Factors Affecting the Appearance of the Hump Charge Movement Component in Frog Cut Twitch Fibers

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ABSTRACT Charge movement was measured in frog cut twitch fibers with the double Vaseline gap technique. Five manipulations listed below were applied to investigate their effects on the hump component (Iv) in the ON segments of TEST minus CONTROL current traces. When external Cl\textsuperscript{−} was replaced by MeSO\textsubscript{4}\textsuperscript{−} to eliminate Cl current, Iv peaked earlier due to a few millivolts shift of the voltage dependence of I\textsubscript{v} kinetics in the negative direction. The Q-V plots in the TEA.Cl and TEA.MeSO\textsubscript{4} solutions were well fitted by a sum of two Boltzmann distribution functions. The more steeply voltage-dependent component (Q\textsubscript{1}) had a V \approx 6 mV more negative in the TEA.MeSO\textsubscript{4} solution than in the TEA.Cl solution. These voltage shifts were partially reversible. When creatine phosphate in the end pool solution was removed, the Iv hump disappeared slowly over the course of 20–30 min, partly due to a suppression of Q\textsubscript{v}. The hump reappeared when creatine phosphate was restored. When 0.2–1.0 mM Cd\textsuperscript{2+} was added to the center pool solution to block inward Ca current, the Iv hump became less prominent due to a prolongation in the time course of Iv but not to a suppression of Q\textsubscript{v}. When the holding potential was changed from −90 to −120 mV, the amplitude of I\textsubscript{a} was increased, thereby obscuring the Iv hump. Finally, when a cut fiber was stimulated repetitively, Iv lost its hump appearance because its time course was prolonged. In an extreme case, a 5-min resting interval was insufficient for a complete recovery of the waveform. In general, a stimulation rate of once per minute had a negligible effect on the shape of I\textsubscript{v}. Of the five manipulations, MeSO\textsubscript{4}\textsuperscript{−} has the least perturbation on the appearance of Iv and is potentially a better substitute for Cl\textsuperscript{−} than SO\textsubscript{4}\textsuperscript{2−} in eliminating Cl current if the appearance of the Iv hump is to be preserved.

INTRODUCTION

In a previous paper, Hui and Chandler (1990) reported that the ON segments of TEST minus CONTROL current traces recorded from cut twitch fibers showed a hump charge movement component, Iv, similar to that observed in intact fibers (Adrian and Peres, 1979), but different from some cut fiber traces published by other
investigators (for the definitions of $I_p$, $I_v$, $Q_v$, and $Q_s$, refer to the preceding paper). $Q_s$ has been hypothesized to be the trigger for intracellular Ca release (Huang, 1982; Hui, 1983a, b; Vergara and Caputo, 1983) or a consequence of the release (Csernoch et al., 1989; Pizarro et al., 1990). The observation that cut fibers which did not show any $I_v$ hump could still release Ca (Melzer et al., 1986) challenged these hypotheses. However, it is possible that $I_v$ might still be present in TEST minus CONTROL current traces when it is not manifested as a hump (see the preceding paper). In any case, the altered kinetics of $I_v$ should be associated with an altered kinetics in Ca release, whether $Q_s$ is the cause or consequence of the release. It is thus of physiological interest to find out the underlying reason(s) for the difference in the manifestations of $I_v$.

One possible cause for the alteration of the shape of $I_v$ was investigated by Hui and Chandler (1990), who showed that a substitution of external Cl\(^-\) by SO\(_4^{2-}\) led to an increase in $I_p$, making $I_v$ less prominent. The steady-state voltage distribution of $Q_v$ was not affected, but whether the kinetics of $I_v$ was affected was difficult to assess. The preceding paper (Hui, 1991) described another factor affecting the appearance of $I_v$ in cut fibers: when the temperature was lowered to < 10°C, $I_v$ in the ON segments of TEST minus CONTROL current traces became very broad and, at ~6°C, completely lost its hump appearance. At this low temperature, the steady-state $Q-V$ plot of the total charge contained a steeply voltage-dependent component, indicating the presence of $Q_s$. Thus, $I_v$ was not suppressed at low temperatures in cut fibers, but was most likely obscured in the decay phase of $I_v$.

This paper shows five other conditions that apparently affect the appearance of $I_v$. These interventions were studied because they have been used separately or jointly by various investigators in studying charge movement. These modifications would have some advantages in the measurement of charge movement and would be preferable if they had no effect on $I_v$. They include the substitution of Cl\(^-\) in the center pool solution by MeSO\(_4^{2-}\) to eliminate Cl current, the removal of creatine phosphate in the end pool solution to simplify the procedure in preparing the solution, the addition of Cd\(^{2+}\) to the center pool solution to block Ca current, a shift of the holding potential to a more negative potential to reduce the amount of charge in the CONTROL trace, and an increase in the stimulation rate of the fiber to collect more data within a certain time period. It was concluded that each of the interventions affected the appearance of the $I_v$ hump.

**METHODS**

All experiments were performed on cut fibers stretched to a sarcomere length of 3.5 μm at 13–14°C. The experimental protocol and method of data analysis were similar to those used in previous papers (Chandler and Hui, 1990; Hui and Chandler, 1990). All traces presented are from single TEST sweeps. Unless otherwise specified, TEST runs in a complete sequence were taken at 1-min intervals. Solutions are given in Table I. For the experiments studying the effect of external Cd\(^{2+}\), the phosphate buffer in the center pool solution (solution D) was replaced with PIPES (solution E) to avoid precipitation of the phosphates by Cd\(^{2+}\). This replacement had no observable effect on either the kinetics of charge movement or the steady-state charge versus voltage distribution (unpublished observations).

The fitting of $Q-V$ plots by a single Boltzmann distribution function or a sum of two
Factors Affecting the Appearance of $I_v$

Boltzmann distribution functions included corrections for the charge movements in the CONTROL current trace (CONTROL charge correction) and for the charge movements in the membranes underneath the Vaseline seals (gap correction), as described in Hui and Chandler (1990) and Hui (1991). To avoid repetitive use of the phrase, the corrections will not be mentioned. When a sum of two Boltzmann distribution functions is used, $V_p$, $k_p$, and $q_{\text{max}}/c_m$ are used to denote the Boltzmann parameters for the less steeply voltage-dependent component and $V_p$, $k_p$, and $q_{\text{max}}/c_m$ for the more steeply voltage-dependent component.

### TABLE 1

<table>
<thead>
<tr>
<th>Solutions</th>
<th>K$_{\text{glutamate}}$</th>
<th>MgSO$_4$</th>
<th>K$_p$ EGTA</th>
<th>K$_p$ PIPES</th>
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<tr>
<td><strong>A</strong></td>
<td>120</td>
<td>1</td>
<td>0.1</td>
<td>5</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>45.5</td>
<td>20</td>
<td>6.8</td>
<td>20</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>75.5</td>
<td>0</td>
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<td>20</td>
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End pool solutions:

<table>
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<tr>
<th>Solutions</th>
<th>Cs$_2$CP</th>
<th>MgSO$_4$</th>
<th>Cs$_2$ EGTA</th>
<th>Cs$_2$ ATP</th>
<th>Glucose</th>
<th>Cs$_2$ PIPES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B</strong></td>
<td>20</td>
<td>6.8</td>
<td>20</td>
<td>5.5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>0</td>
<td>6.8</td>
<td>20</td>
<td>5.5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Center pool solutions:

<table>
<thead>
<tr>
<th>Solutions</th>
<th>TEA Cl</th>
<th>TEA MeSO$_3$</th>
<th>RbCl</th>
<th>CaCl$_2$</th>
<th>Na$_2$ HPO$_4$</th>
<th>NaH$_2$ PO$_4$</th>
<th>PIPES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D</strong></td>
<td>120</td>
<td>0</td>
<td>2.5</td>
<td>1.8</td>
<td>2.15</td>
<td>0.85</td>
<td>0</td>
</tr>
<tr>
<td><strong>E</strong></td>
<td>120</td>
<td>0</td>
<td>2.5</td>
<td>1.8</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td><strong>F</strong></td>
<td>0</td>
<td>120</td>
<td>2.5</td>
<td>1.8</td>
<td>2.15</td>
<td>0.85</td>
<td>0</td>
</tr>
</tbody>
</table>

All concentrations are in millimolar. CP represents creatine phosphate in solution B. MeSO$_3$ represents methane sulfonate in solution F. Solutions D–F contained 1 μM tetrodotoxin. The relaxing and end pool solutions were titrated to pH 7.0 with KOH and CsOH, respectively. Center pool solutions D and F were titrated to pH 7.1 with HCl, whereas solution E was titrated to pH 7.1 with NaOH.

### RESULTS

#### Effect of Substituting Cl$^-$ by MeSO$_3^-$

Resting Cl conductance is known to be twice as large as K conductance in intact frog twitch fibers (Hodgkin and Horowicz, 1959; Hutter and Noble, 1960). To increase the membrane space constant, thereby improving spatial uniformity along the fiber during voltage clamp, SO$_4^{2-}$ has been used to replace Cl$^-$ in the center pool solution. However, Hui and Chandler (1990) showed that when extracellular Cl$^-$ was completely replaced by SO$_4^{2-}$, the $I_v$ hump in the ON segments of TEST minus CONTROL current traces became less resolvable due to a threefold increase in $Q_p$, as estimated from fitting the $Q-V$ plots by a sum of two Boltzmann distribution functions. Since Cl current can also be eliminated by replacing Cl$^-$ with MeSO$_3^-$ (methane sulfonate), experiments were performed to investigate whether MeSO$_3^-$ affects $I_p$ like SO$_4^{2-}$.

