Evidence for Active Chloride Accumulation in Normal and Denervated Rat Lumbrical Muscle

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ABSTRACT Intracellular Cl− activity (aCl−) was measured with Cl−-sensitive microelectrodes in normal and denervated rat lumbrical muscle. In normal muscle bathed in normal Krebs solution, aCl− lay close to that predicted by the Nernst equation. The addition of 9-anthracene carboxylic acid, which blocks Cl− conductance, caused aCl− to increase far above that predicted by a passive distribution. Furosemide (10 µM) reversibly blocked this accumulation. After muscle denervation, aCl− progressively increased for 1–2 wk. The rise occurred in two stages. The initial stage (1–3 d after denervation) reflected passive Cl− accumulation owing to membrane depolarization. At later times, aCl− continued to increase, with no further change in membrane potential, which suggests an active uptake mechanism. This rise approximately coincided with the natural reduction in membrane conductance to Cl− that occurs several days after denervation. Na+ replacement, K+ replacement, and furosemide each reversibly blocked the active Cl− accumulation in denervated muscle. Quantitative estimates suggested that there was little difference between Cl− flux rates in normal and denervated muscles. The results can be explained by assuming that, in normal muscle, an active accumulation mechanism operates, but that Cl− lies close to equilibrium owing to the high membrane conductance to Cl−. The rise in aCl− after denervation can be accounted for by the membrane depolarization, the reduction in membrane Cl− conductance, and the nearly unaltered action of an inwardly directed Cl− “pump.”

INTRODUCTION

In skeletal muscle fibers, the membrane conductance to Cl− ions (GCl) is relatively high, comprising 50–90% of the total resting membrane conductance (Hodgkin and Horowicz, 1959; Adrian and Freygang, 1962; Hagiwara and Takahashi, 1974; Palade and Barchi, 1977a). Direct measurement of intracellular chloride activity (aCl−) with Cl−-sensitive microelectrodes has shown that Cl− is distributed close to the value predicted by the Nernst equation in frog sartorius (Bolton and Vaughan-Jones, 1977) and in rat and mouse extensor digitorum longus (EDL) (McCaig and Leader, 1984; Donaldson and Leader, 1984) muscle fibers. Moreover, in some muscles, removal of external Cl− has little or no effect on the steady state resting membrane potential (frog sartorius, Hodgkin and Horowicz,
These observations are consistent with a purely passive distribution of Cl\textsuperscript{−} across the skeletal muscle fiber membrane. Other results have suggested that Cl\textsuperscript{−} may be actively transported into skeletal muscle fibers. For example, Hutter and Warner (1967) showed that in frog sartorius muscle, the magnitude of the transient membrane depolarization produced by removal of external Cl\textsuperscript{−} was increased if muscles were preincubated in a solution of low pH, which blocked G_{Cl}. Quantitative estimates suggested that, in the low-pH solution, Cl\textsuperscript{−} was accumulated in excess of that predicted by the Nernst equation. Hutter and Warner suggested that blocking G_{Cl} may have revealed an active transport mechanism whose activity was normally masked by the high membrane conductance. This explanation was confirmed by Bolton and Vaughan-Jones (1977). Using intracellular Cl\textsuperscript{−}-sensitive microelectrodes, they showed directly that when external pH was lowered, a_{Cl} rose significantly higher than predicted by a passive distribution. Further evidence in support of active Cl\textsuperscript{−} accumulation was obtained in some muscles whose fibers undergo a relatively large (10–15 mV) steady state membrane hyperpolarization after either removal of external Cl\textsuperscript{−} or pharmacological block of G_{Cl} (rat sternomastoid and mouse soleus and EDL, Dulhunty, 1978; rat lumbrical, Betz et al., 1986b). Thus, while Cl\textsuperscript{−} normally might not lie far from equilibrium, active Cl\textsuperscript{−} transport could effectively raise internal Cl\textsuperscript{−} and exert a significant depolarizing influence on the membrane potential.

In the present study, we have confirmed and extended earlier observations. Using Cl\textsuperscript{−}-sensitive microelectrodes, we found that Cl\textsuperscript{−} is distributed close to equilibrium in normal rat lumbrical muscle. However, when Cl\textsuperscript{−} conductance was reduced pharmacologically (9-anthracene carboxylic acid) or physiologically (denervation), a_{Cl} rose to levels significantly higher than predicted by the Nernst equation. We also studied agents that interfere with active Cl\textsuperscript{−} accumulation. The removal of external Na\textsuperscript{+} or K\textsuperscript{+} or the addition of furosemide reversibly inhibited active Cl\textsuperscript{−} accumulation.

METHODS

Preparation

The second, third, and/or fourth deep lumbrical muscles of adult (~150–250 g) Sprague-Dawley rats were used for all experiments. The hindlimbs muscles on one side were denervated by removing a segment of sciatic nerve of several millimeters from the midthigh region under ether anesthesia. This operation produced a minimal disturbance of the animals' mobility in their environment and they resumed normal feeding and grooming behavior. Control muscles were obtained from either unoperated animals or from the contralateral nondenervated foot of operated animals. For electrical recording, the muscles were excised and pinned to a recording chamber lined with Sylgard (Dow Corning Corp., Midland, MI). The 0.5-ml volume of the chamber was superfused continuously by a gravity flow system, which delivered ~0.1 ml/s, and allowed for solution changes using a rotary switching device. All experiments were performed at room temperature (20–22°C).

Solutions

Normal Krebs solution consisted of (millimolar): 136 NaCl, 5 KCl, 2 or 8 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2},
11 glucose, and 2 PIPES (disodium salt) buffer. The pH was adjusted to 7.4 with H₂SO₄. Cl⁻-free solutions were prepared fresh for each experiment by replacing NaCl and KCl with the respective salts of isethionate, and CaCl₂ and MgCl₂ with SO₄ salts. For Na⁺-free solutions, NaCl was replaced with choline Cl, and muscles were pretreated (15 min) with α-bungarotoxin (4 µg/ml; Sigma Chemical Co., St. Louis, MO) in order to block activation of acetylcholine receptors. K⁺-free solutions contained 141 mM NaCl. Thus, all ion-substituted solutions were isosmolar with normal Krebs. The anthroic acid derivative 9-anthracene carboxylic acid (9-AC) was used to block Gc (Palade and Barchi, 1977b). Solutions of this drug were prepared from a stock solution of 22 mg 9-AC dissolved in 5 ml ethanol. For experiments involving low-Cl⁻ solutions or the use of 9-AC, tetrodotoxin (3 µM; Sigma Chemical Co.) was added to all solutions to prevent muscle fibrillation. The stilbene derivative 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS; Sigma Chemical Co.) was added directly to solutions immediately before use.

