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KINETIC PROPERTIES OF THE CHLORIDE CONDUCTANCE OF FROG MUSCLE

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SUMMARY

1. The anion conductance of frog muscle has been studied at alkaline, neutral and acid extracellular pH values using a voltage clamp technique. Potassium in the extracellular solution was replaced by rubidium in order to simplify the behaviour of the cation conductance.

2. At pH 9·8 the chloride conductance fell exponentially during a hyperpolarizing voltage step. The speed of inactivation was directly proportional to the hyperpolarization from the holding potential; at 60 mV the rate constant was about 0·01 $msec^{-1}$.

3. An exponential fall in chloride current during the voltage pulse also occurred at pH 7·4; the speed of inactivation, which was proportional to the membrane potential, was about 20% greater at neutral than at alkaline pH values.

4. The instantaneous voltage–current relation was approximately linear at pH 7·4 and 9·8; the instantaneous conductance was always greater at the alkaline pH value.

5. At neutral pH values when there were no time-dependent conductance changes the voltage–current relation was linear.

6. In acid solutions (pH 5·0) the chloride current gradually increased during a hyperpolarizing voltage step. The time course of this increase was complex, but it took place at greater speed during large voltage steps.

7. Comparison of the steady-state voltage–current relations measured in the absence and presence of chloride ions confirmed that in alkaline solutions the chloride current could reach a limiting value.

8. The equilibrium potential for the time-dependent conductance changes was close to the holding potential.

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INTRODUCTION

In the preceding paper (Hutter & Warner, 1972) it was shown that in frog muscle alterations in the extracellular pH value change the voltage dependence of the membrane current carried by chloride ions. Time-dependent variations in chloride conductance were also found to take place during the passage of current across the muscle membrane. The present paper contains a description of the properties of these conductance changes determined with a voltage clamp technique (Adrian, Chandler & Hodgkin, 1970*a*).

METHODS

Only additional details applying to experiments using the voltage clamp technique are described here. All of the other relevant information is given in the previous paper (see Methods; Hutter & Warner, 1972).

The technique was identical to that used by Adrian, Chandler & Hodgkin (1970*a*, *b*). Three micro-electrodes were inserted in a line close to the pelvic tendon of a sartorius muscle fibre. The electrode nearest to the tendon was inserted midway between the tendon and the second electrode. As in experiments using constant current pulses the two electrodes nearest to the tendon recorded the membrane potential, the difference in voltage measured between these two electrodes being proportional to the membrane current, while the third electrode injected current into the muscle fibre. Voltage control was maintained with reference to the recording electrode closest to the tendon using a feed-back circuit which allowed the membrane potential to be clamped at a value away from the holding potential within 2 msec.

While the muscle was superfused with rubidium-containing solution at pH 7·4 or 9·8 (solution *B*, Table 1, Hutter & Warner, 1972) the two voltage recording electrodes were inserted into the muscle fibre. Small, hyperpolarizing, constant current pulses were put on to the current passing electrode and it also was lowered into the fibre. If an electrotonic potential was visible at both recording electrodes and the resting membrane potential, E_m, had not fallen below -80 mV the setup was switched over to voltage control. The holding potential, E_h, was always set equal to the resting potential after all three electrodes had been inserted into the fibre, so that initially the holding current was very small. Chloride ions are normally passively distributed across the membrane according to the level set by the membrane potential (Boyle & Conway, 1941; Hodgkin & Horowicz, 1959). With the technique used in these experiments voltage control is maintained only over the portion of the muscle fibre lying between the current passing electrode and the pelvic tendon and when the holding potential is far away from E_m a potential gradient, and therefore a chloride concentration gradient, is set up along the muscle fibre. Setting E_h equal to E_m at the start of a run ensured that initially the internal chloride concentration was uniform along the fibre. In practice so long as the muscle was superfused with solution at neutral and alkaline pH values the holding current remained small and close to the starting value. However, in acid solutions the time available before significant depolarization (cf. Hutter & Warner, 1967*a*), and consequent elevation of $[Cl]_i$ set in over the rest of the fibre was rather short; once this occurred the fibre was discarded.

To prolong the time course of the time-dependent membrane current changes being studied the temperature of the perfusion fluid was often held below room temperature (20–22° C) at either 15 or 18° C. If the temperature was taken much

lower the reduction in the relative chloride contribution to the membrane conductance arising from the difference in the Q_{10} of the anion and cation conductances of frog muscle (Harris, 1958) became too great.

When junction potentials needed to be avoided the voltage reference electrode was replaced with a micro-electrode with large tip diameter ($\simeq 2\,\mu$m) and low resistance ($\simeq 200\,\Omega$) filled with 3 M potassium chloride. This electrode was placed downstream of the preparation to prevent potassium chloride leaking from the electrode affecting the muscle.

RESULTS

Fig. 1 shows records of the current which flowed across the muscle fibre membrane in response to a 36 mV hyperpolarizing voltage step away from the holding potential at pH 9·8, 7·4 and 5·0. The holding potential