In the experiment of Fig. 1, the center pool segment of the fiber was bathed in a TEA MeSO$_3$ solution immediately after saponin treatment. The MeSO$_3^-$ very effec-
tively blocked the positive pedestal in the ON segment arising from the nonlinear ionic current routinely seen with the TEA.Cl solution and actually gave rise to a small negative pedestal. In contrast to the SO₄ experiments, a prominent \( I_v \) hump can be clearly seen in the ON segments of the traces between \(-66\) and \(-45\) mV. At \(-66\) mV, the hump was very broad and had a very small magnitude. When the level of depolarization was increased, the hump became faster and had a larger amplitude, as routinely observed in intact fibers and our cut fibers. At potentials \( \geq -58 \) mV, the peak of \( I_v \) rose above that of \( I_g \). At \(-40\) and \(-30\) mV, the kinetics of \( I_v \) was even faster, such that the peak of \( I_g \) was buried in the rising phase of \( I_v \).

![Figure 1](https://example.com/fig1.png)

The numbers at the right show the potentials during the TEST pulses. Only representative traces are shown.

In the experiment shown in Fig. 2, charge movements in the TEA.Cl and TEA.MeSO₃ solutions were compared. TEST minus CONTROL current traces were recorded in the TEA.Cl solution first (Fig. 2A). Prominent \( I_v \) humps can be seen clearly in the ON segments of the second to the fourth traces. When the TEA.Cl solution was replaced by the TEA.MeSO₃ solution, the positive pedestals due to maintained ionic current in the ON segments were replaced by negatively sloping baselines up to about \(-40\) mV (Fig. 2B). Beyond that potential, the ON segments showed a sloping baseline plus a negative pedestal. In addition to the change in maintained current, the shape of \( I_v \) was altered, with the largest difference detectable at \(-46\) mV. This change in shape could be due to a voltage shift in the kinetics of \( I_v \), as will be explained below. These effects of MeSO₃ were partially reversed when the
center pool solution was changed back to the TEA.Cl solution, as revealed by traces in Fig. 2 C. The negative pedestals in the ON segments disappeared, but not the negatively sloping baselines. $I_h$ humps can be seen in the first four traces, but the shapes of the humps were still different from those in the corresponding traces in Fig. 2 A.

The amounts of ON and OFF charge were estimated (see Hui and Chandler, 1990) by integrating the areas of the ON and OFF transients in the traces of Fig. 2 (and others not shown), and are plotted against the TEST pulse potential in Fig. 3 as diamonds (for control TEA.Cl solution), squares (for TEA.MeSO3 solution), and triangles (for washout TEA.Cl solution). Filled symbols represent OFF charge and
open symbols ON charge. ON charge that overlaps with the corresponding OFF charge is not shown. Also, ON charge at the potentials at which the I, hump was broad (around −60 to −55 mV) was not estimated because baseline fit was unreliable (see Hui and Chandler, 1990; Hui, 1991).

Several observations can be made before detailed data analysis. In the TEA.Cl solution, ON and OFF charges were equal within experimental error, except at 0 mV. When Cl− was replaced by MeSO₃, ON:OFF charge equality was preserved up to −40 mV, beyond which the amount of OFF charge exceeded the corresponding amount of ON charge, probably due to contamination of inward ionic current. The steep portion of the Q−V plot is shifted slightly to the left; the maximum amount of total charge is reduced; and the shoulder where the slope of the Q−V plot turned from the steep portion to the less steep portion is lowered, implying that the maximum

![Figure 3. Steady-state voltage distributions of total charge in a cut fiber bathed in a TEA.Cl or TEA.MeSO₃ solution. Same fiber as in Fig. 2. Diamonds show data taken with TEA.Cl (control), squares with TEA.MeSO₃, and triangles with TEA.Cl (bracketing), in the center pool solution. Open symbols represent ON charge and filled symbols represent OFF charge. The points were obtained from the time integrals of ON or OFF transients in TEST minus CONTROL current traces, some of which are shown in Fig. 2. The smooth curves A–C were obtained by least-squares fits of a sum of two Boltzmann distribution functions to the three data sets. In each data set, ON charge was used for the fit when both ON and OFF charges are shown at the same potential; otherwise, OFF charge was used. The values of the best-fit parameters are listed in the first three rows of Table II.](image)

amount of Q, was reduced. When the center pool solution was changed back to the TEA.Cl solution, the reduction in total charge was reversed. In fact, the amounts of charge above −40 mV in curve C are slightly larger than the corresponding amounts in curve A. However, the voltage shift of the steep portion of the plot is not reversed and the shoulder is less marked. Even in the presence of negatively sloping baselines in the ON segments of the traces in Fig. 2 C, ON:OFF charge equality was still preserved up to −20 mV and the amounts of OFF charge in these traces were similar to the corresponding amounts in the traces of Fig. 2 A. This suggests that there is no correlation between the negatively sloping baseline in the ON segment and the ionic contamination on OFF charge at large depolarizations.

To assure that the maintained, apparently inward ionic current does not generate a tail current to contaminate the OFF charge, charge movement was studied in another...
fiber, bathed in the TEA(MeSO₃) solution, with TEST pulses of varying durations. The negative pedestal began to appear in the ON segments of TEST minus CONTROL current traces at -50 mV. With larger depolarizations, the magnitude of the pedestal was increased and the negative slope of the baseline became more steep, the same as the traces in Fig. 2 B. Three TEST pulses to -45 mV, of durations 200, 300, and 400 ms, were applied in the early phase of the experiment, and the amounts of OFF charge were 8.4, 8.8, and 8.6 nC/µF, respectively, which were almost identical to the amount of ON charge, 8.5 nC/µF, within experimental error. Two other sequences of TEST pulses to -45 mV were applied halfway through and at the end of the experiment by including the durations of 100 and 600 ms. The amounts of OFF charge in all the runs were 8–9 nC/µF, indicating that the fiber was absolutely stable. At -40 and -35 mV, OFF charge was still invariant for pulse durations of 100–300 ms and had values of 9.8–10.4 and 11.1–12.5 nC/µF, respectively.

The TEST current trace from a run at -40 mV and the associated CONTROL current trace were examined at high gain. The ON segment of the CONTROL current trace was absolutely flat and the negative slope of the ON baseline in the TEST minus CONTROL current trace was originated in the TEST current. In addition, although the TEST pulse was 2.5 times as large as the CONTROL pulse, the magnitude of the ionic current at the beginning of the TEST pulse was only 2.35 times the maintained ionic current during the CONTROL pulse. Thus, when the CONTROL current was scaled up 2.5 times and subtracted from the TEST current, the slight mismatch in the ionic current yielded the negative pedestal. This rectifying property is characteristic of MeSO₃⁻, as it does not occur when Cl⁻ is used. The negative slope mentioned above must then be due to a time-dependent change in the flux of MeSO₃⁻. On repolarization, the change should reverse in direction. However, the invariance in OFF charge with increasing pulse durations implied that the change in flux on repolarization probably lasted hundreds of milliseconds, or even longer, and so was buried in the OFF baseline.

The situation was different with more depolarized TEST pulses. At -30 mV, the amounts of OFF charge were 13.3, 14.8, and 18.9 nC/µF for pulse durations of 150, 200, and 300 ms, while the ON charge was 13.6 nC/µF. At -20 mV, the amounts of OFF charge were 17.9, 22.9, and 29.2 nC/µF for pulse durations of 100, 150, and 200 ms, while the ON charge was 17.5 nC/µF. Thus, at these potentials another inward ionic current, distinct from the current described above, was activated and its tail current contaminated the OFF charge progressively more with increasing pulse durations. This latter current is probably related to the slow Ca current and is generally noticeable as it has a waveform similar to those shown in the ON segments of the last two traces in Fig. 7 A. It should be noted that, in this fiber, if the durations of the TEST pulses at -30 and -20 mV were not longer than 150 and 100 ms, respectively, the contamination of OFF charge by the tail Ca current was not substantial.

The smooth curves A–C in Fig. 3 were obtained by fitting a sum of two Boltzmann distribution functions to the three data sets. In each data set, any open symbol shown was used to replace the corresponding filled symbol in the fit. The best-fit parameters of the three curves are listed in Table II as the first entry. The features described above were confirmed by the values of the parameters; namely, \( V \) in curve B was 4–5
mV more negative than that in curve A. The value of \( q_{\text{max}} / C_m \) for either \( Q_s \) or the total charge \( (Q_s + Q_v) \) in curve B was 2–3 nC/\( \mu \)F smaller than the corresponding value in curve A. After washout, the value of \( \bar{V}_s \) in curve C was not restored to the control value but stayed close to the value in curve B. The values of \( q_{\text{max}} / C_m \) for the total charge were similar in curves A and C. The smaller amount of total charge above \(-40\) mV in curve A than in curve C can be explained by the larger value of \( k_s \) in curve A, such that the curve did not reach saturation at \( 0 \) mV but would rise to the same asymptotic value as curve C. The disappearance of the shoulder in curve C can also be explained by a smoother transition from the steeper portion to the less steep portion.

