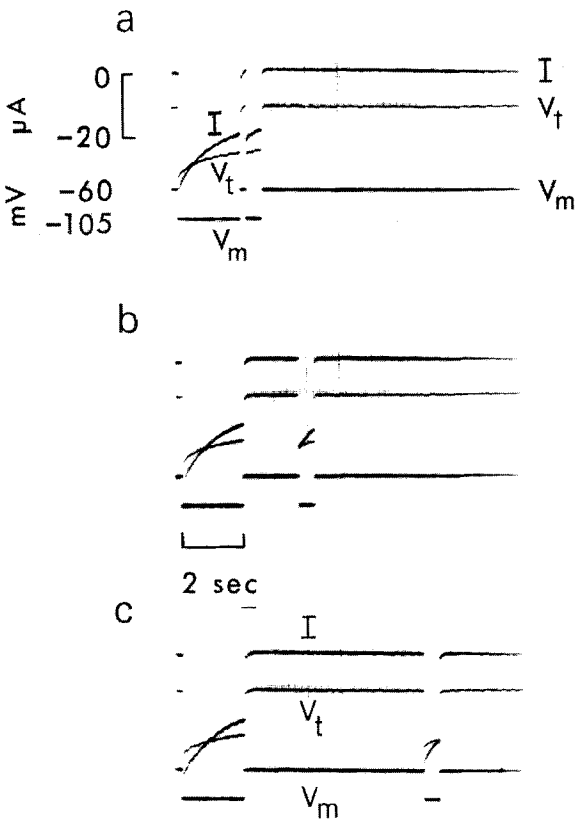


Fig. 5. Recovery of initial current following hyperpolarization, where test pulses were applied following conditioning pulses in order to study the recovery kinetics. The interpulse interval was, from (*a*) to (*c*), 0.3, 1.7 and 5.5 sec. Note the increase in amplitude of the initial current of the test pulse with increasing interpulse intervals. $[K^+]_o = 10.8$ mM; $T = 36$ °C; holding potential −60 mV; bundle 20.12.3

during inward current flow (which negates any simple quantitative treatment of inward current kinetics; *cf.* Section 1), the voltage gradients were found to be markedly reduced following a return to the original holding potential (around $E_R$). Fig. 5*a*−*c* illustrates one of several tests of this uniformity following a hyperpolarizing pulse. Note that in Fig. 5*c*, there is throughout the whole recovery period less than a 5% difference in potential recorded by the voltage control electrode and the second test electrode, even though up to a 40% difference in potentials occurs during the preceding hyperpolarization. Thus one is probably justified in quantifying the *recovery* of the current response following a particular conditioning hyperpolarization, since the kinetics are more likely to reflect the underlying mechanism of recovery than any artifacts from the slight voltage nonhomogeneity.