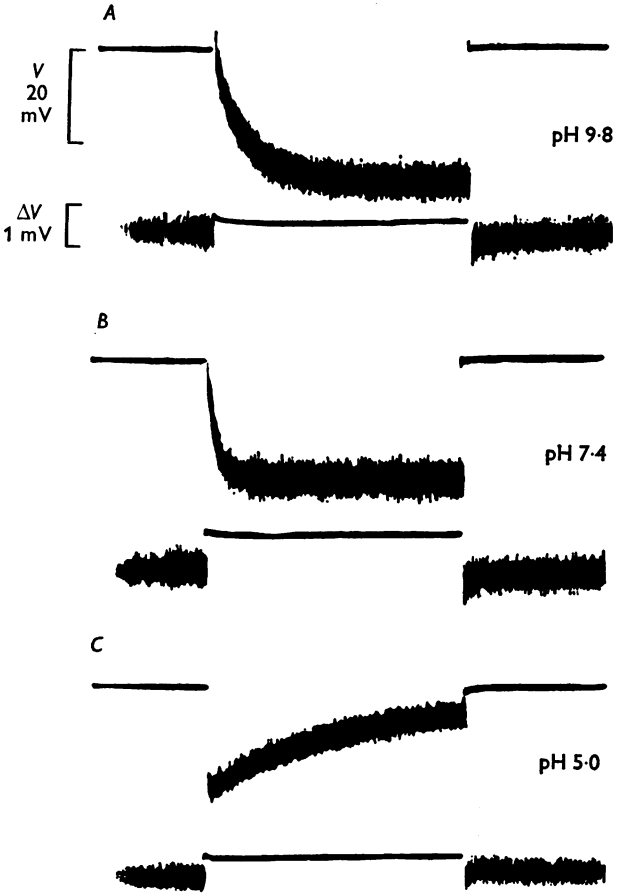

Fig. 1. Membrane currents flowing during a hyperpolarizing voltage clamp step at pH 9·8, 7·4 and 5·0. $E_h = -80$ mV; $l = 500\,\mu$m; $\Delta V = 1$ mV $\equiv 3{\cdot}14 \times 10^{-6}$ A/cm^2; Temperature = 18° C. 1 sec clamp pulse. Note inward current shown as an upward deflexion.

for this fibre, that is, the resting potential after insertion of the third electrode, was −80 mV. In alkaline rubidium-containing solution (Fig. 1*A*) once the transient arising from the charging of the membrane capacitance was over the membrane current fell approximately exponentially from a high value at the beginning of the pulse to a steady level 500 msec later with a time constant of about 100 msec. Little membrane current continued to flow after the voltage step ended. In acid solution (Fig. 1*C*) a *rise* in membrane current, corresponding to an increase in conductance, was seen. The current climbed approximately exponentially throughout the voltage step and a steady state was not reached within the duration of the pulse, confirming that the time constant for this activation process is relatively long (Hutter & Warner, 1972).

Time-dependent alterations in membrane current were found to occur even in neutral solutions (Fig. 1*B*). The current fell less far, but more rapidly, than in the alkaline solution; in Fig. 1*B* the current was steady after about 200 msec.

In rubidium-containing methyl sulphate solution no comparable alterations in membrane current were seen. So long as the cation conductance retains this stability also in the presence of chloride ions it may be concluded that the conductance changes represent pH-dependent inactivation and activation of the chloride current through the membrane.

Analysis of the time-dependent alterations in chloride current followed the pattern set by others during investigation of both active and passive components of the membrane conductance in nerve, cardiac and skeletal muscle (e.g. Hodgkin & Huxley, 1952; Noble & Tsien, 1968, 1969; Adrian, Chandler & Hodgkin, 1970*a*, *b*).

The behaviour of the chloride conductance in alkaline solutions

The potential dependence of the transient currents. The rate at which the chloride conductance approached its steady-state value was determined by plotting semi-logarithmically the difference between the current in the steady state and at other times during the voltage step. The current varied exponentially with time at all voltages. In Fig. 2*A* the time constant for the inactivation has been plotted against the deflexion from the holding potential; only measurements from pulses negative to the holding potential, −84 mV, have been included. The conductance change took place with greater speed as the membrane potential was made more negative and the time constant, τ, fell from 250 msec at −100 mV to 110 msec at −160 mV. On occasion the time course of the membrane current change was not truly exponential and there was some scatter in the time constant measured at each potential, particularly close to the holding potential.

The rate constant for the current change was directly proportional to

the membrane potential over the whole of the voltage range studied, as in all other fibres examined (up to 100 mV negative to E_h; ten fibres). Such behaviour contrasts with that of most other time-dependent systems studied, where a minimum in the τ^{-1} (E_m) relation has generally been observed (e.g. Noble & Tsien, 1968, Fig. 4; Adrian *et al.* 1970*a*, Fig. 14).

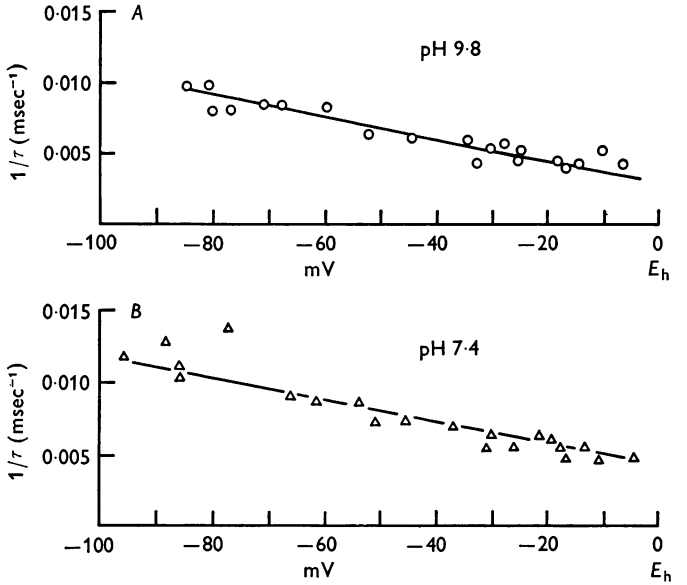

Fig. 2. Relation between time constant for inactivation of the membrane current and the voltage deflexion from the holding potential. *A*, pH 9·8; *B*, pH 7·4. Same fibre. Holding potential = −84 mV, 15° C.

Perhaps in the present experiments only one limb of an extremely shallow τ^{-1} (E_m) relation of the kind illustrated by Noble & Tsien (1968) and Adrian *et al.* (1970*a*) has been covered. But the fit to a straight line was generally good and it seems more probable that the rate constant for the transient chloride current depends directly on the membrane potential, like the potassium current through the inward rectifier (Adrian *et al.* 1970*b*, Fig. 11).

The instantaneous voltage–current relation. The current flowing at the beginning of the pulse was estimated by extrapolating semi-logarithmic plots of membrane current measured at different times during the clamp step back to zero time. The accuracy of the method depended on the speed and magnitude of the current changes being measured and was least close to the holding potential, where the membrane currents were often very small. The instantaneous voltage–current relation so obtained has been plotted, along with the steady-state relationship, in Figs. 6 and 8.

The relation was roughly linear, resembling the fast component of the potassium conductance in skeletal muscle (Adrian *et al.* 1970*a*) rather than the inwardly rectifying component of the potassium conductance, for which both instantaneous and steady-state voltage–current relations are non-linear (Noble & Tsien, 1968; Adrian *et al.* 1970*b*).