### Table II

**Effect of Replacing Cl\(^{-}\) in the Bathing Solution by MeSO\(_3\) on \( Q-V \) Distributions of \( Q_s \) and \( Q_v \) in Cut Fibers**

<table>
<thead>
<tr>
<th>Fiber reference</th>
<th>External solution</th>
<th>( c_{e} ) ( \mu F/cm )</th>
<th>( \bar{V}_s ) mV</th>
<th>( k_s ) mV</th>
<th>( q_{\text{max}}/c_{e} ) nC/( \mu )F</th>
<th>( k_v ) mV</th>
<th>( q_{\text{max}}/c_{e} ) nC/( \mu )F</th>
<th>( % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>85231 Cl(^{-})</td>
<td>0.193</td>
<td>-34.2</td>
<td>10.7</td>
<td>8.6</td>
<td>-60.4</td>
<td>3.3</td>
<td>11.1</td>
<td>56.4</td>
</tr>
<tr>
<td>MeSO(_3)</td>
<td>0.192</td>
<td>-40.1</td>
<td>10.8</td>
<td>9.0</td>
<td>-65.1</td>
<td>3.3</td>
<td>8.3</td>
<td>47.9</td>
</tr>
<tr>
<td>85241 Cl(^{-})</td>
<td>0.194</td>
<td>-38.8</td>
<td>9.6</td>
<td>8.3</td>
<td>-56.6</td>
<td>2.1</td>
<td>13.9</td>
<td>62.6</td>
</tr>
<tr>
<td>MeSO(_3)</td>
<td>0.193</td>
<td>-42.7</td>
<td>9.1</td>
<td>12.5</td>
<td>-63.8</td>
<td>2.5</td>
<td>11.1</td>
<td>47.0</td>
</tr>
<tr>
<td>85311 Cl(^{-})</td>
<td>0.145</td>
<td>-27.7</td>
<td>12.3</td>
<td>11.3</td>
<td>-58.6</td>
<td>3.9</td>
<td>18.3</td>
<td>61.8</td>
</tr>
<tr>
<td>MeSO(_3)</td>
<td>0.150</td>
<td>-26.9</td>
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<td>86241 Cl(^{-})</td>
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<td>54.0</td>
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<tr>
<td>MeSO(_3)</td>
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<tr>
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<td>-63.0</td>
<td>2.6</td>
<td>11.4</td>
<td>48.7</td>
</tr>
</tbody>
</table>

Column 1 gives fiber identifications. Columns 2 and 3 give the major anions in the center pool solution and the values of \( c_{e} \), respectively. Columns 4–9 give the best-fit values of the parameters in the Boltzmann distribution functions of \( Q_s \) and \( Q_v \), with CONTROL charge correction and gap correction. Column 10 gives the fractions of \( Q_s \) in the total charge.

The left shift of \( \bar{V}_s \) when Cl\(^{-}\) was replaced by MeSO\(_3\) can explain, to some extent, the apparent change in the kinetics of \( I_v \) in the ON segments of the traces in Fig. 2 B. To allow for the voltage shift, the first trace in Fig. 2 B should be compared with the second trace in Fig. 2 A, and so forth. Indeed, after the shift, the kinetics of \( I_v \) in the two solutions was comparable. After washout, the shift in \( \bar{V}_s \) was not reversed. This can also explain why the humps in the ON segments of the traces in Fig. 2 C resembled the corresponding ones in Fig. 2 B more than those in Fig. 2 A.

Three other similar experiments were performed and the results are summarized in Table II. In all the fibers, MeSO\(_3\) shifted the value of \( \bar{V}_s \) by 4–7 mV in the hyperpolarizing direction and the effect was not reversed after washout. The slight suppression of \( Q_s \) in Fig. 3 by MeSO\(_3\) was shared by two of the three other fibers.
In seven other experiments, including the one shown in Fig. 1, the fibers were not exposed to CI⁻ and charge movement was measured in the TEA.MeSO₃ solution only. The Q-V plots were fitted by a sum of two Boltzmann distribution functions. The mean values of the parameters for \( Q_b \) and \( Q_v \) from these seven fibers and the four fibers in Table II are listed in Table III. For comparison, the mean values in the TEA.CI solution from Hui (1991) and those in the TEA₂SO₄ solution from Hui and Chandler (1990) are also listed.

The values in the TEA.MeSO₃ solution are quite close to those in the TEA.CI solution. \( \bar{V}_b \) appears to be more negative, and \( q_{b,max}/e_m \) and \( q_{v,max}/e_m \) appear to be smaller, in the TEA.MeSO₃ solution than in the TEA.CI solution, but the differences are statistically insignificant (\( P > 0.05 \) with the two-tailed t test). The fraction of \( Q_v \) in the total charge is almost identical in the two solutions (\( P > 0.5 \) with the two-tailed t test).

Although the shift in \( \bar{V}_b \) is insignificant, it is parallel to the shift in \( \bar{V}_v \). In contrast, the voltage distributions of \( Q_b \) and \( Q_v \) are affected to a larger extent by SO₄²⁻: \( k_v \) and \( q_{v,max}/e_m \) are not changed (\( P > 0.5 \) with the two-tailed t test); \( \bar{V}_b \) and \( \bar{V}_v \) are 13–15 mV less negative in the TEA₂SO₄ solution than in the TEA.CI solution; \( k_b \) is increased by \( \sim 50\% \) in the TEA₂SO₄ solution; and \( q_{b,max}/e_m \) is increased more than twofold in the TEA₂SO₄ solution, making the fraction of \( Q_v \) in the total charge decrease from one-half to one-fourth. The latter differences are highly significant (\( P < 0.001 \) with the two-tailed t test).

Results in this section show that, although the shape of \( I_v \) was affected by replacing CI⁻ with MeSO₃⁻, probably due to a negative shift in \( \bar{V}_v \), prominent \( I_v \) humps could
Effect of Removal of Intracellular Creatine Phosphate on Charge Movement

Cut skeletal muscle fibers have many advantages over intact fibers for studying charge movement and other signals involved in excitation-contraction coupling. Unfortunately, these advantages are undermined by at least one complication: since the plasma membranes of the end pool segments of a cut fiber are either notched or permeabilized, myoplasmic constituents that may be vital in maintaining the physiological state of the fiber can diffuse out of the fiber. This problem can be avoided by making the end pool solution as close in composition to the myoplasm as possible. Since creatine phosphate is normally present in the myoplasm at a concentration as high as 50 mM (Godt and Maughan, 1988), we generally included 20 mM creatine phosphate in the end pool solution (Hui and Chandler, 1990; Hui, 1991), but other workers who measured charge movement in cut fibers did not. Thus, experiments were performed to compare the properties of charge movement in the presence and absence of 20 mM creatine phosphate in the end pool solution.

Fig. 4 A shows TEST minus CONTROL current traces elicited in a cut fiber by a 400-ms TEST pulse to −40 mV. Trace 1 was recorded with 20 mM creatine phosphate in the end pool solution (solution B in Table I). The ON segment shows an early $I_p$ component and an $I_v$ component manifested as a hump in the decay phase of $I_p$, same as in other cut or intact fibers. After trace 1 was taken, creatine phosphate was removed from the end pool solution (solution C in Table I). Five runs were taken, of which three are shown as traces 2–4. In these traces, the amplitude of the hump became progressively smaller, and 25 min after the removal of creatine phosphate no $I_v$ could be resolved in trace 4. In other control experiments in which creatine phosphate was not removed, the $I_v$ hump generally remained unchanged for several hours. After trace 4 was taken, 20 mM creatine phosphate was restored in the end pool solution and many TEST minus CONTROL current traces (not shown) were taken. In trace 5, which was taken 62 min after the restoration of creatine phosphate, an $I_v$ hump can be visualized in the ON segment; however, it is not as prominent as that in trace 1, suggesting that the diminution of the $I_v$ hump with creatine phosphate removal was partially reversible.

The amount of OFF charge in each trace of Fig. 4 A (and other traces not shown), estimated by integrating the area of the OFF current transient, is plotted as a function of time in the upper panel of Fig. 4 C. The concentration of creatine phosphate in the end pool solution is shown by the solid line in the lower panel. 20 mM creatine phosphate was present in the end pool solution from the beginning of the experiment. After equilibration for ~1 h, the concentration of creatine phosphate in the myoplasm in the center pool region was assumed to be 20 mM. When creatine phosphate was removed from the end pool solution, creatine phosphate in the myoplasm presumably diffused into the end pools, lowering its concentration in the center pool region. The approximate average concentration in the center pool region was estimated by Eq. 2.15 in Crank (1956), assuming a diffusion constant of $2 \times 10^{-6}$ cm$^2$/s. The average concentration is plotted as the decaying portion of the
**FIGURE 4.** Effect of 20 mM creatine phosphate in the end pool solutions on TEST minus CONTROL currents in a cut fiber. Fiber identification: 83241. Diameter, 129 μm. Saponin treatment was applied to membrane segments in both end pools at time zero. After rinsing, the solutions in the end pools were replaced by solution B. Then, the solution in the center pool was changed to a TEACl solution (solution D). At the 24th minute, the voltage clamp was turned on and the holding potential was set at −90 mV. From the beginning to the end of the experiment, the holding current changed from −45 to −58 nA and \( r/(r_i + r_e) \) decreased from 0.988 to 0.983. (A) TEST minus CONTROL traces elicited by a TEST pulse to −40 mV. The times at which the traces were taken and the end pool solutions were changed can be obtained from C. (B) Difference traces from A obtained by subtracting trace 4 from each trace. (C) The upper panel shows the amounts of OFF charge estimated from the traces in A (numbered correspondingly) and other traces not shown. The lower panel shows the concentration of creatine phosphate in the end pool solution.
dashed curve in the lower panel. When 20 mM creatine phosphate was restored in the end pool solution, it diffused back into the myoplasm, increasing its concentration in the center pool region. The approximate average concentration in the center pool region was estimated by Eq. 4.17 in Crank (1956) and is plotted as the rising portion of the dashed curve.

The upper panel shows that, after the removal of creatine phosphate, there was a progressive decrease in OFF charge parallel to the slow disappearance of $I_v$ in the ON segment, implying that there was an actual loss of $Q_v$. When 20 mM creatine phosphate was restored, the amount of OFF charge recovered fully, whereas the shape of $I_v$ in the ON segment did not (compare traces 1 and 5 in Fig. 4A).

If creatine phosphate removal has no effect on the ON time course of $I_v$ at $-40$ mV, then the waveform of the $I_v$ hump in traces 1–3 and 5 of Fig. 4A can be obtained by subtracting trace 4 from each trace. The first three difference traces in Fig. 4B show that the $I_v$ humps in the ON segments are bell shaped, as was suggested for $I_v$ in intact fibers (Hui, 1983b). The first three points in the ON segment of trace 1 are below the baseline, implying that there might be some minor change in the magnitude or kinetics of $I_v$ at this potential after the removal of creatine phosphate.