**Cl⁻-sensitive Electrodes**

Cl⁻-sensitive microelectrodes were fabricated from 1.5-mm-o.d. glass microcapillary pipettes pulled to a resistance of 12–20 MΩ when filled with 3.0 M K⁺-acetate. The electrodes were silanized either (a) by dipping the tips in a solution of hexamethyldisilazane, trichloromethylsilane, and chloronaphthalene (1:5:50, by volume), and then heating with the tips down in a predried oven at 200°C for 1 h, or (b) by exposure to trichloromethylsilane vapor at 200°C. The silanized electrodes were filled by first dipping the tips in Cl⁻-sensitive resin (WPI-170, WP Instruments, Inc., New Haven, CT) and then backfilling the remaining tapered portion with more resin. Either 100 or 147 mM KCl was used as a reference solution. Each Cl⁻-sensitive electrode was calibrated before use by recording voltage changes in pure KCl solutions (147, 10, 5, and 1 mM concentration; respective activities: 106, 9, 4.5, and 1.0 mM, calculated from Dean, 1985). Electrode responses were approximately linear over this range with an average slope, at room temperature, of 57.5 ± 0.2 mV/decade (mean ± SEM) KCl activity. Fig. 1A (KCl) shows the calibration records of a typical electrode. Fig. 1B (KCl) is a semilogarithmic plot of the electrode response in A.

We used two methods for measuring aCl⁻. In the first, muscle fibers were impaled with both a Cl⁻-sensitive and a conventional electrode (20–40 MΩ, filled with a solution of 0.5 M K₂SO₄ and 0.2 M KCl) separated by ~50 µm. Current passed through the voltage-recording electrode produced a change in Vm recorded by the Cl⁻ electrode, verifying that both electrodes were in the same cell. The potential difference between the two electrodes was then used to calculate aCl⁻ from the electrode calibration curves. This method was always used in experiments involving large changes in the external Cl⁻ concentration. In the second method, intracellular potentials were sampled with each electrode in turn. First, 10–20 muscle fibers were impaled with the Cl⁻ electrode, and then 10–20 additional fibers were impaled with the voltage-recording electrode. The difference between the means of the sampled fibers was used to calculate aCl⁻. For both methods, the reference electrode was an Na/KCl-filled agar bridge connected to an Ag/AgCl wire, or, for experiments involving large changes in external ion concentrations, a second recording microelectrode positioned near the muscle. The results from the two methods were not significantly different (p > 0.1) and are presented together.

**Sources of Error**

Intracellular anions other than Cl⁻ may interfere with aCl⁻ measurements because of a lack of complete selectivity of the Cl⁻-sensitive resin. The selectivity sequence of the WP-170 resin for a number of anions that we tested was Cl⁻ > isethionate > HCO₃⁻ > SO₄²⁻ > gluconate > glucuronate. The resin was selective ~6:1 for Cl⁻ over HCO₃⁻. The incomplete
FIGURE 1. Effect of cations on the calibration of Cl⁻-sensitive electrodes. (A) Pen recording of a Cl⁻-sensitive electrode response to either pure KCl or pure NaCl solutions. Activities (millimolar) are marked below the recording for KCl (solid lines) and NaCl (dotted lines). Note the 4-5-mV potential change produced by replacing 106 mM KCl with 106 mM NaCl. (B) Semilogarithmic plot of the responses recorded in A. Note the decreased slope of the response to NaCl (dotted line) compared with KCl (solid line) calibration solutions. This effect would lead to a slight underestimate of $a_\text{Cl}^-$.
selectivity may have produced a small overestimate of $a_{Cl}$ because of residual internal HCO$_3^-$, although all experiments were performed in nominally HCO$_3^-$-free solutions. In addition, the combined KCl/K$_2$SO$_4$ solution that we used in our voltage-recording electrodes has been reported to underestimate $V_m$, compared with a pure KCl-filled electrode (Aickin and Brading, 1982). Assuming that the Cl$^-$-sensitive electrode measures the membrane voltage that would be recorded by a pure KCl-filled electrode, then an overestimate of $a_{Cl}$ would be produced.

One factor may have produced an underestimate of $a_{Cl}$. We noted the development of a small potential (2–5 mV, depending on the type of reference electrode) when the calibration solution was changed from KCl to NaCl. This is illustrated in the electrode calibration shown in Fig. 1. The reason for this shift is not known; the activity coefficients for 0.1 molal KCl and NaCl solutions are nearly identical, being 0.770 and 0.778, respectively (Weast, 1971). The slope of the electrode response was less using NaCl than KCl calibration solutions. In practice, an underestimate of $a_{Cl}$ would be produced since, upon impalement, the electrode moves from high [Na$^+$] to high [K$^+$]. Thus, for example, if $V_m = -70$ mV and measured $E_{Cl} = -67$ mV, the "corrected" $E_{Cl}$ would be about $-63$ mV. Assuming $a_{Cl} = 112$ mM, calculated $a_{Cl}$ values would be 7.8 mM (uncorrected) and 9.1 mM ("corrected"). In the results presented, we calculated $a_{Cl}$ directly from the KCl calibration curve for each electrode; we did not apply additional corrections, owing to their uncertain and offsetting magnitudes.

**Membrane Electrical Properties**

$V_m$ was first measured by impalement with a single electrode, and then, in order to measure input resistant ($R_i$), a second electrode was inserted into the fiber within 50 μM of the first. $V_m$ was set at $-80$ mV by passing steady current; superimposed small square pulses (hyperpolarizing; 2 nA for 400 ms) were passed and the change in $V_m$ was recorded. Tetrodotoxin (3 μM for controls; 30 μM for denervated muscles) was added to the bath to block spontaneous twitching. The specific membrane slope conductance, $G_m$, was calculated from the relationship:

$$G_m = 1/R_m = R_i/(R_i^2 \cdot \pi^2 \cdot d^2),$$

where $R_i$ is the internal resistance (assumed to be 100 Ω·cm) and $d$ is the average muscle fiber diameter. To measure $d$, the diameters of 100 randomly selected fibers in each muscle were calculated from the cross-sectional areas of the fibers, assuming cylindrical geometry. Areas were measured on a digitizer from camera lucida drawings of glutaraldehyde-fixed sections. The measured areas were corrected for 30% shrinkage, determined by comparing fixed sections with unfixed, frozen sections. $G_{Cl}$ was calculated as the difference between $G_m$ in normal and in Cl$^-$-free Krebs.