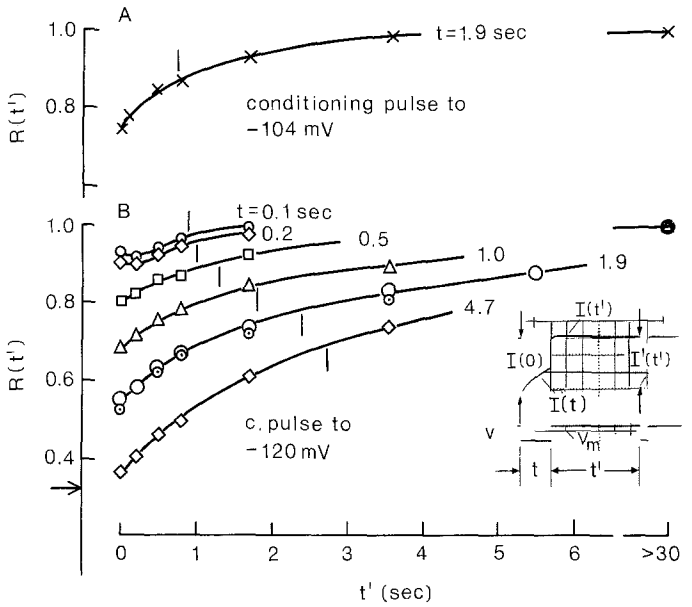


Fig. 6. Complete experiment of Fig. 5, showing recovery kinetics following pulses of varying amplitude and duration. *Inset:* definitions of various parameters used in the figure and text. Recovery is defined as $R(t')=I'(t')/I'(t'>30\text{ sec})$ (Eq. 1), where $I'$ is taken at the beginning of the test pulse, and $t'$ is the interpulse interval. Conditioning pulse to $-104$ mV (*A*), $-120$ mV (*B*), where conditioning pulse durations (in sec) are given next to each curve. The dotted circles in (*B*) correspond to another later microelectrode impalement. Arrow gives the minimum value of $R$ in the experiment, taken after the current reached a steady level. Vertical slashes give the half recovery times, which ranged between 0.8 and 2.7 sec

Fig. 6 illustrates in more detail the recovery of the current response using the two-pulse technique. After a conditioning hyperpolarization, the potential was returned to the holding potential and maintained there for varying times. Short pulses were then applied. Recovery of the initial current was operationally described by the ratio (*cf.* Almers, 1972*a*)

$$R(t')=I'(t')/I'(t'>30\text{ sec}) \tag{1}$$

where $I'(t')$ is the initial inward current during the test pulse given $t'$ sec after the end of the conditioning pulse, and $I'(t'>30\text{ sec})$ is that after at least a 30 sec conditioning-test pulse interval, when the value of $I'$ reaches its maximum.

In general, the time to half-recovery (indicated by the vertical slashes) increased with larger and longer conditioning hyperpolarizations, although the relative increase in half-recovery time was much less following conditioning pulses of more than 2 sec. For example, increasing the conditioning pulse duration from 0.1 to 1.9 sec (clamp to $-120$ mV, Fig. 6) produced

a 2.8× increase in half-recovery time, to 2.3 sec, whereas increasing the conditioning pulse duration from 1.9 to 4.7 sec produced a further increase of only 1.2×. In six bundles, the mean half time of recovery at −60 mV (noting that it does depend on both the magnitude and duration of the conditioning pulse) was 2.45±1.47 (SD) sec, or about 3.7±1.9× greater than the preceding mean $t^{\frac{1}{2}}$ of decline of inward current (in response to various 2 sec hyperpolarizations).

In an attempt to mathematically characterize the recovery time courses following hyperpolarizing pulses of 2 sec or greater in duration, data were fitted to a single exponential or to a sum of two exponentials using a regression method developed by R.K. Wright (*to be published*). Data from 21 recovery time course (10 bundles) were normalized by dividing $1-R(t')$ by $1-R(0)$, where $R(0)$ is the value taken immediately after repolarization, so that the recovery data points ranged from 1 to 0. For these 21 curves, the mean root-mean-square (*rms*) value of the data points fitted to a single exponential function was 0.045±0.018; *i.e.*, approximately 5% of the total range of recovery. The mean exponential time constant was 3.00±1.49 sec. In some cases a closer match of curve to experimental points was possible by using the sum of two exponential functions, but the slight improvement in fit (*e.g.*, in 5 cases, a lower mean *rms* value of 0.017) is probably an insufficient reason to warrant the more complicated analysis using two exponentials.

### *4. Potassium Diffusion and the Recovery Process*

As mentioned previously, when the membrane is clamped near the resting potential following hyperpolarization, the membrane current is generally outward. In 14 bundles, the mean estimated half time of decline of these outward current tails at −60 mV, 2.52±0.56 sec, was of nearly the same magnitude as the half time of recovery of the initial inward current response to similar 2 sec hyperpolarizations.

