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Intracellular Chloride Activity in the Extensor Digitorum Longus (EDL) Muscle of the Rat

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Summary. Measurement of intracellular chloride activity in the *extensor digitorum longus* (EDL) muscle of the rat using liquid ion-exchanger microelectrodes gave an apparent resting value of 10 to 11 mmol liter^{-1}. If chloride ions were distributed passively across the muscle fiber membrane the predicted value would be 4 mmol liter^{-1}. In experiments in which the bathing fluid was changed by (a) reduction of external chloride, or incubation at low external chloride followed by a return to normal concentrations, (b) an increase or reduction in external potassium, (c) alteration in potassium and chloride in the bathing medium so as to maintain a constant [K] × [Cl] product, or in other experiments in which the membrane potential was caused to change by anoxia, or by addition of ouabain to the medium, changes in intracellular chloride activity were invariably consistent with the hypothesis that this ion is passively distributed. Measurements of intracellular chloride activity with recessed-tip solid-state Ag/AgCl electrodes gave a value of 4.6 mmol liter^{-1}. Since the liquid ion-exchanger is known to be poorly selective for chloride, it is concluded that the chloride ion is passively distributed in rat EDL muscle.

Key Words rat EDL muscle · chloride activity · ion-selective microelectrodes

Introduction

The Goldmann-Hodgkin-Katz (GHK) equation, based on the so-called constant field assumption (Goldmann, 1943; Hodgkin & Katz, 1945) describes the resting membrane potential of excitable animal cells in terms of the steady-state distribution of the major permeant ions and their respective permeabilities in the membrane; thus

$$\Delta\psi = \frac{RT}{F} \ln \frac{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o} \quad (1)$$

where $\Delta\psi$ is the membrane potential, R is the gas constant, T the temperature in degrees Kelvin, F is the Faraday, P the permeability of the ion subscripts and $[\ ]_i$ and $[\ ]_o$ refer to the activity of the ion inside and outside the cell.

It is a commonly made assumption that animal cell membranes are in general rather more permeable to chloride ions than to sodium or potassium (Conway, 1957), and thus the passive distribution of chloride approximates to electrochemical equilibrium. If correct, this permits the simpler description (Hodgkin, 1958)

$$\Delta\psi = \frac{RT}{F} \ln \frac{[K]_o + b[Na]_o}{[K]_i + b[Na]_i} \quad (2)$$

where $b = P_{Na}/P_K$.

Furthermore, if b is small, the contribution of sodium to the steady-state membrane potential is minimal, and over a wide range of external concentrations of potassium and chloride the membrane potential will behave as if these two ions were distributed in thermodynamic equilibrium (Donnan equilibrium) (Boyle & Conway, 1941). That is, $\Delta\psi$ will approximate the equilibrium potential of these ions:

$$\Delta\psi = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i} = \frac{RT}{F} \ln \frac{[Cl]_o}{[Cl]_i} \quad (3)$$

and

$$[K]_i \times [Cl]_i = [K]_o \times [Cl]_o. \quad (4)$$

(It should be noted that such a treatment ignores any active potassium transport which must occur, via the (Na,K)-ATPase, across the membrane. This approach can be justified if K permeability is high and the ion is distributed close to electrochemical equilibrium).

Such behavior has been demonstrated in experiments in which the effect upon the membrane po-

tential of excitable tissues of changing external concentrations of potassium and chloride, or both, has been studied (e.g. Boyle & Conway, 1941; Hodgkin & Horowicz, 1959; Adrian, 1960; Harris, 1963).

Direct proof of the passive distribution of the chloride ion has proved difficult, in part because of the problem of distinguishing between intracellular and extracellular chloride. With the advent of chloride-sensitive electrodes suitable for intracellular recording (Walker, 1971; Neild & Thomas, 1973), there has been renewed interest in obtaining direct evidence concerning the distribution of chloride ions.

In frog skeletal muscle, measurements with ion-selective electrodes have shown that intracellular chloride activity (aCl_i) always appears to exceed the value predicted for a purely passive distribution (Kernan, MacDermott & Westphal, 1974; Armstrong, Wojtkowski & Bixenman, 1977; Bolton & Vaughan-Jones, 1977; Baumgarten & Fozzard, 1978; Macchia & Baumgarten, 1979). It is difficult to decide whether this difference represents a true excess of intracellular chloride or is the result of interference from other intracellular anions, since the microelectrodes which have been used are imperfectly selective (Saunders & Brown, 1977).

Active accumulation of chloride in frog muscle has also been inferred from the studies of Hutter and Warner (1967) but only in conditions where chloride conductance was artificially reduced by exposure to acidic media. Recently, however, Hironaka and Morimoto (1980) have claimed that chloride accumulation is apparent under physiological conditions in this muscle. In addition, Dulhunty (1978) has reported dependence of the membrane potential on chloride concentration in some mammalian muscles, which can be interpreted as evidence for the active pumping of chloride into the fibers.

