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INTRACELLULAR CHLORIDE AND THE MECHANISM FOR ITS ACCUMULATION IN RAT LUMBRICAL MUSCLE

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SUMMARY

1. Double-barrelled Cl^--sensitive microelectrodes have been used to measure the intracellular Cl^- activity (a^i_{Cl}) and membrane potential (E_m) in rat lumbrical muscles. The mean Cl^- equilibrium potential (E_{Cl}), calculated from the measured a^i_{Cl} in sixty fibres, was $2{\cdot}9 \pm 2{\cdot}5$ mV (S.D. of an observation) less negative than E_m. The value of a^i_{Cl} was higher than would be expected for a passive distribution, by a mean $1{\cdot}4 \pm 1{\cdot}2$ mM. The mean E_m was $-59{\cdot}5 \pm 8{\cdot}2$ mV.

2. Removal of external Cl^- (Cl^-_o) resulted in a rapid fall in a^i_{Cl} and a transient depolarization. a^i_{Cl} stabilized at an apparent level of $1{\cdot}7 \pm 1{\cdot}0$ mM ($n = 24$) while E_m became substantially more negative than in normal Krebs solution (mean, $-80{\cdot}1 \pm 12{\cdot}4$ mV). Readdition of Cl^-_o caused a rapid rise in a^i_{Cl} and transient hyperpolarization. E_{Cl} quickly became less negative than E_m and both then fell in parallel towards the levels previously recorded in normal Krebs solution.

3. If lack of selectivity of the Cl^--sensitive ion exchanger and the intracellular presence of interfering anions, assumed to be responsible for the apparent a^i_{Cl} recorded in Cl^--depleted fibres, were also responsible for the apparently non-passive Cl^- distribution recorded under normal conditions, the difference between the calculated E_{Cl} and E_m would increase at more negative potentials. This was not observed over a range of E_m values between -46 and -84 mV.

4. Inhibition of the Cl^- permeability by application of 9-anthracene carboxylic acid (9-AC) resulted in an immediate rise in a^i_{Cl} and hyperpolarization. An a^i_{Cl} up to 40 mM higher, or eleven times higher, than that predicted by a passive distribution was recorded. Application of 9-AC after depletion of intracellular Cl^- in Cl^--free solution had no effect on either the apparent a^i_{Cl} or E_m.

5. It is concluded that Cl^- ions are actively accumulated by the skeletal muscle fibre and that the Cl^- distribution therefore normally exerts a depolarizing influence.

6. In the presence of 9-AC and nominal absence of CO_2 and HCO_3^-, readdition of Cl^-_o to Cl^--depleted fibres resulted in a substantial rise in a^i_{Cl} and a small, maintained

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depolarization. This clear demonstration of active accumulation was used to investigate the mechanism responsible for inward transport of Cl^- ions.

7. Neither application of CO_2 and HCO_3^- nor application of DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid) had any effect on the accumulation of Cl^- ions. This suggests that Cl^-–HCO_3^- exchange is not involved.

8. Removal of Na_o^+ or K_o^+ resulted in a limited rise in a_{Cl}^i such that E_{Cl} approximated to E_m. Removal of both cations had no greater effect than the absence of only one. Readdition of the cations caused an immediate accumulation of Cl^- ions and divergence of E_{Cl} from E_m. This was largely inhibited by the presence of frusemide. These results suggest that a Na^+, K^+, Cl^- co-transport mechanism is responsible for the inward transport of Cl^-.

INTRODUCTION

Chloride ions are widely believed to be passively distributed across the skeletal muscle sarcolemma. In other words, the intracellular Cl^- is believed to be governed by the extracellular Cl^- and the membrane potential (E_m) with the Cl^- equilibrium potential (E_{Cl}) equal to E_m. Certainly, the dominating Cl^- conductance (50–90% of the resting membrane conductance; e.g. see Hodgkin & Horowicz, 1959; Palade & Barchi, 1977*a*) would compromise the effectiveness of any mechanism actively transporting Cl^- ions. Yet indirect evidence from observation of changes in E_m has suggested that Cl^- ions may be actively transported into the sarcoplasm (Hutter & Warner, 1967; Dulhunty, 1978; Betz, Caldwell & Kinnamon, 1984). Direct measurement of the intracellular Cl^- activity (a_{Cl}^i) under normal conditions has, however, failed to produce convincing evidence for or against a non-passive Cl^- distribution (Kernan, MacDermott & Westphal, 1974; Bolton & Vaughan-Jones, 1977; Macchia & Baumgarten, 1979; McCaig & Leader, 1984; Donaldson & Leader, 1984; Harris & Betz, 1987). But technical problems, mainly due to the lack of specificity of the Cl^--sensitive microelectrodes, prevent sufficiently accurate or trustworthy determination of low levels of Cl^- for these measurements to be conclusive.

If Cl^- ions are actively transported into skeletal muscle, the effect of the transport should become unequivocally detectable with Cl^--sensitive electrodes when the Cl^- permeability (P_{Cl}) is reduced and a_{Cl}^i thus elevated. Indeed, active Cl^- accumulation has been clearly demonstrated with Cl^--sensitive electrodes in both cardiac (Vaughan-Jones, 1979*a*) and smooth muscle (Aickin & Brading, 1982) where P_{Cl} is naturally low. Bolton & Vaughan-Jones (1977) were the first to measure the development of an undeniably non-passive Cl^- distribution in skeletal muscle following reduction of P_{Cl}. Their measurements of a_{Cl}^i in frog sartorius muscle showed a notable inequality between E_{Cl} and E_m after reduction of P_{Cl} in solutions of low pH. This finding directly confirmed the earlier conclusion of Hutter & Warner (1967), formed from the observation of a transient depolarization on return to solutions of more alkaline pH (higher P_{Cl}). Recently, Harris & Betz (1987) have shown that reduction of P_{Cl} in rat lumbrical muscles, either by application of 9-anthracene carboxylic acid (9-AC; Palade & Barchi, 1977*b*) or by denervation (Camerino & Bryant, 1976), causes a significant intracellular accumulation of Cl^- ions. A

difference between E_{Cl}, determined by direct measurement of a^i_{Cl}, and E_m of nearly 50 mV was recorded after prolonged exposure to 9-AC, while a difference of about 10 mV was recorded ten or more days after denervation.

Clear demonstration of active Cl^- accumulation raises the question of the underlying mechanism. Cl^-–HCO_3^- exchange has been shown to be largely responsible for the high a^i_{Cl} in both cardiac (Vaughan-Jones, 1979*b*, 1986) and smooth muscle (Aickin & Brading, 1984) but seems unlikely to be so in skeletal muscle (Bolton & Vaughan-Jones, 1977; Harris & Betz, 1987). In experiments relying mainly on brief, independent impalements with voltage and Cl^--sensitive electrodes, Harris & Betz (1987) showed that the high a^i_{Cl} of denervated muscle was reversibly reduced to that predicted from a passive distribution by the removal of external Na^+ or K^+ or by application of frusemide. Thus Na^+, K^+, Cl^- co-transport seems likely to be responsible (see also Betz *et al.* 1984; Betz, Caldwell & Harris, 1986).

