ABSTRACT In an attempt to determine the mechanism by which rat skeletal muscle endplates generate a steady outward current, we measured the effects of several drugs (furosemide, bumetanide, 9-anthracene carboxylic acid [9-AC]) and changes in external ion concentration (Na⁺, K⁺, Cl⁻, Ba++) on resting membrane potential (Vₘ) and on the steady outward current. Each of the following treatments caused a 10–15-mV hyperpolarization of the membrane: replacement of extracellular Cl⁻ with isethionate, addition of furosemide or bumetanide, and addition of 9-AC. These results suggest that Cl⁻ is actively accumulated by the muscle fibers and that the equilibrium potential of Cl⁻ is more positive than the membrane potential. Removal of external Na⁺ also caused a large hyperpolarization and is consistent with evidence in other tissues that active Cl⁻ accumulation requires external Na⁺. The same treatments greatly reduced or abolished the steady outward current, with a time course that paralleled the changes in Vₘ. These results cannot be explained by a model in which the steady outward current is assumed to arise as a result of a nonuniform distribution of Na⁺ conductance, but they are consistent with models in which the steady current is produced by a nonuniform distribution of Gₐ or Gₖ. Other treatments (Na⁺-free and K⁺-free solutions, and 50 μM BaCl₂) caused a temporary reversal of the steady current. Parallel measurements of Vₘ suggested that in none of these cases did the electrochemical driving force for K⁺ change sign, which makes it unlikely that the steady current arises as a result of a nonuniform distribution of Gₖ. All of the results, however, are consistent with a model in which the steady outward current arises as a result of a nonuniform distribution of Cl⁻ conductance, with Gₜ lower near the endplate than in extrajunctional regions.

INTRODUCTION

In the previous paper (Caldwell and Betz, 1984), we described a steady electric current generated by rat lumbrical muscle fibers. Current leaves the muscle fibers at the endplate region and re-enters in the flanking regions. We presented evidence that the steady current does not depend on activation of acetylcholine channels, voltage-gated Na⁺ channels, or increased electrogenic Na⁺ pumping in the endplate region.
In the present paper we describe further experiments designed to study the physiological mechanism by which the current is generated. We have focused attention on several "conductance models," in each of which the steady current is produced as a result of a nonuniform spatial distribution of membrane conductance to a particular ion ($G_{Na}$, $G_K$, or $G_{Cl}$), with all other pathways for transmembrane charge movement assumed to be uniformly distributed along the length of the muscle. Thus, for instance, a relative increase in $G_K$ at the endplate region (the $G_K$ model) could generate a steady outward current at that site. The $G_{Na}$ model and the $G_{Cl}$ model require a reduction of their respective conductances at the endplate region in order to generate a steady outward current at that site. Each model of course requires that the ion in question not be distributed at equilibrium. This condition is clearly met for $Na^+$ and $K^+$, and evidence will be given that $Cl^-$ is accumulated in muscle fibers via a cation-coupled cotransport process.

To test these conductance models, we measured the effects of several drugs (furosemide, bumetanide, and 9-anthracene carboxylic acid [9-AC]) and of changes in external ion concentrations on both membrane potential ($V_m$) and the steady outward current. The results suggest that the steady current is produced as a result of a nonuniform membrane conductance to chloride ions. Specifically, it appears that $Cl^-$ is actively transported into the muscle fibers and can leak out more easily in the extrajunctional regions than at the endplate. This nonuniform leak apparently generates the steady current. A preliminary account of these results has been given (Betz et al., 1983).

**METHODS**

The fourth deep lumbrical muscle of the rat hindfoot was used in all experiments. When the steady outward current was studied with the vibrating probe, the methods were similar to those described previously (Jaffe and Nuccitelli, 1974; Betz and Caldwell, 1984; Caldwell and Betz, 1984). Briefly, the vibrating probe was oriented perpendicular to the axis of the muscle, and signals, processed by a lock-in amplifier, were recorded on chart paper. Records were digitized and analyzed with a Hewlett-Packard (Palo Alto, CA) digitizer and computer. Voltages were converted to current density knowing the probe vibration distance and the resistivity of the medium (measured in a conductivity meter).