We have recently initiated experiments to examine the effect of denervation on the composition and membrane properties of the rat *extensor digitorum longus* (EDL) muscle (Bray et al., *submitted for publication*). Since there are no reports of aCl_i in mammalian muscle, the first step in characterizing the behavior of chloride was to determine the aCl_i in normal muscle. We report here the results of experiments in which the aCl_i was determined under a number of conditions to allow us to decide whether or not chloride distribution was passively determined in this muscle.

Materials and Methods

Preparation

Extensor digitorum longus (EDL) muscles were dissected from adult Wistar rats (weighing 200 g) and pinned through their tendinous ends to the silastic base of the recording bath. Liley's solution (composition in mmol liter^{-1}: Na^+, 140; K^+, 5; Ca^{2+}, 2; Mg^{2+}, 1; Cl^-, 132; HCO_3^-, 18; $H_2PO_4^-$, 1; glucose, 11) was used in one series of experiments. In most experiments, however, a bicarbonate-free solution was used in which HEPES (10 mmol liter^{-1}) was substituted for $NaHCO_3$. In one other series of experiments a phosphate-buffered solution was used; here 8 mmol liter^{-1} NaH_2PO_4 replaced $NaHCO_3$. HEPES- and phosphate-buffered solutions were equilibrated with 100% O_2, or in one instance, 100% N_2; bicarbonate-buffered solution was bubbled with a mixture of 95% O_2, 5% CO_2. The pH of all solutions was adjusted to 7.25, and the solutions flowed through the experimental bath at 1 to 2 ml min^{-1}. All experiments were carried out at room temperature (17 to 20°C).

Microelectrodes

For measurement of membrane potentials electrodes were made from capillary tubing containing an internal glass fiber (Clarke Electromedical) and which had an impedance of 15 to 20 MΩ when filled with 3 mol KCl and tested in Liley's solution. The reference half-cell was a large chlorided silver plate in 3 mol KCl, making electrical contact with the bath through a bridge of 3 mol KCl in 3% agar. No electrode was used if the initial potential measured between the bath and the indifferent electrode was greater than 3 mV. Potentials were recorded on an electrometer (WPI, KS701), and a permanent record made on a Grass recorder. Criteria for acceptance of a transmembrane potential measurement were that the rapid change in potential on entering a fiber should remain stable (within 0.5 mV) for at least 1 min, and that upon withdrawal the potential should return to within 2 mV of its initial value.

Chloride-sensitive electrodes were made from thin-walled inner-filament-containing glass tubing (Clark Electromedical). The electrodes were rendered hydrophobic by exposure to the vapor of dichlorodimethyl silane for 5 sec followed by baking at 100°C for 40 min. They were then back-filled to the shank with liquid ion-exchanger (Orion chloride resin). A fine chlorided silver wire was inserted into the resin and connected to one probe of a dual differential amplifier (WPI, F223A). The amplifier output was displayed on an oscilloscope and a permanent record obtained using a Grass pen recorder.

Electrodes were calibrated using a series of solutions of sodium or potassium chloride (1 to 0.001 mol liter^{-1}). Usable electrodes had an average slope of 55 mV (range 53 to 58) per tenfold change in chloride activity. Intracellular chloride activity was calculated using the empirical relation

$$aCl_i = aCl_o \cdot 10^{\frac{-\Delta E - \Delta\psi}{S}} \quad (5)$$

where aCl_i and aCl_o are the intracellular and extracellular activities of chloride, respectively, ΔE is the change in potential given by the chloride electrode on entering the fiber, $\Delta\psi$ is the mean membrane potential recorded from 10 to 15 fibers in the same muscle before and after chloride measurements, and S the empirically determined slope of the electrode.

The selectivity of the electrodes for Cl^- over a number of other anions was examined by calibrating the electrodes first in pure solutions of NaCl and then in appropriate mixtures of NaCl and the sodium salt of the particular anion. A somewhat better selectivity ratio was found between chloride and glucuronate than gluconate, propionate, acetate, or isethionate. Hence, in experiments where external chloride was altered, glucuronate was used as an anion substitute. In these experiments a term correcting for interference was introduced into Eq. (5):

