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

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ABSTRACT Intracellular Cl^- activity (a^i_{Cl}) was measured with Cl^--sensitive microelectrodes in normal and denervated rat lumbrical muscle. In normal muscle bathed in normal Krebs solution, a^i_{Cl} lay close to that predicted by the Nernst equation. The addition of 9-anthracene carboxylic acid, which blocks Cl^- conductance, caused a^i_{Cl} to increase far above that predicted by a passive distribution. Furosemide (10 μM) reversibly blocked this accumulation. After muscle denervation, a^i_{Cl} 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, a^i_{Cl} 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 a^i_{Cl} 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 (G_{Cl}) 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, 1977*a*). Direct measurement of intracelluar chloride activity (a^i_{Cl}) 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,

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1959; rat diaphragm, Palade and Barchi, 1977*a*; Dulhunty, 1978; rat EDL, McCaig and Leader, 1984). These observations are consistent with a purely passive distribution of Cl^- across the skeletal muscle fiber membrane.

Other results have suggested that Cl^- 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^- 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^- 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^--sensitive microelectrodes, they showed directly that when external pH was lowered, a^i_{Cl} rose significantly higher than predicted by a passive distribution. Further evidence in support of active Cl^- 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^- or pharmacological block of G_{Cl} (rat sternomastoid and mouse soleus and EDL, Dulhunty, 1978; rat lumbrical, Betz et al., 1986*b*). Thus, while Cl^- normally might not lie far from equilibrium, active Cl^- transport could effectively raise internal Cl^- and exert a significant depolarizing influence on the membrane potential.

In the present study, we have confirmed and extended earlier observations. Using Cl^--sensitive microelectrodes, we found that Cl^- is distributed close to equilibrium in normal rat lumbrical muscle. However, when Cl^- conductance was reduced pharmacologically (9-anthracene carboxylic acid) or physiologically (denervation), a^i_{Cl} rose to levels significantly higher than predicted by the Nernst equation. We also studied agents that interfere with active Cl^- accumulation. The removal of external Na^+ or K^+ or the addition of furosemide reversibly inhibited active Cl^- 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 hindlimb 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 $CaCl_2$, 1 $MgCl_2$,

11 glucose, and 2 PIPES (disodium salt) buffer. The pH was adjusted to 7.4 with H_2SO_4. Cl^--free solutions were prepared fresh for each experiment by replacing NaCl and KCl with the respective salts of isethionate, and $CaCl_2$ and $MgCl_2$ with $SO_4^=$ 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 G_{Cl} (Palade and Barchi, 1977*b*). 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. 1*A* (KCl) shows the calibration records of a typical electrode. Fig. 1*B* (KCl) is a semilogarithmic plot of the electrode response in *A*.

We used two methods for measuring a_{Cl}^i. 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 K_2SO_4 and 0.2 M KCl) separated by ~50 μm. Current passed through the voltage-recording electrode produced a change in V_m 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 a_{Cl}^i 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 a_{Cl}^i. 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 a_{Cl}^i 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 > HCO_3^- > $SO_4^=$ > gluconate > glucuronate. The resin was selective ~6:1 for Cl^- over HCO_3^-. The incomplete