We have now further investigated the Cl^- distribution in rat lumbrical muscles, both under normal conditions and in the presence of 9-AC when undeniably active accumulation of Cl^- is revealed. Double-barrelled Cl^--sensitive microelectrodes (Aickin, 1981) were used which enabled continuous recording of a^i_{Cl} and E_m while external Cl^- was removed and reapplied under a variety of conditions. The results confirm that a Na^+- and K^+-dependent, frusemide-sensitive mechanism is responsible for Cl^- accumulation in the presence of 9-AC. Observations in the absence of 9-AC are consistent with effective accumulation of Cl^- ions under normal conditions.

A preliminary report of some of these results has been published (Aickin, Betz & Harris, 1988).

METHODS

Preparation and solutions

These were generally the same as those described previously by Harris & Betz (1987). The second, third and fourth deep lumbrical muscles of adult Sprague–Dawley rats (150–250 g) were used. They were pinned through the tendons to the bottom of a Sylgard-lined chamber (volume, 0·5 ml) and continually superfused at about 6 ml/min with a modified Krebs solution of the following composition (mM): NaCl, 136; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 11; buffered with 2 mM-PIPES (piperazine-*N*,*N*′-bis(2-ethanesulphonic acid)) and H_2SO_4 to pH 7·4 at room temperature (20–22 °C) and equilibrated with 100% O_2. Chloride-free solutions were made with equimolar replacements with sodium and potassium isethionate and $MgSO_4$ while $CaSO_4$ was increased to 10 mM to compensate for binding by isethionate. Sodium-free solution was made by substitution with choline and the preparation was pre-treated by exposure to α-bungarotoxin (Sigma; 4 μg/ml) for 15 min to prevent activation of aceltylcholine receptors. Potassium-free solutions contained 141 mM of the relevant sodium salt. Carbon dioxide–bicarbonate-buffered solutions were equilibrated with 5% CO_2, 95% O_2 and contained 24 mM-HCO_3^-. 9-Anthracene carboxylic acid (9-AC; Sigma) was added from a 2×10^{-2} M stock solution dissolved in ethanol to give a final concentration of 100 μM. Although control experiments to determine whether ethanol itself had any effect were not performed, the fact that application of the standard dose of 9-AC had no effect on either E_m or a^i_{Cl} in Cl^--free conditions (see Fig. 4) suggests these would have proved negative. Tetrodotoxin (Sigma; 1 μg/l) was present in all solutions to prevent fibrillation on removal of Cl^- or application of 9-AC. Frusemide (LyphoMed, Inc.) was added from a stock solution (10 mg/ml) to give a concentration of 10 μM. DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; Eastman Kodac) was added directly to the required solutions immediately prior to use.

Chloride-sensitive microelectrodes

Double-barrelled microelectrodes, as previously described (Aickin, 1981), were used in an attempt to alleviate the problems of instability encountered in this preparation with independent

voltage- and Cl^--sensitive electrodes (Harris & Betz, 1987). Micropipettes, of total resistance around 15 MΩ when filled with 3 M-KCl, were silanized by dipping their tips in a dry 1% solution of tri-*n*-butylchorosilane in 1-chloronaphthalene for 20 s and then baking for at least 24 h at 180 °C. The Cl^--sensitive ion exchanger (Corning Code 477315) was introduced into the back of one barrel and the electrode stored under reduced pressure for 40 min before the reference liquid ion exchanger (Thomas & Cohen, 1981) was added to the other barrel. The electrode was then stored under reduced pressure overnight. Any bubbles remaining in the columns of ion exchanger were removed by judicious application of heat from a fine soldering iron. The remainder of both barrels was then filled with 147 mM-KCl, taking care to ensure a single interface.

Electrodes were calibrated both before and after impalement in modified Krebs solution against a 3 M-KCl–agar electrode. A small shift (2–5 mV) was recorded between the reference liquid ion exchanger and this bath reference on removal of Cl_o^- and a similar shift was observed if a flowing 3 M-KCl reference was used. The origin of this shift was not determined and no correction was made for its occurrance. Thus E_m recorded in Cl^--free solution may have been overestimated (and consequently the apparent a_{Cl}^i underestimated) but the changes in E_m recorded on alteration of Cl_o^- were always larger and of a different time course to the extracellularly recorded shift. Glucuronate and gluconate were used to substitute for Cl^- in the calibrating solutions since these anions cause minimal interference with the Cl^--sensitive ion exchanger (see Bolton & Vaughan-Jones, 1977). Unfortunately, insufficient quantities of these substitutes were available during the period in which these experiments were performed for them to be used other than for calibration purposes. Isethionate does cause significant interference (the same potential was recorded in Cl^--free, isethionate-substituted solution as in the 10% Cl^- calibration solution; see also Saunders & Brown, 1977) and so is much less convenient for electrode calibration, but it is unlikely to cross the fibre membrane and add to the intracellularly recorded signal: the ion probably does not permeate Cl^- channels (see Coombs, Eccles & Fatt, 1955) and the pK_a' is sufficiently low for permeation in the associated form to be negligible (Sharp & Thomas, 1981). The electrodes used gave a 49·5–56·5 mV response (mean 54·6 ± 1·7 mV, $n = 23$, S.D. of an observation) on changing Cl^- from 100 to 10%, and a 73–103 mV response (mean 92·6 ± 6·3 mV, $n = 23$) on changing Cl^- from 100 to 1%. The deviation from a Nernstian response was equivalent to that predicted in the presence of a constant interference. Thus the electrode's response could be described by the following equation:

$$V_{Cl} = 58 \log \frac{(112 + I_o)}{(a_{Cl} + I_o)},$$

where V_{Cl} is the potential recorded by the electrode relative to that recorded in normal Krebs solution (a_{Cl} of 112 mM, calculated from an assumed activity coefficient of 0·764; Aickin & Brading, 1982), a_{Cl} is the prevailing Cl^- activity and I_o, the interference. I_o was considered to represent an apparent interference due to the diminished sensitivity of the ion exchanger in fine-tipped electrodes (see Aickin & Brading, 1982) and was between 0·8 and 5·3 mM a_{Cl} equivalents in the electrodes used. This equation was used to calculate the intracellular Cl^- activity from the recorded intracellular V_{Cl}. The Cl^- equilibrium potential, E_{Cl}, could then be calculated.

The Cl^--sensitive ion exchanger is sensitive to 9-AC, DIDS and frusemide and their presence at the concentrations used adds significantly to the extracellularly recorded Cl^- signal. Intracellular permeation at these concentrations is, however, not sufficient to add to the apparent a_{Cl}^i recorded in Cl^--depleted fibres.

All means are given ± S.D. of an observation.

RESULTS

Measurement of a_{Cl}^i and the effect of removal of external Cl^-

Despite the fact that the double-barrelled Cl^--sensitive microelectrodes often caused significant damage in the rat deep lumbrical muscle fibres, as judged from the relatively low membrane potentials, stable impalements lasting for more than 2 h were not uncommon. One such impalement is illustrated in Fig. 1. The mean E_m recorded in sixty fibres was −59·5 ± 8·2 mV with a range of −46 to −84 mV.