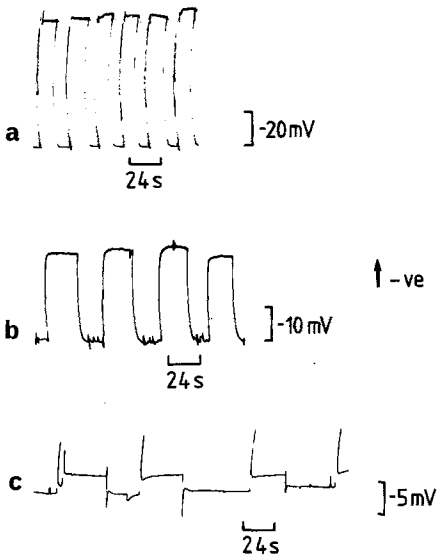

Fig. 1. Typical records of impalements of rat EDL muscles with (*a*) a conventional microelectrode, (*b*) a liquid-resin Cl-sensitive microelectrode and (*c*) a solid-state recessed-tip Ag/AgCl microelectrode

$$aCl_i = (aCl_o + k \cdot G^-)\, 10^{\frac{\Delta E - \Delta\psi}{S}} \quad (6)$$

where G^- is the concentration of glucuronate in the bathing medium, and k is the empirically determined selectivity coefficient for chloride over gluconate.

Solid-State Chloride Electrodes

Solid-state chloride electrodes were constructed by the method of Leader (1982). These electrodes behaved ideally in pure chloride solutions and did not appear to be subject to interference from other anions present in the experimental solutions.

Experimental Procedure

To measure the intracellular chloride activity, the following procedure was adopted. The muscle was first pinned out and a series of impalements (10 to 15) made with conventional microelectrodes to determine the mean resting membrane potential. A series of impalements of other fibers was then made using a previously calibrated chloride electrode. The long time constant of the chloride electrodes (up to 1 min) meant that the criterion for acceptable impalements was a relatively rapid change in the chloride electrode potential which remained stable for at least 5 min, and which returned to the original value when the electrode was withdrawn. To establish that the membrane potential had not changed during chloride activity measurement, a second series of impalements was then performed with a standard microelectrode. Finally the chloride electrode was recalibrated. If the potential given by the electrode in the standard solutions had changed by more than 2 mV the results were not used.

Table 1. Measured and predicted values of aCl_i and E_m in muscle incubated in differently buffered solutions

Buffer	Electrode	Apparent aCl_i	Predicted aCl_i	E_m
HEPES (40)[a]	liquid ion-exchanger	10.5 ± 0.2[b]	4.3	−79 ± 0.3
HEPES (5)	AgCl	4.6 ± 0.1	4.3	−79 ± 0.5
HCO_3^- (8)	liquid ion-exchanger	11.5 ± 0.2[b]	4.0	−81 ± 0.7
PO_4^- (15)	liquid ion-exchanger	11.1 ± 0.4	4.3	−79 ± 0.5

[a] Numbers in parentheses are the number of muscles studied. Approximately 15 cells per muscle were impaled with standard microelectrodes and 10 with Cl-sensitive microelectrodes.
[b] Values significantly different at $P < 0.01$.

Results

Intracellular Chloride Activity

The mean membrane potential in a series of 40 muscles incubated in HEPES-buffered solutions was −79 ± 0.3 mV. Although individual muscles gave potentials which ranged from −75 to −81 mV, in any one muscle acceptable impalements were always within 1 to 2 mV of the mean value for that muscle. Impalement of a fiber with a resin-filled electrode produced a negative deflection of about 25 mV. Some typical recordings are shown in Fig. 1. The recordings were stable and reproducible. The intracellular chloride activity calculated from 10 impalements in each of 40 preparations was 10.5 ± 0.2 mmol (Table 1). The activity did not change during repeated measurements in one muscle (up to 3 series of measurements over 2.5 hr).

When impalements were made using solid-state electrodes a much smaller deflection was observed (Fig. 1*c*). In this case the intracellular chloride activity was calculated to be 4.6 ± 0.1 mmol, a value close to that predicted from the Donnan relation (4.3 mmol). Resin-filled electrodes are known to be subject to a number of interferences from organic anions, and the higher value determined using these electrodes may be a result of this lack of specificity.

Intracellular Chloride Activity in Different Buffered Solutions

When muscles were incubated in phosphate-buffered solution, aCl_i was not significantly different from that measured in HEPES-buffered solution (Table 1). On the other hand when the bathing solution contained bicarbonate a significant increase in apparent aCl_i was found. The selectivity of the electrodes for chloride over bicarbonate was not a constant but was dependent upon the ionic strength of the solution. At chloride levels approaching those

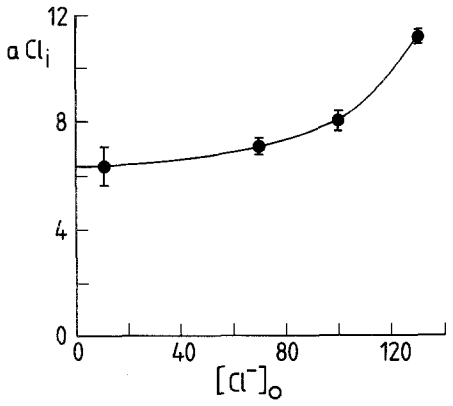

Fig. 2. The relationship between external chloride concentration and intracellular chloride activity in rat EDL muscle, measured with liquid-resin Cl^--sensitive microelectrodes. No correction has been made for possible interference

found intracellularly, selectivity was about 10:1, and thus an increase of 10 mmol in intracellular bicarbonate would increase estimated intracellular chloride by 1 mmol. For this reason, experiments involving changes in extracellular chloride were carried out using bicarbonate-free media.