Usually, the efflux of charge following hyperpolarization was much less than the influx of charge across cell membranes during hyperpolarization (note, *e.g.*, *inset* in Fig. 6). The area ($A$) under the declining portion of the inward current time course (the integral of $I(t)-I(t\geqq 2$ sec); *cf.* Fig. 6 *inset*) should give an upper limit of the amount of charge on the $K^+$ that leaves the restricted space. Similarly, after returning to approximately the resting potential, the area ($A'$) under the declining outward current trace (the integral of $I(t')-I(t'>30$ sec)) should give the amount of charge

on the $K^+s$ which re-enter the restricted space from the myoplasm. In 14 bundles (bathed in 10.8 mM $K^+$ Tyrode) under various clamp conditions, the mean ratio of these two areas ($A'/A$) was $0.23 \pm 0.23$. This means that less than 23% of the $K^+s$ re-enters the restricted space *via* outward current across cell membranes.

If appreciable $K^+$ should re-enter the restricted space by diffusing in from the bath surrounding the whole bundle, then the half-recovery times would depend directly on the bundle radius. This, however, appears not to be the case. Although half times tended to be greater with thicker bundles, this tendency was not significant since a least squares regression analysis of data from 13 bundles showed that there was no significant dependence ($p < 0.05$) of recovery time on bundle radius. Thus one can conclude that $K^+$ primarily re-enters the depleted space by diffusing in from less restricted extracellular spaces *within* the muscle bundle. In these tests, recovery was determined after a standard pulse of 2 sec, when depletion was estimated to be (in the mean) 80% complete.

### 5. *Recovery Kinetics have a Low $Q_{10}$*

If recovery in fact depends primarily upon $K^+$ re-entering the depleted space by diffusing in from a less restricted (nondepleted) space, it is of interest to check to see if the $Q_{10}$ of the recovery phase is consistent with the low $Q_{10}$ expected of diffusion limited processes (Robinson & Stokes, 1959).

Fig. 7 illustrates an experiment of the kind used to study the temperature dependence of recovery, where the normalized recovery time course, $\frac{1-R(t')}{1-R(0)}$, was plotted semilogarithmically against time. As mentioned previously, (*cf.* Section 3), recovery was in general not strictly exponential so that the estimated half times, rather than exponential time constants, were used to calculate $Q_{10}$'s. The $Q_{10}$ for half-recovery was 1.3 in Fig. 7.

For 7 bundles, the mean $Q_{10}$ of the half-recovery times was $1.2 \pm 0.2$. (For comparison, mean $Q_{10}$'s for the initial current amplitude and half-decline time were $1.2 \pm 0.2$ and $1.3 \pm 0.2$, respectively, in the same seven bundles; however, the data from which these $Q_{10}$'s were derived were subjected to greater uncertainties due to greater loss of voltage control). The low recovery $Q_{10}$ can be explained if $K^+$ replenishment in the restricted spaces occurs predominantly by extracellular diffusion, since the measured values were close to the $Q_{10}$ of 1.27 calculated on the basis of the effect of temperature on ionic mobility (Robinson & Stokes, 1959).