**Statistics**

Values are given as means ± SEM. The significance of differences between sample means was assessed by Student's two-tailed $t$ test.

**RESULTS**

**Cl$^-$ Distribution in Normal and Denervated Muscle**

The average value of $E_{Cl}$ measured in 35 normal muscles (428 fibers) was $-64.2$ ± 0.7 mV, which was 3.1 mV less negative than the average $V_m$ ($-67.3$ ± 0.6 mV). The average value of $a_{Cl}$ was $7.8$ ± 0.2 mM. If Cl$^-$ were distributed
passively, the fibers should have contained 6.9 mM Cl\(^-\), or 0.9 mM less than we measured. This value is not corrected for possible interference from other intracellular anions. Thus, as others have shown previously using different muscles, \(a_{c1}\) lies close to equilibrium in normal lumbrical muscle.

In denervated muscle, the results were different. We found that \(a_{c1}\) rose steadily after denervation, and, more importantly, that the difference between \(E_{c1}\) and \(V_m\) increased by a large amount. The results are shown in Fig. 2. \(a_{c1}\) began to rise shortly after denervation (Fig. 2A). The initial rise reflected the membrane depolarization that occurs within 1 d after denervation (for references, see Leader et al., 1984). Lumbrical muscle fibers were depolarized by ~10 mV soon after denervation; the average \(V_m\) of all denervated fibers in this study was ~58.5 mV. By itself, this depolarization caused \(a_{c1}\) to rise passively to ~12 mM. Consistent with this, Fig. 2B shows that \(E_{c1} - V_m\) did not change for several days after denervation. After ~5 d, however, \(E_{c1} - V_m\) increased, and in muscles denervated \(\geq10\) d, \(E_{c1} - V_m\) had more than tripled (to ~10 mV).
difference between the observed $a_{\text{cl}}$ and the calculated passive values of $a_{\text{cl}}$ increased more than sixfold (to $\sim5.5$ mM). In summary, $a_{\text{cl}}$ more than doubled (to $16.5 \pm 0.6$ mM) after denervation, and the rise occurred in two phases, an early phase of passive accumulation caused by membrane depolarization, and a later active phase. As described below, the later phase may reflect the unmasking of normal active accumulation by the natural reduction of $p_{\text{cl}}$ that follows denervation.

![Figure 3](image)

**Figure 3.** Effect of 9-AC (100 $\mu$M, applied at zero time) on $V_m$ (A, solid line), $E_{\text{cl}}$ (A, dotted line), measured $a_{\text{cl}}$ (B, solid line), and calculated $a_{\text{cl}}$ assuming a passive distribution (B, dotted line). Voltage-recording and Cl$^-$-sensitive electrodes were in the same muscle fiber. If Cl$^-$ were passively distributed, the hyperpolarization caused by 9-AC should be caused $a_{\text{cl}}$ to decrease, following the curve marked "passive"; the fact that it actually increased strongly suggests the presence of active Cl$^-$ accumulation. During the brief gap in the $E_{\text{cl}}$ and $a_{\text{cl}}$ traces, the Cl$^-$-sensitive electrode was partly dislodged from the fiber.

**Effects of Inhibiting $G_{\text{cl}}$ in Normal Muscle**

As noted by Hutter and Warner (1967), if a Cl$^-$-accumulating mechanism is normally shunted by the relatively high membrane $G_{\text{cl}}$, it should be possible to unmask its activity by blocking $G_{\text{cl}}$. Bolton and Vaughan-Jones (1977) confirmed this in frog sartorius muscle by reducing external pH, which reduces $G_{\text{cl}}$. In the present study, we used 9-AC, which appears to be a selective inhibitor of $G_{\text{cl}}$ in rat skeletal muscle (Palade and Barchi, 1977b). The drug itself is not detected by the Cl$^-$-sensitive resin (Aickin, C. C., W. J. Betz, and G. L. Harris, unpublished observations). The results of a typical experiment are shown in Fig. 3. The
application of 9-AC (100 µM) caused a rapid membrane hyperpolarization and a rise in $a_{Cl}$. The hyperpolarization itself suggests that Cl$^-$ is not distributed at equilibrium; if it were, the steady state $V_m$ should not have changed in the presence of 9-AC (assuming that 9-AC only blocks $G_{Cl}$). If, on the other hand, 9-AC hyperpolarized the membrane by some other mechanism, then the increased internal negativity should have passively driven Cl$^-$ out of the fiber through any remaining conductance pathways, leading to a fall in $a_{Cl}$. Instead, $a_{Cl}$ actually increased in 9-AC, moving further from equilibrium. The action of an active Cl$^-$ uptake mechanism offers a relatively simple explanation of these results: the reduction of $G_{Cl}$ by 9-AC caused $V_m$ to move away from $E_{Cl}$ (toward $E_K$); the low $G_{Cl}$ also reduced the loss of Cl ions, which were subsequently "pumped" into the fiber, thereby producing a rise in $a_{Cl}$.

In muscle fibers from four muscles similarly exposed to 9-AC for 10 min, $V_m$ hyperpolarized by an average of 5.4 mV, and the difference between $E_{Cl}$ and $V_m$ increased from ~1 to 13.9 mV ($E_{Cl}$ less negative than $V_m$). The effects of 9-AC were even more pronounced with longer treatments. For example, in fibers from one muscle exposed for 8 h to 100 µM 9-AC, the average difference between $E_{Cl}$ and $V_m$ was 49.9 mV; $a_{Cl}$ had apparently risen to 40–50 mM.

Effects of Removing External Cl$^-$

When external Cl$^-$ is replaced with an impermeant anion, $a_{Cl}$ should decrease to a very low level. Under this condition, interference from other intracellular anions could contribute significantly to the signal recorded with the Cl$^-$-sensitive microelectrode. Fig. 4 shows the effect in a normal muscle on $V_m$ (A) and $a_{Cl}$ (B) of replacing normal Krebs with Cl$^-$-free solution. In this cell, the apparent $a_{Cl}$ fell to 4.3 mM. We made similar recordings from 10 cells (five muscles). On average, the apparent $a_{Cl}$ fell to 4.1 ± 0.2 mM in Cl$^-$-free Krebs. In the same cells, the observed value of $a_{Cl}$, measured in normal Krebs before exposure to Cl$^-$-free solution, was 0.7 mM greater than predicted for a passive distribution. That is, the apparent interference level that we measured was greater than the difference between the observed and equilibrium values for $a_{Cl}$ (see Discussion).