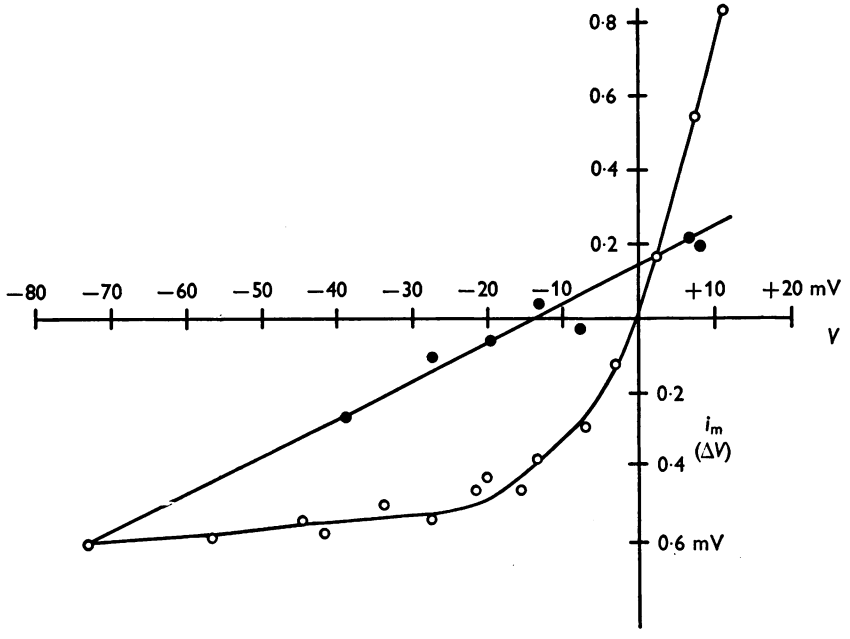

Fig. 3. Steady-state voltage–current relation and instantaneous 'off' relation in chloride-containing solution at pH 9·8. ○, steady-state relation; ●, instantaneous 'off' relation. $E_h = -90$ mV; $l = 500$ μm; $\Delta V = 1$ m$V \equiv 3{\cdot}14 \times 10^{-6}$ A/cm^2.

To determine the instantaneous relation when the voltage step was removed, and the chloride conductance reactivated, a second voltage step, V_2, was imposed immediately following the first, V_1. With V_1 held constant and large V_2 was varied and the current at the beginning of the second clamp pulse estimated as described for a single clamp step. Fig. 3 shows such an experiment in which V_1 was held at -165 mV and V_2 varied between -130 and -80 mV. Because the measurements are based on an extrapolation procedure there must be some uncertainty about the exact position of this instantaneous voltage–current relation. But it is roughly linear and crosses the voltage axis about 10 mV negative to the holding potential (-90 mV), suggesting that during the passage of current through the membrane some distribution of charge takes place which cannot immediately be reversed.

The equilibrium potential for the transient current changes. The fact that little current flowed after a single voltage step despite large transient alterations in current during the pulse, suggested that the equilibrium potential for this system lay in the region of the holding potential; this is confirmed by the experiment of Fig. 4*A*. A large hyperpolarizing voltage pulse, V_1, was applied to the membrane and once the current had become steady a second voltage pulse, V_2, brought the membrane potential close to E_h, the initial resting potential of the fibre, which was found to be the equilibrium potential regardless of the absolute level of E_h.

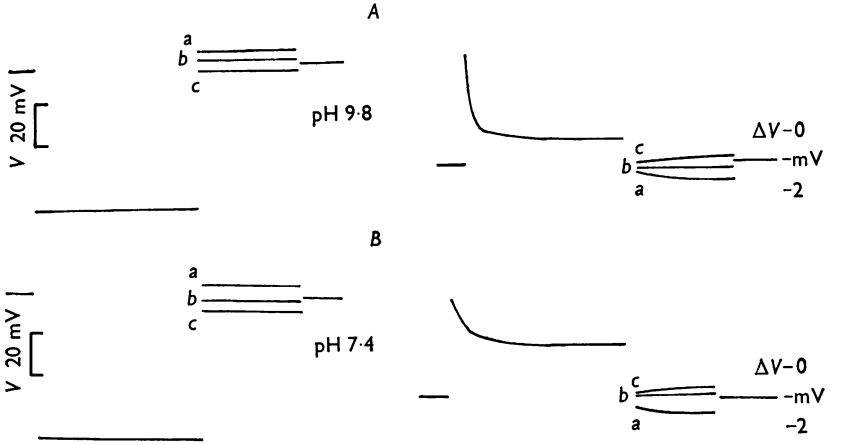

Fig. 4. The reversal potential of the transient chloride currents. *A*, pH 9·8; *B*, pH 7·4. V_1 = 68 mV. l = 500 μm; ΔV = 1 mV $\equiv$ $3{\cdot}14 \times 10^{-6}$ A/cm^2. Records traced from originals.

The availability of the chloride conductance. The tail currents following a single voltage step were too small to allow measurement of the fraction of the system still available at the end of the pulse. The current flowing at the beginning of a second clamp pulse, V_2, to a fixed level about 20 mV away from the holding potential, was therefore measured for various values of V_1. Such measurements are plotted in Fig. 5*A*, where the results are also expressed as fractions of the maximum instantaneous current at V_2. The relation is sigmoid, as for the activation and inactivation variables of the sodium and potassium conductances (Hodgkin & Huxley, 1952; Weidmann, 1955; Noble & Tsien, 1968, 1969; Adrian *et al.* 1970*a*) with a slope of 0·5/10 mV over the steepest part. Maximal activation occurred about 15 mV positive to the holding potential, which was -84 mV, and a steady level of inactivation of about 0·3 was reached for potentials more than 55 mV negative to the holding potential. Either the time-dependent system under study never completely inactivates, or it is superimposed upon another current component which is still activated at this potential.