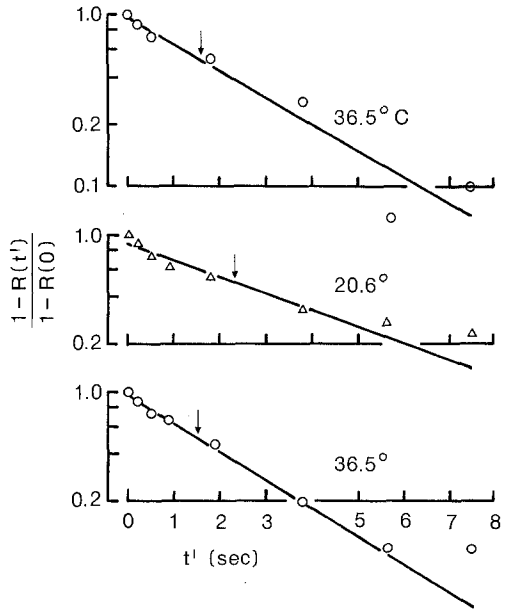


Fig. 7. The effect of temperature on the recovery of the initial current response to a hyperpolarizing pulse which follows an identical conditioning pulse. Normalized data points of recovery *vs.* time ($\{(1-R(t'))\}/\{1-R(0)\}$), where $R(t')$ is defined by Eq. (1) are plotted semilogarithmically at 36.5°, 20.6, and once again at 36.5 °C. Conditioning and test pulses (2 sec duration) were to − 120 mV. Recovery was allowed to occur at −60 mV (holding potential). $Q_{10}$'s were calculated according to the formula $t^{\frac{1}{2}}/t^{\frac{1}{2}\prime}=Q_{10}{}^{(T'-T)/10}$, where $t^{\frac{1}{2}}$ is the half-recovery time, and $T$ is the temperature in °K. All measurements were taken between 5 and 10 min after changing the bath temperature. Arrows indicate half-recovery times, which in this case are associated with a $Q_{10}$ of 1.3. If, for comparison, the time courses are approximated by a single exponential (straight lines) by using a regression routine (rms values less than 7%), the respective exponential time constants are 2.8, 4.1 and 2.5 sec (top to bottom). The $Q_{10}$ using these values (where the time constants at 36 ° are averaged) works out to be 1.33. $[K^+]_o$= 10.8 mM. Bundle 6.12.3B.

## 6. *Estimating the Magnitude of the $K^+$ Depleted Space*

Assuming that $K^+$ depletion and replenishment occur in some restricted extracellular spaces during and following membrane hyperpolarization, the magnitude of the $K^+$ depleted space can be estimated by the following methods:

A. From previous results, the initial current was found to be roughly proportional to the external $[K^+]_o$ (from 10.8 to 2.7 mM) over a range of potentials negative to $E_K$. Under these conditions, Almers (1972*b*, p. 77) showed that the initial inward current ($I'(t')$) should, in general, be proportional to the average $K^+$ concentration in the extracellular space ($[\hat{K}^+]$) even if concentration *differences* exist throughout, as long as the

extracellular spaces are homogeneously distributed. Morphological studies show that the extracellular spaces in ventricular tissues do indeed form a relatively fine, homogeneous meshwork (Page & McAllister, 1973). Assuming, then, that at a fixed potential $I'(t')$ is linearly related to $[\hat{K}^+]$ one is probably justified in expanding Eq. (1) to:

$$R(t') = \frac{I'(t')}{I'(t' > 30 \text{ sec})} = \frac{[\hat{K}^+]_{t'}}{[\hat{K}^+]_{t' > 30 \text{ sec}}}. \tag{2}$$

Adapting a method of Almers' (1972*b*), one can approximate the normalized time course of recovery by an exponential (*cf.* Section 3, *also* Fig. 7), letting the corresponding exponential time constant be given by $\tau$. Since the re-entry of $K^+$ occurs primarily by diffusion (*cf.* Section 4),

$$\frac{d[\hat{K}^+]_{t'}}{dt'} \cong \frac{1}{\tau}\{[\hat{K}^+]_{t' > 30 \text{ sec}} - [\hat{K}^+]_{t'}\}. \tag{3}$$

Consider the events immediately preceding the recovery process. Once the current has declined to a steady-state level, the net charge carried by $K^+$ entering the restricted spaces by diffusion ought to be less than the recorded current, $I(t)$, since $I(t)$ also contains a component which is shunted through the sucrose gap (New & Trautwein, 1972). In other words, letting $I(t = t^*)$ be the current which is recorded at the end of the conditioning pulse,

$$I(t = t^*) > \rho v F \frac{d[\hat{K}^+]_{t'}}{dt} \tag{4}$$

where $\rho$ is the fraction of muscle volume occupied by the $K^+$ depleted space, $v$ is the volume of the muscle in the Tyrode's solution, and $F$ is the Faraday.