Effect of Reduction of Extracellular Chloride

The Hodgkin-Huxley interpretation of the Boyle-Conway theory predicts that altering external chloride should only have transient effects upon the membrane potential of muscle fibers. Since in the steady state $\Delta\psi$ is close to the equilibrium potentials for potassium and chloride (E_K and E_{Cl}, respec-

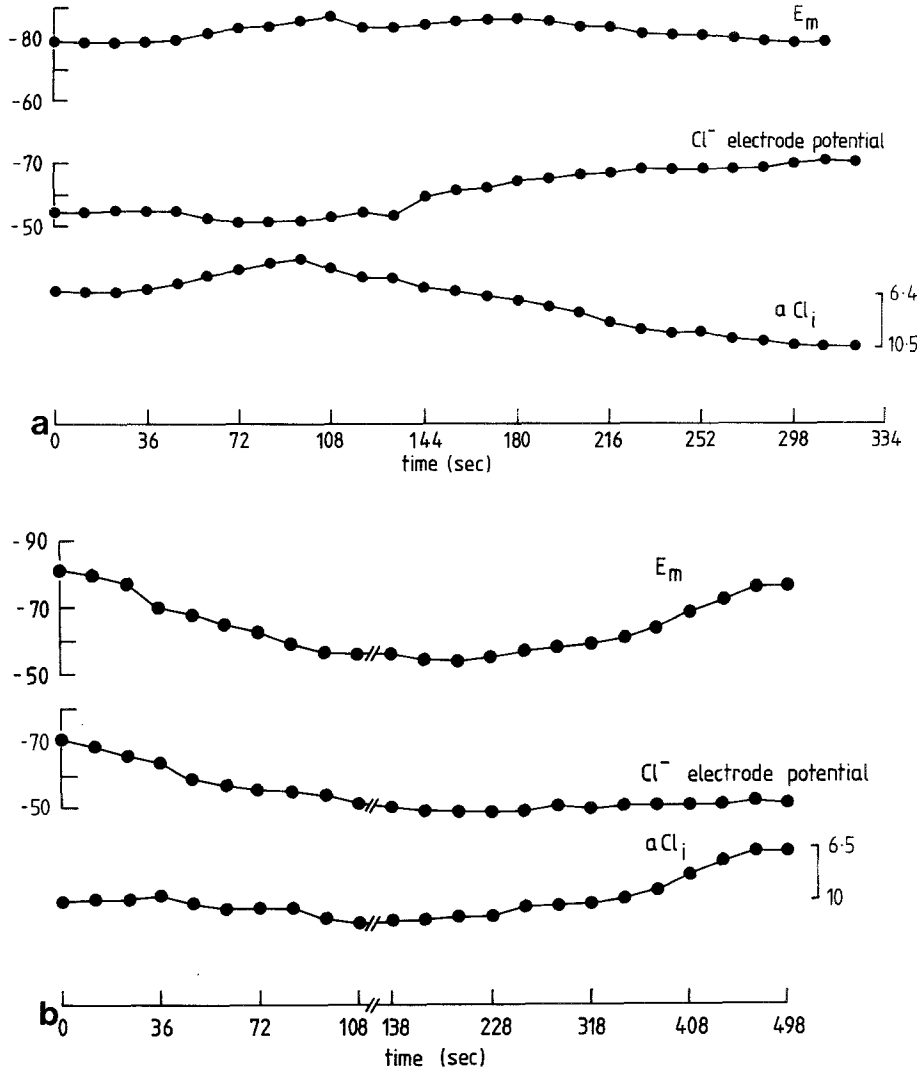

Fig. 3. Records of the effect of changing external chloride concentration upon membrane potential and measured intracellular chloride activity of rat EDL muscle. (*a*) at $t = 0$, external chloride was reduced from 129 mmol liter^{-1} to 11 mmol liter^{-1} by isosmotic substitution of sodium glucuronate for sodium chloride. (*b*) at $t = 0$, a muscle previously incubated for 2 hr in low chloride solution (as in (*a*) above), was transferred to normal bathing solution (Cl = 129 mmol liter^{-1}). Left-hand scales – electrode potential (mV); right-hand scales – intracellular chloride activity (mmol liter^{-1}) measured with liquid-resin microelectrodes, and calculated assuming no interference from other anions

tively), a sudden lowering of external chloride makes E_{Cl} more positive than $\Delta\psi$ while E_K remains unchanged. The membrane potential will thus fall initially to a value intermediate between E_{Cl} and E_K. This will occasion an outward diffusion of KCl, repolarizing the membrane. When a new steady state is established, $\Delta\psi$ should be virtually unchanged from its original value, intracellular potassium concentration hardly altered, and intracellular chloride reduced.

