Intracellular Chloride Concentration and Evidence for the Existence of a Chloride Pump in Frog Skeletal muscle

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Abstract Intracellular chloride concentration in frog sartorius muscle was determined at 20°C utilizing ionic permeability change of the membrane for an ion-selective electrode. The membrane potential and external potassium concentration relation was obtained with changes in pH. Using the constant field equation with a condition given by the crossing point in the two relations obtained with the two different pH, the intracellular chloride concentration was estimated to be 3.7 ± 0.18 mM (mean $\pm$ S.E.M.) with a 0.76 of the activity coefficient for chloride in Ringer's solution. The chloride potential (-88.5 ± 1.26 mV) was significantly positive to the resting potential (-94.8 ± 1.09 mV), suggesting that a chloride pump may be working in the frog skeletal muscle. The existence of the chloride pump was supported further by the fact that the depolarization induced by the pH increase became smaller with cooling and finally disappeared when cooled below 5°C.

In frog sartorius muscle, the chloride conductance of the membrane is about twice as large as the potassium conductance, and the distribution of chloride may be determined by a purely passive mechanism (HODGKIN and HOROWICZ, 1959; HUTTER and NOBLE, 1960; ADRIAN, 1960, 1961). On the other hand, an active movement of chloride was suggested from the fact that the membrane potential varied with the pH in a medium which specifically alters the chloride conductance (HUTTER and WARNER, 1967), although LING and GERARD (1949) found no clear variation of the resting potential with changes in pH.

Recently direct measurements of chloride activity in frog sartorius muscle using ion-selective electrodes have also shown that the intracellular chloride concentration appears to be higher than the value predicted on the basis of a passive distribution of chloride (KERNAN *et al.*, 1974; BOLTON and VAUGHAN-JONES, 1977). The major uncertainties in these measurements are the liquid junction potentials between the KCl salt bridges and the myoplasm (COLE and MOORE, 1960; HIRONAKA and MORIMOTO, 1979), and the possible contribution of interfering anions to the electrode potentials (WALKER and BROWN, 1977). Inserting the large elec-

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trode into the cell also might cause sufficient damage to allow leakage. As the difference between the chloride equilibrium potential and the resting membrane potential was generally small, it might reasonably be ascribed to some experimental error (BOLTON and VAUGHAN-JONES, 1977).

The present study describes an entirely different kind of determination of intracellular chloride concentration and offers evidence for the existence of a chloride pump in frog sartorius muscle with special reference to accurate potential measurements (HIRONAKA and MORIMOTO, 1979).

METHODS

Theoretical. If pH specifically alters the chloride permeability of the membrane (HUTTER and WARNER, 1967), the ratio of change in the membrane potential to change in its permeability in the constant field equation (GOLDMAN, 1943; HODGKIN and KATZ, 1949), Eq. 1, is given as a derivative of the membrane potential (mV) with respect to chloride permeability, Eq. 2:

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

$$\frac{RT}{F} \left(\frac{[Cl]_i}{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i} - \frac{[Cl]_o}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o} \right) \quad (2)$$

where P refers to the ion permeability in the subscript, $[]$ to the ion activity outside (o) or inside (i) the muscle fiber, and R , T and F have the conventional meanings.

From the condition of $dV_m/dP_{Cl}=0$, the following equation is derived to calculate the intracellular chloride activity:

$$[Cl]_i = [Cl]_o \exp (V_m F / RT) \quad (3)$$

Material. Sartorius muscles of *Rana nigromaculata* were carefully removed to avoid damaging the pelvic end of the muscle, and they were dissected into small parts to ensure rapid perfusion around the muscle fibers within several seconds (*cf.* HODGKIN and HOROWICZ, 1959). The muscle was mounted on a block in a chamber with a capacity of 0.6 ml. The block had a drain in the middle through which the muscle fibers were continuously perfused crosswise with solutions at a rate of 4 ml/min. The experiments were carried out on the muscles in 1 hr after the dissection at 20°C.