Assume that $[K^+]_{t' > 30 \text{ sec}} = [K^+]_o$, the concentration of $K^+$ in the Tyrode's solution. It follows then from Eqs. (2), (3) and (4) that an upper limit for $\rho$ is given by the right-hand side of Eq. (5),

$$\rho < I(t = t^*)\, \tau / F v [K^+]_o \{1 - R(\emptyset)\} \tag{5}$$

where $R(\emptyset)$ is taken as $I(t = t^*)/I(t = 0)$.

In ten bundles (where $t^* \geqq 2$ sec), $\tau$ was estimated by fitting the recovery time course to a single exponential (as mentioned in Section 3). In 18 of the 21 measurements, the *rms* value of the exponential fit to $\{1 - R(t')\}/\{1 - R(\emptyset)\}$ was less than 6%, and it was these data that were used to calculate the upper limit of $\rho$. The mean of the upper limit of $\rho$, calculated according to Eq. (5) under a variety of conditions, was $0.082 \pm 0.041$ for

cat (7 bundles; 13 measurements); and $0.050 \pm 0.030$ for sheep (3 bundles; 5 measurements). There appeared to be no consistent effect of $[K^+]_o$ or temperature on $\rho$.

B. In a second method, one assumes that when sufficiently large hyperpolarizations are applied, inward $K^+$ movement across the membrane is rapid compared to the re-entry of $K^+$ by diffusion from larger extracellular spaces. Under these conditions, one would expect the total quantity of charge (including that carried by the shunt current) which flows in the transient during hyperpolarization to be more than the total charge on the $K^+$ depleted from the restricted extracellular spaces. It follows (Adrian, Chandler & Hodgkin, 1970) that another upper limit for $\rho$ is given by the right-hand side of (6),

$$\rho < \int_{t=0}^{t>6\,\text{sec}} \frac{\{I(t) - I(t>6\,\text{sec})\}}{F[K^+]_o(1-R(\emptyset))\,v} \tag{6}$$

where the integral is the area (in coulombs) under the declining portion of the current. From declining inward current records of the kind in Fig. 1, upper limits for $\rho$ (calculated from (6) under a variety of conditions) were $0.037 \pm 0.019$ for cat (7 bundles; 34 measurements) and $0.034 \pm 0.025$ for sheep (3 bundles; 20 measurements). As with the previous method, there was no consistent effect of $[K^+]_o$ or temperature on $\rho$.

Method *B* is subject to the criticism that some re-entry of $K^+$ does in fact occur concurrent with $K^+$ depletion, since the half-decline time of the inward current is a significant fraction of that of recovery (even for large hyperpolarizations). This error would cause the right-hand side of Eq. (6), and thus $\rho$, to be further overestimated.

In order to satisfy the requirement that $\rho$ be constant, Eq. (6) predicts that inward current declines more rapidly if the magnitude of the inward current increases, as would happen, *e.g.*, with greater hyperpolarizations. The results tended to be consistent with this (*cf.* Fig. 1). Eq. (6) also predicts that the current decline becomes less rapid with increased $[K^+]_o$. This behavior was seen in many bundles at a given level of hyperpolarization; however, the opposite occurred in others. There is at present no adequate explanation for this inconsistency.

### 7. *Hyperpolarization and the Cardiac Action Potential*

Van der Walt and Carmeliet (1971) showed that increasing the duration or magnitude of a conditioning hyperpolarization produced an

increase in the duration of a subsequent action potential in cow Purkinje fibers. They preferred to interpret this effect primarily in terms of voltage and time dependent conductance changes, noting similarities between the kinetics of the prolongation and recovery of the action potentials during and following a hyperpolarizing pulse and the kinetics of the K2 $K^+$ conductance system studied by McAllister and Noble (1967) and Noble and Tsien (1968). However, $K^+$ depletion may also play a role in Van der Walt and Carmeliet's experiments, since the prolongation of the action potential in low $[K^+]$ media (hypokalemia) is a well-known phenomenon in Purkinje fibers.