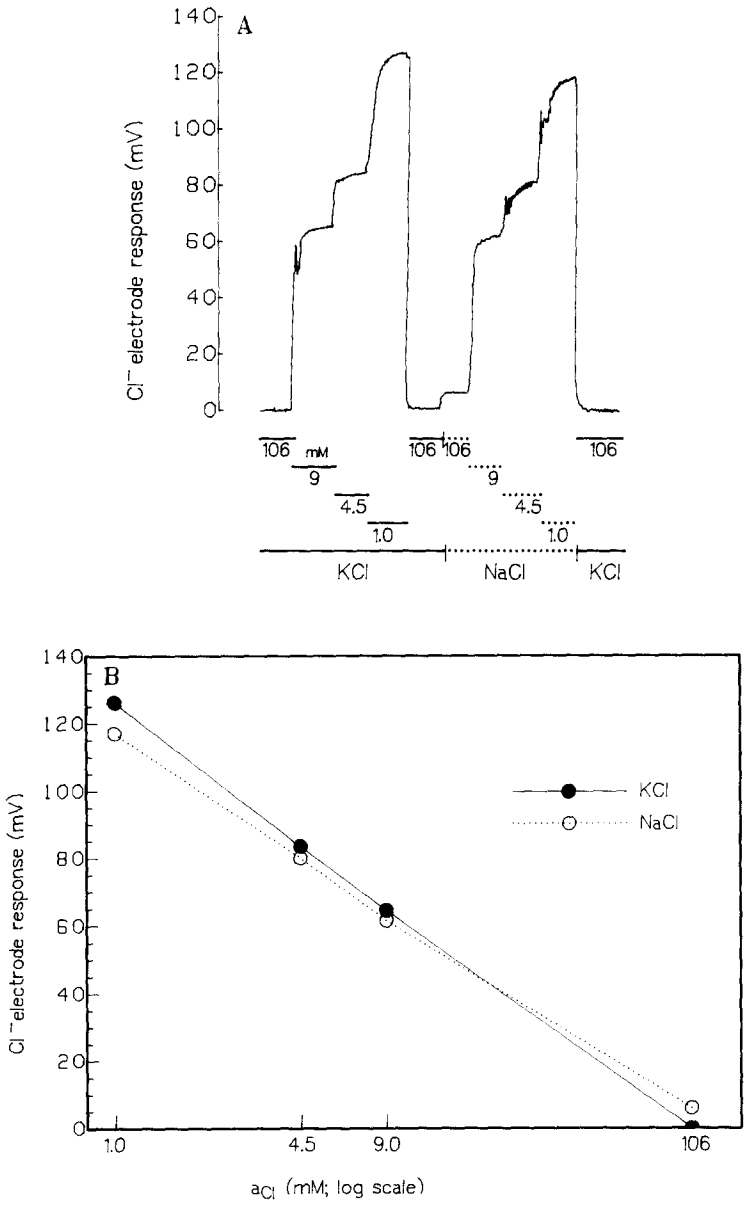

FIGURE 1. Effect of cations on the calibration of Cl^--sensitive electrodes. (*A*) Pen recording of a Cl^--sensitive electrode response to either pure KCl or pure NaCl solutions. Activities (millimolar) are marked below the recording for KCl (solid lines) and NaCl (dotted lines). Note the 4–5-mV potential 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_{Cl}^i.

selectivity may have produced a small overestimate of a^i_{Cl} because of residual internal HCO_3^-, although all experiments were performed in nominally HCO_3^--free solutions. In addition, the combined KCl/K_2SO_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^i_{Cl} would be produced.

One factor may have produced an underestimate of a^i_{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^i_{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 $a^o_{Cl} = 112$ mM, calculated a^i_{Cl} values would be 7.8 mM (uncorrected) and 9.1 mM ("corrected"). In the results presented, we calculated a^i_{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_{in}), 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:

$$G_m = 1/R_m = R_i/(R_{in}^2 \cdot \pi^2 \cdot d^3),$$

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^i_{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^i_{Cl} lies close to equilibrium in normal lumbrical muscle.

In denervated muscle, the results were different. We found that a^i_{Cl} rose steadily after denervation, and, more importantly, that the difference between E_{Cl} and V_m increased by a large amount. The results are shown in Fig. 2. a^i_{Cl}