Impalements in which E_m declined to values less negative than -46 mV were discarded. In all of these fibres, and even in those discarded because of an excessively low E_m, E_{Cl}, calculated as described in the Methods, was never more negative than E_m. The mean difference between E_{Cl} and E_m was $2{\cdot}9 \pm 2{\cdot}5$ mV with a range of 0–12·8 mV. The mean value of a^i_{Cl} was $1{\cdot}4 \pm 1{\cdot}2$ mM (with a range of 0–5·6 mM) higher than that predicted for a passive distribution.

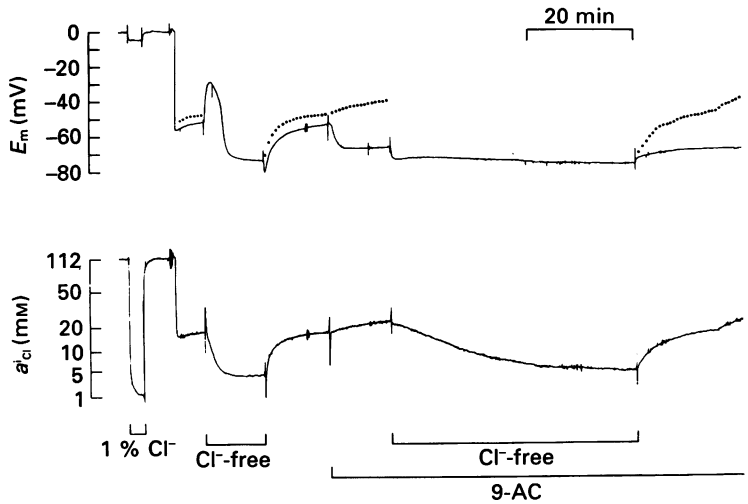

Fig. 1. Pen recordings of the simultaneous measurement of membrane potential (E_m, shown at the top) and intracellular Cl^- activity (a^i_{Cl}, shown at the bottom) in a rat deep lumbrical muscle, both before and after inhibition of the Cl^- permeability by application of 9-anthracene carboxylic acid (9-AC; 100 μM). Initially the double-barrelled Cl^--sensitive microelectrode was extracellular and the response to reduction of Cl^- to 1 % was determined. Following impalement of a fibre, E_m and a^i_{Cl} stabilized at $-52{\cdot}0$ mV and 17·4 mM respectively. The Cl^- equilibrium potential (E_{Cl}, plotted as dots on the recording of E_m) stabilized at $-47{\cdot}0$ mV. Note that application of 9-AC caused an immediate and pronounced departure of E_{Cl} from E_m, as a^i_{Cl} rose in the face of a hyperpolarization. Its presence slowed the changes in a^i_{Cl} observed on removal and readdition of external Cl^- (isethionate substituted) and greatly modified the changes in E_m. The value of a^i_{Cl} fell to the same apparent level in Cl^--free solution in both the presence and absence of 9-AC.

Since the Cl^--sensitive ion exchanger is not selective for Cl^- but sees a large number of other ions (see Saunders & Brown, 1977), it is possible that the observed disparity between E_{Cl} and E_m was caused by the intracellular presence of interfering anions and not by an active accumulation of Cl^-. We therefore investigated the effect of removing external Cl^- (Cl^-_o). As shown in Fig. 1, this resulted in a rapid decline of a^i_{Cl} to a mean apparent level of $1{\cdot}7 \pm 1{\cdot}0$ mM ($n = 24$), with a range of 0·6–4·1 mM. Assuming that all the Cl^- ions were washed out of the sarcoplasm, the remaining apparent Cl^- must represent interference from other intracellular anions. At first sight, this degree of intracellular interference seems a perfect explanation for the apparently non-passive distribution of Cl^-. Nevertheless, if this were the case, the difference between E_m and the calculated E_{Cl} should increase with increasing E_m (see Deisz & Lux, 1982). Figure 2 shows that the experimental data did not conform to

this prediction. Linear regression of these data revealed that there was no correlation between E_m and the amount by which E_{Cl} differed from E_m ($r = 0{\cdot}04$).

The rapid fall in a^i_{Cl} on removal of Cl^-_o was accompanied by a marked transient depolarization, indicative of the high Cl^- permeability (P_{Cl}) of skeletal muscle (see Hodgkin & Horowicz, 1959). However, as previously observed in this (Betz *et al.*

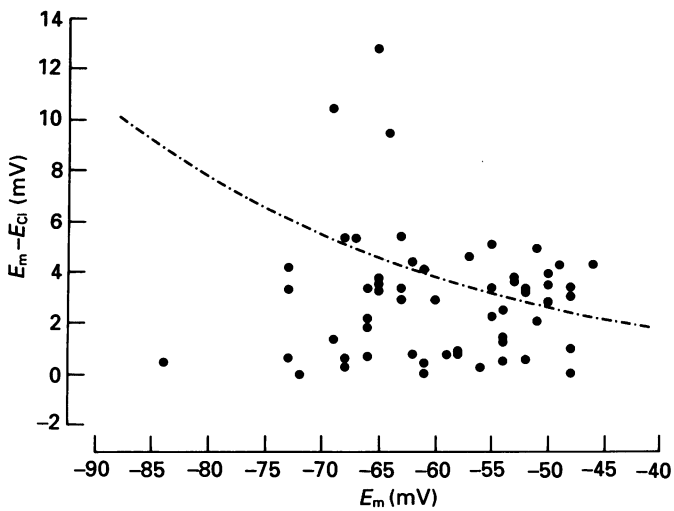

Fig. 2. The relationship of E_m to the difference between the calculated E_{Cl} and E_m. Each point represents the data from a single fibre. The dashed and dotted line shows the relationship predicted if the difference between E_{Cl} and E_m was caused by intracellular interference (taken as the mean of 1·7 mM) and Cl^- was passively distributed.

1984, 1986; Harris & Betz, 1987) and other mammalian skeletal muscles (Dulhunty, 1978), E_m then became considerably more negative than in the presence of normal Cl^-_o. A mean value of $-80{\cdot}1 \pm 12{\cdot}4$ mV ($n = 24$) was recorded with a range of -53 to -109 mV, the lower values being associated with a low E_m in normal conditions. Readdition of Cl^-_o resulted in a rapid restoration of a^i_{Cl} and a small transient hyperpolarization before E_m declined towards the value previously recorded in normal Krebs solution. Notably, the time course of the fall in E_m was very similar to that of the fall in E_{Cl} (see also Fig. 4).

Estimation of P_{Cl} from the fall in a^i_{Cl} observed on removal of Cl^-_o

The net Cl^- flux can be calculated from these recordings with the simplistic assumption that the rate of fall of a^i_{Cl} on removal of Cl^-_o represents the net transmembrane movement of Cl^- ions, that influx of Cl^- ions is negligible and that the volume to surface area ratio remains constant. This flux can then be used to calculate P_{Cl} from the constant-field theory (see Vaughan-Jones, 1979*a*; Aickin & Brading, 1983). Semilogarithmic plots of the data reveal that, after a delay of 0·5–1 min during which a^i_{Cl} fell rather slowly, the fall in a^i_{Cl} approximated to a single exponential with a mean time constant of $1{\cdot}01 \pm 0{\cdot}28$ min ($n = 8$). This yields a mean

P_{Cl} of $5{\cdot}8 \times 10^{-6}$ cm s^{-1}. However, it should be noted that the surface area to volume ratio will not remain constant on removal of Cl_o^-. Assuming loss of all intracellular Cl^-, these cells will shrink by 7–8%. This will cause an underestimate of P_{Cl}. Allowing for shrinkage, the data would suggest a P_{Cl} of $6{\cdot}2 \times 10^{-6}$ cm s^{-1}, close to the value derived from measurements of input conductance in normal and Cl^--free solutions (7×10^{-6} cm s^{-1}; Harris & Betz, 1987).