Attempts to repeat Van der Walt and Carmeliet's (1971) experiments using the present preparations produced equivocal results. At 10.8 mM $[K^+]_o$, 2–5 sec conditioning hyperpolarizations in two cat bundles produced significant (15–20%) prolongations of the action potentials, but in two other bundles (one cat, one sheep) there was either no change or a slight decrease under similar conditions. The responses to hyperpolarizing pulses of variable amplitude and duration, as well as the responses to variable conditioning pulse-test response intervals, were too small or inconsistent to study systematically.

Lowering external $[K^+]$ from 10.8 to 5.4 mM produced at most only a slight prolongation of the action potential (elicited by a 5 msec depolarizing suprathreshold current test pulse, without a conditioning pulse). In six experiments of this kind, the action potential durations increased by a factor of $1.1 \pm 0.1$, which is considerably less than comparable experiments with cow Purkinje fibers. However, these results are not particularly surprising since it has been reported that in mammalian ventricle $[K^+]_o$ has less effect on the duration of the action potential than in mammalian Purkinje fibers (Gettes & Surawicz, 1968). Thus, one would expect that any $K^+$ depletion during hyperpolarizing pulses in the present experiments would have little or no effect on subsequent action potentials.

## Discussion

The decline in inward current investigated in this paper may well be a general feature of cardiac muscle, since similar changes occur in such diverse tissues as dog ventricle (Maughan, *et al.*, 1973), calf, cow and pig ventricle (*unpublished observations*), frog atrium (Maughan, 1973) and cow Purkinje fibers (Van der Walt & Carmeliet, 1971).

Several factors point to $K^+$ being the primary charge carrier during hyperpolarization of the membrane. Foremost among them is the observation that the magnitude of the inward current response to a voltage step depends on the extracellular concentration of $K^+$. A similar dependency was noted in frog atrium (Maughan, 1973) and cow Purkinje fibres (Van der Walt & Carmeliet, 1971).

Sodium and chloride are not likely to play a predominant role in the hyperpolarizing responses since replacing sodium with choline (or tetramethylammonium) and replacing chloride with sulphate (or glutamate) does not produce any significant changes in the hyperpolarizing responses to current-(Ehara, 1971) (or voltage-(Maughan, 1971)) steps. Furthermore in the present experiments, there is no detectable decline in current when the voltage is clamped at the presumed value of $E_K$, where $Na^+$ and $Cl^-$ are probably the dominant charge carriers. In addition, the $Na^+$ channel-blocker tetrodotoxin ($10^{-5}$ g/liter) does not significantly affect the current responses to a hyperpolarizing voltage step in frog atrium (Maughan, 1971) or sheep, cat and pig ventricle (*unpublished observations*).

Several important features of both decline and recovery of current appear to be consistent with a model of extracellular $K^+$ depletion and replenishment: (a) Hyperpolarization can cause a measurable negative shift of the zero current potential (which is closely related to the $K^+$ equilibrium potential), suggesting $K^+$ depletion in some diffusion limited extracellular space. (b) At a given level of hyperpolarization, the half-decline time of the shift in zero-current potential is in reasonable agreement with the half-decline time of the inward current. (c) Recovery of the current-passing characteristics of the muscle system has a small temperature dependence, consistent with the idea that $K^+$ replenishment in the restricted spaces occurs primarily by diffusion from larger extracellular spaces.

The chain of events that are likely to affect $K^+$ movement as measured by the present voltage-clamp technique and which would be consistent with the above points are as follows:

During hyperpolarization to below the resting $E_K$ (1) $K^+$ is probably depleted from some restricted, diffusion-limited extracellular space because while $K^+$ is the main charge carrier across the membrane, $Na^+$ and $Cl^-$ carry nearly all the charge in the extracellular fluid. Thus, the amount of $K^+$ available to move across the membrane is progressively reduced, until the amount of $K^+$ diffusing in from larger extracellular spaces exactly equals the amount of $K^+$ moving inwardly across the membrane; (2) $K^+$ depletion decreases the $K^+$ driving force; and (3) since it is known

that lowering external $[K^+]$ in sheep ventricular bundles decreases $^{42}K^+$ efflux (Weidmann, 1966; Kleber, 1973), a diminution of extracellular $[K^+]$ during hyperpolarization would be expected to decrease the $K^+$ membrane permeability.