The OFF transients of the traces show a marked rising phase, which has not been observed in intact or cut fibers. The half-widths, times-to-peak, and areas of the ON and OFF transients of the traces in Fig. 4B are listed in Table IV. The numbers in columns 5 and 8 show that the amounts of ON and OFF charge in each trace were almost equal, within experimental error. It will be shown in the following experiment (Figs. 5 and 6) that this equality might not be held at more negative potentials. The amount of charge in trace 1 of Fig. 4B provided a lower limit to the amount of $Q_v$ in trace 1 of Fig. 4A, but the actual amount of $Q_v$ could be larger if trace 4 of Fig. 4A contained some residual $Q_v$ or an increased amount of $Q_p$.

Table IV also shows that, after the removal of creatine phosphate, the amount of ON or OFF charge diminished progressively and the time-to-peak of the ON

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<th>Area</th>
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Same fiber as in Fig. 4. Columns 1 and 2 give the trace numbers in Fig. 4B and the concentrations of creatine phosphate in the end pool solution when the trace was taken. Column 3 gives the values of $c_\alpha$. Columns 4–6 give the half-widths, the times-to-peak, and the amounts of charge in the ON transients of the traces. Columns 7–9 give the corresponding information for the OFF transients.
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The transient was somewhat prolonged. When creatine phosphate was restored, the amount of $Q_o$ recovered completely but the time course of $I_v$ did not. The change in the shape of $I_v$ from trace 1 to trace 5 could be due to a chronic effect of creatine phosphate removal. Four other experiments showed similar reversible disappearance of $I_v$ in the ON segment and reversible reduction in the amount of OFF charge due to creatine phosphate removal.

The experiment in Fig. 4 was performed with a constant TEST pulse to $-40\,\text{mV}$, which is usually at the upper edge of the steep portion of a $Q-V$ curve. If creatine phosphate removal causes the $Q_v-V$ curve to be shifted in the depolarizing direction such that the lower edge of the steep portion is at $-40\,\text{mV}$ after the shift, that would result in the apparent reduction of $Q_o$. The experiment in Figs. 5 and 6 was carried out to rule out this possibility by comparing the steady-state voltage distribution of charge in the presence and absence of 20 mM creatine phosphate.

With creatine phosphate in the end pool solution, a distinct $I_v$ hump can be visualized in the ON segments of the traces (thick traces in Fig. 5 A) recorded at TEST pulse potentials between $-60$ and $-40\,\text{mV}$. At $\geq -30\,\text{mV}$, it is difficult to resolve $I_v$ from $I_{p}$. After the removal of creatine phosphate from the end pool solution, no humps can be visualized in the ON segments (thin traces in Fig. 5 A) over a wide range of potential, indicating that the disappearance of the $I_v$ hump was not due to a shift in the voltage dependence of the kinetics of $I_v$. In addition, the OFF currents in the thin traces decay faster than those in the thick traces. This difference in OFF kinetics could be due to a direct effect of creatine phosphate on the kinetics of OFF current. Alternatively, if $I_v$ has slower OFF kinetics than $I_{p}$ (Hui and Chandler, 1991), that would make the OFF currents in the thick traces decay slower than those in the thin traces. A similar difference in the rate of decay of OFF current also existed between traces 1 and 4 of Fig. 4 A, but was less marked, probably because that fiber had less $I_v$.

In Fig. 6A, the amounts of ON charge (open symbols) and OFF charge (filled symbols) from the traces in Fig. 5 (and others not shown) are plotted against the potentials during the TEST pulses. The diamonds represent the amounts of charge in the presence of creatine phosphate. Between $-55$ and $-45\,\text{mV}$, ON charge is smaller than OFF charge. The difference is caused by the broad waveforms of the $I_v$ humps at these potentials, resulting in unreliable fittings of the ON baselines (Hui and Chandler, 1990). Thus, from $-80$ to $-30\,\text{mV}$, OFF charge is taken to represent the total charge. At $-20$ and $-10\,\text{mV}$, OFF charge is larger than ON charge, probably because of contamination by tail Ca current (Hui and Chandler, 1990). ON charge is therefore taken to represent the total charge at these two potentials. Curve 1 was fitted to the mentioned filled and open diamonds by a sum of two Boltzmann distribution functions. The values of $V_{\text{p}}$, $k_{\text{p}}$, $q_{b,\text{max}}/C_m$, $V_{\text{p}}$, $k_{\text{p}}$, and $q_{b,\text{max}}/C_m$ are $-38.2\,\text{mV}$, $8.2\,\text{mV}$, $10.5\,\text{nC}/\mu\text{F}$, $-58.2\,\text{mV}$, $2.4\,\text{mV}$, and $8.9\,\text{nC}/\mu\text{F}$, respectively. Thus, in the presence of creatine phosphate, $Q_a$ and $Q_o$ existed in this fiber in approximately equal amounts, in agreement with the results in preceding papers (Hui and Chandler, 1990; Hui, 1991).

After the removal of creatine phosphate, the amount of ON or OFF charge at each potential (squares) was reduced. Between $-60$ and $-40\,\text{mV}$, ON charge is smaller than OFF charge, similar to the situation with creatine phosphate, but the removal of
creatine phosphate appears to enhance the discrepancy between ON and OFF charges, suggesting that, without creatine phosphate, more $I_v$ is buried in the baseline of the ON segment, particularly around $-50$ mV. Indeed, in the thin trace of the fourth pair in Fig. 5 A, although the ON transient only shows a fast $I_h$ component, it is impossible to rule out the presence of an $I_v$ hump having an extremely broad time course and an unresolvable amplitude. Only with the presence of this obscured

![Figure 5](image)

**FIGURE 5.** TEST minus CONTROL currents in a cut fiber with and without 20 mM creatine phosphate in the end pool solution. Fiber identification: 83231. Diameter, 121 μm. Saponin treatment was applied to membrane segments in both end pools at time zero. After rinsing, the solutions in the end pools were replaced by solution B. Then the solution in the center pool was changed to a TEA·Cl solution (solution D). At the 21st minute, the voltage clamp was turned on and the holding potential was set at $-90$ mV. The holding current and $r_h/(r_h + r_e)$ remained constant at $-29$ nA and 0.991, respectively, throughout the experiment. (A) Thick traces were taken with 20 mM creatine phosphate (solution B) in the end pools from the 58th to the 86th minute. At the 90th minute, the end pool solutions were replaced by a creatine phosphate–free solution (solution C). Thin traces were taken from the 113th to the 130th minute. (B) Difference traces obtained by subtracting each thin trace in A from the superimposed thick trace. The numbers at the right show the potentials during the TEST pulses (same pulse for the traces in A and B). Only representative traces are shown in each panel.

$I_v$ would the ON-OFF charge equality be preserved. At $-20$ and $-10$ mV, OFF charge (filled squares) in Fig. 6 A is larger than ON charge (open squares), the same as with creatine phosphate. Thus, the filled squares up to $-30$ mV and the open squares at $-20$ and $-10$ mV are taken to represent the total charge in the absence of creatine phosphate.

A comparison of the two sets of data in Fig. 6 A show that the steep rise of the filled
diamonds between -65 and -55 mV and the shoulder at around -55 mV disappear in the filled squares. An attempt was made to fit a sum of two Boltzmann distribution functions to the squares, but the fitting routine did not converge. The points were therefore fitted by a single Boltzmann distribution function, as shown by curve 2. The best-fit values for $V$, $k$, and $q_{\text{max}}/C_m$ were -45.2 mV, 6.5 mV, and 15.4 nC/µF, respectively. The value of $q_{\text{max}}/C_m$ in curve 2 is 4 nC/µF smaller than the sum of $q_{\alpha,\text{max}}/C_m$ and $q_{\beta,\text{max}}/C_m$ in curve 1, implying that the total amount of charge was reduced after the removal of creatine phosphate. It should be noted that this difference is larger.
than the difference between the open diamond and the open square at -10 mV, which is \( \sim 2 \text{nC/\mu F} \). This apparent discrepancy arises as a result of gap correction and CONTROL charge correction, as explained in the Methods section of the preceding paper (Hui, 1991).

The 4 \( \text{nC/\mu F} \) decrease in total charge was too small to account for all the \( Q_v \) in curve 1, which amounted to 8.9 \( \text{nC/\mu F} \). To understand the reason for the difference, each thin trace in Fig. 5A was subtracted from the thick trace at the same potential. In the difference traces shown in Fig. 5B, the ON transients are bell shaped and the OFF transients have a pronounced rising phase, similar to the traces in Fig. 4B. For potentials \( >-48 \text{mV} \), the OFF transients show a positive deflection preceding the usual negative deflection. This biphasic nature is also present in the ON transients, but to a smaller extent. The positive (negative) deflections in the OFF (ON) transients could be due to an increase in the peak amplitude of \( I_\text{Na} \) when creatine phosphate was removed.

The amount of charge in each trace of Fig. 5B is plotted as a function of potential in Fig. 6B. The squares, representing ON charge, were obtained by subtracting the open squares in Fig. 6A from the corresponding open diamonds. Likewise, the diamonds in Fig. 6B, representing OFF charge, were obtained by subtracting the filled squares in Fig. 6A from the corresponding filled diamonds. For potentials \( <-50 \text{mV} \), ON charge and OFF charge are roughly equal. The points rise sharply at \(-60 \text{mV} \). OFF charge begins to decline at \(-50 \text{mV} \), while ON charge continues to increase before declining. The largest difference between ON and OFF charges is at around \(-50 \text{mV} \), as explained above.