The alternative possibility that the increase in chloride conductance, which at very long times succeeds the inactivation process even in neutral and alkaline solutions (Hutter & Warner, 1972), lifts the fractional activation (E_m) relation away from zero for potentials greater than -110 mV cannot be ignored. Measurement of the membrane resistance using constant current pulses (Hutter & Warner, 1972) suggested that in alkaline solution any activation process did not occur within the duration of a 1 sec pulse until potentials more than 80 mV negative to the resting potential were reached. In the experiment of Fig. 5*A* the membrane potential was not driven more than 55 mV negative to E_h, -84 mV, so that contributions from such an activation process should have been negligible. In addition the membrane current at the end of both voltage steps fell along the same steady-state voltage–

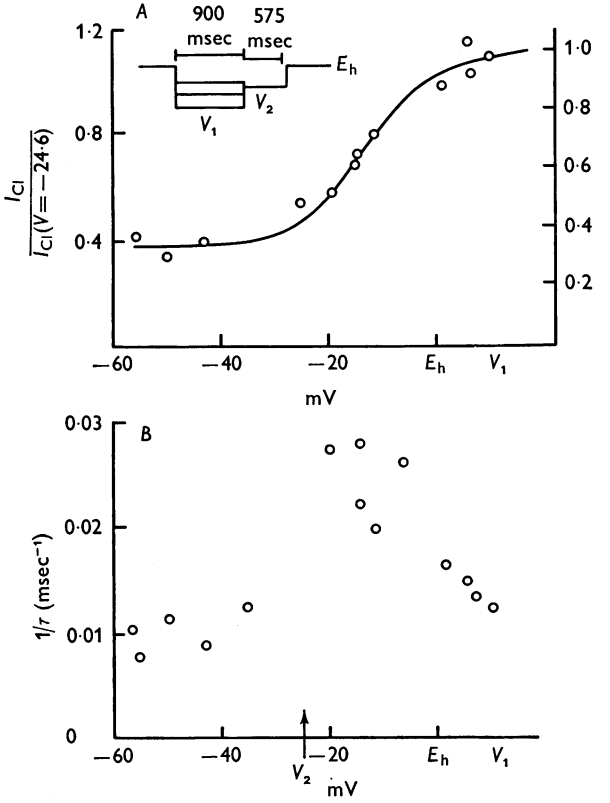

Fig. 5. *A*, the availability of the chloride conductance at different membrane potentials in alkaline solution. Ordinates: L.H.S. Current at beginning of second clamp step (24 mV negative to the holding potential). R.H.S. fraction of the maximum instantaneous current flowing at V_2. Abscissa: potential during first clamp step as deflexions from the holding potential. $E_h = -84$ mV; 21° C.

B, the time constant after conditioning clamp to varying membrane potentials. Ordinate: rate constant for conductance change (msec^{-1}). Abscissa: potential during conditioning clamp step (mV). Same expt. as in Fig. 5*A*.

current relation despite the different pulse widths ($V_1 \doteq 1$ sec; $V_2 \doteq 600$ msec) and it therefore seems reasonable to suppose that for potentials about 50 mV negative to E_h the chloride conductance at the end of the first voltage step was in a steady state.

If the availability of the chloride conductance is a unique function of E_m then the time constant for variations in chloride conductance should not depend on the degree of activation at a given value of E_m. Fig. 5*B* shows the rate constants for the approach of the current in V_2 to a steady state, plotted against the membrane potential, V_1. When V_1 is less than -110 mV (V_2 held constant at -110 mV) the membrane current decreases with time and the rate constant for decay of the current still activated at the beginning of the second pulse becomes progressively less, falling from about 0·028 msec^{-1} when the system is 50 % available to 0·013 msec^{-1} with the system fully available. When V_1 is greater than -110 mV and the fraction of the system available has fallen to a steady level, the rate constant for the reactivation of the chloride conductance is more or less constant at about 0·01 msec^{-1}. Thus the kinetic properties of the time-dependent chloride conductance are a function not only of the membrane potential, but also of the magnitude of the current carried by chloride ions. As a corollary the kinetic behaviour of the chloride conductance after a voltage step should be influenced by the duration of the step for pulse widths less than the time taken for the system to come to steady state. In view of these results it is surprising that during a single voltage step the chloride current inactivated along a simple exponential time course, since the time constant is itself a function of the degree of inactivation. However, the effect would be greatest over the voltage range -30 to $+10$ mV around E_h (Fig. 5*B*), where the membrane currents are small and most difficult to measure accurately.

In systems in which time-dependent alterations in membrane current have been described by the Hodgkin–Huxley equations 50 % activation or inactivation occurred at about the same membrane potential as the minimum in the relation between membrane potential and the time constant for the conductance change, and the degree of activation or inactivation was a function of the membrane potential alone (cf. Noble & Tsien, 1968, 1969; Adrian *et al.* 1970*a*). Figs. 2*A* and 5 show that this is *not* the case for the anion current studied here, for the speed of inactivation increased more or less linearly to potentials beyond that at which the current was 50 % inactivated and the speed of the conductance changes varied with the degree of activation at a particular membrane potential. This suggests that the kinetic behaviour of the anion conductance of frog muscle does not fall into the range of phenomena to which the Hodgkin–Huxley equations can be applied.

The position of the steady-state voltage–current relation. The voltage clamp technique incidentally made measurement of the voltage–current relation in rubidium-containing methyl sulphate solution (solution *D*, Hutter & Warner, 1972) more feasible and allowed comparison of voltage–current relations determined in the absence and presence of chloride ions in the same fibre. Fig. 6 illustrates one of such experiments, which were done to establish whether in alkaline solution the chloride current reaches a limiting value for large negative membrane potentials (cf. Hutter & Warner, 1972). In the absence of chloride ions the membrane conductance