Following repolarization, $K^+$ replenishment probably occurs primarily by extracellular diffusion from larger, less depleted spaces. Outward movement of $K^+$ through the cardiac membrane is impeded by the current rectifying property of the membrane. Note that a further increase in $[K^+]$-dependent permeability and a decrease in driving force during recovery have opposite modulating effects on what $K^+$ current is able to pass through the rectifier.

From the above considerations, one would expect $K^+$ accumulation would also occur in the same restricted spaces during membrane depolarizations in mammalian heart muscle. Indeed, McGuigan (1974) showed that $K^+$ probably does accumulate in some extracellular space during prolonged depolarization in sheep and calf ventricle. In McGuigan's experiments, the potential where the instantaneous current-voltage relation crossed the voltage axis (equivalent to $V_o$ in this paper) shifted in a positive direction as the clamp duration increased.

McGuigan (1974), however, showed that a change in the instantaneous current-voltage relation in various $[K^+]$ could not exactly mimic the changes during a depolarizing clamp, so an additional conductance change, not associated with accumulation, was postulated in order to account for the discrepancy. It is possible then, that as a result of switching to a more negative potential, the $K^+$ permeability of the membrane could also change as a function of time and voltage *per se*. However, it is not readily apparent in the present experiments that $K^+$ permeability does depend directly on time and voltage. In particular, it is unlikely that an ionic gating system of the type described by Hodgkin and Huxley (1952) plays a significant role in the current changes during hyperpolarization since the observed half-recovery times had an unusually low temperature sensitivity compared with other $K^+$ channels of the Hodgkin-Huxley type (Hodgkin & Huxley, 1952; Frankenhauser & Moore, 1963). Furthermore, the half-decline times for current recorded at a given level of hyperpolarization were usually markedly different for bundles in 5.4 and 10.8 mM $K^+$ Tyrode (*cf.* Fig. 1). Finally, the half-recovery time of the current response depended on the magnitude and duration of the conditioning hyperpolarization – not just on the potential level during recovery (*cf.* Fig. 6). Both of these latter observations are inconsistent with what one would generally expect from a Hodgkin-Huxley conductance system.

According to Hodgkin-Huxley analysis, the decline time of current should not depend on the amount of $K^+$, but only on the corresponding potential level; furthermore, the recovery times of such systems usually depend on the potential level at which recovery takes place, rather than on previous potential levels. (However, an unusual situation can be imagined where inactivation and activation time constants of approximately the same magnitude might give a different appearance to the recovery curves, depending on the amount of activation during the preceding clamp).

There was at least no obvious time- and voltage-dependent decline in $K^+$ permeability of the kind studied by Almers (1972*b*) in analogous experiments with skeletal muscle. Almers noted effects of a time- and voltage-dependent permeability system which played a prominent role in membrane conductance (current) changes during extreme hyperpolarization. One important effect of this permeability system was to produce a negative slope in the steady state current voltage relation ($V < -140$ mV), something which was not observed in ventricular bundles using the present techniques (Maughan, *et al.*, 1973).