For studies using intracellular microelectrodes, conventional micropipettes filled with 3 M K acetate were used to record membrane potentials. For pulsing solutions of different compositions on localized regions of a muscle fiber, a large (10–50 μm)-tipped micropipette was filled with the test solution. Pressure (<2 psi) was applied from a gas cylinder, with pulses gated by a solenoid valve. Pulses typically lasted 0.5–1.0 s. The pipette tip was positioned ~50 μm above the fiber and 100–200 μm away from the intracellular pipette. Membrane potential was recorded on chart paper and later digitized and analyzed.

Normal Krebs consisted of (mM): 136 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 11 glucose, and HEPES or PIPES buffer. In most experiments in which membrane potentials were recorded, the CaCl$_2$ concentration was elevated to 4 or 8 mM in order to achieve more stable membrane potentials. In experiments with low [$Cl^-$], a second microelectrode (3 M K acetate) positioned near the muscle or a salt bridge was used as reference electrode, and NaCl was replaced with sodium isethionate. No correction was made for electrode junction potentials, which ranged up to ~10 mV in low-$[Cl^-]$ solutions. The drug 9-AC was dissolved in ethanol, and all solutions, including controls, in these experiments
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contained 4% ethanol. For intracellular recordings, all solutions contained tetrodotoxin (0.3 μM). For sodium replacement, NaCl was replaced with equimolar concentrations of LiCl or choline chloride. When sodium was replaced with choline, muscles were pretreated with α-bungarotoxin (4 μg/ml) in order to block activation of acetylcholine receptors. For solutions with elevated potassium, an equimolar amount of NaCl was omitted. Thus, all solutions were isosmolar. In all experiments with the vibrating probe, muscles were pretreated with α-bungarotoxin. For experiments involving changes in pH, the Krebs solution contained citrate (4 mM), PIPES (4 mM), and glycine (4 mM), thereby ensuring adequate buffering from pH 4 to pH 10. The pH in the bath was checked in a few cases and did not change during the experiment. All experiments were performed at room temperature (~25°C).

**RESULTS**

**Effects of Cl⁻ Replacement and Drugs Affecting Transmembrane Cl⁻ Movement**

Fig. 1A shows the effect of replacing external Cl⁻ on membrane potential (Vₘ). At zero time, most of the Cl⁻ in the external bathing solution was replaced with isethionate. The initial transient depolarization is due to the large positive shift of E_Cl (Hodgkin and Horowicz, 1959). Then the membrane repolarized to a level ~10 mV more negative than the initial resting potential. The 10-mV hyperpolarization may reflect the presence of inwardly directed active chloride transport, which normally maintains E_Cl at a level more positive than Vₘ (Dulhunty, 1978). Similar results were obtained in experiments in which Vₘ was sampled in a number of different fibers before, during, and after exposure to low [Cl⁻].

The effect of Cl⁻ replacement on the steady outward current is shown in Fig. 1B. The current transiently increased and then decreased to a very low level. To obtain further evidence about the possible existence of active chloride transport, we investigated the effects of several drugs that have been shown to interfere with Cl⁻ fluxes. Furosemide (10 μM) and bumetanide (1 μM), which block active Cl⁻ transport in other tissues (Frizzell et al., 1979; Russell, 1979), caused a 10–15-mV hyperpolarization of the membrane, which developed over a period of 4–8 min (cf. Fig. 5). The same drugs also reversibly abolished the steady outward currents (Fig. 2).

Palade and Barchi (1977b) showed that 9-anthracene carboxylic acid (9-AC) selectively blocked G_Cl in rat diaphragm. We tested its effects on Vₘ and on the steady current; results are shown in Fig. 3. Normal Krebs containing 0.1 mM 9-AC caused a rapid 10–15-mV hyperpolarization of the membrane (Fig. 3A). The same treatment also inhibited the steady outward current (Fig. 3B).