Effect of reduction of P_{Cl} on a_{Cl}^i and E_m

If Cl^- ions are actively accumulated in the normal muscle fibre, reduction of P_{Cl} should cause an increase in a_{Cl}^i. As described by Harris & Betz (1987) and shown in Fig. 1, application of the P_{Cl} inhibitor, 9-AC (Palade & Barchi, 1977*b*), caused a rise in a_{Cl}^i and a marked hyperpolarization. These observations clearly confirm both the reduction in P_{Cl} by 9-AC, since hyperpolarization would otherwise cause a fall in a_{Cl}^i, and the presence of an inward Cl^- transport mechanism, since inhibition of P_{Cl} would otherwise result in a constant a_{Cl}^i.

In order to test that the rise in a_{Cl}^i on application of 9-AC was not caused by an increase in intracellular interference, we investigated the effect of removal of Cl_o^- in the presence of 9-AC. As illustrated in Fig. 1, a_{Cl}^i fell much more slowly but reached the same apparent level in Cl^--free solution as observed in the absence of 9-AC. Furthermore, application of 9-AC after stabilization in Cl^--free solution had no effect on the apparent a_{Cl}^i (see Fig. 4). It is notable that in this case, application of 9-AC had no effect on E_m either (see Fig. 4). Thus, 9-AC seems unlikely to affect a_{Cl}^i or E_m by alteration of parameters other than P_{Cl}.

It has been reported that 100 μM-9-AC virtually completely blocks P_{Cl} in the rat diaphragm (Palade & Barchi, 1977*b*). The absence of any depolarization on removal of Cl_o^- in the presence of this concentration (see Fig. 1) suggests a substantial block in rat lumbrical muscles. However if P_{Cl} was fully blocked, both E_m and a_{Cl}^i might be expected to be unaffected by the change in transmembrane Cl^- gradient, yet removal of Cl_o^- caused a maintained hyperpolarization and a slow decline of a_{Cl}^i. The rate of fall of a_{Cl}^i would be consistent with an 80–90% inhibition of P_{Cl}, and it is notable that measurements of input conductance in this preparation have suggested a lower value in the presence of 100 μM-9-AC in Cl^--free, rather than Cl^--containing, solution (G. L. Harris & W. J. Betz; unpublished observations). A residual P_{Cl} could also explain the hyperpolarization on removal of Cl_o^-. Nevertheless, the fall in a_{Cl}^i could also be caused by reversal of the mechanism normally responsible for accumulation of Cl^- ions (see Vaughan-Jones, 1979*b*, 1986; Aickin & Brading, 1983, 1984; Altamirano & Russell, 1987).

Whatever the cause of the fall, the rise in a_{Cl}^i on readmission of Cl_o^- in the presence of 9-AC can only be explained by active uptake of Cl^- ions because E_{Cl} rapidly became less negative than E_m. Levels of a_{Cl}^i up to 40 mM higher (eleven times higher) than that predicted by a passive distribution were observed. The initial rate of rise of a_{Cl}^i was approximately half that observed in the absence of 9-AC.

Hutter & Warner (1967) were the first to suggest that Cl^- ions are actively accumulated in skeletal muscle when P_{Cl} is reduced. Their evidence was based on the observation of a transient depolarization in frog muscle when external pH (pH_o) was raised (P_{Cl} increased), particularly after pH_o had first been lowered (P_{Cl} decreased). We attempted to reproduce their findings in the rat

lumbrical muscle using two conventional microelectrodes to monitor E_m and membrane conductance (G_m). Exposure to Krebs solution at pH 5·4 caused a slow fall in G_m and a depolarizing drift but switching to pH_o 7·4 or 9·4 caused only a slow recovery of E_m and G_m. Application of solution at pH 9·4, after equilibration at normal pH_o, had virtually no effect on E_m or G_m but there was a transient hyperpolarization on return to normal pH_o (7·4). Clearly, alteration of pH_o did not have large or rapid enough effects on P_{Cl} in mammalian muscle to cause sudden transients in E_m. These results confirm the earlier findings of Palade & Barchi (1977*a*) in rat diaphragm where 15–20 min were required for the effects of pH_o on P_{Cl} to develop fully.

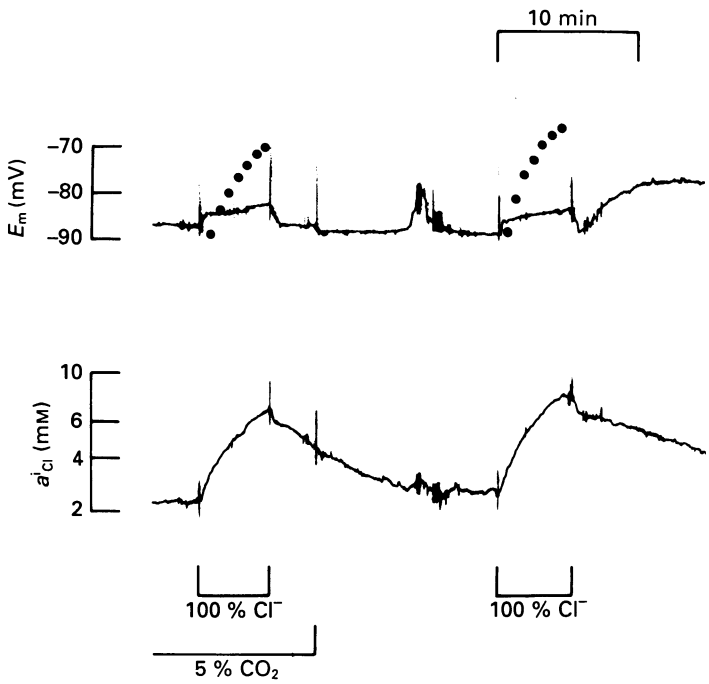

Fig. 3. Pen recordings of part of an experiment showing the lack of effect of the presence or nominal absence of CO_2 and HCO_3^- on the rate of Cl^- accumulation in the presence of 9-AC (100 μM). The preparation was superfused with Cl^--free, nominally CO_2 and HCO_3^--free solution except where indicated otherwise.

Investigation of the mechanism responsible for active accumulation of Cl^- ions

Indirect evidence in this preparation has previously led to the suggestion of inward Na^+-dependent, and possibly also K^+-dependent Cl^- transport (Betz *et al.* 1984). More recently Harris & Betz (1987) showed that removal of Na_o^+ or K_o^+, or addition of frusemide, reversibly decreased a^i_{Cl} to the level predicted by a passive distribution in denervated rat lumbrical muscles. However, Cl^- accumulation in both mammalian cardiac (Vaughan-Jones, 1979*b*, 1986) and smooth muscle (Aickin & Brading, 1984) is largely dependent upon Cl^-–HCO_3^- exchange. We therefore set out to test for the involvement of cation-dependent co-transport and of anion exchange in the active uptake of Cl^- ions observed on readdition of Cl_o^- in the presence of 9-AC.