When muscles were incubated in solutions of 100 or 70 mmol liter^{-1} chloride, in which the rest of the chloride had been replaced with glucuronate, $\Delta\psi$ was virtually unchanged from control values 15 min after the reduction (control -130 mmol liter^{-1} Cl^-: -79 ± 0.4 mV; 100 mmol liter^{-1} Cl^-: -80 ± 1.0 mV; 70 mmol liter^{-1} Cl^{-1}: -77 ± 1.0 mV). When chloride was further reduced to 11 mmol liter^{-1} there was a significant reduction in $\Delta\psi$ to -72 ± 1.1 mV. As external chloride was reduced, there was a reduction in the steady-state intracellular chloride activity, as shown in Fig. 2. Calculated intracellular chloride activity shows a direct relationship with the $\log_{10}$ of external chloride concentration, as would be expected if chloride ions are passively distributed across the fiber membrane. If the line joining the measured points is extrapolated to zero external chloride, the apparent value of intracellular chloride at the intercept is 6.3 mmol liter^{-1}. This is the amount by which calculated intracellular chloride activity exceeds that expected in normal media for a passive distribution of the ion.

The aCl_i measured 15 min after a change in external chloride was stable and did not alter during a further 60 min. In order to examine the time course of the effects of lowering external chloride on $\Delta\psi$ and aCl_i, a further series of experiments was performed in which continuous recordings were made from fibers during changes in solution. A liquid-resin chloride electrode was inserted into one fiber and a standard electrode into an adjacent fiber. Since $\Delta\psi$ varies little between neighboring cells, aCl_i could be continuously estimated from the difference in recorded potential between the two electrodes. Results from a typical experiment are shown in Fig. 3 and data summarized in Table 2 and Fig. 4. On decreasing external chloride concentration from 130 to 11 mmol liter^{-1}, there was a rapid depolarization of 20 mV. The apparent increase in aCl_i at this time probably reflects the relatively slow response time of the chloride electrode, which could not follow the change in with sufficient rapidity. This suggestion is supported by the gradual return to control values. Over the next few minutes (average 7.5 min) the membrane potential rose again to reach a value close to the initial measurement. Simultaneously, measured aCl_i fell from 10.5

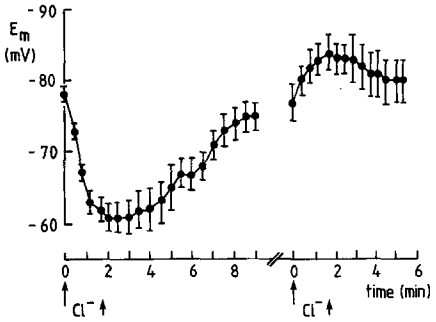

Fig. 4. The effect of a change in external chloride concentration on the membrane potential of the rat EDL muscle. Left: at $t = 0$, external chloride was reduced from 129 to 11 mmol liter^{-1} by isosmotic substitution of sodium glucuronate for sodium chloride. Right: at $t = 0$, muscles previously equilibrated for 2 hr in low chloride solution (11 mmol liter^{-1}) were transferred to normal bathing solution (Cl = 129 mmol liter^{-1}). Each point is the mean ± SEM of 10 measurements

Table 2. Changes in E_m and aCl_i produced by reducing the external chloride concentration[a]

	A				B		
	Control	Maximum depolarization	Start repolarization	Steady-state repolarization	Control	Maximum hyperpolarization	Steady-state repolarization
E_m (mV)	-79 ± 1	-59 ± 2.5	-59 ± 2.6	-74 ± 1.7	-77 ± 2.3	-84 ± 2.0	-79 ± 1.4
aCl_i (mmol liter^{-1})	5.9 ± 0.4	6.6 ± 0.6	6.2 ± 0.6	6.5 ± 0.5	0.6 ± 0.1	0.8 ± 0.4	5.1 ± 0.7
time (sec)	0	100 ± 8	209 ± 35	452 ± 54	0	83 ± 12	269 ± 28
$[Cl]_o$ (mmol liter^{-1})	130	11	11	11	11	130	130

[a] A: Reduction from 130 to 11 mmol liter^{-1} external chloride. Values are mean ± SEM in 9 cells from 6 different muscles. aCl_i values were determined by subtracting 6.5 mmol liter^{-1} from values measured with liquid ion-exchanger microelectrodes. B: Incubation in low external chloride (11 mmol liter^{-1}) and then exposure to normal (130 mmol liter^{-1}) chloride. Values are mean ± SEM for 5 cells from 3 muscles.