Because of all the complications, the difference \( Q_v \) plots of both ON and OFF charges were studied. Each plot was fitted by a single Boltzmann distribution function. The points represented by the open symbols were excluded from the fits because they correspond to the ON or OFF transients that are biphasic. For the ON charge (curve 1) the values of \( \bar{V} \), \( k \), and \( q_{\text{max}}/c_m \) are \(-57.8 \text{mV}, 2.4 \text{mV}, \) and 8.0 \( \text{nC/\mu F} \), respectively, whereas for the OFF charge (curve 2) the values are \(-61.0 \text{mV}, 0.2 \text{mV}, \) and 5.1 \( \text{nC/\mu F} \), respectively. The value of \( k \) in curve 2 is exceptionally small and the value of \( q_{\text{max}}/c_m \) is smaller than the value of \( q_{\text{max}}/c_m \) of the \( Q_v \) component in curve 1 of Fig. 6A. On the other hand, the values of the parameters in curve 1 of Fig. 6B are very close to the values of the \( Q_v \) component in curve 1 of Fig. 6A, but some of the points used for the fit lie in the potential range in which the fitting of baseline is uncertain. Thus, there is some uncertainty about the exact amount of charge suppressed as a result of creatine phosphate removal. Nonetheless, there is no doubt that at least 4 \( \text{nC/\mu F} \) of charge was suppressed when creatine phosphate was removed. The exact amount suppressed is likely to be less than the 8.9 \( \text{nC/\mu F} \) of \( Q_v \) in the presence of 20 mM creatine phosphate, because creatine phosphate in the myoplasm had not completely diffused out (see dashed curve in the lower panel of Fig. 4C). Moreover, there could be an increase in \( Q_{\text{Na}} \) when creatine phosphate was removed, as hinted by the biphasic nature of the current transients at large depolarizations. Whether the increase in \( Q_{\text{Na}} \) is due to an interconversion from \( Q_v \) remains to be clarified (see Discussion), but the increase in \( Q_{\text{Na}} \), if it occurred, was definitely smaller than the decrease in \( Q_v \).

Two other experiments were performed with the order of solution change reversed. Since the fibers were equilibrated in creatine phosphate--free end pool
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solution from the beginning of the experiment, the residual concentration of creatine phosphate in the myoplasm of the center pool region should be negligible. TEST minus CONTROL current traces from both fibers did not show any $I_v$ hump in the ON segments. When the $Q-V$ plots were fitted by a sum of two Boltzmann distribution functions, the fitting routine did not converge. When the plots were fitted by a single Boltzmann distribution function, the best-fit values for $V_1$, $k_1$, and $q_{max}/C_m$ were $-44.3$ mV, $7.5$ mV, and $10.6$ nC/µF for the second fiber and $-39.1$ mV, $11.2$ mV, and $16.6$ nC/µF for the third fiber.

After the addition of 20 mM creatine phosphate to the end pool solution, ~60 min were allowed for equilibration. The concentration of creatine phosphate in the myoplasm of the center pool region should be close to 20 mM. $I_v$ humps appeared in the ON segments of TEST minus CONTROL current traces in both experiments. The $Q-V$ plots could be fitted very well by a sum of two Boltzmann distribution functions. The best-fit values for $V_1$, $k_1$, $q_{max}/C_m$, $V_2$, $k_2$, and $q_{max}/C_m$ are $-41.1$ mV, $9.7$ mV, $10.4$ nC/µF, $-54.5$ mV, $2.8$ mV, and $4.8$ nC/µF for the second fiber and $-22.7$ mV, $16.1$ mV, $8.1$ nC/µF, $-49.0$ mV, $4.7$ mV, and $12.7$ nC/µF for the third fiber. In the second fiber, the values of the Boltzmann parameters for the $Q_\beta$ component are similar to those for the total charge before the addition of creatine phosphate, but the amount of $Q_\beta$ reprimed is less than the average amount in control fibers. In the third fiber, the amount of total charge in the absence of creatine phosphate is larger than the amount of $Q_\beta$ in 20 mM creatine phosphate, as in the first fiber.

Following the procedure applied to the first experiment, difference current traces were obtained from TEST minus CONTROL current traces before and after the addition of creatine phosphate. For the second fiber, ON and OFF charges were equal in the difference traces, within experimental error, whereas for the third fiber, ON charge was larger than OFF charge as in Fig. 6 B. The difference $Q-V$ plots of OFF charge were fitted by a single Boltzmann distribution function. The plot of the second fiber is sigmoidal and the values of $V_1$, $k_1$, and $q_{max}/C_m$ are $-57.3$ mV, $4.0$ mV, and $6.5$ nC/µF. The plot of the third fiber is bell shaped and the values of the parameters are $-48.0$ mV, $3.2$ mV, and $5.8$ nC/µF. The value of $q_{max}/C_m$ for the difference charge is larger (smaller) than the value of $q_{max}/C_m$ in the presence of creatine phosphate in the second (third) fiber.

Results in this section show that when creatine phosphate was removed from the end pool solution, the $I_v$ hump disappeared. Comparison of $Q-V$ plots before and after solution change and of difference $Q-V$ plot suggests qualitatively that the disappearance of $I_v$ is not primarily due to a change in the time course of $I_v$, but to an actual suppression of $Q_\gamma$ as well. This indicates that creatine phosphate is required in the end pool solution for the maintenance of $Q_\gamma$. Removal of creatine phosphate from the end pool solution also has the secondary effect of increasing $I_\beta$ or altering the waveform of the residual $I_v$. The dose dependencies of these effects have not been studied.

Effect of Addition of External Cd$^{2+}$

When a depolarizing pulse of sufficiently large magnitude is applied to a muscle fiber, many ionic channels, including Ca channels, are opened. The slow inward Ca current makes the ON segment baseline in a TEST minus CONTROL current trace.
deviate from a straight line. Also, the channels do not close instantaneously after the pulse. The decaying ionic current contaminates the OFF transient, as described by Horowicz and Schneider (1981). The potential at which the OFF charge begins to exceed the ON charge varies from fiber to fiber, but in our hands is usually > -30 mV. One way to reduce the inward Ca current is to add Cd\(^{2+}\) to the center pool solution (Cota et al., 1983; Donaldson and Beam, 1983).

**Figure 7.** Effect of extracellular Cd\(^{2+}\) on TEST minus CONTROL currents in a cut fiber. Fiber identification: 87081. Diameter, 93 \(\mu m\). Saponin treatment was applied to membrane segments in both end pools at time zero. After rinsing, the solutions in the end pools were replaced by solution B. Then, the solution in the center pool was changed to a TEA.CI solution (solution E). At the 21st minute, the voltage clamp was turned on and the holding potential was set at -90 mV. From the beginning to the end of the experiment the holding current changed from -35 to -47 nA and \(r_f/(r_c + r_f)\) decreased from 0.985 to 0.981. (A) Traces taken without Cd\(^{2+}\) in the center pool solution from the 55th to the 74th minute. At the 108th minute, 0.5 mM Cd\(^{2+}\) was added to the center pool solution. (B) Traces taken from the 120th to the 139th minute. At the 172nd minute, the [Cd\(^{2+}\)] in the center pool solution was changed to 1 mM. (C) Traces taken from the 181st to the 200th minute. Only representative traces are shown in each panel. The numbers at the right show the potentials during the TEST pulses (same pulse for the traces in each row).

The traces in Fig. 7 were taken when the center pool solution contained 0 (A), 0.5 (B), and 1 mM (C) Cd\(^{2+}\). In the absence of Cd\(^{2+}\), the inward Ca current becomes noticeable at -20 mV (sixth trace in Fig. 7A), as reflected by the negative deflection in the later part of the ON segment, and increases progressively with increasing levels of depolarization. When 0.5 mM Cd\(^{2+}\) was added to the center pool solution, the
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Inward Ca current was blocked, flattening the baselines in the ON segments of the traces in Fig. 7 B. In other fibers in which inward Ca current had a larger magnitude, 0.5 mM Cd\(^{2+}\) was insufficient to block the current completely. In this fiber, 0.5 mM was sufficient and 1 mM did not bring about much more change. However, further increase of [Cd\(^{2+}\)] to 2 mM generally caused the baseline of the OFF segment to droop (not shown), making it very difficult to estimate the area of the OFF transients. Thus, 0.5–1 mM seems to be the optimal [Cd\(^{2+}\)] to block inward Ca current in our cut fibers.

Although Cd\(^{2+}\) is useful in suppressing the inward Ca current, it is not without effect on charge movement. In the absence of Cd\(^{2+}\), the ON segments of the first two traces in Fig. 7 A clearly show an \( I_v \) hump. At -45 and -40 mV, the peak of \( I_v \) actually rises above the peak of \( I_h \). At potentials > -40 mV, \( I_v \) peaks very early and merges with the \( I_h \) component. Fig. 7 B shows that 0.5 mM Cd\(^{2+}\) made the \( I_h \) humps in the first four traces much less prominent. At -30 mV, the peak of \( I_h \) is above the shoulder in the rising phase, which presumably is the peak of \( I_h \). This suggests that \( I_h \) had not been completely suppressed, but its kinetics was slowed by 0.5 mM Cd\(^{2+}\). This retarding effect was further magnified by 1 mM Cd\(^{2+}\) (traces in Fig. 7 C).

The amounts of ON or OFF charge with 0 (diamonds), 0.5 (squares), and 1 mM (triangles) Cd\(^{2+}\) are plotted as a function of TEST pulse potential in Fig. 8. Filled symbols represent OFF charge and open symbols represent ON charge. Undoubtedly, the difference between ON and OFF charges at -10 or 0 mV is reduced by Cd\(^{2+}\). (At -20 mV, ON and OFF charges without Cd\(^{2+}\) overlap each other, which is probably due to scatter of the data). Unlike the situation with creatine phosphate removal, the steep portion in the \( Q-V \) plot was still present after the addition of 0.5 or...
1 mM Cd$^{2+}$. Cd$^{2+}$ simply shifted the plot to the right without affecting the total amount of charge appreciably. The three sets of data were well fitted by a sum of two Boltzmann distribution functions, represented by the smooth curves A–C. The best-fit parameters are listed in Table V under fiber 87081. In the presence of Cd$^{2+}$, $V_r$ was shifted to a less negative value and the value of $q_{\text{max}}/C_m$ remained practically unchanged, confirming the presence of $Q_v$. 1 mM Cd$^{2+}$ did not appear to have much more effect than 0.5 mM Cd$^{2+}$ in this fiber.