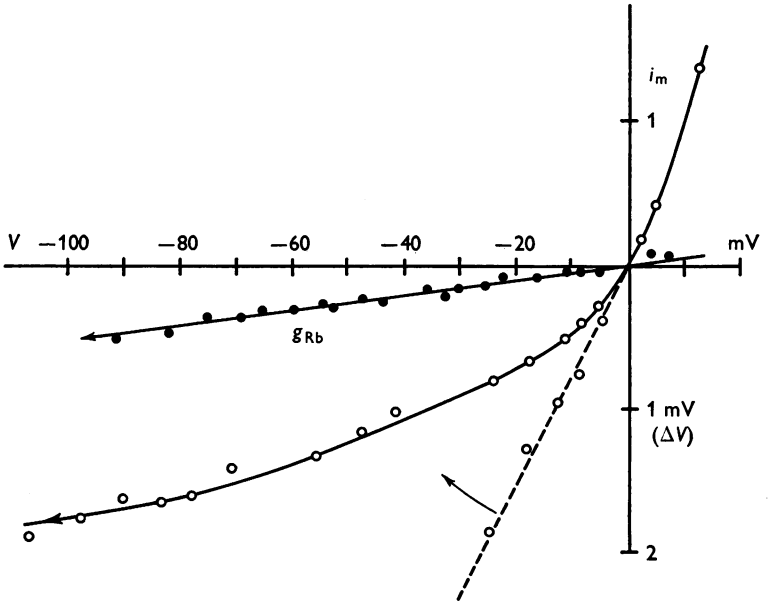

Fig. 6. Steady-state voltage–current relations in alkaline solutions determined in the absence and presence of chloride ions. ○ : chloride-containing solution; ● : methyl sulphate-containing solution. $E_h = -87$ mV; $l = 500$ μm; $\Delta V = 1$ mV $\equiv 3{\cdot}14 \times 10^{-6}$ A/cm²; 15° C.

was low, 12 μmho/cm², and the voltage–current relation linear. When chloride ions also carried current the conductance fell steeply as the membrane potential was made more negative and the two steady-state voltage–current relations became parallel for large hyperpolarizations suggesting that the maximum chloride current was limited. No sign of any activating current was seen in this fibre. The dotted line marks the position of the instantaneous voltage–current relation. The chloride current for small voltage deflexions from the holding potentials was 141 μmho/cm², which when substituted into the constant field equation gave the permeability constant for chloride, P_{Cl}, as $3{\cdot}4 \times 10^{-6}$ cm/sec. This is close to that

measured by Hodgkin & Horowicz (1959) for single muscle fibres from semitendinosus at pH 7·2 and seems low for a muscle in alkaline solution, but it fits the values of about $2{\cdot}0 \times 10^{-6}$ cm/sec previously measured in sartorius fibres at neutral pH (Adrian & Freygang, 1962; Hubbard, 1963).

The effect of temperature on the transient chloride currents. In Fig. 7*A* average values of the rate constant for inactivation of the chloride conductance measured 60 and 20 mV negative to the holding potential, are plotted on a semi-logarithmic scale for temperatures of 15, 18 and 21° C. At 60 mV the Q_{10} was 2·3 and at 20 mV 1·8. These values are similar to that previously found in alkaline solutions when measuring the efflux of ^{36}Cl under equilibrium conditions (O. F. Hutter, unpublished).

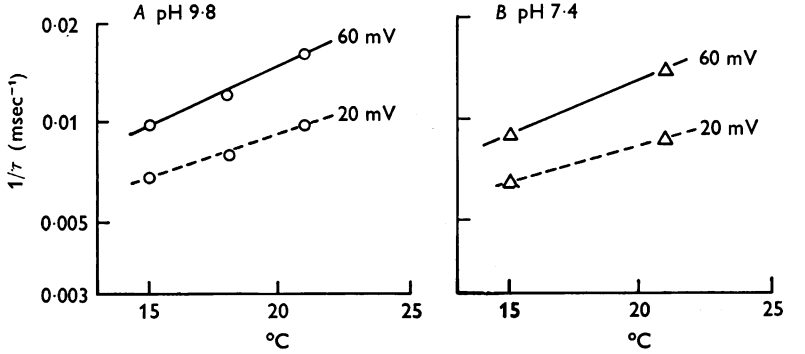

Fig. 7. Temperature dependence of transient currents at pH 9·8 (*A*) and 7·4 (*B*). Ordinates: rate constant for decline of transient current: $msec^{-1}$ (means of several measurements). Abscissa: temperature in °C; — measurements made 60 mV negative to E_h; - - - - - measurements made 20 mV negative to E_h.

The chloride conductance in neutral solutions

When a step potential change was imposed on the membrane of a muscle fibre in solution at pH 7·4 time-dependent alterations in chloride conductance, which shared the properties of the system described in alkaline solutions, were observed.

The effect of potential on the speed of the conductance changes. The transient variations in chloride current became faster as the membrane potential was made more negative; the conductance fell exponentially during the voltage step and the rate constant for the conductance change is plotted against the membrane potential in Fig. 2*B*. When measurements were made in the same fibre, at each value of the membrane potential the rate constant was greater in the neutral than in the alkaline solution. Thus in Fig. 2, 85 mV negative to the holding potential the rate constant at pH 7·4 was 0·011 $msec^{-1}$ and at pH 9·8 0·0095 $msec^{-1}$. 17 mV away from E_h the neutral rate constant was 0·006 $msec^{-1}$ as compared with 0·004 $msec^{-1}$ at

pH 9·8. Because the magnitude of the transient current changes were smaller than at pH 9·8, as well as occurring with greater speed, the errors in the rate constant determination were larger than in the alkaline solution, but again a linear relation between τ^{-1} and E_m was usually seen, as in the illustrated experiment. The chloride currents produced by the same voltage step at pH 7·4 and 9·8 in another fibre are illustrated in Fig. 8. By the time this membrane potential was reached (40 mV away from E_h, −80 mV) the steady-state voltage–current relations had crossed over (Fig. 8); this is reflected by the fall in chloride current at pH 9·8 from an initial value almost twice that at pH 7·4 to a steady-state level below the final current in the neutral solution.