As with Cl⁻ replacement, the hyperpolarizing effects of furosemide, bumetanide, and 9-AC suggest that Cl⁻ is not distributed at equilibrium, but rather is accumulated by the muscle fibers. The effect of such a transport mechanism would be to shift E_Cl and Vₘ in a positive direction. Then, with inhibition of active Cl⁻ transport or block of G_Cl, the membrane would hyperpolarize as chloride redistributed. In addition, if the Cl⁻ transport mechanism were electrogenic (depolarizing), its inhibition could also contribute to the observed hyperpolarization.
The same treatments also greatly reduced or abolished the steady outward current. Moreover, the time course of the change in steady current was similar to the time course of change in $V_m$ for each treatment. The effects on the steady current are predicted by both the $G_C$ model and the $G_K$ model, but not by the

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**Figure 1.** Effects of low-Cl$^-$ Krebs solution. (A) Effect on $V_m$. At zero time, low-Cl$^-$ Krebs was added. Cl$^-$ was replaced by isethionate; in the low-Cl$^-$ Krebs, $[Cl^-] = 2$ or 4 mM (cell giving smallest depolarization). At the times marked by the arrowheads, normal Krebs was reintroduced. (B) Effect on the steady endogenous current. Low-Cl$^-$ Krebs was introduced at zero time. At the time marked by the arrow, normal Krebs was reintroduced.
$G_{Na}$ model (see Discussion). Experiments that distinguish between the $G_{Cl}$ and $G_{K}$ models are described later.

**Effects of Furosemide and 9-AC on Cl Conductance**

In order to gain further information about the actions of furosemide and 9-AC, their effects on $G_{Cl}$ were measured. The protocol was as follows (see also Methods). First, the preparation was bathed in a low-Cl$^-$ solution ($[Cl^-]_o = 11$ mM). Then brief pulses of normal Krebs were applied (by pressure ejection from a micropipette) to a local area of membrane, and the resulting hyperpolarization (reflecting the entry of Cl$^-$) was recorded with an intracellular microelectrode. The drug was then added to the bathing solution, without moving either micropipette, and the pulsing procedure was repeated. Typical recordings are shown in Fig. 4A (inset).

Furosemide (Fig. 4A) had relatively little effect on the hyperpolarizing responses, whereas 9-AC (Fig. 4B) rapidly abolished the responses. In each case, exposure to the drug began at zero time. Usually the response in 9-AC reversed sign and became depolarizing (Fig. 4B). A possible explanation for the reversed (depolarizing) responses is as follows: if Cl$^-$ accumulation continued (by active transport) in the low-Cl$^-$ solution, then with $G_{Cl}$ blocked by 9-AC, $[Cl^-]$ would increase; consequently, when a pulse of normal Krebs ($[Cl^-] = 159$ mM) was applied, $E_{Cl}$ would be driven to a less negative level than in the absence of 9-AC. For instance, when no response was obtained to the pulse in 9-AC, $E_{Cl}$ must have been equal to $V_m$, which was about $-75$ mV.
Then from the Nernst equation, \([\text{Cl}]_i = 8.1 \text{ mM}\). This means that in the low-\(\text{Cl}^-\) solution (\([\text{Cl}] = 11 \text{ mM}\)) between pulses, \(E_{\text{Cl}} = -12 \text{ mV}\). From this, the \(P_{\text{Cl}}/P_K\) ratio can be calculated from the Goldman (1943) equation: assuming that \([\text{Na}]_i\),

\[
E_{\text{Cl}} = E_{\text{Na}} + \frac{R_T}{F} \ln \left(\frac{[\text{Na}]}{[\text{Cl}]} \frac{P_K}{P_{\text{Cl}}}ight)
\]

where \(R_T\) is the gas constant and \(F\) is the Faraday constant. If \([\text{Na}] = 10 \text{ mM}, [K] = 157 \text{ mM},\) and \(P_{\text{Na}}/P_K = 0.015\) (cf. Robbins, 1977), then \(P_{\text{Cl}}/P_K = 0.1\) in the presence of 9-AC. This represents a 60-fold reduction in \(G_{\text{Cl}}\) in the presence of 9-AC (cf. Palade and Barchi, 1977a, b).