Measurement of the membrane potential. Micro-electrodes with resistances between 5 and 10 MΩ and with tip potentials less than 1 mV when filled with 3 M KCl were used for potential recordings. A coarse micro-electrode filled with 3 M KCl was used as a reference electrode. A special precaution was taken to eliminate "tip potentials" in the potential measurements (*cf.* HIRONAKA and MORIMOTO, 1979). No appreciable change in potential was observed in the electrodes when the pH of the solution was altered.

Solutions. Normal Ringer's solution was composed of (mM): NaCl, 115, KCl, 2.5, CaCl_2 , 1.8, tris maleate, 2.0, acetylglutamine, 2.0, and was titrated with NaOH for the necessary pH (HUTTER and WARNER, 1967). To alter potassium concentration in the solution; Na was substituted for K. In the cooling experiments, a high Ca Ringer's solution (4 mM) was used for stability of the muscle membrane in terms of the potential measurements.

RESULTS

Relation between membrane potential and external potassium concentration at different pH values

The potential measurements were made on 5 fibers of a muscle 2–3 min after changing into the solution containing each K concentration for pH 7.2, and a quick measurement was done on the 5 fibers for pH 8.2 between 1 and 2 min after changing into the solution because of the transient effect of the pH increase (HUTTER and WARNER, 1967). After washing (30 min), the experiments were repeated for another K concentration.

The resting membrane depolarized with an increase in external K concentration as shown in Fig. 1 (filled circles). When the pH in each solution was raised, the membrane potential depolarized in the Ringer's solution containing 2.5 mM K, but it hyperpolarized in a higher K concentration in Fig. 1 (filled squares).

Fig. 1. Membrane potential-external potassium concentration relation in neutral and high pH Ringer's solutions at 20°C. Ordinate: membrane potential in mV. Abscissa: external potassium concentration in mM. Filled circles, pH 7.2; filled squares, pH 8.2. The curve was drawn by a polynomial curve fitting. -86.7 mV and 3.85 mM at the cross. When the membrane potentials at 2.5 and 5 mM of external potassium with pH 8.2 were not close, two points were further added to fit a polynomial curve.

Calculation of intracellular chloride concentration

At the crossing point in Fig. 1, the condition of $dV_m/dP_{Cl}=0$ derived in the theoretical section is fulfilled. A derived equation Eq. (3) was used to calculate the intracellular chloride concentration. The membrane potential obtained in Fig. 1 was corrected for the liquid junction potentials between Ringer's solution and 3 M KCl, and the myoplasm and 3 M KCl solution by -2.6 mV (HIRONAKA and MORIMOTO, 1979), and incorporated into Eq. (3). The results of 5 experiments are summarized in Table 1.

This equation can be rewritten to give:

$$V_m = \frac{RT}{F} \ln \frac{[Cl]_i}{[Cl]_o} \quad (4)$$

where V_m represents the chloride equilibrium potential (E_{Cl}). The E_{Cl} was -88.5 mV on the average, which was clearly positive to the resting membrane potential by 6.3 mV (Table 1). The clear difference in the potentials between the E_{Cl} and the E_m in Table 1 strongly suggests that a chloride pump may exist in the frog skeletal muscle.

Further evidence for the existence of a chloride pump

The membrane depolarization with pH increase was measured over a wide range of temperature (2.5 to 22°C) in Fig. 2. The membrane potential was firstly measured on 5 fibers of a muscle in the high Ca-Ringer's solution with pH 7.2, and secondly the potential change induced by the pH increase (pH 9.2) was measured on the 5 fibers as in Fig. 1 at a temperature. The muscle was then cooled down to a relevant temperature by circulating water with the temperature around the chamber for 30 min, and the potential change induced by the pH increase was measured as above.

With cooling, the depolarization induced by the pH increase became smaller and finally disappeared when cooled below 5°C (Fig. 2), indicating that the chloride pump had been inhibited to attain a passive distribution of the chloride across the membrane. A slight depolarization observed with cooling was that

Table 1. Intracellular chloride concentration $[Cl]_i$, chloride potential E_{Cl} , and resting membrane potential E_m .