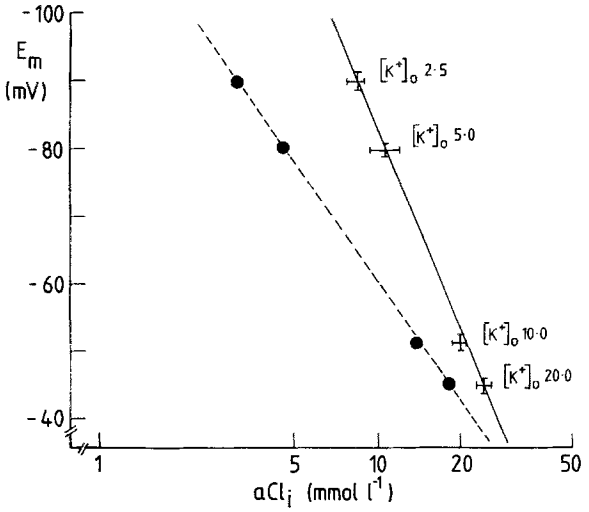

Fig. 5. The relationship between membrane potential and intracellular chloride activity of rat EDL muscle in solutions in which potassium concentration was altered by isosmotic substitution for sodium. Each point gives the mean ± SEM (n = 40) of calculated chloride activity, measured with liquid ion-exchanger chloride-sensitive microelectrodes assuming no interference from other ions; and the mean ± SEM (n = 40) of the membrane potential measured with conventional microelectrodes. Potassium concentrations of the bathing medium in each series of determinations are shown. Solid circles show the intracellular Cl^- activity calculated for the mean membrane potential from each set of data, assuming that the chloride ion is passively distributed

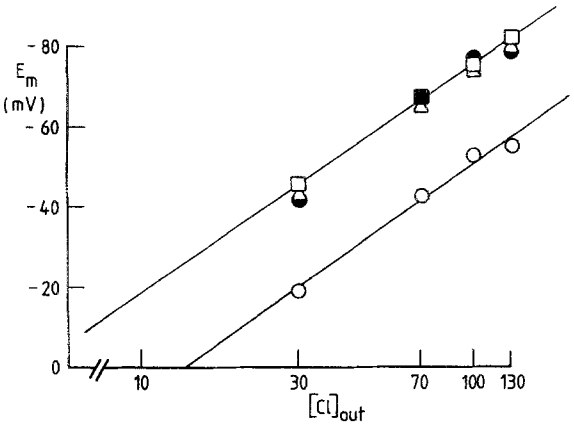

Fig. 6. The relationship between membrane potential, measured intracellular chloride activity, and calculated chloride activity, when external potassium and chloride are altered isosmotically at constant [K] × [Cl] product. ■ transmembrane potential measured with KCl-filled microelectrodes; ● transmembrane potential calculated from measured intracellular potassium concentrations determined in control muscles, assuming that a Donnan relation holds; ○ transmembrane potential calculated from aCl_i, measured with liquid ion-exchange chloride-sensitive microelectrodes, assuming no anion interference; △ transmembrane potential calculated from measured aCl_i, assuming that the liquid ion-exchanger is subject to a constant offset equivalent to 6.3 mmol $liter^{-1}$. *See* text for details

to 6.7 mmol $liter^{-1}$. Muscle fibers equilibrated in low external chloride and then exposed to normal chloride responded as shown in Figs. 3 and 4 and Table 2. There was first a hyperpolarization of about 7 mV, and then a gradual decrease into a steady-state value generally somewhat higher than the value in low chloride. The aCl_i increased steadily until a new steady-state value was reached in 4.5 min.

Effect on aCl_i of Altering K_o

If both potassium and chloride are passively distributed across the muscle fiber membrane, then raising K_o will cause the fibers to become depolarized to a new steady level. E_{Cl} will then be more negative than $\Delta\psi$, thus creating a driving force for entry of KCl into the fibers until E_K, E_{Cl} and $\Delta\psi$ are equal. On the other hand lowering K_o will hyperpolarize the cells, and, since E_{Cl} is now positive, a new steady state can only be achieved by loss of fiber KCl. Results of experiments in which steady-state values of $\Delta\psi$ and aCl_i were measured in fibers exposed to external potassium concentrations ranging from 2.5 to 20 mmol $liter^{-1}$ are shown in Fig. 5. It can be seen that, as external potassium is raised, the membrane potential falls, and calculated aCl_i rises, although at reduced potassium levels the hyperpolarization is less than expected. On Fig. 5 is also plotted the calculated value for intracellular chloride activity assuming that the ion is passively distributed. It can be seen, again, that there is an elevation of apparent aCl_i above the expected value of an almost constant 6 to 7 mmol $liter^{-1}$.