**Figure 3.** Effects of 9-AC (0.1 mM). (A) Effect on \(V_m\). (B) Effect on the steady current. One muscle (circles) was returned to normal Krebs (arrow).
Figure 4. Effects of furosemide (A) and 9-AC (B) on $G_{Cl}$. Muscles were first equilibrated with low-Cl $^-$ Krebs ($[Cl^-] = 11$ mM). Then normal Krebs ($[Cl^-] = 159$ mM) was applied to a small patch of membrane by pressure ejection from a pipette. This caused a hyperpolarization (examples shown in inset in A; calibrations = 2 mV and 10 s) as $Cl^-$ entered the cell. The size of the hyperpolarization provided a relative measure of $G_{Cl}$; this is plotted on the ordinates. (A) Effect of 10 $\mu$M furosemide applied at zero time. (B) Effect of 0.1 mM 9-AC applied at zero time. In this case, the hyperpolarizing responses reversed and became depolarizing (see text).
This interpretation was tested in a few experiments in which the experiment was repeated with active chloride transport blocked (all solutions containing 10 \( \mu M \) furosemide). The depolarizing responses were reduced, and in some cases abolished, by furosemide.

**Effects of Na\(^+\) Replacement**

Normal \( V_m \) in rat lumbrical muscle is about \(-70 \) mV. Choline Krebs (normal Krebs with all Na\(^+\) replaced by choline) caused a large (15–20 mV) hyperpolarization, which developed monotonically over \(~5\) min. This effect is larger than predicted by the Goldman equation, assuming that internal ion concentrations do not change. Using values for \( P_{Na}/P_K \), [Na], and \( E_K \) obtained in rat muscle by Robbins (1977), a hyperpolarization of 5–10 mV is predicted.

This discrepancy could reflect an additional effect of Na\(^+\) replacement on \( V_m \). In this regard, it has been shown in several other tissues that Na\(^+\) replacement interferes with active Cl\(^-\) uptake (Frizzell et al., 1979; Russell, 1979). Thus, the large hyperpolarization we observed in choline Krebs may reflect the inhibition of active Cl\(^-\) transport and a consequent negative shift in \( E_{Cl} \) as Cl\(^-\) diffuses out of the cell. An electrogenic component (depolarizing) of the Cl\(^-\) transport process could also contribute to the change in membrane potential.

To test the idea that much of the large hyperpolarization in Na-free Krebs was due to interference with active Cl\(^-\) transport, we measured the effect of choline Krebs on \( V_m \) in muscles pretreated with furosemide to block active chloride transport. The results are shown in Fig. 5A. At the time marked by the arrowheads, 10 \( \mu M \) furosemide was added, causing a large hyperpolarization. Then, at zero time, choline Krebs (containing furosemide) was added; this caused an additional hyperpolarization of several millivolts, which is in reasonable agreement with that predicted by the Goldman equation. Normal Krebs (containing furosemide) was restored at the times marked by the arrows. Thus, treatment with furosemide prevented most of the choline Krebs effect on \( V_m \). This could reflect the presence of an inwardly directed Na\(^+\)/Cl\(^-\) cotransport system in rat muscle.

Fig. 5B shows the effect of Na\(^+\) replacement on the steady current. The current transiently increased and then decreased to zero. When normal Krebs was restored, a transient inward current preceded the return of the normal outward current. These effects were practically indistinguishable from those produced by Cl\(^-\) replacement (cf. Fig. 1B). However, the transient responses in Fig. 5B are particularly important, since they are predicted by the \( G_{Cl} \) model, but not by the \( G_K \) model of the steady current (see Discussion).

Replacement of Na\(^+\) with Li\(^+\) produced effects similar to, though less marked than, replacement with choline (e.g., a 5–10-mV hyperpolarization of \( V_m \) and a 50% reduction in the steady current in Li\(^+\) Krebs). This might reflect the ability of Li\(^+\) to substitute for Na\(^+\) better than choline can in driving active chloride accumulation, as is the case in squid giant axon (Russell, 1979).