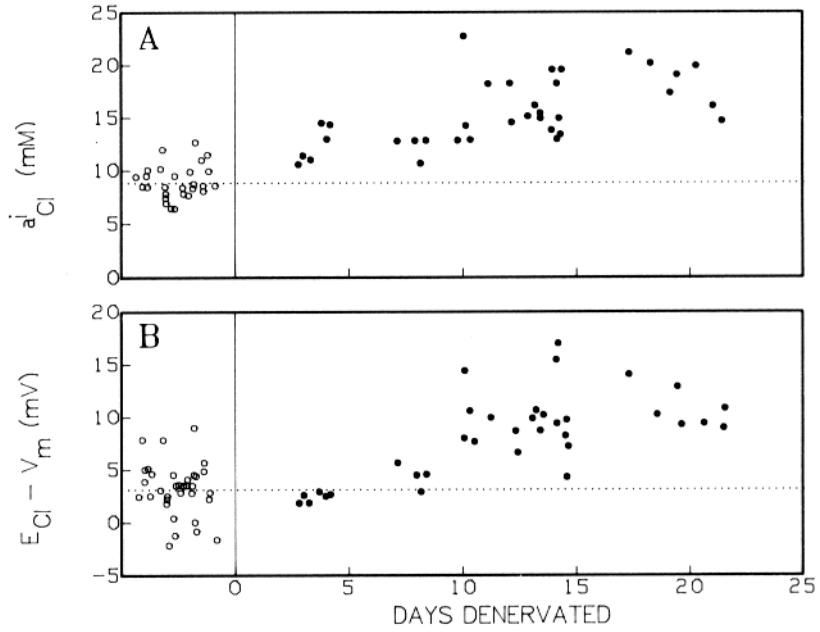

FIGURE 2. Time course of changes in Cl^- distribution after denervation. (*A*) Each symbol represents the averaged value of a^i_{Cl} in 10–20 fibers in one muscle. Data from 35 control muscles (428 fibers; open circles; the time scale has no significance for controls) and 35 muscles (471 fibers; filled circles) denervated from times ranging from 3 to 21 d. The dotted line shows the average value for control muscles. a^i_{Cl} increased steadily after denervation. (*B*) Each symbol shows the average values of $E_{Cl} - V_m$ for one muscle (same fibers as in *A*). The dotted line shows the average value for all control muscles. The increase in $E_{Cl} - V_m$ after denervation reflects increased active accumulation of the ion. This did not begin to change until 5–7 d after denervation. Note that the rise in a^i_{Cl} (*A*) observed at early times after denervation was not accompanied by an increase in $E_{Cl} - V_m$.

began to rise shortly after denervation (Fig. 2*A*). 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^i_{Cl} to rise passively to ~12 mM. Consistent with this, Fig. 2*B* shows that $E_{Cl} - V_m$ did not change for several days after denervation. After ~5 d, however, $E_{Cl} - V_m$ increased, and in muscles denervated ≥10 d, $E_{Cl} - V_m$ had more than tripled (to ~10 mV). The

difference between the observed a_{Cl}^{i} and the calculated passive values of a_{Cl}^{i} increased more than sixfold (to ~5.5 mM). In summary, a_{Cl}^{i} more than doubled (to 16.5 ± 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_{Cl} that follows denervation.

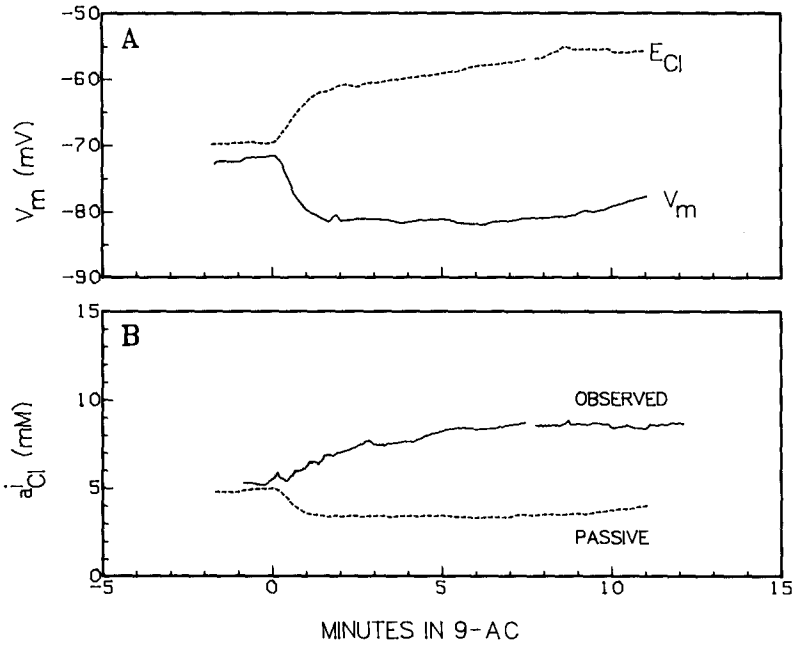

FIGURE 3. Effect of 9-AC (100 μM, applied at zero time) on V_m (*A*, solid line), E_{Cl} (*A*, dotted line), measured a_{Cl}^{i} (*B*, solid line), and calculated a_{Cl}^{i} assuming a passive distribution (*B*, dotted line). Voltage-recording and Cl^--sensitive electrodes were in the same muscle fiber. If Cl^- were passively distributed, the hyperpolarization caused by 9-AC should be caused a_{Cl}^{i} 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_{Cl} and a_{Cl}^{i} traces, the Cl^--sensitive electrode was partly dislodged from the fiber.

Effects of Inhibiting G_{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_{Cl}, it should be possible to unmask its activity by blocking G_{Cl}. Bolton and Vaughan-Jones (1977) confirmed this in frog sartorius muscle by reducing external pH, which reduces G_{Cl}. In the present study, we used 9-AC, which appears to be a selective inhibitor of G_{Cl} in rat skeletal muscle (Palade and Barchi, 1977*b*). 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}^{i}. 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}^{i}. Instead, a_{Cl}^{i} 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}^{i}.