To understand why $I_v$ did not appear as a hump in the presence of Cd$^{2+}$ while $Q_v$ apparently had not been abolished, each trace in Fig. 7 B was subtracted from the corresponding trace at the same potential in Fig. 7 A and the difference traces are shown in Fig. 9. At $-54$ mV, the ON transient of the difference trace is bell shaped. The amount of charge in the ON transient, listed in the legend, is equal to the amount in the OFF transient, and these quantities represent the amount of $Q_v$ that disappeared at $-54$ mV in the presence of 0.5 mM Cd$^{2+}$. At $-50$ mV, the decay of the ON transient undershoots somewhat before returning to the baseline. This biphasic nature is more apparent in the ON segments of the bottom three traces. The net amounts of ON charge in these three difference traces are close to zero, suggesting that 0.5 mM Cd$^{2+}$ did not suppress any $Q_v$ but slowed the kinetics of $I_v$ in this potential range. In contrast, the OFF segment in this potential range does not show much current transient, suggesting that the OFF current was not affected by the addition of Cd$^{2+}$. However, at higher levels of depolarization (not shown), the OFF segments of the difference traces show an inward transient, which is the ionic current blocked by 0.5 mM Cd$^{2+}$.

The difference in appearance between the first trace and the bottom three traces of Fig. 9 can be explained by the voltage shift of the $Q_v$–$V$ curve by 0.5 mM Cd$^{2+}$. In

### Table V

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Column 1 gives fiber identifications. Columns 2 and 3 give the [Cd$^{2+}$] in the center pool solution and the values of $c_m$, respectively. Columns 4–9 give the best-fit values of the parameters in the Boltzmann distribution functions of $Q_v$ and $Q_v$, with CONTROL charge correction and gap correction.
Fig. 8, $Q_v$ is close to saturation at $-54$ mV in curve A but slightly above threshold in curve B. The $I_v$ component in the first trace of Fig. 9 represents the current lost due to this voltage shift. At potentials $\geq -45$ mV, $Q_v$ has reached saturation in both $Q_v-V$ curves. Hence, the difference traces reflect a change in the kinetics of $I_v$ during depolarization. As $V_v$ was shifted a few millivolts in the positive direction by 0.5 mM Cd$^{2+}$, it would be better to obtain the difference traces with a few millivolts offset between the traces in Fig. 7, A and B. However, such an offset might not be appropriate for the subtraction of $I_k$. Even with the shift, the $I_v$ components in the traces of Fig. 7 B still do not match those in the traces of Fig. 7 A.

Two other experiments were performed with varying $[\text{Cd}^{2+}]$. In both fibers, the $I_v$ hump was progressively slowed by increasing $[\text{Cd}^{2+}]$. The $Q-V$ plots at different $[\text{Cd}^{2+}]$ were fitted by a sum of two Boltzmann distribution functions. The best-fit parameters are listed in Table V. The parameters in each fiber were affected somewhat differently by Cd$^{2+}$. The positive shift of $V_v$ observed in the experiment of Fig. 8 was shared by only one of the two other fibers. Nonetheless, a common feature shared by all three fibers is that a substantial amount of $Q_v$ was present in 1 mM Cd$^{2+}$, although $q_{\text{v, max}}/c_m$ was suppressed somewhat in two of the three fibers. Hence, the disappearance of the $I_v$ hump with Cd$^{2+}$ in the center pool solution was not primarily due to a suppression of $Q_v$, as in the case of removing creatine phosphate from the end pool solution, but rather to the prolongation of the time course of $I_v$. (An alternative explanation will be given in the Discussion.) Just 0.5–1 mM Cd$^{2+}$ is effective in

![Figure 9](image.png)
blocking inward Ca current, but this advantage is compromised by an alteration in the kinetics of \( I_v \).

When the experiments were performed, emphasis was put on obtaining the dose–response relationship of the effect of Cd\(^{2+}\) on charge movement and Ca current so as to gain information about the optimal [Cd\(^{2+}\)] to be used. No attempts were made to wash out the Cd\(^{2+}\) and bracket the control runs. Nonetheless, it is unlikely that the retardation of the kinetics of \( I_v \) in the presence of Cd\(^{2+}\) was due to a rundown of the fibers, because at each [Cd\(^{2+}\)] two complete sequences of runs were taken. The shape of \( I_v \) at each potential was the same in the two sequences. The time course of \( I_v \) was prolonged at each potential only when the [Cd\(^{2+}\)] was increased. Recently we studied the effect of Co\(^{2+}\), another Ca blocker, on charge movement. We focused on the reversibility of the effect of 20 mM Co\(^{2+}\) at the expense of the dose–response relationship. We found that this [Co\(^{2+}\)] also slowed the time course of \( I_v \) and the effect was partially reversible (Hui, C.S., and W. Chen, unpublished results).

Effect of Maintained Hyperpolarization

In the measurements of charge movement and other electrophysiological signals, linear capacitive and ionic currents are routinely removed by subtracting a properly scaled CONTROL current taken in some subthreshold voltage range from the TEST current. If charge movement obeys the two-state Boltzmann distribution, some charge movement should exist in the CONTROL current trace and is inevitably subtracted from the TEST current trace. This problem can be minimized by setting the holding potential at 0 mV and taking CONTROL current traces in the positive potential range (Brum and Rios, 1987). However, with such a protocol, not too many CONTROL runs can be taken and one has to rely on interpolation to obtain a CONTROL current trace for every TEST current trace. Besides, if the fiber runs down at the end of the experiment, no reliable bracketing CONTROL current trace can be recorded. Moreover, there is worry about the physiological state of the fiber after maintained depolarization at 0 mV for an extended period of time. Another approach to minimize CONTROL charge is to set the holding potential at a highly negative level and take the CONTROL pulse in that potential range, but this results in an undesirably large holding current. Even a holding potential around −120 mV has some drawbacks for cut fibers mounted in a double Vaseline gap chamber as it affects the shape of the foot in the Q-V curve. This is explained by the theoretical curves shown in Fig. 10.

Curve A of Fig. 10 was generated by Eq. 2 in the preceding paper (Hui, 1991), with gap correction but without CONTROL charge correction, using the mean values of the Boltzmann parameters listed in Table II of that paper. As explained in detail in Fig. 2 of Hui and Chandler (1990), the finite and slowly rising foot of the curve is contributed by charge movement underneath the Vaseline seals. If CONTROL pulses are elicited from −110 to −90 mV, as in most of our cut fiber experiments, the CONTROL charge between these potentials is scaled up and subtracted from the TEST charge. The net charge measured at any potential is represented by curve C, which is given by the difference between curve A and straight line 1, which intersects curve A at −110 and −90 mV. However, if CONTROL pulses are elicited from −140
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To \(-120\) mV, the net charge measured at any potential is then represented by curve B, which is given by the difference between curve A and straight line 2, which intersects curve A at \(-140\) and \(-120\) mV. Thus, even without changing the holding potential and with the magnitude of the CONTROL pulse remaining constant, switching the CONTROL pulse potential to a more negative range alone would decrease the amount of CONTROL charge and increase the amount of charge moved at the same TEST potential. Without gap correction (e.g., if the resistance of the Vaseline seals is infinite), the slopes of lines 1 and 2 should be very close to zero and curves A–C should almost superimpose on each other.

In principle, subtraction of straight line 3, which intersects curve B at \(-110\) and \(-90\) mV, from curve B should also give curve C. In practice, the values of \( Q \) in a \( Q-V \) plot are normalized by \( c_m \), which is estimated from the CONTROL current transient.

For CONTROL pulses from \(-110\) to \(-90\) mV (\(-140\) to \(-120\) mV), \( c_m \) is effectively measured at \(-100\) mV (\(-130\) mV). Since the voltage-dependent component of \( c_m \) at each potential is given by the first derivative of the \( Q-V \) plot, it is easy to visualize that the foot of the \( Q-V \) plot leads to a smaller value of \( c_m \) at \(-130\) mV than its value at \(-100\) mV (see Fig. 2 C of Hui and Chandler, 1990). Hence, subtraction of straight line 3 from curve B should yield a curve that is scaled up from curve C by a factor equal to \( c_m(-100)/c_m(-130) \).

The above theoretical predictions explain some, but not all, of the effects when the holding potential was changed from \(-90\) to \(-120\) mV, as illustrated in the experiment of Fig. 11. A shows TEST minus CONTROL current traces taken when the holding potential was set at \(-90\) mV and CONTROL pulses taken from \(-110\) to
−90 mV. They look typical, with an $I_0$ hump clearly visible in the ON segments of the traces between −50 and −30 mV. After the holding potential was changed to −120 mV, CONTROL pulses were taken from −140 to −120 mV and the traces in B were recorded. The amplitudes of the ON and OFF transients were increased (note the change in vertical gain). At −50 and −44 mV, the $I_0$ hump disappears. At −40 mV, the ON current decays with a fast and a slow exponential. The latter component could be $I_0$, but its shape is very different from that in the corresponding trace in A. At −35 and −30 mV, a small hump can be visualized in the decay phase of $I_0$. In addition, the positive pedestals in the ON segments of all the traces are greatly increased.