Effects of addition of CO_2 and HCO_3^- and of application of DIDS

If Cl^-–HCO_3^- exchange was involved in the active accumulation of Cl^- ions, changing from the normal, nominally CO_2-free solutions to CO_2–HCO_3^--buffered solutions should accelerate the rise in a^i_{Cl} on readdition of Cl^-_o (see Vaughan-Jones, 1979*b*, 1986; Aickin & Brading, 1984). Figure 3 shows that the rise in a^i_{Cl} or, more importantly, the increasing divergence between E_{Cl} and E_m, on readmission of Cl^-_o was unaffected by the presence or nominal absence of CO_2 and HCO_3^-. In fact in this experiment, the rate of rise of a^i_{Cl} was, if anything, faster in the nominal absence of CO_2. No acceleration of Cl^- accumulation was observed in three other experiments.

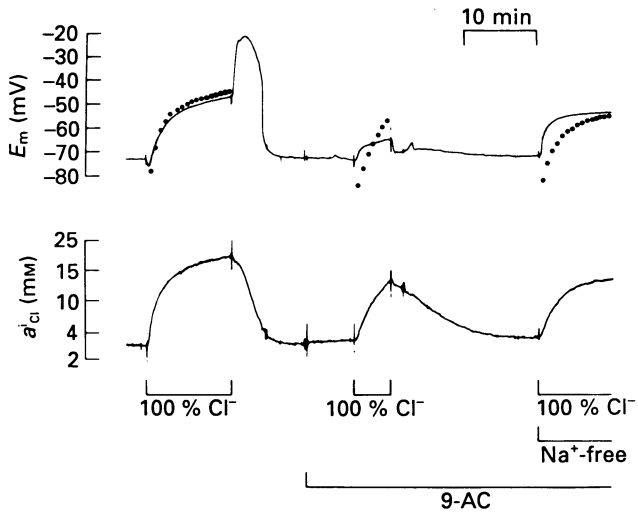

Fig. 4. Pen recordings of an experiment to show the effect of the absence of Na^+_o on the accumulation of Cl^- in the presence of 9-AC (100 μM). Initially the effect of addition and removal of Cl^-_o was recorded in the absence of 9-AC. 9-AC was then applied after equilibration in Cl^--free solution, when it had no effect on either E_m or a^i_{Cl}. Note that following simultaneous addition of Cl^-_o and removal of Na^+_o, E_{Cl} did not become less negative than E_m. Na^+_o was substituted by choline and the preparation pre-treated with α-bungarotoxin.

Further evidence for the lack of participation of Cl^-–HCO_3^- exchange was provided by the failure of the anion exchange inhibitor DIDS (100 μM; Knauf & Rothstein, 1971) to affect the attainment of a non-passive Cl^- distribution (not illustrated, $n = 3$).

Effect of removal of external Na^+

Due to the problems of making a Na^+-free, Cl^--free solution, investigation of the dependence of Cl^- accumulation on Na^+_o was carried out by simultaneous readmission of Cl^-_o and removal of Na^+_o. Despite this non-ideal experimental design, Fig. 4 clearly shows that a^i_{Cl} did not rise above the level predicted by a passive distribution in the absence of Na^+_o. In this experiment Cl^-_o was first reapplied in the absence of 9-AC. The

level of a^{i}_{Cl} rose rapidly as the fibre depolarized. The change in E_{Cl} closely matched that in E_m, although after the first few minutes E_{Cl} was slightly less negative than E_m. After application of 100 μM-9-AC, readdition of Cl^-_o caused a slower rise in a^{i}_{Cl} (at about half the rate) and a small depolarization. E_{Cl} became considerably less negative than E_m. Simultaneous removal of Na^+_o and readdition of Cl^-_o in the presence of 9-AC caused a similar initial rate of rise in a^{i}_{Cl} to that observed in the presence of Na^+_o but this then declined. E_m depolarized more, but most significantly, E_{Cl}

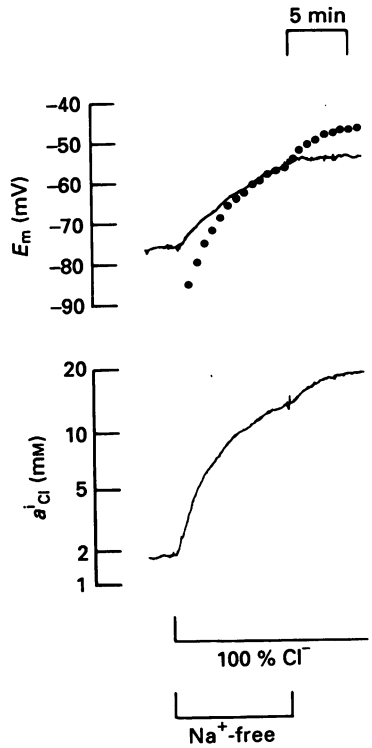

Fig. 5. Pen recordings of part of an experiment illustrating the effect of readdition of Na^+_o on the accumulation of Cl^- in the presence of 9-AC (100 μM). After equilibration in Cl-free solution, Cl^-_o was added and Na^+_o simultaneously removed. The level of a^{i}_{Cl} rose such that E_{Cl} approximated to E_m. Readdition of Na^+_o caused an immediate rise in a^{i}_{Cl} such that E_{Cl} became less negative than E_m. Na^+_o was substituted by choline and the preparation was pre-treated with α-bungarotoxin.

remained more negative than E_m. This result not only demonstrates the dependence of active Cl^- accumulation on Na^+_o but also suggests that the small apparent accumulation of Cl^- under normal conditions is real. If it was caused by the intracellular presence of interfering anions, the same disparity between E_{Cl} and E_m should be recorded in the presence of 9-AC when the accumulating mechanism was inhibited.

Another example of the dependence of Cl^- accumulation on Na^+_o is illustrated in Fig. 5. In this experiment, E_{Cl} approximated to E_m in the

absence of Na_o^+. Readdition of Na_o^+ then caused an immediate rise in a_{Cl}^i and divergence of E_{Cl} from E_m.

Effect of removal of external K^+

The absence of K_o^+ also severely impaired accumulation of Cl^- ions in the presence of 9-AC, as illustrated in Fig. 6. In this experiment Cl^- uptake in K^+-free solution fell short of the level predicted for a passive distribution: E_{Cl} remained considerably more negative than E_m. In three other experiments, E_{Cl} approximated to E_m. The rapidity with which this considerable inhibition was observed seems to rule out the