Since $G_{Cl}$ falls in denervated muscle, the rate at which Cl$^-$ leaves the denervated fibers in Cl$^-$-free solutions should be reduced. Fig. 5 shows the effect in a 10-d-denervated muscle fiber of replacing normal Krebs with a Cl$^-$-free solution. As described previously (Betz et al., 1986), the effect on $V_m$ (A) in a denervated fiber is quite different from that observed for normal fibers. We never observed in denervated fibers the transient depolarization that, in normal muscle, precedes the steady state hyperpolarization. Instead, the fibers underwent a rapid, monotonic hyperpolarization. $a_{Cl}$ fell monotonically (Fig. 5B), the maximum rate of loss being about one-half that in normal fibers (~1.8 and 3.3 mM/min, respectively). In the cell shown, $a_{Cl}$ fell to 6.8 mM in Cl$^-$-free solution. On average (eight cells), $a_{Cl}$ appeared to decrease to 8.1 ± 1.1 mM after exposure for ~10 min to the Cl$^-$-free solution. This is higher than the amount by which Cl$^-$ appeared to be out of equilibrium in normal Krebs (5.5 mM for muscles denervated ≥10 d). It is unlikely, however, that the 8.1-mM value is an accurate estimate of the level of internal interfering anions; rather, internal Cl$^-$ was
probably not fully depleted by the rather brief exposure to Cl\(^{-}\)-free Krebs. Unfortunately, it was difficult to maintain stable impalements in denervated fibers for longer periods, since some fibers began to contract several minutes after being exposed to Cl\(^{-}\)-free Krebs. The conclusion that there is incomplete depletion of internal Cl\(^{-}\) is further supported by the observation (described below) that a longer (20–30 min) exposure to K\(^+\)-free solutions (containing normal external Cl\(^{-}\)) reduced \(a_{\text{Cl}}\) to an even lower level (6.3 mM, compared with 8.1 mM after \(\sim\)10 min in Cl\(^{-}\)-free Krebs).

**Figure 4.** Effect in a normal muscle of Cl\(^{-}\)-free solution on \(V_m\) (A) and \(a_{\text{Cl}}\) (B). During the time indicated by the horizontal line, Cl\(^{-}\)-free Krebs was applied while recording from a single fiber with both voltage-recording and Cl\(^{-}\)-sensitive electrodes. \(V_m\) transiently depolarized and then repolarized to a hyperpolarized steady level. \(a_{\text{Cl}}\) fell from \(\sim11\) to \(\sim4.5\) mM in \(\sim5\) min. When the cell was returned to normal Krebs, both \(V_m\) and \(a_{\text{Cl}}\) recovered fully.

**Effects of Cl\(^{-}\) Transport Inhibitors on \(a_{\text{Cl}}\)**

In normal muscle bathed in normal Krebs, Cl\(^{-}\) lies so close to equilibrium that the effects of potential inhibitors of active Cl\(^{-}\) uptake would not be expected to be easily measurable. Table I shows that this was indeed the case; exposure for 20–30 min to Na\(^+\)- and K\(^+\)-free solutions, and to furosemide, a drug that blocks Na\(^+\)/K\(^+\)/Cl\(^{-}\) cotransport, had little effect on \(E_{\text{Cl}} - V_m\). In addition, in one muscle, the effect of SITS (80 \(\mu\)M) was studied; it, too, had no significant effect on Cl\(^{-}\) distribution.

The increased difference between \(V_m\) and \(E_{\text{Cl}}\) in the presence of 9-AC provided a larger signal for detecting inhibition of any Cl\(^{-}\)-accumulating mechanism in normal muscle. As shown in Fig. 6, furosemide completely abolished the ability
of normal fibers to accumulate Cl\textsuperscript{−} in the presence of 9-AC, and the effect was reversible. More detailed studies of the inhibitory effects of furosemide and cation replacement are currently in progress in collaboration with C. C. Aickin.

In denervated muscle, clear inhibitory effects of Na\textsuperscript{+} and K\textsuperscript{+} substitution and furosemide were observed. Treatment with SITS, however, did not significantly alter Cl\textsuperscript{−} distribution. The results are summarized in Table II. All muscles were denervated ≥10 d. In the absence of either external cation, the amount by which Cl\textsuperscript{−} was out of equilibrium decreased significantly. Both effects were reversible. One noteworthy difference between the responses of normal and denervated muscles was that innervated fibers depolarized and denervated fibers hyperpolarized in K\textsuperscript{+}-free solutions (cf. Betz et al., 1986); the inhibitory effects on Cl\textsuperscript{−} accumulation, however, were qualitatively similar. Furosemide produced a membrane hyperpolarization and also greatly reduced the amount by which Cl\textsuperscript{−} was out of equilibrium. All of these effects were reversible. In summary, Na\textsuperscript{+} removal, K\textsuperscript{+} removal, and furosemide all markedly reduced the amount by which Cl\textsuperscript{−} appeared to be out of equilibrium.

These results also provide evidence that the amount by which Cl\textsuperscript{−} appeared to be out of equilibrium (E\textsubscript{Cl} − V\textsubscript{m}) in denervated muscle was real, and was not due to anion interference. Suppose, for example, that Cl\textsuperscript{−} was actually distributed
Values for Cl⁻ distribution in normally innervated muscles, and the effects of Na⁺ replacement (0 Na⁺), K⁺ replacement (0 K⁺), and furosemide (10 or 50 μM). N is the number of muscle fibers (number of muscles); Vm is the membrane potential; ECl is the Cl⁻ equilibrium potential (calculated from measured aCl); passive aCl is the predicted aCl assuming a passive distribution; observed – passive is millimolar units out of equilibrium. Measurements were made 20–30 min after solution changes. The only statistically significant (p < 0.05) change was produced by K⁺ replacement (marked by an asterisk).