The instantaneous voltage–current relation. The semi-logarithmic plots used to determine the rate constant were extrapolated back to give the

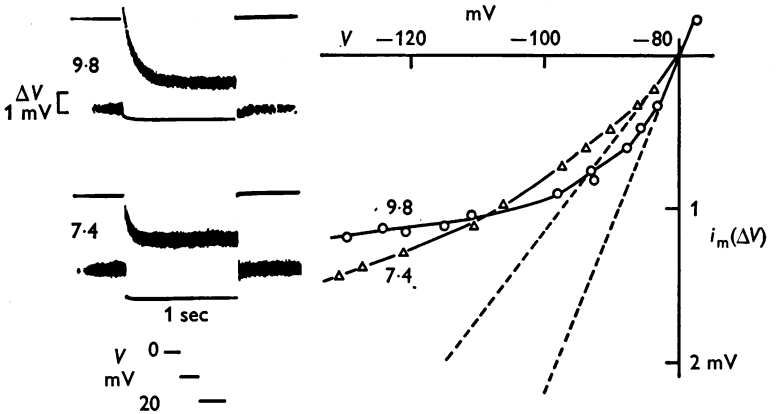

Fig. 8. Comparison of steady-state and instantaneous voltage–current relations at pH 9·8 (○) and 7·4 (△). Abscissa: potential during clamp step, mV. Ordinate: membrane current density plotted as ΔV. ΔV = 1 mV ≡ $3{\cdot}14 \times 10^{-6}$ A/cm². $E_h = -80$ mV; $l = 500$ μm; 18° C. Continuous line, current at end of 1 sec clamp step; interrupted line, current at beginning of clamp step. Individual clamp steps to the same potential at pH 9·8 and 7·4 are shown on the left.

current at the beginning of the pulse. The relation between voltage and the instantaneous current so obtained is plotted as the dashed line in Fig. 8, which compares the voltage–current relations obtained in neutral and alkaline solutions in one fibre. In the neutral, as in the alkaline solution, the instantaneous relation was roughly linear, so that, in contrast to the steady-state membrane conductances, the instantaneous conductance at pH 7·4 was less than at pH 9·8 for all values of the membrane potential.

The position of the instantaneous voltage–current relation measured after removal of the voltage step proved difficult to determine with

accuracy. The magnitude of the current change produced by a second voltage step was always rather small and it quickly decayed back to the steady-state value appropriate for V_2.

The equilibrium potential of the transient current changes. The current produced by a second voltage step often did not reverse direction until V_2 was just positive to the holding potential (Fig. 4*B*) suggesting that in the neutral solution the equilibrium potential could be slightly displaced from the resting potential. Since the muscle fibre of Fig. 4 had been under voltage control for 27 min when these measurements were made, away from the pelvic tendon the membrane potential might well have fallen a little below E_h, the initial resting potential of the fibre, with consequent elevation of $[Cl]_i$, so that even in the region of voltage control E_{Cl} might have been just less than E_h.

The availability of the chloride conductance at different membrane potentials. The small size and the speed of the time-dependent changes made reliable measurement of the degree of activation at different membrane potentials difficult. But the relation was sigmoid, as in the alkaline solution, and activation seemed to be complete in the region of the holding potential.

Temperature dependence of the conductance changes. The rate constant for current inactivation measured at different temperatures in the neutral solution is plotted in Fig. 7*B* for two potentials. The Q_{10} was 2·1 at 60 mV and 1·7 at 20 mV, i.e. similar to the alkaline values. Measurement of the Q_{10} for chloride fluxes under equilibrium conditions also showed little difference in temperature dependence between neutral and alkaline solutions (O. F. Hutter, unpublished).

In two fibres no time-dependent changes in anion conductance were seen in the neutral solution. On both occasions the voltage–current relation was linear and the absolute value of the chloride conductance, given by the difference in g_m measured in the absence and presence of chloride ions, was relatively low. In these fibres the voltage dependence in the alkaline solution was less steep than normally observed, as if the mid-point of the g_{Cl}–pH relationship (cf. Hutter & Warner, 1967*a*) had been shifted to alkaline pH values. Although the voltage dependence at pH 7·4 in these fibres was more like that usually found in acid solutions, there was no sign of the time-dependent increase in chloride conductance characteristic of low extracellular pH values, perhaps because the resting chloride conductance was higher than at pH 5·0.

The behaviour of the chloride conductance in acid solutions

The information which could be obtained about the properties of the chloride currents at pH 5·0 was limited. After some time there was a dramatic increase in the holding current; the membrane properties altered

concomitantly and the fibre was then discarded. This probably reflects a rise in the intracellular concentration of chloride ions in response to depolarization in the region of the fibre not under voltage control (cf. Hutter & Warner, 1967*a*). Often rather less than 10 min elapsed after the solution change before the experiment came to an end. Detailed kinetic information allowing a full description of the membrane current changes could not therefore be collected, but nevertheless some interesting features emerged.

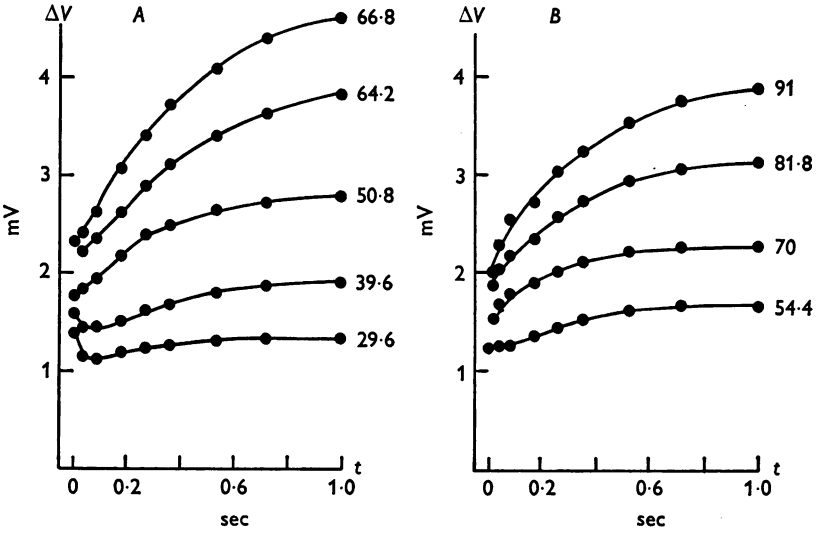

Fig. 9. Time-dependent increases in conductance in acid solution produced by hyperpolarizing voltage steps. Two fibres (*A* and *B*). Ordinate: membrane current density as ΔV. $\Delta V = 1$ mV $\equiv 3{\cdot}14 \times 10^{-6}$ A/cm^2. Abscissa: time after beginning of clamp pulse. *A*, $E_h = -80$ mV; $l = 500$ μm; 18° C; *B*, $E_h = -96$ mV; $l = 500$ μm; 18° C. The figures beside each plot give the deflexion from the holding potential (mV).