**Effects of Changes in External K\(^+\) Concentration**

Increases in [K\(^+\)]\(_o\) (Na\(^+\) replaced by K\(^+\)) depolarized the membrane by amounts that were accurately predicted by the Goldman equation. A reduction in [K\(^+\)]\(_o\)
also depolarized the membrane; K⁺-free Krebs, for instance, caused a 5–7-mV depolarization. The changes occurred monotonically and were reversible. The effects of changes in [K⁺]₀ on the steady current are shown in Fig. 6. K⁺-free

**Figure 5.** Effects of Na⁺ replacement. (A) Effect on Vₘ. At times marked by the arrowheads, normal Krebs containing 10 μM furosemide was applied. At zero time, choline Krebs (also containing furosemide) was applied. At times marked by the arrows, the muscles were returned to normal Krebs (plus 10 μM furosemide). (B) Effect of Na⁺ replacement on the steady current. At zero time, choline Krebs was applied; the muscles were returned to normal Krebs at the time marked by the arrow. Note the transient responses (see text).
Figure 6. Effects of changes in external [K⁺] on the steady current. (A) K⁺-free Krebs (added at zero time) caused the steady outward current to reverse sign; the inward current then declined to zero. When normal Krebs was reintroduced (arrow), the outward current was promptly restored. (B) Effects of high-[K⁺] solutions on the steady current. At zero time, test solutions were added (squares = 20 mM K⁺; circles = 40 mM K⁺; triangles = 80 mM K⁺; in each solution Na⁺ was reduced by a corresponding amount). The current became large and inward, and then spontaneously declined to zero. In one experiment (squares), the muscle was returned to normal Krebs (arrow). (C) Effects of various [K⁺], on the steady state amplitude of
the endogenous current. The vertical lines are drawn to indicate the large transient inward currents that occurred in high-K⁺ solutions (Fig. 6B). Note that steady currents exist only at or near the physiological range of [K⁺]ₑ. (D) Effect of high [K⁺] in the absence of external Cl⁻. At zero time, Cl⁻-free Krebs was added; the steady current transiently increased and then decreased to a low level. Then, at the times marked by the arrows, the muscle was bathed in a high-K⁺/Cl⁻-free solution (K⁺ concentration marked on graph). No large inward current developed in the absence of Cl⁻ (compare with B).
Krebs rapidly abolished the steady current (Fig. 6A), and usually the current became inward and then decayed to zero. The transient inward current provides another distinction between the $G_C$ and $G_K$ models of the steady current.

High-[K+] solutions (Fig. 6B) produced large inward currents that spontaneously decayed to zero. Thus, as summarized in Fig. 6C, a steady current exists only at or near the physiological range of [K+]o (transients are denoted in Fig. 6C by vertical lines). The inward current with high [K+] depended on the presence of Cl-. As shown in Fig. 6D, when the effect of high [K+]o was tested in the absence of external Cl-, no inward current developed (contrast with Fig. 6B).

**Effects of Barium Ions**

Ba++ has been reported to block resting $G_K$ (Sperelakis et al., 1967), and so it was of interest to investigate its effect on $V_m$ and on the steady outward current. Normal Krebs containing 50 μM BaCl2 produced a 10–20-mV depolarization of the membrane, which developed monotonically (not shown).

The effects of Ba++ on the steady outward current were more complex. Results from one experiment are shown in Fig. 7. The outward current was rapidly abolished and became large and inward. Then, even in the continued presence of Ba++, the inward current decayed and reversed, and the final current was again outward. When normal Krebs was reintroduced, the outward current returned to its former level. The inward current in Ba++ offers a third way of
distinguishing between the $G_{\text{Cl}}$ and $G_{\text{K}}$ models of the steady current (see Discussion).

**Effects of pH Changes**

Since pH has been shown to affect $G_{\text{Cl}}$ in skeletal muscle (Hutter and Warner, 1967; Palade and Barchi, 1977a), we investigated its effect on $V_m$ and on the steady current in the rat lumbrical muscle. At pH 5 and 10, there was little change in $V_m$. At pH 4, large depolarizations occurred, which required 20–40 min to develop and were only partly reversible. Longer exposure at pH 4 sometimes led to the development of contraction clots in the muscle. The steady outward current varied approximately linearly with changes in pH, being reduced to ~40% at pH 4 and increased to 140% at pH 10. These changes occurred slowly, requiring ~20–30 min to develop fully, and they reversed with a similar time course. The slow time course is consistent with that found by Palade and Barchi (1977a) for the inhibition of $G_{\text{Cl}}$.