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}^{i} had apparently risen to 40–50 mM.

Effects of Removing External Cl^-

When external Cl^- is replaced with an impermeant anion, a_{Cl}^{i} 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}^{i} (*B*) of replacing normal Krebs with Cl^--free solution. In this cell, the apparent a_{Cl}^{i} fell to 4.3 mM. We made similar recordings from 10 cells (five muscles). On average, the apparent a_{Cl}^{i} fell to 4.1 ± 0.2 mM in Cl^--free Krebs. In the same cells, the observed value of a_{Cl}^{i}, 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}^{i} (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}^{i} fell monotonically (Fig. 5*B*), 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}^{i} fell to 6.8 mM in Cl^--free solution. On average (eight cells), a_{Cl}^{i} 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^i_{Cl} to an even lower level (6.3 mM, compared with 8.1 mM after ~10 min in Cl^--free Krebs).

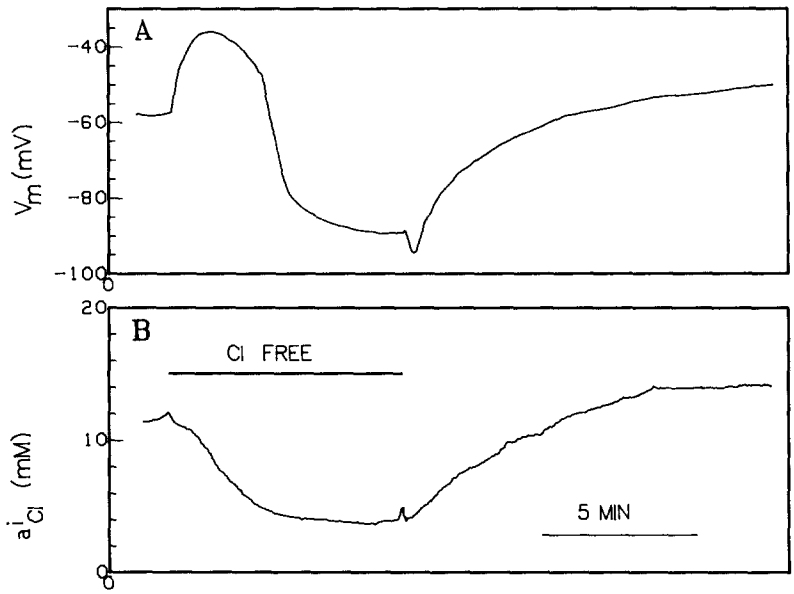

FIGURE 4. Effect in a normal muscle of Cl^--free solution on V_m (*A*) and a^i_{Cl} (*B*). During the time indicated by the horizontal line, Cl^--free Krebs was applied while recording from a single fiber with both voltage-recording and Cl^--sensitive electrodes. V_m transiently depolarized and then repolarized to a hyperpolarized steady level. a^i_{Cl} fell from ~11 to ~4.5 mM in ~5 min. When the cell was returned to normal Krebs, both V_m and a^i_{Cl} recovered fully.

Effects of Cl^- Transport Inhibitors on a^i_{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 $E_{Cl} - V_m$. In addition, in one muscle, the effect of SITS (80 μM) was studied; it, too, had no significant effect on Cl^- distribution.

The increased difference between V_m and E_{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^- 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^+ and K^+ substitution and furosemide were observed. Treatment with SITS, however, did not significantly alter Cl^- 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^- was out of equilibrium decreased significantly. Both effects were reversible. One noteworthy difference between the responses of normal and denervated