Muscle	$[Cl]_i$ (mM)	E_{Cl} (mV)	E_m (mV)
1	3.3	-91.4	-96.6
2	3.4	-90.6	-96.4
3	4.2	-85.0	-95.4
4	4.0	-86.0	-90.6
5	3.5	-89.3	-94.8
Mean $\pm$ S.E.	3.7 ± 0.18	-88.5 ± 1.26	-94.8 ± 1.09

In calculation the membrane potential as obtained in Fig. 1 was corrected for the liquid junction potentials. See text for the details.

Fig. 2. Effect of temperature on the depolarization induced by the pH increase (7.2 to 9.2). Ordinate: change in membrane potential in mV. Abscissa: temperature in °C.

predicted on the physical basis from the amount of the cooling temperature. The potential was restored to the original level by rewarming the muscle fibers, but the depolarization effect by the pH increase was not recovered in 30 min after rewarming when the muscle was drastically cooled down, say below 5°C.

DISCUSSION

In the present study, a specific permeability change of the membrane induced by a pH change was utilized for ion-selective electrode so that only a micro-electrode filled with 3 M KCl needed to be introduced to measure the membrane potential, thereby keeping the intracellular ionic perturbation relatively small. Since the potential measurements were performed with special reference to accurate potential measurements without introducing the "tip potentials" (HIRONAKA and MORIMOTO, 1979), the error involved in Table 1 would be less than 4% in terms of the potential measurements (1 mV at most in Eq. 3).

When the experiment as in Fig. 1 and Table 1 was performed with a higher pH of 9.2, essentially the same result was obtained, *e.g.*, the average $[Cl]_i$, E_{Cl} and E_m were 3.8 mM, -87.2 mV and -94.1 mV respectively ($n=3$). However, the reversibility of the measurements was worse at the high pH so that only the results obtained with pH 8.2 were summarized in Table 1.

Accumulation and depletion effects of chloride ion should be negligible based on the magnitude of the chloride permeability (HODGKIN and HOROWICZ, 1959; HUTTER and NOBLE, 1960) and the time required for the potential measurements.

Especially the crossing point in Fig. 1 at which the intracellular chloride concentration was determined corresponds to the E_{Cl} , resulting in no net flux of chloride.

It is interesting to find that the upper and lower limit of the intracellular chloride concentration determined by radio-active tracer techniques (3.1–3.8 m mole/kg fiber water; ADRIAN, 1961) well involves the value determined with this method (Table 1). Our measurement further revealed that the E_{Cl} was significantly positive to the resting membrane potential (Table 1, $P < 0.01$ with t -test), strongly suggesting that there exists a chloride pump in the frog skeletal muscle. This was further supported by the experimental fact provided in Fig. 2. The difference between this result and the previous studies (LING and GERARD, 1949; HODGKIN and HOROWICZ, 1959; HUTTER and NOBLE, 1960; ADRIAN, 1960, 1961) may be ascribed to a matter of the experimental resolution. In this connection, it is also interesting to have found that the chloride pump was not active without cooling when the frogs were in hibernation (unpublished observation).

The marked temperature dependency of the pumping activity around 5–10°C as seen in Fig. 2 suggests a molecular mechanism for the chloride pump (*cf.* also HIRONAKA, 1974). It is worthy of note that some proteins denature when cooled below 10–15°C and some membrane lipids have their melting point in the range of the temperature concerned (LEHNINGER, 1970).

Active transport of chloride in neurons has been shown to be outwardly directed, and E_{Cl} serves as a hyperpolarizing battery for inhibitory postsynaptic potentials (LUX, 1971; RUSSELL and BROWN, 1972; LLINAS *et al.*, 1974). In a number of other excitable cells, on the other hand, it has been proposed to occur inwardly, for instance in squid giant axon (KEYNES, 1963; RUSSELL, 1976), smooth muscle (CASTEELS, 1971), and cardiac muscle (LAMB, 1961; LADLE and WALKER, 1975). Some one of the active transports seems related to regulate intracellular pH (RUSSELL and BORON, 1976). As the chloride conductance is relatively high in frog sartorius muscle (HODGKIN and HOROWICZ, 1959; HUTTER and NOBLE, 1960), considerable work may be required to pump chloride against the potential difference. Further study is necessary to establish the molecular basis of the pumping mechanism and the biological significance of the inwardly-directed chloride pump in the frog skeletal muscle.

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