passively, and that a constant amount of interfering anion made ECl appear more positive than it really was. Membrane hyperpolarization such as that produced by Na⁺ removal, K⁺ removal, or furosemide would passively drive Cl⁻ from the cell. The apparent ECl would hyperpolarize, reflecting the loss of Cl⁻, but if the

|          | N  | Vm  | ECl    | ECl - Vm | Passive aCl | Observed aCl | Observed - passive |
|----------|----|-----|--------|----------|-------------|--------------|-------------------|
|          |    |     | mV     | mM       | mM          | mM           |                   |
| Krebs    | 25 (2) | -71.7±0.8 | -69.7±0.6 | 2.0 | 6.5±0.1 | 7.1±0.2 | 0.6 |
| 0 Na⁺    | 25 (2) | -81.5±0.8 | -77.0±0.9 | 4.4 | 4.4±0.1 | 5.3±0.2 | 0.9 |
| Krebs    | 61 (4) | -68.9±0.7 | -66.4±0.4 | 1.8 | 7.5±0.2 | 8.1±0.2 | 0.6 |
| 0 K⁺     | 59 (4) | -58.7±0.7 | -58.9±0.2 | -0.2 | 10.9±0.1 | 10.8±0.2 | -0.1* |
| Wash     | 16 (1) | -68.8±1.0 | -65.7±0.3 | 3.1 | 7.5±0.1 | 8.2±0.1 | 0.9 |
| Krebs    | 41 (3) | -64.4±0.6 | -62.2±0.4 | 2.2 | 8.7±0.1 | 9.5±0.2 | 0.8 |
| Furosemide | 46 (3) | -80.6±1.0 | -76.9±0.9 | 3.7 | 4.6±0.2 | 5.5±0.2 | 0.9 |

**TABLE I**

**Normal Muscle**

*Figure 6.* Effect of furosemide on aCl. Furosemide (10 μM) and 9-AC (100 μM) were applied during the times indicated by the heavy lines. The filled symbols show the observed aCl; the open symbols show the aCl values for a passive Cl⁻ distribution. Calculations for each symbol were made from average values recorded from 10–15 impalements with a conventional intracellular microelectrode and 10–15 additional impalements with a Cl⁻-sensitive microelectrode. The application of 9-AC increased the amount by which Cl⁻ was out of equilibrium (aCl did not increase in this particular muscle, although it did in others; cf. Fig. 4). Furosemide application in the continued presence of 9-AC reduced aCl to the passive level and the effect was partially reversible.
anion interference remained constant, the hyperpolarization of \( E_{\text{Cl}} \) would be less than the hyperpolarization of \( V_m \). Thus, active Cl\(^-\) accumulation \( (E_{\text{Cl}} - V_m) \) would appear to increase. In fact, just the opposite was observed: Na\(^+\) removal, K\(^+\) removal, and furosemide all reduced the amount by which Cl\(^-\) appeared to be out of equilibrium (Table II).

**Calculation of Cl\(^-\) Flux**

A simple qualitative explanation for the above results is that reduction of \( G_{\text{Cl}} \) after denervation leads to increased \( a_{\text{Cl}} \) through continued active Cl\(^-\) accumulation. In order to investigate this explanation more quantitatively, we calculated the magnitude of the passive Cl\(^-\) efflux (which, of course, must be equal and opposite to active Cl\(^-\) influx in the steady state). As shown in Table III, flux values were similar in normal and denervated muscles. The procedure was as follows. First, input conductance in normal and Cl\(^-\) free Krebs was measured (muscles were equilibrated with Cl\(^-\) free Krebs for 30 min before measurements were begun). From these values and from measured muscle fiber diameters, the specific membrane slope conductance to Cl\(^-\) \( (G_{\text{Cl}}) \) was calculated (see Methods). Cl\(^-\) flux \( (i_{\text{Cl}}, \text{C}/\text{cm}^2\cdot\text{s}) \), was calculated according to the relation given by Goldman (1943) and Hodgkin and Katz (1949):

\[
i_{\text{Cl}} = \frac{p_{\text{Cl}}}{b_{\text{Cl}}} \int \frac{dV}{V} \left[ a_{\text{Cl}} e^{b_{\text{Cl}}V} - a_{\text{Cl}}^0 \right]
\]

\[
e^{b_{\text{Cl}}V} = 1
\]

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**TABLE II**

|                | Passive \( a_{\text{Cl}} \) | Observed \( a_{\text{Cl}} \) | Observed - passive |
|----------------|-----------------------------|-----------------------------|-------------------|
| **Denervated Muscle** |                             |                             |                   |
| **N** | **\( V_m \)** | **\( E_{\text{Cl}} \)** | **\( E_{\text{Cl}} - V_m \)** | **\( a_{\text{Cl}} \)** | **\( a_{\text{Cl}}^0 \)** | **\( a_{\text{Cl}}^0 - a_{\text{Cl}} \)** |
| Krebs 64 (4) | -54.2±0.6 | -49.5±0.7 | 4.7 | 13.0±0.2 | 16.0±0.4 | 3.0 |
| 0 Na\(^+\) 58 (4) | -64.9±0.5 | -65.4±0.7 | -0.4 | 8.5±0.2 | 8.4±0.2 | -0.1* |
| Wash 15 (1) | -51.5±1.0 | -44.6±1.1 | 7.0 | 14.4±0.1 | 19.4±0.1 | 5.0 |
| Krebs 43 (3) | -60.3±0.6 | -53.4±0.4 | 7.0 | 10.2±0.1 | 15.5±0.2 | 3.3 |
| 0 K\(^+\) 40 (3) | -71.9±0.5 | -72.5±0.3 | -0.4 | 6.4±0.1 | 6.3±0.1 | -0.1* |
| Wash 30 (2) | -60.7±0.8 | -54.5±0.6 | 6.2 | 10.0±0.1 | 12.9±0.3 | 2.9 |
| Krebs 87 (6) | -59.7±1.2 | -51.6±1.9 | 8.1 | 10.5±0.5 | 14.7±1.1 | 4.2 |
| Furosemide 83 (6) | -67.8±2.1 | -66.8±1.7 | 1.0 | 7.8±0.7 | 8.0±0.6 | 0.2* |
| Wash 28 (2) | -61.2±0.8 | -50.7±2.1 | 10.4 | 9.9±0.3 | 15.0±1.2 | 5.1 |
| Krebs 25 (2) | -58.9±0.9 | -52.0±0.6 | 6.9 | 10.6±0.2 | 14.3±0.2 | 3.7 |
| SITS 24 (2) | -65.9±0.8 | -57.9±0.9 | 8.0 | 8.2±0.1 | 11.4±0.4 | 3.2 |
| Wash 19 (2) | -64.9±1.3 | -56.7±0.7 | 8.2 | 8.5±0.1 | 11.9±0.4 | 3.4 |