Effect of Varying $[K]_o$ and $[Cl]_o$ at Constant $[K]_o \times [Cl]_o$ Product

If potassium and chloride are passively distributed across the fiber membrane then it follows that if the external concentrations of these two ions are altered in such a way that their product remains constant, there should result a rapid change in $\Delta\psi$, with no transmembrane redistribution of either potassium or chloride. Experiments were performed to test this directly, by measuring $\Delta\psi$ and aCl_i in muscle fibers incubated in media in which external potassium was raised and chloride correspondingly reduced, so as to maintain a constant KCl product. The results, shown in Fig. 6, show that there is good

agreement between measured $\Delta\psi$ and that predicted from the Donnan relation, using control values to calculate K_i. E_{Cl}, however, calculated from measured aCl_i is consistently lower than measured $\Delta\psi$ by an amount which decreases as external chloride concentration is lowered.

Effects of Hypoxia, Ouabain and Sodium-Free Solutions on aCl_i

If chloride ions were actively accumulated by the muscle fibers by an energy-requiring mechanism, then any process which denied energy to the fibers should cause aCl_i to decrease towards electrochemical equilibrium. If, on the other hand, chloride was passively distributed, then as electrochemical gradients were dissipated $\Delta\psi$ would fall and aCl_i would rise. After 2 hr of exposure to 1 to 0% nitrogen, muscles were depolarized (Table 3). Intracellular chloride activity had risen during this period by 4.3 mmol liter^{-1}, compared with a rise of 3.6 mmol liter^{-1} predicted on the basis of a passive distribution.

Incubation of muscles in 10^{-2} mol liter^{-1} ouabain for one hour caused changes in $\Delta\psi$ and aCl_i similar to those occurring during hypoxia. The fibers became depolarized, and aCl_i rose by 2.5 mmol liter^{-1}, compared with the predicted 3.3 mol liter^{-1}.

Finally the possibility that chloride might enter the cells by a coupled mechanism was investigated by incubating muscle fibers in a sodium-free medium. If inward accumulation of chloride was coupled to the inward diffusion of sodium, then in sodium-free medium aCl_i should decrease towards electrochemical equilibrium. As shown in Table 3, however, aCl_i was unaffected for at least one hour following removal of external sodium.

Table 3. aCl_i and E_m under different experimental conditions[a]

	No. muscles	aCl_i[b]	Predicted aCl_i	E_m	Calculated E_{Cl}
Control	40	4.2 ± 0.2	4.3	−79 ± 0.3	−81
Nitrogen	4	8.3 ± 0.3	7.6	−65 ± 0.6	−63
Ouabain	4	7.1 ± 0.3	7.9	−65 ± 0.6	−47
Na^+-free	3	4.4 ± 0.1	4.5	−78 ± 0.8	−79

[a] N_2: after 2-hr exposure to 100% N_2; Ouabain: after 1-hr incubation with 10 mmol liter^{-1}; Na^+-free: 1 hr after change to Na^+-free solution.
[b] Corrected for anion interference.

Discussion

The initial aim of these experiments was to examine, using ion-selective microelectrodes, the assumption that the chloride ion is passively distributed across the mammalian muscle fiber plasma membrane. Direct measurements of intracellular chloride activity using liquid-ion exchange electrodes and solid-state recessed-tip Ag/AgCl electrodes gave conflicting results. Chloride activity determined with the Orion resin approximated 10 mmol liter^{-1}, while the Ag/AgCl electrode gave a much smaller value of 4.6 mmol liter^{-1}.

Many authors have pointed out that absolute measurement of intracellular chloride using ion-selective electrodes is difficult if not impossible, since the ion is present within cells at low activities, and thus measurement is influenced profoundly by interfering substances present within cells, e.g. bicarbonate, propionate, isethionate (Walker, 1971; Saunders & Brown, 1977; Walker & Brown, 1977). In the present experiments, for example, measurements of aCl_i with ion-exchange electrodes in bicarbonate-buffered media were about 1 mmol liter^{-1} higher than that in bicarbonate-free media. Since the selectivity of this electrode was found to be 10 : 1 for chloride over bicarbonate, the higher apparent intracellular chloride activity in bicarbonate-containing media resulted from the presence intracellularly of about 10 mmol liter^{-1} of bicarbonate. This is similar to that calculated for frog sartorius by Bolton and Vaughan-Jones (1977).