**DISCUSSION**

**Evidence for Active Cl$^-$ Transport**

Direct measurement of internal Cl$^-$ activity with Cl$^-$-selective microelectrodes in frog skeletal muscle has shown that $E_{\text{Cl}}$ is a few millivolts more positive than the resting potential (Bolton and Vaughan-Jones, 1977). In mammalian skeletal muscle, evidence for active Cl$^-$ accumulation is less direct. In our experiments, all of the following treatments hyperpolarized the membrane by 10–20 mV: replacement of external Cl$^-$ (Fig. 1; cf. Dulhunty 1978); treatment with 9-AC (Fig. 3), which blocks $G_{\text{Cl}}$ in rat muscle (Palade and Barchi, 1977b); and treatment with furosemide (Fig. 5) and bumetanide, which block active Cl$^-$ transport in several cell types (Frizzell et al., 1979; Russell, 1979). The results are consistent with the presence of active Cl$^-$ accumulation in rat lumbrical muscle. In other tissues, active Cl$^-$ transport requires external Na$^+$, and consistent with this, we found that removal of Na$^+$ (replaced with choline) also caused a large hyperpolarization of the membrane. The hyperpolarization most likely reflected a negative shift of $E_{\text{Cl}}$ as Cl$^-$ diffused out of the fiber when active Cl$^-$ accumulation was blocked by Na$^+$ removal. An additional contribution could occur if the Cl$^-$ transporter were electrogenic (depolarizing), as has been suggested by Musch et al. (1982). Russell (1983), on the other hand, studying ion fluxes in dialyzed squid axons, measured a stoichiometry of cation-coupled Cl$^-$ flux that suggests an electrically neutral process. The time course of the hyperpolarization observed in the present experiments did not show an initial rapid component, as might be expected if the Cl$^-$ transport mechanism were electrogenic, which suggests that most and possibly all of the hyperpolarization was due to Cl$^-$ redistribution.

Recently, it has been found that active Cl$^-$ transport in intestinal epithelium (Musch et al., 1982), in erythrocytes (Ellory et al., 1982), and in squid giant axon (Russell, 1983) also requires external K$^+$. Russell (1983) has shown that in squid giant axon all three ions are transported into the cell with a stoichiometry of $2\text{Na}^+:1\text{K}^+:3\text{Cl}^-$. If a similar mechanism operates in rat muscle, it might be
predicted that removal of external $K^+$ should hyperpolarize the membrane as it blocked active $Cl^-$ transport. In fact, $K^+$-free Krebs caused a 5–10-mV depolarization. This is similar to that observed in mouse soleus muscle (Aickin and Thomas, 1977), although Akaike (1982) observed a hyperpolarization of rat soleus muscle in $K^+$-free solution. The reasons for these contrasting results are not clear, but $K^+$ removal has multiple effects, with hyperpolarization predicted by the Goldman equation (assuming no changes in intracellular ion concentrations) and by the inhibition of active $Cl^-$ transport, and depolarization predicted by the inhibition of electrogenic $Na^+$ pumping (Thomas, 1969) and the blocking effect on the inward rectifier (Katz, 1949; Hodgkin and Horowicz, 1959; Almers, 1972; Gadsby and Cranefield, 1977). For present purposes, however, the important point is simply that in the present experiments rat lumbrical muscle depolarized in $K^+$-free Krebs.