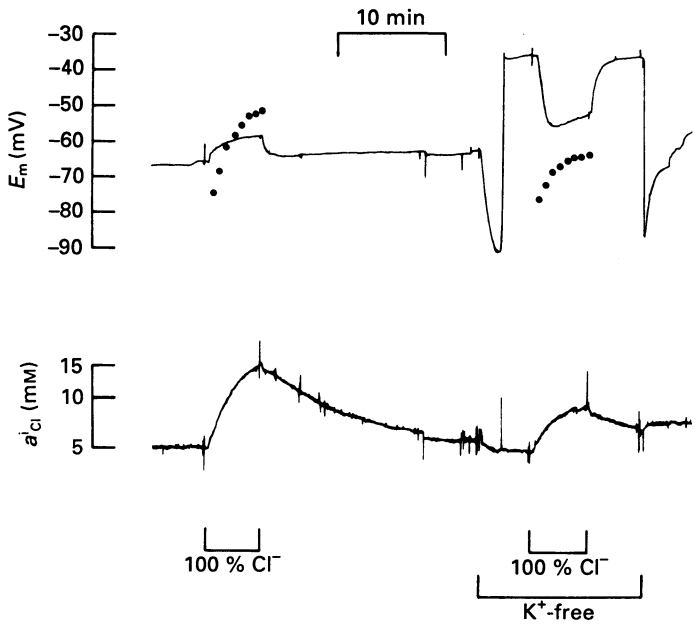

Fig. 6. Pen recordings of an experiment to show the effect of the absence of K_o^+ on the accumulation of Cl^- in the presence of 9-AC (100 μM). Note that in the absence of K_o^+, E_{Cl} did not become less negative than E_m. K_o^+ was substituted by Na^+. The preparation was maintained in Cl^--free solution except where indicated otherwise.

possibility that it was mediated indirectly via the run-down of the transmembrane Na^+ gradient resulting from inhibition of the Na^+ pump.

In the presence of 9-AC and absence of Cl_o^-, K_o^+ removal had dramatic effects on E_m. There was an initial hyperpolarization of about 30 mV before E_m suddenly dropped to stabilize at around -35 mV (cf. Betz *et al.* 1986). This behaviour is similar to that of the cardiac Purkinje fibre under normal conditions, where K_o^+ removal causes an immediate drop in E_m to around -35 mV (e.g. see Ellis, 1977; Gadsby & Cranefield, 1977) and is possibly caused by reduction of the K^+ conductance (inward rectification). Readdition of K_o^+ resulted in a large and rapid hyperpolarization in which E_m became more negative than before the removal of K_o^+. E_m then decayed back to the original level. This hyperpolarization may reflect

increased activity of the electrogenic Na^+ pump during the restoration of the intracellular Na^+ activity or a slow reactivation of the K^+ conductance.

Effect of removal of both external Na^+ and K^+

Since removal of either Na_o^+ or K_o^+ prevented accumulation of Cl^- ions to a level above that predicted by a passive distribution, it seems likely that Na^+, K^+, Cl^- co-transport is responsible for the active accumulation Cl^-. This mechanism underlies the active uptake of Cl^- in the squid giant axon (Russell, 1983), is involved in inward

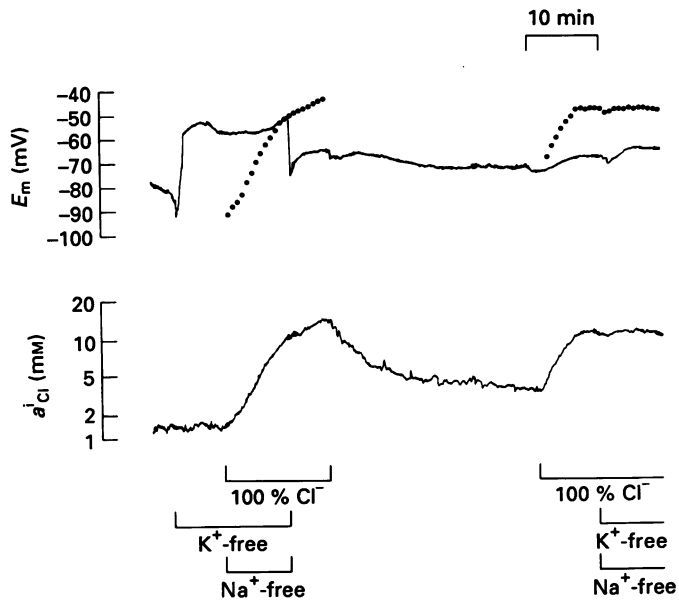

Fig. 7. Pen recordings showing the effect of the absence of both Na_o^+ and K_o^+ on the accumulation of Cl^- in the presence of 9-AC (100 μM). The level of a^i_{Cl} rose on readdition of Cl_o^- in the absence of both cations such that E_{Cl} equalled E_m. Readmission of the cations caused an immediate departure of E_{Cl} from E_m. Note that removal of both cations after significant accumulation of Cl^- resulted in little change in the difference between E_{Cl} and E_m. Na_o^+ and K_o^+ were replaced by choline and the preparation was pre-treated with α-bungarotoxin.

Cl^- transport in smooth muscle (Owen, 1985; Aickin, 1987) and is present in a variety of other cell membranes (for review see Chipperfield, 1986). The alternative explanation is that separate Na^+-dependent and K^+-dependent mechanisms co-exist. The slow accumulation to the passive level in the absence of one of the cations possibly argues in favour of this latter explanation. Removal of both cations should distinguish between these alternatives. In the former case, removal of both would be no more effective than removal of one, whereas in the latter, removal of both would cause a greater inhibition. As shown in Fig. 7, readmission of Cl_o^- in the absence of both Na_o^+ and K_o^+ caused a slow fall in E_{Cl} towards E_m. After about 8 min E_{Cl} equalled E_m but did not fall below E_m. Thus the absence of both cations

seems no more effective at inhibiting Cl^- accumulation than the absence of only one.

Readdition of Na_o^+ and K_o^+ in the presence of Cl_o^- resulted in the rapid development of a substantial disequilibrium in the Cl^- distribution. On some occasions there was a marked hyperpolarization (see Fig. 7) but on others there was a sharp rise in a_{Cl}^i (see Fig. 9). E_{Cl} became up to 55 mV less negative than E_m. It is of interest that the simultaneous removal of Na_o^+ and K_o^+ in the presence of Cl_o^- had little effect on a_{Cl}^i, although it sometimes caused a significant depolarization (see Figs 7 and 9). In the experiment shown in Fig. 7, the difference between E_{Cl} and E_m only decreased by 4 mV after a 10 min absence of Na_o^+ and K_o^+. This suggests that the Na^+- and K^+-dependent mechanism for Cl^- accumulation is not readily reversed.

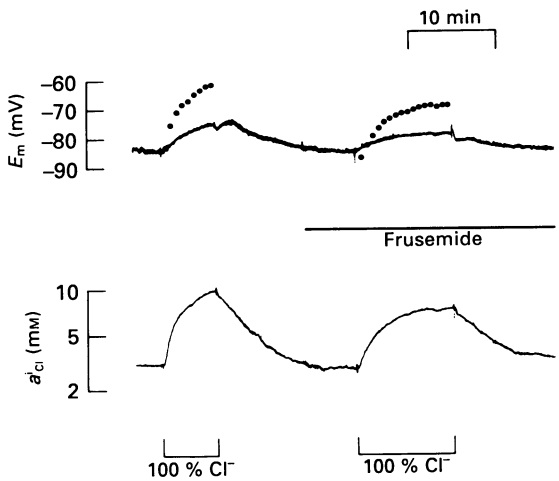

Fig. 8. Pen recordings illustrating the effect of 10 μM-frusemide on the accumulation of Cl^- in the presence of 9-AC (100 μM). Note that the presence of frusemide slowed the initial rate of rise of a_{Cl}^i on readmission of Cl_o^- and decreased the amount by which a_{Cl}^i rose above the passive level. The preparation was superfused with Cl^--free solution except where indicated otherwise.