Values for Cl\(^-\) distribution in muscles denervated ≥10 d, presented as in Table I. In denervated muscles, Na\(^+\) replacement, K\(^+\) replacement, and furosemide each produced significant (p < 0.05, marked by an asterisk) and reversible reductions in the amount by which Cl\(^-\) appeared to be out of equilibrium (millimolar units out). SITS, however, did not significantly reduce this value.
where \( b = \frac{zF}{RT} \). The membrane permeability to \( \text{Cl}^- \), \( p_{\text{Cl}} \), was calculated from \( G_{\text{Cl}} \) by equating measured \( G_{\text{Cl}} \) with the first derivative of Eq. 1:

\[
G_{\text{Cl}} = \frac{di_{\text{Cl}}}{dV_m} = \frac{p_{\text{Cl}} b zF}{e^{(bV_m)} - 1} \left[ \frac{bV_m(a_{\text{Cl}} - a_{\text{Cl}}) e^{(bV_m)}}{e^{(bV_m)} - 1} + a_{\text{Cl}} e^{(bV_m)} - a_{\text{Cl}} \right].
\] (2)

This equation was then solved for \( p_{\text{Cl}} \). Finally, with this value of \( p_{\text{Cl}} \), the \( \text{Cl}^- \) flux was calculated according to Eq. 1 and converted to moles per square centimeter per second. The resulting values (Table III, last column) differed by only 24%, which suggests a modest reduction in pump and leak fluxes after denervation.

Thus, the increased \( a_{\text{Cl}} \) after denervation can be accounted for with reasonable quantitative accuracy by assuming that as \( G_{\text{Cl}} \) falls by nearly fourfold after denervation, continued inward pumping causes \( a_{\text{Cl}} \) to rise, increasing the driving force for passive \( \text{Cl}^- \) efflux by over threefold. The increased driving force almost completely offsets the conductance decrease, so that the flux rate is not changed very much.

**TABLE III**

| Flux Calculations |
|-------------------|
| Type | Solution | \( N \) | \( V_m \) | \( R_m \) | Diameter | \( G_m \) | \( G_{\text{Cl}} \) | \( p_{\text{Cl}} \) | Flux |
|------|----------|------|------|------|---------|-------|--------|-------|------|
|      |          |      |      |      |         |       |        |       |      |
| Control | Krebs | 37 (4) | -70.1±0.3 | 1.1±0.1 | 24.5±1.1 | 24.5±1.1 | 666±176 | 622±175 | 7.0 | 22.1 |
| Control | 0 Cl | 42 (4) | -80.6±1.1 | 4.1±0.3 | -- | 42±2 | -- | -- | -- |
| Denervated | Krebs | 40 (4) | -60.4±0.6 | 2.4±0.4 | 19.6±0.6 | 245±25 | 164±40 | 1.27 | 16.9 |
| Denervated | 0 Cl | 40 (4) | -69.9±0.4 | 4.1±0.3 | -- | 80±8 | -- | -- | -- |

Effect of denervation on membrane properties and calculated \( \text{Cl}^- \) flux. Measured values of \( V_m \), input resistance \( (R_m) \), fiber diameter, and specific membrane \( (G_m) \) and \( \text{Cl}^- \) \( (G_{\text{Cl}}) \) conductances are shown, together with calculated \( \text{Cl}^- \) permeability \( (p_{\text{Cl}}) \) and \( \text{Cl}^- \) flux (efflux positive). \( N \) gives the number of muscle fibers and (in parentheses) the number of muscles. Recordings were made in control muscles and muscles denervated for 10–12 d in normal (Krebs) and Cl-free (0 Cl) solutions.

The values in Table III can also be used to calculate the maximum rate of fall of \( a_{\text{Cl}} \) upon switching to a Cl-free solution (Figs. 4 and 5). The rate of change in \( a_{\text{Cl}} \) is given simply as (units in parentheses):

\[
d(a_{\text{Cl}})/dt \text{ (mM/min)} = -2.4 \times 10^8 \text{ flux (mol/cm}^2\cdot\text{s)/diameter (cm).}
\]

Flux was calculated according to Eq. 1 with \( a_{\text{Cl}}^2 \) = 0. Since \( V_m \) changes during removal of external \( \text{Cl}^- \), the calculations were performed over a range of values of \( V_m \) (-70 to -40 mV for normal muscles; -60 to -75 mV for denervated muscles); values of \( p_{\text{Cl}} \) (assumed to remain constant) and muscle fiber diameter were taken from Table III. The calculated rates (-11 to -16 mM/min in control fibers; -6.7 to -8.1 mM/min in denervated fibers) are considerably faster than the observed maximum rates of fall (about -3.3 and -1.8 mM/min in control and denervated fibers, respectively). The lower observed rates undoubtedly reflect the relatively slow rate of perfusion of the preparation (see Methods), together with the use of whole muscles, rather than isolated fibers or small bundles of fibers.
**Effect of Furosemide on $G_{\text{Cl}}$ in Denervated Muscle**

A key event in this sequence of events after denervation is clearly the decrease in $G_{\text{Cl}}$. The stimulus for this change is not known. Gold and Martin (1983) described a synaptic Cl− channel in lamprey neurons, the conductance of which decreases with elevated internal Cl−. This offers an explanation of the present results, if the Cl− channel in skeletal muscle operates similarly. According to this model, the early depolarization after denervation (which may be caused by a relative increase in Na+ permeability; Robbins, 1977; Wareham, 1978; Shabunova and Vyskocil, 1982; Leader et al., 1984) triggers initial Cl− accumulation passively. This in turn would reduce $G_{\text{Cl}}$, leading to further Cl− accumulation and a further reduction in $G_{\text{Cl}}$ in a cycle of positive feedback. Therefore, in this scheme, the fall in $G_{\text{Cl}}$ is triggered by the initial rise in $a_{\text{Cl}}$.

If this were correct, then reducing $a_{\text{Cl}}$ in denervated muscle should produce a large increase in input conductance ($G_{\text{m}}$) as Cl− channels become unblocked. To test this idea, we exposed denervated muscles to 10 μM furosemide, which caused $a_{\text{Cl}}$ to fall to nearly its equilibrium value. We found that $G_{\text{m}}$ did not change significantly after exposure to furosemide. In randomly sampled denervated fibers, $G_{\text{m}} = 415 \pm 25$ mS (control); 385 ± 19 mS 30 min after exposure to furosemide, and 414 ± 45 mS after 2 h in furosemide (all $p$ values >0.35). Thus, reducing $a_{\text{Cl}}$ in denervated muscles for 2 h with furosemide did not unmask Cl− channels.