**Mechanism of the Steady Outward Current**

**NONUNIFORM DISTRIBUTION OF ACTIVE $Cl^-$ TRANSPORT** If active $Cl^-$ transport occurred to a greater extent in extrajunctional regions than at the endplate, [$Cl^-$] would be highest in extrajunctional regions, and $E_{Cl}$ (and $V_m$) could be shifted to more positive levels at the extrajunctional locations. This nonuniform $Cl^-$ accumulation in the fiber could produce a steady electric current like the one we measured with the vibrating probe. If the $Cl^-$ transport mechanism were electrogenic (depolarizing), this could additionally contribute to the steady current. In its simplest form (i.e., assuming that all other transport pathways are uniformly distributed), this model cannot predict a reversal of the steady current since $E_{Cl}$ (and hence $V_m$) in extrajunctional regions could not become more negative than at the endplate for any of our experimental manipulations. In fact, the steady current did reverse and became inward under several different experimental circumstances (e.g., $K^+$-free solutions and in Krebs containing 50 $\mu$M BaCl$_2$). Thus, a nonuniform spatial distribution of an active $Cl^-$-transporting pathway is not a likely mechanism for generation of the steady outward current.

**NONUNIFORM DISTRIBUTION OF PASSIVE ION CONDUCTANCE PATHWAYS** Each experimental result was also analyzed in terms of three conductance models described earlier (see Introduction). If $V_m$ moved closer to the equilibrium potential of the ion in question, then the steady current should decrease (assuming no change in equilibrium potential or membrane conductance to the ion) because of the reduced driving force on the ion. Of particular importance were experiments in which the steady current reversed sign and became inward, which would require that $V_m$ has crossed the equilibrium potential of the relevant ion.

We found many examples in which the observed results were not predicted by the $G_{Na}$ model (in which $G_{Na}$ is assumed to be reduced in the endplate region). For instance, $Cl^-$ removal (Fig. 1), 9-AC addition (Fig. 3), and bumetanide and furosemide addition (Figs. 2 and 5) caused $V_m$ to move away from $E_{Na}$. This should have increased the steady current, if it were due to nonuniform spatial distribution of $G_{Na}$. Instead, the steady current was greatly reduced or abolished. Similarly, the effects of $K^+$ removal (Fig. 6A) and Ba$^{++}$ (Fig. 7) are not consistent with this model.
Distinguishing between the \( G_K \) model (increased \( G_K \) at the endplate) and the \( G_{Cl} \) model (reduced \( G_{Cl} \) at the endplate) was not as simple because a change in the driving force for chloride was often accompanied by a similar change in the driving force for potassium. There were, however, three observations that are not predicted by the \( G_K \) model but are consistent with the \( G_{Cl} \) model. These are illustrated in Fig. 8 and described below. In each case, the important observation is that the steady current became inward without \( V_m \) becoming more negative than \( E_K \).

**Figure 8.** Diagrams illustrating three experimental results (and their interpretations), which distinguish between the \( G_{Cl} \) and \( G_K \) models of the steady current. Solid lines show observed changes in \( V_m \) and the steady current; dashed lines show probable but not directly measured values of \( E_{Cl} \) and \( E_K \). Hatched areas show times when the steady current was inward and are interpreted as reflecting conditions when \( V_m \) was more positive than \( E_{Cl} \). (A) Effect of choline Krebs and return (arrow) to normal Krebs. (B) Effect of K+-free Krebs. (C) Effect of 50 \( \mu \)M \( \text{BaCl}_2 \) in normal Krebs.

**External Na\(^+\) Replacement**

Choline Krebs caused a large hyperpolarization of the membrane (see Fig. 8A). This was interpreted as reflecting the inhibition of active Cl\(^-\) transport, with a subsequent drift of \( E_{Cl} \) to \( V_m \). The steady current was greatly reduced (Fig. 5B). This reduction by itself does not distinguish between the \( G_K \) and \( G_{Cl} \) models since the driving force on both ions was reduced. However, when normal Krebs was reintroduced, the current became transiently inward, while \( V_m \) monotonically depolarized. Assuming that the choline Krebs did not affect the value of \( E_K \), the transient inward current is not consistent with the \( G_K \) model, since \( V_m \) never
became more negative than $E_K$. On the other hand, the $G_{CI}$ model does predict the transient inward current. When $Na^+$ was reintroduced, the membrane depolarized, making $V_m$ more positive than $E_{CI}$, and thereby giving rise to an inward current. Then, with the active $Cl^-$ transport reactivated by the external $Na^+$, the fibers accumulated $Cl^-$, $E_{CI}$ moved in a positive direction, and the normal outward current was eventually restored. A similar argument can be made for the transient increase in the steady current when choline Krebs was first applied.