Effect of frusemide

The foregoing results clearly support the previous suggestions of the involvement of Na^+, K^+, Cl^- co-transport in active Cl^- accumulation (Betz *et al.* 1984; Harris & Betz, 1987). We therefore investigated the effect of frusemide, a well-established inhibitor of this transport mechanism (see Ellory, Dunham, Logue & Stewart, 1982; Chipperfield, 1986). Figure 8 shows that the presence of 10 μM-frusemide indeed slowed Cl^- accumulation but, interestingly, had no effect on the fall in a_{Cl}^i on removal of Cl_o^-. Its presence did not add to the inhibition of Cl^- uptake observed in the absence of Na_o^+ and K_o^+ but greatly reduced the stimulation of Cl^- accumulation when Na_o^+ and K_o^+ were reapplied, as illustrated in Fig. 9. This relatively low concentration of frusemide was used both because it was found to be sufficient to reduce E_{Cl} to E_m in denervated muscle (Harris & Betz, 1987) and because of the problems caused

by the substantial sensitivity of the Cl^--sensitive microelectrode to frusemide (up to 150 times greater than the sensitivity to Cl^-; Chao & Armstrong, 1987). Frusemide slowly crosses the cell membrane and, when applied at higher concentrations, adds considerably to the apparent a^i_{Cl} recorded in Cl^--free conditions (C. C. Aickin; unpublished observations). This was not observed with the low dose used here (see

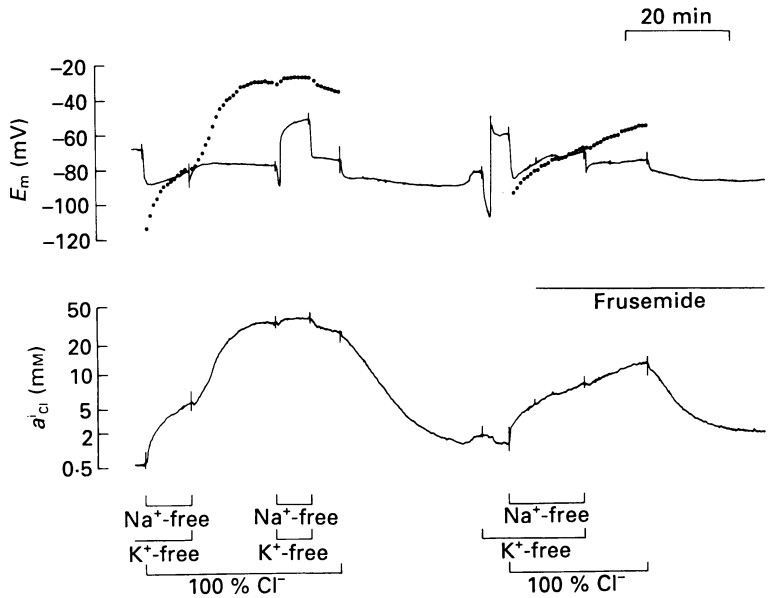

Fig. 9. Pen recordings of part of an experiment showing the effect of 10 μM-frusemide on the accumulation of Cl^- observed after readmission of Na^+_o and K^+_o. 9-AC (100 μM) was present throughout and the preparation was superfused with Cl^--free solution except where indicated otherwise. Na^+_o and K^+_o were substituted with choline and the preparation was pre-treated with α-bungarotoxin.

Fig. 8). However, 10 μM is close to the K_i found in other preparations (see Chipperfield, 1986) and this probably accounts for the incomplete inhibition observed in these experiments. Nevertheless, the results are clearly consistent with Na^+_o removal, K^+_o removal and frusemide application all inhibiting a single mechanism.

DISCUSSION

Is a^i_{Cl} higher than or equal to that predicted by a passive distribution?

Our consistent finding of a small apparent accumulation of Cl^- ions in normal skeletal muscle is in agreement with all previous measurements in both amphibian and mammalian preparations (Kernan *et al.* 1974; Bolton & Vaughan-Jones, 1977; Macchia & Baumgarten, 1979; Donaldson & Leader, 1984; McCaig & Leader, 1984; Harris & Betz, 1987). However, as pointed out in many of the previous studies, the small discrepancy between E_{Cl} and E_m begs the question as to whether it could be an artifact caused by the errors inherent in the method. The present use of double-barrelled electrodes eliminates the lack of isopotentiality at voltage- and Cl^--sensitive

electrode impalement sites as a possible source of error. Equally, the use of the reference liquid ion exchanger eliminates the problem of artificial elevation of the apparent a_{Cl}^{i} by the intracellular leakage of Cl^- or of an interfering anion from the voltage electrode. Nevertheless, the lack of selectivity of the Cl^--sensitive electrode still prohibits any great assurance of the absolute a_{Cl}^{i}. Indeed, the apparent residual a_{Cl}^{i} recorded in Cl^--free solution (mean, 1·7 mM) may suggest that intracellular interference is large enough to account for the apparent Cl^- accumulation (mean of 1·4 mM) under normal conditions and that Cl^- ions are passively distributed. However, it does seem likely that the interference level would be greater during Cl^- depletion than under normal conditions. Firstly, shrinkage, resulting from the loss of intracellular Cl^-, will concentrate any interfering anions, and secondly, the loss of intracellular Cl^- may cause some compensatory gain in other anions. If interference was responsible for the apparent accumulation, the disparity between the calculated E_{Cl} and E_m would be greater at more negative potentials (see Deisz & Lux, 1982). This was not observed. Further, the disparity between E_{Cl} and E_m recorded under normal conditions should also have been observed when the accumulating mechanism was inhibited in the presence of 9-AC. But under these conditions (Na^+ and/or K^+-free solutions) E_{Cl} did not become less negative than E_m.

Although we cannot be certain that the small discrepancy between E_{Cl} and E_m is entirely real, there can be little doubt that Cl^- ions are actively accumulated by the skeletal muscle fibre. The immediate, clear departure of E_{Cl} from E_m as a_{Cl}^{i} rises in the face of hyperpolarization on application of 9-AC is not easily explained in any other way. Increased intracellular interference cannot be held responsible since application of 9-AC in Cl^--free conditions had no measureable effect on the apparent a_{Cl}^{i}. Nor can there be much doubt that P_{Cl} exerts a depolarizing influence under normal conditions since reduction of the Cl^- conductance, either by removal of Cl_o^- or by application of 9-AC, caused a steady-state hyperpolarization. Although other explanations of the hyperpolarization are not excluded, it seems unlikely that Cl^- removal and 9-AC application would have the same effect on E_m other than by their common inhibition of the Cl^- conductance. Significantly, application of 9-AC had no observable effect on E_m in the absence of Cl^- ions. Dulhunty (1978) also concluded that an alternative explanation of the steady-state change in E_m on alteration of Cl_o^- was unlikely. She showed that for modulation of the P_{Na}/P_K ratio to underlie the change, a complex relationship between P_{Na}/P_K and both K_o^+ and Cl_o^- would be required. Interestingly, inhibition of the mechanism responsible for Cl^- accumulation by application of frusemide in normal muscle also causes a marked hyperpolarization (Betz *et al.* 1984; Harris & Betz, 1987). Although frusemide has been reported to inhibit P_{Cl} (e.g. Bretag, Dawe, Kerr & Moskwa, 1980) which would contribute to the hyperpolarization, this was at a much higher concentration than used in the present study (2·5 mM cf. 10 μM). Finally, it is notable that the reaccumulation of a_{Cl}^{i} by Cl^--depleted fibres was not greatly slowed when P_{Cl} was blocked by the presence of 9-AC. This suggests that under normal conditions, active accumulation of Cl^- ions is largely responsible for the concomitant depolarization rather than the depolarization causing the rise in a_{Cl}^{i}. Examination of the records reveals that E_{Cl} became less negative than E_m shortly after readdition of Cl_o^- and thereafter appeared to lead the fall in E_m.