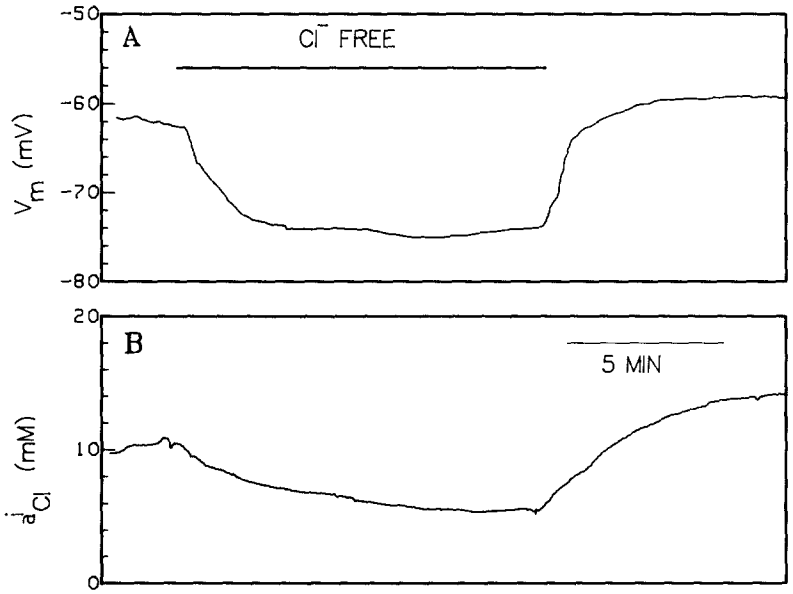

FIGURE 5. Effect in an 11-d-denervated muscle of Cl^--free solution on V_m (*A*) and a_{Cl}^i (*B*). During the time indicated by the horizontal line, Cl^--free solution was applied while recording from a single fiber with both voltage-recording and Cl^--sensitive electrodes. V_m monotonically hyperpolarized and a_{Cl}^i fell from ~10 to ~5 mM. The rate of Cl^- loss from the denervated fiber was about one-half that from the normally innervated fiber (cf. Fig. 4*B*). When the cell was returned to normal Krebs, both V_m and a_{Cl}^i recovered.

muscle was that innervated fibers depolarized and denervated fibers hyperpolarized in K^+-free solutions (cf. Betz et al., 1986); the inhibitory effects on Cl^- accumulation, however, were qualitatively similar. Furosemide produced a membrane hyperpolarization and also greatly reduced the amount by which Cl^- was out of equilibrium. All of these effects were reversible. In summary, Na^+ removal, K^+ removal, and furosemide all markedly reduced the amount by which Cl^- appeared to be out of equilibrium.

These results also provide evidence that the amount by which Cl^- appeared to be out of equilibrium ($E_{Cl} - V_m$) in denervated muscle was real, and was not due to anion interference. Suppose, for example, that Cl^- was actually distributed

TABLE I

Normal Muscle

	N	V_m	E_{Cl}	$E_{Cl} - V_m$	Passive a^i_{Cl}	Observed a^i_{Cl}	Observed − passive
			mV			*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)	−68.2±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	7.3±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

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); V_m is the membrane potential; E_{Cl} is the Cl^- equilibrium potential (calculated from measured a^i_{Cl}); passive a^i_{Cl} is the predicted a^i_{Cl} 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 E_{Cl} 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 E_{Cl} would hyperpolarize, reflecting the loss of Cl^-, but if the

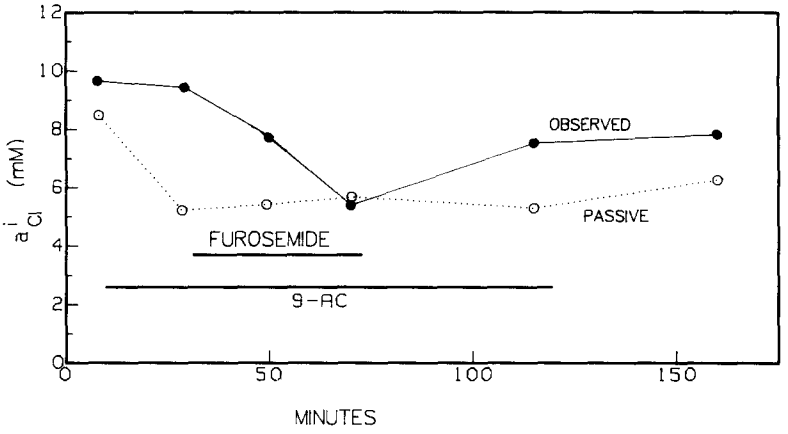

FIGURE 6. Effect of furosemide on a^i_{Cl}. Furosemide (10 μM) and 9-AC (100 μM) were applied during the times indicated by the heavy lines. The filled symbols show the observed a^i_{Cl}; the open symbols show the a^i_{Cl} values for a passive Cl^- distribution. Calculations for each symbol were made 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 (a^i_{Cl} 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 a^i_{Cl} to the passive level and the effect was partially reversible.

anion interference remained constant, the hyperpolarization of E_{Cl} would be less than the hyperpolarization of V_m. Thus, active Cl^- accumulation ($E_{Cl} - V_m$) 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_{Cl} after denervation leads to increased a^i_{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

TABLE II

Denervated Muscle

	N	V_m	E_{Cl}	$E_{Cl} - V_m$	Passive a^i_{Cl}	Observed a^i_{Cl}	Observed − passive
			mV			*mM*	
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	13.5±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)	−61.1±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.