Aside from the effect of bicarbonate, residual measured activity was about 6 mmol liter^{-1} higher than required for chloride to be in thermodynamic equilibrium. For this reason, a recessed-tip Ag/AgCl electrode was built and, as Saunders and Brown (1977) point out, if results obtained using two different kinds of electrodes with different selectivity properties are similar, then there are good grounds for belief in their validity. Solid-state Ag/AgCl electrodes have not always behaved in a predictable fashion intracellularly. Neild and Thomas (1977), for example, found that such electrodes could develop a large offset. In the present work, however, it was found that the aCl_i determined with Ag/AgCl electrodes was 4.6 mmol liter^{-1}, a value substantially less than that given by the resin-filled electrodes, but close to that predicted for an equilibrium transmembrane distribution (4.3 mmol liter^{-1}). Although it can be argued that the values obtained with the solid-state electrodes are more likely to be valid because of the known lack of selectivity of the liquid ion-exchange resin, it was felt that further studies using both electrodes were required.

It was necessary, therefore, to alter the distribution of chloride in mammalian muscle in a variety of ways. As Hodgkin and Horowicz pointed out (1957), if chloride and potassium are passively distributed across the muscle fiber membrane, then re-

duction in external chloride should have predictable effects. As outlined in Results, initially the equilibrium potential for chloride will exceed the membrane potential and there will be an outward movement of potassium chloride until a new equilibrium is established with little alteration in membrane potential but an appreciable decrease in cellular chloride activity. Such behavior was observed except at low external concentrations of chloride. This may have been due to the fact that glucuronate, used as a chloride substitute, was not completely impermeant. Nevertheless, in bathing media with reduced chloride, the magnitude of the change in intracellular chloride activity was exactly that predicted from the assumption of passive behavior. Furthermore, when the plot of the measured aCl_i is extrapolated to zero external chloride, the value at the intercept, where aCl_i would also be zero, if the ion was passively distributed, is 6.3 mmol liter^{-1}. This value is similar to the difference between the activity measurements given by the two kinds of chloride-sensitive electrodes. Thus these results are consistent with the hypothesis that the Ag/AgCl electrode gives an accurate measure of aCl_i, but that the liquid ion-exchanger electrode is subject to interference which results in an erroneously high reading. Spring and Kimura (1978), studying *Necturus* renal proximal tubule, found an offset of similar magnitude when aCl_i was measured under conditions where intracellular chloride activity should have been zero.

When the muscles are exposed to changes in the potassium concentration of the bathing medium, the prediction based on passive behavior of potassium and chloride is that the fibers will reach a new equilibrium following transmembrane movement of potassium chloride in which the membrane potential and the intracellular activities of potassium and chloride are all changed. Again, except at low external potassium concentrations, the changes in intracellular chloride were entirely as predicted for passive behavior.

The hypothesis of passive distribution of chloride is further reinforced by the results of experiments in which the concentrations of potassium and chloride in the bathing medium were altered in such a way as to give a constant [K] × [Cl] product. If the membrane potential is determined largely by the permeability to these two ions, then such changes should change the transmembrane potential without net ion movement. Again, behavior was exactly as predicted. However, as before, changes in E_{Cl} measured using liquid ion-exchanger electrodes paralleled the changes in membrane potential, but fell about 23 mV below it. If it is assumed that the electrode is subject to a constant offset caused by interference from some unknown source, then corrected values fall almost exactly on the line predicted from the Nernst relation.

If the distribution of the chloride ion was determined in part by an energy-dependent mechanism, then interference with metabolism should affect the transmembrane distribution of chloride. Anoxia caused no direct effect on chloride distribution; as ionic gradients became dissipated the membrane potential fell and intracellular chloride activity rose at the expected rate. A similar result was found in other experiments, not reported here, in which the metabolic poison iodoacetate was used. In the presence of ouabain, similarly, the fall in membrane potential caused by poisoning of the (Na,K)-ATPase was paralleled by an equivalent rise in intracellular chloride. Finally, the absence of medium sodium did not affect cellular chloride activity (Table 3). Therefore, it is unlikely that intracellular chloride is displaced from equilibrium by a mechanism involving the entry of neutral NaCl followed by energy-dependent outward pumping of sodium ions.

Through the experiments reported here cannot prove conclusively the absence either of coupled movement between chloride and other ions, or of any energy-dependent mechanism, all the results support the hypothesis that the chloride ion is passively distributed across the mammalian muscle fiber membrane. Measurements of the rate of change of intracellular chloride in response to changes in the bathing medium indicate that the transmembrane chloride conductance is high, and thus that the chloride ion must be distributed close to equilibrium. That chloride distribution can be accounted for by passive behavior may, however, merely reflect the high membrane permeability to this ion.

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