**DISCUSSION**

**Cl− Transport in Normal Muscle**

The present results are in general agreement with previous studies of Cl− distribution in skeletal muscle using Cl−-sensitive microelectrodes (frog sartorius, Bolton and Vaughan-Jones, 1977; Vaughan-Jones, 1982a, b; rat EDL, McCaig and Leader, 1984; mouse EDL, Donaldson and Leader, 1984). Our recordings consistently suggested that $a_{\text{Cl}}$ is ~1 mM higher than that predicted by a purely passive distribution. However, even though the bathing solutions contained no added HCO−3, the likely presence of this and other intracellular interfering anions could have generated such a signal. After exposure to Cl−-free solutions, the measured value of $a_{\text{Cl}}$ decreased, but if one assumes that the residual amount (~4 mM) represents the activity of interfering anions for muscles bathed in normal Krebs solution, then one would conclude that Cl− is actively extruded, not accumulated. A further technical difficulty is that the Cl−-sensitive electrodes responded differently in NaCl and KCl calibrating solutions (see Methods). The effect of this would be to cause $a_{\text{Cl}}$ to be underestimated by 1–2 mM. Given these offsetting uncertainties, these experiments by themselves do not resolve the question of whether active Cl− transport is present or absent in skeletal muscle.

The difficulties in detecting active Cl− transport in skeletal muscle arise in part because of the relatively high membrane conductance to Cl−, which would counteract the ability of any active transport mechanism to drive $E_{\text{Cl}}$ away from $V_{\text{m}}$. When we reduced $G_{\text{Cl}}$ by application of 9-AC, we obtained clear evidence of
active Cl\(^-\) accumulation (cf. Bolton and Vaughan-Jones, 1977). First, 9-AC caused the membrane to hyperpolarize. Assuming that 9-AC does not affect other transport pathways (Palade and Barchi, 1977b), the hyperpolarization suggests that Cl\(^-\) is actively accumulated in normal muscle. Moreover, \(a_{Cl}\), which would normally decrease passively upon membrane hyperpolarization, actually increased in 9-AC. Assuming that 9-AC does not increase the concentration of internal interfering anions, the observed increase in \(a_{Cl}\) provides strong evidence for the presence of active Cl\(^-\) accumulation.

Other observations also support this conclusion. The increase in \(a_{Cl}\) produced by 9-AC was reversibly abolished by furosemide, a drug that interferes with active Cl\(^-\) transport in several tissues (squid axon, Russell, 1983; intestinal epithelium, Musch et al., 1982). In addition, the membrane was hyperpolarized 10–15 mV by removal of external Cl\(^-\) (replaced with isethionate) and by addition of furosemide. Assuming that Cl\(^-\) removal and furosemide do not affect membrane permeabilities to other ions, the observed hyperpolarization suggests that Cl\(^-\) is normally accumulated actively (cf. Dulhunty, 1978; Betz et al., 1984b, 1986).

**Cl\(^-\) Transport in Denervated Muscle**

The measured \(a_{Cl}\) was significantly higher in denervated muscles than in control muscles. The time course of the increase occurred in two phases. The initial rise, occurring within a few days after denervation, was purely passive, and reflected the membrane depolarization that occurs soon after denervation. The amount by which Cl\(^-\) appeared to be out of equilibrium did not change during this time. Similar findings have been reported by Leader et al. (1984), who studied muscles denervated for up to 3 d. We found that a second increase in \(a_{Cl}\) ensued, unaccompanied by further change in \(V_m\). This second process caused a large (about fivefold) increase in the amount by which Cl\(^-\) was out of equilibrium. This rise could be accounted for by the fall in \(G_{Cl}\) that occurs several days after denervation (Camerino and Bryant, 1976; Lorkovic and Tomanek, 1977). In the lumbrical muscle, \(G_{Cl}\) decreased by about fourfold by 10 d after denervation. The calculated membrane permeability to Cl\(^-\) fell by about fivefold (the fall in permeability was greater than the fall in conductance owing to the rise in \(a_{Cl}\) after denervation). Interestingly, the net flux calculated according to the Goldman-Hodgkin-Katz equation did not change very much after denervation (it fell by 24%). In other words, the fall in \(G_{Cl}\), which would reduce Cl\(^-\) efflux, was largely offset by the increased driving force (\(E_{Cl} - V_m\)).

**Mechanism of Active Cl\(^-\) Accumulation**

The difference between \(E_{Cl}\) and \(V_m\) was greater in denervated muscle than in innervated muscle. This provided a larger signal for studying the actions of potential blocking agents. Na\(^+\) replacement, K\(^+\) replacement, and furosemide all significantly reduced the amount by which Cl\(^-\) was out of equilibrium in denervated muscle; the effects were reversible. SITS, however, did not alter Cl\(^-\) distribution significantly.

These findings further support the idea that Cl\(^-\) accumulation occurs primarily
via an Na+-K+-dependent process rather than via Cl-/HCO₃⁻ exchange. This is somewhat surprising, since in both cardiac (Vaughan-Jones, 1982a, b) and smooth (Aickin and Brading, 1983, 1984) muscle, anion exchange has been demonstrated to be the primary mechanism of Cl⁻ accumulation. The present experiments were performed in the absence of added HCO₃⁻, however, and whether Cl⁻/HCO₃⁻ exchange normally plays a role that we failed to detect will require further study.

**Steady Electric Current: Role of Active Cl⁻ Accumulation in Skeletal Muscle**

These findings help to explain another observation. We have previously described a steady electric outward current generated by the rat lumbrical muscle membrane (Caldwell and Betz, 1984; Betz et al., 1984a). The outward current is localized precisely at the neuromuscular junction. While the function of the steady current is unknown, its mechanism has been studied in some detail. Indirect evidence suggested that the current is carried by Cl⁻. The model proposed for the steady current generator requires that Cl⁻ be actively accumulated by muscle fibers, and that G(Cl) be reduced in the endplate region, compared with the extrajunctional regions (Betz et al., 1984b). The present results provide further direct evidence for the first of these requirements, namely that Cl⁻ be actively accumulated. In addition, the steady current is altered very little after denervation (Betz et al., 1986). This persistence can now be seen as reflecting continued Cl⁻ flux, owing to the offsetting changes in Cl⁻ conductance and driving force, as described above.