Fig. 9 shows the family of membrane currents produced by increasing hyperpolarizing voltage steps away from the holding potential in two fibres. For small voltage steps the membrane current increased along an S-shaped curve; as the step became larger the conductance changes took place more rapidly and the early delay was no longer apparent. The potential at which an increase in membrane current with time was first seen varied greatly from fibre to fibre; sometimes a hyperpolarization of 30 mV sufficed, but on other occasions the time-dependent increase in membrane current was not seen until potentials 50 or 60 mV negative to the holding potential were reached. Two points are worth noting. First, in some fibres an early *fall* in membrane current was seen, as in Fig. 9*A*.

Secondly, even when the current increase was not delayed the change in membrane current ($i_\infty - i_t$) could not be described by a single exponential term. An early fall in membrane current could introduce a delay into the rise time of a subsequent small exponential increase but would not account for the non-exponential behaviour of the large membrane currents produced by big voltage deflexions.

Thus the membrane current in acid solutions seems to be made up of several components. The first, not seen in all fibres, was small and rapidly inactivating, perhaps related to the inactivating currents seen in neutral and alkaline solutions. The subsequent increase in membrane current could have come from a single component activating with a delay. Alternatively it could be the sum of two currents each of which increases exponentially, but with different time constants, the early part of the first exponential being masked by the initial inactivating component. One way of deciding between these two possibilities is to look at the inactivation of the current switched on during the hyperpolarizing voltage step. If two components are switched on then the time course of the inactivation process is also made up of two exponential processes. If there is only a single process activating with a delay then the inactivation time course will be a single exponential. In a few experiments a second voltage step was applied after the first and the time course of the inactivation plotted semi-logarithmically against time, but no consistent picture emerged.

Because the time course of the increase in membrane current was complex the instantaneous voltage–current relation in the acid solution could not be assessed with any accuracy, but its position could be estimated on the basis of the change in membrane current with time. For small voltage steps the instantaneous current could not have been far from the earliest membrane current measurement, as initially the current increased very slowly. For larger voltage deflexions the early portion of a semi-logarithmic plot of ($i_\infty - i_t$) was extrapolated back to the beginning of the pulse. Plots based on these estimates showed the instantaneous voltage–current relation to be roughly linear.

The equilibrium potential for the increase in membrane current in acid solution was always depolarized to the holding potential. For a current carried by chloride ions the equilibrium potential might well be slightly less than the membrane potential at pH 5·0, as there is evidence that in acid solution the internal chloride concentration rises above the level expected on the basis of passive distribution of chloride ions (Hutter & Warner, 1967*a*). But the displacement of the equilibrium potential away from the holding potential, the initial resting potential of the fibre, was probably the consequence of an increase in $[Cl]_i$ arising from depolarization over the rest of the muscle fibre. In one fibre the equilibrium potential

was −65 mV with a holding potential of −80 mV. The holding current was immediately removed and the true resting potential of the fibre found to be −60 mV. Thus the equilibrium potential was close to the resting potential of the fibre at the time that the measurements were made.

DISCUSSION

The steady-state chloride conductance of frog muscle in solutions near to neutral pH has hitherto been found to behave in a manner consistent with the requirements of the constant field theory (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960; Adrian & Freygang, 1962; Hubbard, 1963; Harris, 1965; Hutter & Warner, 1972). The finding that the chloride current was time as well as voltage dependent at pH 7·4 came, therefore, as a surprise. The voltage–current relation could be swinging from a position appropriate for a high value of P_{Cl} to one for a low value of P_{Cl}, but in that case some rectification of the instantaneous voltage–current relation would be expected. Errors inherent in the estimation of the instantaneous relation might have masked a small degree of rectification, but this criticism cannot be levelled at the finding that when there was no time dependence, voltage dependence of the chloride conductance was also absent. If, at neutral pH values, alterations in P_{Cl} underlay the variations in chloride conductance with time then, on the constant field assumptions, even in the absence of time-dependent conductance changes some voltage dependence would still be expected.

It has already been pointed out (Hutter & Warner, 1972) that the steady-state voltage–current relations found in acid and alkaline solutions cannot easily be accounted for on the basis of the constant field theory and the findings described in the present paper call into question the applicability of the constant field assumptions in neutral solutions. The question therefore arises: can an alternative hypothesis, which will account for both the time and voltage dependence of the chloride conductance at different pH values, be found?

One way of interpreting time-dependent changes in ionic current is to suppose that they arise as a result of accumulation or depletion of solute in a layer close to the cell membrane. This approach has been used to account for the slow time-dependent changes in potassium current which occur in frog muscle during hyperpolarizing current pulses (Adrian & Freygang, 1962) and for time-dependent conductance changes in cells of *Chara australis* (Barry & Hope, 1969*a*, *b*). Local changes in salt concentration can arise either because the transport numbers of anions and cations in the membrane differ from those in the bulk solutions (cf. Barry & Hope, 1969*a*) or because ion movement close to the membrane is restricted by a

second barrier (cf. Adrian & Freygang, 1962). For chloride ions moving across frog muscle membrane neither effect is likely to be large. The currents applied during the present experiments were small (up to 10×10^{-6} A/cm^2) so that even if the thickness of the unstirred layer were large (say equivalent to the fibre diameter) the magnitude of the concentration changes produced by the transport number effect would be insignificant at all pH values both at the external and the internal faces. Adrian & Freygang (1962) supposed the second barrier to potassium movement to be present at the mouth of the transverse tubules of the sarcoplasmic reticulum. If the same situation existed for chloride ions then the chloride concentration in the limited region would rise by about 20 mM during a hyperpolarizing current pulse. At present there is no compelling evidence to suggest that most, or indeed, any of the chloride current flows across the membrane of the transverse tubules; in fact the evidence points to chloride movements taking place largely across the surface membrane (Hodgkin & Horowicz, 1960; Gage & Eisenberg, 1969).

Although accumulation and depletion effects must accompany the passage of current across the cell membrane it seems therefore unlikely that such effects alone could explain the experimental findings.