Assuming a model of $K^+$ depletion, the $K^+$ depleted space works out to be less than 8% of the total bundle volume (Methods *A* and *B*, Section 6, Results), and probably less than approximately 3% (Method *B*) for both sheep and cat. Since this $K^+$ depleted space, $\rho$, is much lower than the estimate of 24% for the total extracellular space in cat ventricular muscle (Page, 1962), $K^+$ should not be significantly depleted in most of the collagenous spaces which characteristically separate cells or groups of cells in mammalian ventricular tissues (Sommer & Johnson, 1968). The restricted spaces which are most likely to contribute to $\rho$ are the transverse tubular system (Pager, 1971), the longitudinal boundaries between cells (Fawcett & McNutt, 1969, *cf.* Fig. 1), and to a lesser extent the transverse boundaries between cells which include the intercalated disks (Page & McAllister, 1973). The ratio of the sum of the extracellular volumes of these spaces to the total muscle volume works out to be approximately 0.02–0.03 (Page & McAllister, 1973; Pager, 1971) which, in spite of the uncertainties of the morphological measurements, can account for a large part of the above estimate of about 0.03 (3%) for the upper limit of $\rho$.

One limitation of the depletion model is that in order to account for the very long (2–3 sec) recovery half-times, $K^+$ replenishment must occur in the restricted spaces by unusually slow diffusion from larger, adjacent extracellular spaces within the bundle. One would have expected that recovery would be 1–2 orders of magnitude faster than it is,

since the appropriate diffusion distances within the bundle are of the order of microns. Potassium binding in the restricted spaces or obstacles in the diffusion pathway could have the effect of increasing the apparent $K^+$ diffusion co-efficient (Crank, 1956).

In view of the long recovery times mentioned above, one additional site in which $K^+$ could conceivably be depleted should be considered. Makino and Page (1975) have reported that $Na^+$, $Li^+$, and $Cl^-$ are able to move between the interstitial space and sarcoplasmic reticulum (*SR*) in rat ventricle. If $K^+$ (untested in their experiments) is able to move as well, and if $K^+$ were depleted in the *SR* during hyperpolarization, then $K^+$ could be replenished by diffusing in from the interstitial spaces following repolarization. One drawback to this interpretation is that the movement half-times as measured by Makino and Page are at least an order of magnitude longer than the half-recovery times of approximately 2–3 sec measured in the present study. Another drawback is that present measurements of the membrane capacity in ventricular muscle do not allow for appreciable coupling (low resistance connections) between surface and sarcoplasmic reticular membranes (Weidmann, 1966; Page & McAllister, 1973).

If the extracellular diffusivity of $K^+$ in the restricted spaces is approximately that in Tyrode's solution, then only if replenishment of $K^+$ is assumed not to occur from within the bundle, but from outside, do predicted recovery half-times work out to be of the same order of magnitude as the observed half-times. On this assumption, diffusion distances are on the order of hundreds of microns, so that radial $K^+$ diffusion from the bundle surface into the core of the bundle take a couple of seconds, assuming a tortuosity factor for $K^+$ to be close to that of $Na^+$, between 1.44 (Page & Bernstein, 1964) and 2.03 (Suenson, Richmond & Bassingthwaighte, 1974). However, there are problems with this idea. A radial diffusion model of this type requires that the diffusion limited restricted space should roughly correspond to the total extracellular volume within the bundle (*unpublished calculations*), but, in fact, the magnitude of the $K^+$ depleted space estimated above is at most 10–30% of the total *extracellular* space. An equally serious problem is that, in general, recovery times do not appear to depend on bundle diameter (Section 4, Results). Consequently, models based on diffusion of $K^+$ from outside the bundle are inadequate, if not invalid.

How do these $K^+$ movements relate to inward rectification? Assuming a model of $K^+$ depletion and replenishment, it appears that in the hyperpolarizing range of potentials the decline in $K^+$ current through the inward rectifier is largely due to a progressive reduction of extracellular $K^+$, rather than a time dependent permeability change *per se*. Although the exact nature of the rectifier is still unknown, an additional statement can be made about its location. According to the ideas developed in this paper, one major component of the cardiac inward rectifier is located in those

membranes surrounding the $K^+$ depleted spaces, based on the major effect $K^+$ has on the magnitude of the inward rectifying current. As mentioned above, it appears that these membranes are likely to be associated with the transverse tubules and longitudinal and transverse spaces between cells. However, nothing can as yet be said about whether $K^+$ rectification is uniform throughout the preparation.

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