**Figure 11.** Effect of maintained hyperpolarization on TEST minus CONTROL currents in a cut fiber. Fiber identification: 83221. Diameter, 127 μm. Saponin treatment was applied to membrane segments in both end pools at time zero. After rinsing, the solutions in the end pools were replaced by Solution B. Then, the solution in the center pool was changed to a TEA.Cl solution (solution D). At the 24th minute, the voltage clamp was turned on and the holding potential was set at −90 mV. (A) Traces taken at −90 mV from the 68th to the 86th minute. At the 95th minute, the holding potential was changed to −120 mV. (B) Traces taken from the 100th to the 115th minute. At the 116th minute, the holding potential was restored to −90 mV. (C) Traces taken from the 120th to the 138th minute. The CONTROL pulses were always from 20 mV below the holding potential to the holding potential. Only representative traces are shown in each panel. The numbers at the right show the potentials during the TEST pulses (same pulse for the traces in each row). From the beginning to the end of the experiment, holding current changed from −22 to −32 nA and $r/(r_e + r)$ decreased from 0.993 to 0.991, except during maintained hyperpolarization, when they were −50 nA and 0.985, respectively.
After the restoration of the holding potential to −90 mV, the traces in Fig. 11 C were taken. \(I_r\) and the maintained ionic currents returned to their original magnitudes but \(I_r\) did not recover completely. At −50 and −44 mV, the \(I_r\) humps in the ON segments are still smaller than the corresponding ones in Fig. 11 A. It is unlikely that this irreversibility of \(I_r\) was due to a rundown of the fiber because control fibers without going through hyperpolarization generally showed little rundown in similar time periods.

The amounts of charge in the traces of Fig. 11 (and other traces not shown) are plotted against the TEST pulse potential in Fig. 12 A as diamonds (control −90 mV), squares (−120 mV), and triangles (bracketing −90 mV). Filled symbols represent OFF charge and open symbols represent ON charge. The three data sets were fitted by a sum of two Boltzmann distribution functions, represented by curves A–C. Curves A and C were corrected for CONTROL charge between −110 and −90 mV and curve B between −140 and −120 mV. As the holding potential was changed from −90 to −120 mV, the amount of charge in the foot and the maximum amount of charge were increased, as predicted by the theoretical curve B in Fig. 10, but both increases in Fig. 12 A are larger than the theoretical prediction. On returning to a holding potential of −90 mV, the changes were almost completely reversed.

The best-fit parameters of the three curves in Fig. 12 are listed in Table VI under fiber 83221. Comparison of the first and second rows of this entry shows that when the holding potential was changed from −90 to −120 mV, the value of \(q_{\beta, \text{max}}/c_m\) was increased and \(\bar{V}_{\beta}\) was shifted from −32 to −49 mV. This explains the drastic increase in the peak amplitude of \(I_r\) from the traces in Fig. 12 A to the corresponding traces in Fig. 12 B. The decrease in the value of \(c_m\) from 0.195 to 0.187 µF/cm can be explained by the shape of the foot of curve B in Fig. 12 A. A \(Q-V\) curve with gap correction but without CONTROL charge correction was generated with the parameters obtained for this fiber (listed in Table VI). The values of the voltage-dependent part of \(c_m\) at −90 and −120 mV were calculated from the tangents to the curve at these potentials and came out to be 0.0189 and 0.0075 µF/cm, respectively. Thus, the change in the slopes of the tangents is sufficient to account for the observed decrease in \(c_m\). Two other similar experiments were performed and the results are included in Table VI. In all three fibers, \(k_r\) and \(q_{\beta, \text{max}}/c_m\) were increased, \(\bar{V}_{\beta}\) was shifted in the negative direction, and \(c_m\) was decreased when the holding potential was changed from −90 to −120 mV. In the two fibers in which the holding potential was returned to −90 mV, the above effects were mostly reversed.

According to the theoretical prediction shown in Fig. 10, if hyperpolarization has no other effect on charge movement, then curve B in Fig. 12 A can be converted to curve A by subtracting the scaled amount of charge between −110 and −90 mV, represented by the dashed straight line, and by scaling the resulting curve by \(c_m(-100)/c_m(-130)\). This idea was tested as shown in Fig. 12 B. The diamonds and curve A in Fig. 12 B were replotted from Fig. 12 A, with the two filled diamonds at the largest depolarizations omitted. Filled squares in Fig. 12 B were calculated from the differences between the filled squares and the corresponding values on the dashed line in Fig. 12 A. Curve B in Fig. 12 B was obtained by fitting a sum of two Boltzmann distribution functions to the squares. The best-fit values for \(\bar{V}_{\beta}, k_p, q_{\beta, \text{max}}/c_m, \bar{V}_{\gamma}, k_{\gamma}\), and \(q_{\gamma, \text{max}}/c_m\) are −45.5 mV, 11.5 mV, 17.8 nC/µF, −59.3 mV, 4.6 mV,
and 12.3 nC/μF. The values for $Q_a$ are similar in curves A and B, but $q_{\beta,\text{max}}/C_m$ is still larger and $V_\beta$ is still 14 mV more negative in curve B than in curve A. As explained above with Fig. 10, part of the difference in $q_{\beta,\text{max}}/C_m$ can be explained by a difference in $C_m$. After multiplying $q_{\beta,\text{max}}/C_m$ by $C_m$ from column 3 of Table VI, $q_{\beta,\text{max}}$ became 1.93 nC/cm for curve A and 3.33 nC/cm for curve B, which are still different. Possible explanations for the remaining difference will be given in the Discussion.

The main finding in this section is that, during maintained hyperpolarization to
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$-120$ mV, $I_v$ was not manifested as a prominent hump due to an increase in the amplitude of $I_p$, reminiscent of the effect of replacing external $Cl^-$ with $SO_4^{2-}$. However, with $SO_4^{2-}$ the increase in the amplitude of $I_p$ was caused by a large increase in $q_{p,max}/C_m$ together with a positive shift in $\bar{V}_p$, whereas, for maintained hyperpolarization, it was caused by a smaller increase in $q_{p,max}/C_m$ together with a negative shift in $\bar{V}_p$.

Effect of Repetitive Stimulation

It has always been of concern how frequently a cell can be stimulated without noticeably affecting a physiological signal. In charge movement experiments, a higher stimulation rate is desirable because it allows more traces to be recorded within a certain time period. With the present apparatus and protocol for measuring charge movement in cut fibers, single sweep TEST traces can be recorded that are relatively free of noise, eliminating the necessity for signal averaging. Hence, TEST minus CONTROL current traces can be recorded at a much faster rate than in intact fibers. However, the experiment shown in Fig. 13 alerts some caution against fast stimulation rates.

All the traces in the figure were elicited by a TEST pulse to $-40$ mV. The ON segment of the first trace shows a distinct $I_v$ hump having a peak as high as that of $I_p$ and a time-to-peak of $\sim 17$ ms. The hump in the second trace, taken $1$ min later, becomes less prominent. Its time-to-peak is prolonged to $\sim 21$ ms and its peak amplitude is reduced. The third trace, taken another minute later, is similar to the second. After a 13-min rest, the fourth trace shows an $I_v$ hump almost identical to that in the first trace, with the same time-to-peak. The bottom trace shows that after a 5-min rest the shape of the hump is still altered, with a time-to-peak of $\sim 19$ ms.
Although the shapes of the $I_v$ humps in the second, third, and fifth traces are different from those in the first and fourth traces, the amounts of ON or OFF charge are not different (see legend of Fig. 13). The difference trace (not shown) obtained by subtracting the second trace from the first trace showed a biphasic ON transient and a flat OFF segment, similar to the bottom three traces in Fig. 9. These suggest that when the $I_v$ hump became less prominent as a result of incomplete recovery after stimulation, it was not suppressed but was prolonged in time course. This fiber represents an extreme case in which the shape of $I_v$ did not recover fully even after a 5-min rest, and is chosen for the figure to emphasize the point. In most of the cut fibers studied, the shape of $I_v$ recovered almost completely in 1 min. However, if the stimulation rate is faster than once per minute, the recovery might not be complete, depending on the condition of the fiber.

**DISCUSSION**

Several manipulations resulted in the disappearance of the $I_v$ hump in TEST minus CONTROL current traces, but the underlying causes for the disappearance were different. Replacement of extracellular Cl$^-$ by SO$_4^{2-}$ caused the disappearance by an increase in $I_h$ (Hui and Chandler, 1990) and lowering the temperature from 14 to 6°C...
caused the disappearance by prolonging the time course of \( I_a \) (Hui, 1991). This paper describes five additional manipulations that affected the appearance of \( I_v \). Replacement of extracellular \( \text{Cl}^- \) by \( \text{MeSO}_4^- \) caused a negative shift of the voltage dependence of \( I_v \) kinetics, but prominent \( I_v \) humps could still be observed in the TEA.\( \text{MeSO}_4^- \) solution. Removal of creatine phosphate from the end pool solution suppressed the \( I_v \) hump by reducing the amount of \( Q_v \). Maintained hyperpolarization resulted in an increase in \( I_p \), thereby obscuring the \( I_v \) hump, similar to the effect of \( \text{SO}_4^{2-} \). Addition of \( \text{Cd}^{2+} \) to the center pool solution and repetitive stimulation prolonged the time course of \( I_v \) such that \( I_v \) was not manifested as a prominent hump, similar to the effect of cooling. \( I_v \) was originally defined as the delayed hump component in the ON segment of a TEST minus CONTROL current trace (Adrian and Peres, 1979; Hui, 1991). This definition only holds when \( I_v \) is manifested as a hump but loses its meaning when \( I_v \) has a much broader time course. In the latter case, \( I_v \) should be identified with the current generated by the flow of the steeply voltage-dependent charge component.

Among the five manipulations studied in this paper, \( \text{MeSO}_4^- \) has the least effect on the appearance of the \( I_v \) hump. In fact, after allowing for the negative voltage shift, the kinetics of \( I_v \) might not be different in the TEA.\( \text{Cl}^- \) and TEA.\( \text{MeSO}_4^- \) solutions. Table III shows that the fraction of \( Q_v \) in the total charge was the same in both solutions. In contrast, the amount of \( Q_a \) was greatly increased when \( \text{Cl}^- \) was replaced by \( \text{SO}_4^{2-} \), reducing the fraction of \( Q_v \) in the total charge from one-half to one-fourth (Hui and Chandler, 1990). Hence, to visualize \( I_v \) as a hump, \( \text{MeSO}_4^- \) is a better substitute for \( \text{Cl}^- \) than \( \text{SO}_4^{2-} \). It is also worth noting that the negative (positive) shift in \( \bar{V}_v \), when \( \text{Cl}^- \) is replaced by \( \text{MeSO}_4^- \) (\( \text{SO}_4^{2-} \)), is parallel to the negative (positive) shift in contraction threshold (Kao and Stanfield, 1968; Foulks and Perry, 1977).