**Effects of $K^+$ Removal**

$K^+$-free Krebs depolarized the membrane by 5–10 mV and caused the steady current to reverse sign (see Fig. 8B). The inward current then decayed to zero (Fig. 6A). Again, the inward current is not predicted by the $G_K$ model, since $V_m$ could not possibly have crossed $E_K$ (which was shifted to a very negative level). The result is, however, consistent with the $G_{CI}$ model, if $V_m$ became more positive than $E_{CI}$. If correct, this means that $E_{CI}$ is normally ~5 mV more positive than the resting potential.

According to the $G_{CI}$ model, if the only effect of $K^+$ removal were membrane depolarization, then an outward current should have eventually reappeared, once sufficient internal $Cl^-$ had been accumulated to make $E_{CI}$ more positive than $V_m$. In fact, the current never became outward in $K^+$-free solutions. This suggests that $K^+$ removal inhibits active $Cl^-$ accumulation, as has been shown in other tissues (Musch et al., 1982; Russell, 1983).

**Effect of Barium Ions**

$Ba^{++}$ has been reported to block resting $G_K$ (Sperelakis et al., 1967), and consistent with this, we found that 50 $\mu$M $BaCl_2$ caused a large depolarization of the muscle membrane (see Fig. 8C). The steady current became transiently inward and then declined and reversed sign again (Fig. 7). As in the previous cases, the inward current is not predicted by the $G_K$ model, since it is difficult to imagine that $V_m$ could have crossed $E_K$. The results are, however, consistent with the $G_{CI}$ model, for reasons similar to those described for $K^+$-free solutions above. The return of the outward current in $Ba^{++}$ suggests that the active $Cl^-$ transport eventually caused $E_{CI}$ to become more positive than $V_m$.

In summary, this analysis has treated several simple models, in each of which a single nonuniformity in the distribution of a particular transport pathway is assumed to underlie the generation of the steady outward current. More complex mechanisms, particularly those involving spatial nonuniformities of several pathways, offer alternative explanations, which are not discussed here.

The effects of $Na^+$ replacement, $K^+$ replacement, and $Ba^{++}$ suggest that the steady outward current is not produced solely as a result of a nonuniform distribution of $G_K$. The results are, however, consistent with a model in which the only spatial nonuniformity in membrane pathways is a relative reduction of $G_{CI}$ in the endplate region.

The $G_{CI}$ model predicts that the internal $Cl^-$ concentration is higher at the endplate than in extrajunctional regions. We have made preliminary estimates
of the magnitude of this effect, using a computer to calculate \([\text{Cl}]_i\) at different points along a model cable. (Membrane fluxes were calculated with the flux equation [Goldman, 1943; Hodgkin and Katz, 1949] and internal fluxes were calculated with the Fick diffusion equation; \(G_{\text{Cl}}\) was assumed to be reduced by 50% and \(V_m\) was assumed to be 1 mV more negative at the endplate, compared with extrajunctional regions.) The results predict that \([\text{Cl}]_i\) is \(\approx 25\%\) greater at the endplate than extrajunctionally (\(\approx 15\) and \(12\) mM, respectively).

An anatomical basis for this model is suggested by the observations that in mammalian muscle, \(G_K\) appears to be relatively restricted in its distribution to the surface membrane, whereas \(G_{\text{Cl}}\) is distributed in both surface and transverse (T) tubule membranes (Palade and Barchi, 1977; Dulhunty, 1979). Thus, a reduction in the amount of T system in the endplate region could account for virtually all of our results. However, in preliminary experiments (unpublished), we have counted horseradish peroxidase-filled T tubules in longitudinal sections of rat lumbrical muscle fibers in the electron microscope and have not observed fewer T tubule profiles at the endplate. Thus, it may be that the specific membrane conductance to \(\text{Cl}^-\), rather than T tubule membrane surface area, is reduced in the endplate region.

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