This evidence therefore indicates that Cl^- ions are not in equilibrium across the skeletal muscle sarcolemma but actively accumulated to a level slightly higher than that expected for a passive distribution. How much higher remains unclear due to the lack of selectivity of the Cl^--sensitive electrode. It is, however, notable that the apparent accumulation of a mean 1·4 mM a^i_{Cl} is not unreasonable from theoretical considerations. From the constant-field equation, the mean E_m recorded in Cl^--free solution yields a P_{Na}/P_K ratio of 0·015, assuming that E_m is then governed by P_{Na} and P_K only and that a^i_{Na} and a^i_K are 7·0 and 110 mM respectively (see Shabunova & Vyskočil, 1982; Ward & Wareham, 1983, 1986; Leader, Bray, MacKnight, Mason, McCaig & Mills, 1984; MacDermott & Mulryan, 1986). This value is in agreement with those calculated previously in mammalian skeletal muscle (see Dulhunty, 1978; Shabunova & Vyskočil, 1982). If these parameters are unaltered in normal Krebs solution, the mean E_m of −59·5 mV and a^i_{Cl} of 13·2 mM (1·4 mM higher than that predicted by a passive distribution at −59·5 mV) yields a P_{Cl}/P_K ratio of 4·569. This, too, is in good agreement with previously reported values (e.g. Lipicky & Bryant, 1966; Palade & Barchi, 1977*a*). Damage caused by the double-barrelled electrode in this study, as judged from the low membrane potentials, may have resulted in a higher a^i_{Na} and lower a^i_K than the values assumed and in addition Cl^--free conditions may have altered these parameters, but differences of 10 or even 20 mM would not greatly affect the ratios calculated. Finally it is worth pointing out that the damage caused by these electrodes was likely to have decreased transmembrane ionic gradients, not only of Na^+ on which it would appear that the Cl^- gradient depends, but also of Cl^- itself. Thus the present results would, if anything, underestimate the normal Cl^- disequilibrium.

The mechanism for Cl^- accumulation

The present, continuous recording of the rapid accumulation of Cl^- ions in the presence of 9-AC to a level unquestionably higher than that predicted for a passive distribution, not only adds convincingly to the evidence for inward transport of Cl^- ions in skeletal muscle (e.g. Hutter & Warner, 1967; Bolton & Vaughan-Jones, 1977; Dulhunty, 1978), but also provides an ideal approach for the investigation of the transport mechanism. Inhibition of the accumulation by the absence of Na^+_o and/or K^+_o, together with the marked acceleration of the accumulation on readdition of these cations, strongly indicates their involvement in the inward transport of Cl^-. This confirms the earlier suggestions from indirect evidence (Betz *et al.* 1984) and from steady-state measurements of a^i_{Cl} (Harris & Betz, 1987) and is in line with observation of a Cl^--dependent, frusemide-sensitive fraction of Na^+ influx (Kernan, 1986). It seems likely that the requirement for Na^+_o and K^+_o is on the same process because removal of both ions from the superfusing solution gave no greater inhibition that the removal of only one. This is to some extent supported by the inhibitory effect of frusemide, an inhibitor of Na^+, K^+, Cl^- co-transport rather than of Na^+, Cl^- or K^+, Cl^- co-transport (Ellory *et al.* 1982). Tightly coupled co-transport of Na^+, K^+ and Cl^- has been shown to be responsible for the higher than passive internal Cl^- of the squid giant axon (Russell, 1983) and the same mechanism has also been implicated in the establishment of a high a^i_{Cl} in mammalian smooth muscle (Owen, 1985; Aickin, 1987; Brading, 1987) and avian cardiac cells (Liu, Jacob, Piwnica-

Worms & Lieberman, 1987). It is interesting that the mechanism in rat lumbrical muscles does not appear to reverse readily. a^{i}_{Cl} did not decline significantly towards the passive level when Na^{+}_{o} and K^{+}_{o} were removed (Figs 7 and 9) and the fall in a^{i}_{Cl} on removal of Cl^{-}_{o} was not affected by the presence of frusemide (Fig. 8). It seems likely, therefore, that 100 μM-9-AC caused an 80–90% block of P_{Cl} rather than a complete inhibition and that some passive movements of Cl^{-} remained. This would explain the slow rise in a^{i}_{Cl} towards the passive level observed when Cl^{-}_{o} was reapplied in the absence of Na^{+}_{o} and/or K^{+}_{o}.

Perhaps surprisingly from its major contribution to the active accumulation of Cl^{-} ions in both cardiac (Vaughan-Jones, 1979*b*) and smooth muscle (Aickin & Brading, 1984), Cl^{-}–HCO_3^{-} exchange appeared to play no part in the inward transport of Cl^{-} in the rat lumbrical muscle. Neither the presence or nominal absence of CO_2 and HCO_3^{-}, nor the presence of DIDS, had any effect on the rate of Cl^{-} accumulation. It is notable that Bolton & Vaughan-Jones (1977) reached the same conclusion for frog sartorius. Nevertheless, Cl^{-}–HCO_3^{-} exchange does exist in the skeletal muscle sarcolemma. It is involved in the regulation of intracellular pH in mouse soleus (Aickin & Thomas, 1977) and, when intracellular Cl^{-} is elevated, in frog semitendinosus (Abercrombie, Putnam & Roos, 1983). As such, it has been interpreted to *remove* Cl^{-} ions from the sarcoplasm during the effective extrusion of acid equivalents. Significantly, in neither preparation does turnover of the exchanger depend simply upon the Cl^{-} and HCO_3^{-} gradients, as it does in both cardiac and smooth muscle (Vaughan-Jones, 1979*b*, 1986; Aickin & Brading, 1984). The mechanism in mouse soleus has a high Q_{10}, consistent with an active process dependent upon metabolic energy, while that in the frog semitendinosus is dependent on Na^{+}_{o}.

Conclusion

These results clearly show that Cl^{-} ions are actively transported into rat lumbrical muscle fibres. The underlying mechanism is Na^{+}_{o}- and K^{+}_{o}-dependent and sensitive to a relatively low concentration of frusemide. It is therefore likely to be Na^{+}, K^{+}, Cl^{-} co-transport. The high P_{Cl} prevents establishment of a large Cl^{-} disequilibrium under normal conditions and E_{Cl} is held close to E_m. Nevertheless, the combination of a high P_{Cl} and inward transport of Cl^{-} ions exerts a substantial depolarizing influence. This is witnessed by the marked steady-state hyperpolarization observed in Cl^{-}-free solution, on application of frusemide (Betz *et al.* 1984; Harris & Betz, 1987) and on application of 9-AC.

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