In summary, a relatively simple explanation of nearly all of these results is that active Cl⁻ accumulation occurs in normal lumbrical muscle, and causes α(Cl) to be 3–6 mM higher and Vₓ to be 10–15 mV less negative than they would be if Cl⁻ were passively distributed. Owing to the high G(Cl), the ion is never very far out of equilibrium in innervated muscle; Vₓ lies close to E(Cl). After denervation, as G(Cl) falls, the “pump” apparently continues to operate at about the same rate. When the new steady state is reached, α(Cl) is increased, and Cl⁻ comes to lie further from equilibrium than in innervated muscle.

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**REFERENCES**

Adrian, R. H., and W. H. Freygang. 1962. The potassium and chloride conductance of frog muscle membrane. *Journal of Physiology*. 163:61–103.

Aickin, C. C., and A. F. Brading. 1982. Measurement of intracellular chloride in guinea-pig
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vas deferens by ion analysis, \(^{36}\)chloride efflux and microelectrodes. *Journal of Physiology.* 326:139–154.

Aickin, C. C., and A. F. Brading. 1983. Towards an estimate of chloride permeability in the smooth muscle of guinea-pig vas deferens. *Journal of Physiology.* 336:179–197.

Aickin, C. C., and A. F. Brading. 1984. The role of chloride-bicarbonate exchange in the regulation of intracellular chloride in guinea-pig vas deferens. *Journal of Physiology.* 349:587–606.

Betz, W. J., J. H. Caldwell, and G. L. Harris. 1986. Effect of denervation on a steady electric current generated at the end plate region of rat skeletal muscle. *Journal of Physiology.* 373:97–114.

Betz, W. J., J. H. Caldwell, G. L. Harris, and S. C. Kinnamon. 1984a. Properties of a steady electric current generated at rat lumbrical muscles end plates. In Development and Plasticity in the Nervous System. M. Kuno, editor. University of Tokyo Press, Tokyo. 97–117.

Betz, W. J., J. H. Caldwell, and S. C. Kinnamon. 1984b. Physiological basis of a steady electric current in rat skeletal muscle. *Journal of General Physiology.* 83:175–192.

Bolton, T. B., and R. D. Vaughan-Jones. 1977. Continuous direct measurement of intracellular chloride and pH in frog skeletal muscle. *Journal of Physiology.* 270:801–833.

Caldwell, J. H., and W. J. Betz. 1984. Properties of an endogenous steady current in rat muscle. *Journal of General Physiology.* 83:157–173.

Camerino, D., and S. H. Bryant. 1976. Effects of denervation and colchicine treatment on the chloride conductance of rat skeletal muscle fibers. *Journal of Neurobiology.* 7:221–228.

Dean, J. A., editor. 1985. Lange’s Handbook of Chemistry. McGraw-Hill, New York. 5.3–5.7.

Donaldson, P. J., and J. P. Leader. 1984. Intracellular ionic activities in the EDL muscle of the mouse. *Pflügers Archiv.* 400:166–170.

Dulhunty, A. F. 1978. The dependence of membrane potential on extracellular chloride concentration in mammalian skeletal muscle. *Journal of Physiology.* 276:67–82.

Gold, M. R., and A. R. Martin. 1983. Analysis of glycine-activated inhibitory postsynaptic channels in brain-stem neurones of the lamprey. *Journal of Physiology.* 342:99–117.

Goldman, D. E. 1943. Potential, impedance and rectification in membranes. *Journal of General Physiology.* 27:37–60.

Hagiwara, S., and K. Takahashi. 1974. Mechanism of anion permeation through the muscle fiber membrane on an elasmobrach fish *Taeniura lymma.* *Journal of Physiology.* 238:109–127.

Hodgkin, A. L., and P. Horowicz. 1959. The influence of potassium and chloride ions on the membrane potential of single muscle fibers. *Journal of Physiology.* 148:127–160.

Hodgkin, A. L., and B. Katz. 1949. The effect of sodium on the electrical activity of the giant axon of the squid. *Journal of Physiology.* 108:37–77.

Hutter, O. F., and A. E. Warner. 1967. The pH sensitivity of the chloride conductance of frog skeletal muscle. *Journal of Physiology.* 189:403–425.

Leader, J. P., J. J. Bray, A. D. C. Macknight, D. R. Mason, D. McCaig, and R. G. Mills. 1984. Cellular ions in intact and denervated muscles of the rat. *Journal of Membrane Biology.* 81:19–27.

Lorkovic, H., and R. J. Tomanek. 1977. Potassium and chloride conductances in normal and denervated rat muscles. *American Journal of Physiology.* 232:C109–C114.

McCaig, D., and J. P. Leader. 1984. Intracellular chloride activity in the extensor digitorum longus (EDL) muscle of the rat. *Journal of Membrane Biology.* 81:9–17.

Musch, M. W., S. A. Orellana, L. S. Kimberg, M. Field, D. R. Halm, E. J. Krasny, and R. A. Frizzell. 1982. Na⁺-K⁺-Cl⁻ co-transport in the intestine of a marine teleost. *Nature.* 300:351–353.
Palade, P. T., and R. L. Barchi. 1977a. Characteristics of the chloride conductance in muscle fibers of the rat diaphragm. Journal of General Physiology. 69:325–342.

Palade, P. T., and R. L. Barchi. 1977b. On the inhibition of muscle membrane chloride conductance by aromatic carboxylic acids. Journal of General Physiology. 69:879–896.

Robbins, N. 1977. Cation movements in normal and short-term denervated rat fast twitch muscle. Journal of Physiology. 271:605–624.

Russell, J. M. 1983. Cation coupled chloride influx in squid axon: role of potassium and stoichiometry of the transport process. Journal of General Physiology. 81:909–925.

Shabunova, I., and F. Vyskocil. 1982. Postdenervation changes of intracellular potassium and sodium measured by ion-selective microelectrodes in rat soleus and extensor digitorum longus muscle fibers. Pflügers Archiv. 394:161–164.

Vaughan-Jones, R. D. 1982a. Chloride activity and its control in skeletal and cardiac muscle. Philosophical Transactions of the Royal Society of London, Series B. 299:537–548.

Vaughan-Jones, R. D. 1982b. Chloride-bicarbonate exchange in the sheep cardiac Purkinje fiber. In Intracellular pH: Its Measurement, Regulation and Utilization in Cellular Functions. R. Nuccitelli and D. Deamer, editors. Alan R. Liss, New York. 239–252.

Wareham, A. C. 1978. Effect of denervation and ouabain on the response of the resting membrane potential of rat skeletal muscle to potassium. Pflügers Archiv. 373:225–228.

Weast, R. C., editor. 1971. Handbook of Chemistry and Physics. The Chemical Rubber Co., Cleveland, OH. D-123.