We have investigated other anion substitutes for \( \text{Cl}^- \) and they all affected charge movement to some extent. To our surprise, gluconate greatly suppressed \( Q_a \) (Chen and Hui, 1991b), opposite to the effect of \( \text{SO}_4^{2-} \). Thus, gluconate would be a better substitute for \( \text{Cl}^- \) if one wants to study \( Q_a \) exclusively.

It is possible that the appearance of \( I_v \) might depend on other factors, such as the species of frog (\textit{Rana temporaria} vs. \textit{Rana pipiens} or \textit{Rana catesbiana}), the source of supply (Northern vs. Southern \textit{Rana pipiens}), the season of the shipment, the mode of adaptation (warm vs. cold), and the mode of feeding. In this series of papers, \textit{Rana temporaria} were used and they were always cold-adapted for at least a week before experimentation.

Possible Origin of \( I_a \) and \( I_v \)

In the parallel model of charge movement (Hui and Chandler, 1990), \( Q_a \) and \( Q_v \) are considered as two separate species of charge moving independently. An alternative view is that both \( Q_a \) and \( Q_v \) belong to the same charge species. \( Q_a \) triggers Ca release, which in turn induces the movement of more charge (i.e. \( Q_v \) [Csernoch et al., 1989; Pizarro et al., 1990]). Both models can explain some of the results in this paper. Recently we experienced some cut fibers having a usual amount of \( Q_v \) but very little \( Q_a \) (Chen and Hui, 1991a). This finding is consistent with the parallel model in which \( Q_v \) can move irrespective of the presence of \( Q_a \). We are thus tempted to speculate that
Q<sub>t</sub> plays the role of triggering Ca release, although it remains to be demonstrated that the fibers with little Q<sub>t</sub> can release normal amount of Ca.

A molecular mechanism was proposed in the preceding paper (Hui, 1991) to explain the complicated kinetics of I<sub>v</sub>. Chen and Hui (1990) reported that nifedipine suppressed both Q<sub>β</sub> and Q<sub>t</sub>. The tetramers of dihydropyridine binding proteins might indeed provide the charge movement that is observed as I<sub>v</sub>, but the origin of I<sub>β</sub> still remains open. It is quite likely that I<sub>β</sub> consists of several components, including sodium channel gating current, potassium channel gating current, Ca channel gating current, and charge movement that has no known physiological function yet. It is also possible that the movement of charge in the monomeric form of the dihydropyridine binding protein contributes to I<sub>v</sub>. This latter component is speculative, but it accommodates any interconversion between Q<sub>β</sub> and Q<sub>t</sub>, as was suggested by the experiments studying the effect of creatine phosphate in the internal solution. It is premature to speculate that an interconversion between Q<sub>β</sub> and Q<sub>t</sub> occurred. Nonetheless, in another group of experiments there was an indication that perchlorate might increase the amount of Q<sub>t</sub> at the expense of Q<sub>β</sub> (Hui, C.S., and W. Chen, unpublished results).

Effects of the Interventions on I<sub>β</sub> and I<sub>v</sub>

Part of the increase in charge movement during maintained hyperpolarization in cut fibers is a consequence of the enhancement of the foot of the Q-V curve contributed by the charge in the membranes underneath the Vaseline seals (Hui and Chandler, 1990). This characteristic does not apply to intact fibers, but is inevitable in cut fibers unless the resistance of the seals could be made infinite. After correcting for the charge movement between −110 and −90 mV, Q<sub>β</sub,max/C<sub>m</sub> of curve B in Fig. 12 B is still larger than that of curve A. Part of the remaining difference is due to a decrease in ε<sub>n</sub> at −120 mV. Although a decrease in Q<sub>α</sub> at −120 mV (Brum and Rios, 1987) could explain this decrease in ε<sub>n</sub>, the change in slope at the foot of curve B in Fig. 12 A is sufficient to explain the decrease, as described in the Results. After correcting for the difference in ε<sub>n</sub>, the value of Q<sub>β</sub,max for curve B of Fig. 12 B is still larger than that for curve A. Again, this final difference could be explained by a decrease of Q<sub>α</sub> at −120 mV (Brum and Rios, 1987). Alternatively, if Q<sub>β</sub> indeed consists of several components as speculated above, it is possible that hyperpolarization reprimps one or more components that have a more negative voltage distribution relative to those of the other components. Although the cause of the increase in Q<sub>β</sub> is not understood, the experiments in this paper clearly show that maintained hyperpolarization to −120 mV obscures the appearance of the I<sub>v</sub> hump by an increase in I<sub>β</sub>. If the effect is graded, maintained hyperpolarization to −100 or −110 mV would still obscure the hump, although to a lesser extent.

Cd<sup>2+</sup> was found to slow the kinetics of I<sub>v</sub>. If I<sub>v</sub> is caused by Ca release, then the slowing of the kinetics of I<sub>v</sub> could be related to the slowing of the kinetics of Ca release by Cd<sup>2+</sup>. On the other hand, if Ca release is triggered by Q<sub>t</sub>, it is equally likely that the slowing of the kinetics of Ca release is due to the slowing of the triggering mechanism. To pin down which alternative is correct, it is important to find out whether Q<sub>t</sub> is the cause or the consequence of Ca release. For the present moment, caution should be taken in using Cd<sup>2+</sup> to block Ca current if the kinetics of charge...
movement or Ca release is under study. Co\textsuperscript{2+} has similar effect on the kinetics of \( I_v \) (Hui, C.S., and W. Chen, unpublished results). It may be possible to find a Ca channel blocker that does not have this effect. More experiments are required to achieve this goal.

Repetitive stimulation also slowed the kinetics of \( I_v \). In the extreme case shown in Fig. 13, the \( I_v \) hump did not recover fully even after a 5-min rest. In general, the recovery time depends on the condition of the fiber and the experimental conditions. In our hands, one stimulation per minute is considered as optimal. Results of experiments by Schneider et al. (1987) suggested that the SR could be depleted of Ca\textsuperscript{2+} after a conditioning TEST pulse when [EGTA] in the internal solution was only 0.1 mM. Garcia et al. (1989) also observed a substantial depletion of Ca\textsuperscript{2+} in the SR by repetitive stimulation at 0.03 Hz when [EGTA] in the internal solution was 1 mM. Since the experiments in this paper were performed with 20 mM EGTA in the internal solution, the SR should be depleted of Ca\textsuperscript{2+} more readily than with lower [EGTA]. However, in a healthy cut fiber prominent \( I_v \) humps could be observed for as long as 6 h. Thus, superficially, one might conclude that \( I_v \) could not arise as a consequence of Ca release. On the other hand, \( I_v \) could still be the trigger for Ca release because the trigger can be unaffected even when the SR is depleted of Ca\textsuperscript{2+}.

This conclusion can be challenged by arguing that the apparent depletion of Ca\textsuperscript{2+} in the SR was based on the Ca-dye signal recorded globally from the myoplasm of many sarcomeres. If the [Ca\textsuperscript{2+}] in the restricted region between the tubular membrane and the junctional SR membrane is high after Ca release, \( I_v \) humps can still be observed. To chelate the Ca\textsuperscript{2+} in the putative restricted region, Garcia et al. (1990) raised the [EGTA] in the internal solution to 62.5 mM and observed a parallel suppression of the \( I_v \) hump and the intrinsic optical signal that is thought to reflect Ca release. The result does not necessarily prove that the \( I_v \) hump is the consequence of Ca release, because in the presence of such high [EGTA] the trigger for Ca release might not be in a normal state after the first activation. A perturbation on the recovery of the trigger could lead to a parallel perturbation on the recovery of the release. In addition, we have studied the effect of repetitive stimulations on the \( I_v \) hump in the presence of 50 mM EGTA in the end pool solution. The fibers were stimulated by a train of up to 10 identical TEST pulses at a rate of once per 6 s. In the usual potential range, very pronounced \( I_v \) humps could still be observed during the last TEST pulse (Hui, C.S., and W. Chen, unpublished results), different from the finding of Garcia et al. (1990). Although pronounced \( I_v \) humps were present in the later runs of the train, the shape of the hump in the second run was somewhat different from that in the first run, suggesting that the \( I_v \) hump might not have completely recovered after 6 s. It is still preferable to perform experiments at a stimulation rate of once per minute.

Hence, the exceptionally slow recovery of the shape of the \( I_v \) hump in Fig. 13 was probably related to the condition of the fiber. Although this extreme case is very rare, it alerts us that the \( I_v \) hump might take a longer time to recover in some fibers than others.

The slow recovery of \( I_v \) kinetics in cut fibers after stimulation has not been observed in intact fibers. To acquire charge movement traces signal-averaged 8 or 16 times, intact fibers were generally stimulated at rates faster than once per minute; otherwise,
there might not be enough time to complete the experiment. At such a fast rate, there was no noticeable change in the shape of $I_v$ between successive traces (unpublished observations). This adds another item to the list of differences between the two preparations.

The author would like to thank Drs. Steve Baylor, Knox Chandler, Judith Heiny, and Eduardo Rios for reading the manuscript, Dr. Fred Sigworth and the staff of Yale Electronics Lab for designing and fabricating the voltage clamp module and interface card for laserjet printer, and Dr. Siu Hui for expert advice on statistics.

This project was supported by grants from the National Institutes of Health (NS-21955), the Muscular Dystrophy Association, and the American Heart Association. The author was a recipient of a Research Career Development Award (NS-00976) from the NIH.

Original version received 30 March 1990 and accepted version received 15 January 1991.

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