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_{Cl}) was calculated (see Methods). Cl^- flux (i_{Cl}, $C/cm^2 \cdot s$), was calculated according to the relation given by Goldman (1943) and Hodgkin and Katz (1949):

$$i_{Cl} = \frac{p_{Cl}\, bzFV_m[a^i_{Cl}\, e^{(bV_m)} - a^o_{Cl}]}{e^{(bV_m)} - 1}, \quad (1)$$

where $b = zF/RT$. The membrane permeability to Cl^-, p_{Cl}, was calculated from G_{Cl} by equating measured G_{Cl} with the first derivative of Eq. 1:

$$G_{Cl} = di_{Cl}/dV_m = \frac{p_{Cl}\, bzF}{e^{(bV_m)} - 1}\left[\frac{bV_m(a^o_{Cl} - a^i_{Cl})\, e^{(bV_m)}}{e^{(bV_m)} - 1} + a^i_{Cl}\, e^{(bV_m)} - a^o_{Cl}\right]. \quad (2)$$

This equation was then solved for p_{Cl}. Finally, with this value of p_{Cl}, the 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^i_{Cl} after denervation can be accounted for with reasonable quantitative accuracy by assuming that as G_{Cl} falls by nearly fourfold after denervation, continued inward pumping causes a^i_{Cl} to rise, increasing the driving force for passive 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_{in}	Diameter	G_m	G_{Cl}	p_{Cl}	Flux
			mV	*MΩ*	*μm*	*μS/cm²*	*μS/cm²*	*cm/s × 10⁻⁶*	*pmol/cm²·s*
Control	Krebs	37 (4)	−70.1±0.3	1.1±0.1	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±36	164±40	1.27	16.9
Denervated	0 Cl	40 (4)	−69.8±0.4	4.1±0.3	—	80±6	—	—	—

Effect of denervation on membrane properties and calculated Cl^- flux. Measured values of V_m, input resistance (R_{in}), fiber diameter, and specific membrane (G_m) and Cl^- (G_{Cl}) conductances are shown, together with calculated Cl^- permeability (p_{Cl}) and 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^i_{Cl} upon switching to a Cl^--free solution (Figs. 4 and 5). The rate of change in a^i_{Cl} is given simply as (units in parentheses):

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

Flux was calculated according to Eq. 1 with $a^o_{Cl} = 0$. Since V_m changes during removal of external 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_{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 doubtlessly 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_{Cl} in Denervated Muscle

A key event in this sequence of events after denervation is clearly the decrease in G_{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_{Cl}, leading to further Cl^- accumulation and a further reduction in G_{Cl} in a cycle of positive feedback. Therefore, in this scheme, the fall in G_{Cl} is triggered by the initial rise in a^i_{Cl}.

If this were correct, then reducing a^i_{Cl} in denervated muscle should produce a large increase in input conductance (G_{in}) as Cl^- channels become unblocked. To test this idea, we exposed denervated muscles to 10 μM furosemide, which caused a^i_{Cl} to fall to nearly its equilibrium value. We found that G_{in} did not change significantly after exposure to furosemide. In randomly sampled denervated fibers, G_{in} = 415 ± 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^i_{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, 1982*a*, *b*; rat EDL, McCaig and Leader, 1984; mouse EDL, Donaldson and Leader, 1984). Our recordings consistently suggested that a^i_{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^i_{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^i_{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_{Cl} away from V_m. When we reduced G_{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, 1977*b*), the hyperpolarization suggests that Cl^- is actively accumulated in normal muscle. Moreover, a^i_{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^i_{Cl} provides strong evidence for the presence of active Cl^- accumulation.

Other observations also support this conclusion. The increase in a^i_{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., 1984*b*, 1986).

Cl^- Transport in Denervated Muscle

The measured a^i_{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^i_{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^i_{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^-/HCO_3^- exchange. This is somewhat surprising, since in both cardiac (Vaughan-Jones, 1982*a*, *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 HCO_3^-, however, and whether Cl^-/HCO_3^- 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., 1984*a*). 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., 1984*b*). 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 a_{Cl}^i to be 3–6 mM higher and V_m 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; V_m 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, a_{Cl}^i is increased, and Cl^- comes to lie further from equilibrium than in innervated muscle.

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