The decrease in anion permeability on acidification of the external solution does not conform to the classical model (Michaelis, 1926) where the anion permeability increases as the membrane takes on positive charge. Hutter & Warner (1967*a*) considered the possibility that chloride ions became bound to a molecule within the membrane in the process of penetration, in proportion to the positive charge on the molecule, and supposed that in acid solution retardation of chloride at the binding site outweighed more complete site occupation. The chloride permeability was supposed to depend on the proportion of sites unoccupied by anions. Their reaction scheme did not imply any particular location of the anion binding site, or which step in the anion permeation process is rate limiting. A molecule which binds chloride ions could be fixed at one of the membrane faces or within the membrane, or it could move through the membrane, acting as a 'carrier' for chloride ions. In the latter case either entry of the anion into the membrane or movement of the carrier/carrier-chloride complex across the membrane could be the rate limiting process. In all three cases complex steady-state voltage–current relations can be generated (Adrian, 1969; Sandblom, Eisenman & Walker, 1967*b*). The similarity between the experimental voltage–current relations and those generated for a liquid ion exchange membrane showing varying degrees of association between ion and carrier molecule (Sandblom *et al.* 1967*b*, Fig. 2), together with the finding that in acid solutions the isotopic flux of ^{36}Cl is larger than would be expected from electrical measurements (Hutter & Warner, 1967*b*) suggested

that it would be worth exploring how far the kind of model proposed by Sandblom *et al.* (1967*a*, *b*) is compatible with the present experimental findings. In their model a monovalent site, which is restricted to the membrane phase, acts as a carrier for its freely penetrating counter-ion by forming ion pairs; co-ions to the site are supposed to be excluded. The relative membrane concentrations of ion, site and ion pair are inter-related by the Law of Mass Action in proportions determined by the association constant for the formation of the ion pair; the fluxes of ion, site and neutral complex are determined by their membrane concentrations and mobilities. When the association constant is small the membrane current reaches a limiting value for large voltages, as seen in alkaline solutions, because the quantity of carrier is limited. Increasing the degree of association between ion and site produces a voltage–current relation which is linear over a wide voltage range. When the mobility of the ion pair is greater than the mean mobility of free site and counter-ion the voltage–current relations at the different degrees of association cross over in the manner observed in the present experiments, if it is assumed that a high degree of association pertains in acid solution. For the anion flux to be greatest in the alkaline solution the site and ion complex would have to be largely dissociated and the relative mobilities of ion, site and ion pair in the order $u_{\text{ion}} > u_{\text{ion pair}} > u_{\text{site}}$.

For the case considered by Sandblom *et al.* the voltage–current relation is symmetrical about the resting potential for all degrees of association and resting potential. The experimental situation is not so clear cut. A symmetrical voltage–current relation was seen at neutral pH values in depolarized fibres (Hutter & Warner, 1972), although at acid and alkaline pH values the v–i relation was asymmetric. In polarized fibres some rectification of the steady-state voltage–current relation was seen in alkaline solutions (Figs. 3 and 6), but it was not possible to impose depolarizing voltages sufficiently large to test the point properly before the permeability changes underlying the generation of the action potential took over.

The conductance of such an ion exchange membrane *falls* during the passage of current through the membrane because some current flows as the charged sites redistribute according to the imposed potential. As association between site and ion rises the carrier current, and so the fall in membrane current, is proportionately reduced. This kind of time dependence was seen in alkaline solution, to a lesser extent at pH 7·4 and occasionally, for small voltage deflexions only, at pH 5·0. If the decay in membrane current in alkaline and neutral solutions represents carrier current then the time constant for inactivation suggests that the mobility of the site within the membrane is extremely small; this is a necessary

condition if the requirements of ion pair mobility > mean mobility of site and free ion are to be met.

The increase in membrane current with time seen in acid solution, and for very large voltage deflexions in neutral and alkaline solutions, has no counterpart in the mobile site system outlined by Sandblom *et al.* (1967*a*, *b*). However, Ciani & Gliozzi (1968) have pointed out that in such a system an increase in membrane current with time can ensue when the condition of exclusion of co-ions from the membrane is not enforced. If the co-ion, which for frog muscle would be potassium or rubidium, is present at different concentrations on the two sides of the membrane then the voltage–current relation becomes asymmetric about the zero current position. The variable nature of the current increase with time seen experimentally would fit such a notion. G. Eisenman (personal communication) has observed an increase in current following an initial decrease of the kind seen in the present experiments in an artificial chloride permeable liquid ion-exchange membrane. Alternatively, the activation of the chloride conductance seen in acid solutions, and at long times in neutral and alkaline solutions, may be a completely separate phenomenon. The present results are not sufficiently comprehensive to allow an alternative system to be specified.

A number of the features shown by the anion voltage–current relations at different extracellular pH values are consistent with the supposition that anions combine with a carrier molecule to an extent which is determined by the extracellular concentration of hydrogen ions. The presence of some residual charge at the end of the current pulse, an exchange-diffusion component of chloride exchange in acid solutions and a limiting current under conditions when an anion-site complex could be largely dissociated are all consistent with a model invoking carrier-mediated transport of chloride ions. The cross-overs between the voltage–current relations obtained at different pH values are consistent with the particular carrier model described by Sandblom *et al.* (1967*a*, *b*). The reaction scheme used by Hutter & Warner (1967*a*) might then determine the relative proportions of free ion, site and ion pair.

Voltage–current relations which resemble those plotted in Fig. 5*A* of Hutter & Warner (1972) and in Figs. 8 and 9 of the present paper can be calculated from eqn. (24) of Sandblom *et al.* (1967*b*) if the acid voltage–current relation is assumed to be linear over the whole of the voltage range studied experimentally. The dissociation constant, K, in that equation, which determines the relative proportions of free ion, site and ion pair, can be supposed to be equivalent to the reciprocal of the pH dependent anion affinity coefficient, k'_A, defined by Hutter & Warner (1967*a*). The voltage–current relations at the different pH values, the chloride conductance-pH relation (Hutter & Warner, 1967*a*) and chloride flux–pH relation (Hutter & Warner, 1967*b*) are matched using the particular values for k'_A employed by Hutter &

Warner (1967*a*, Fig. 14) when the mobilities of the ion, site and ion pair are in the ratios 1:0·05:0·1. Precise comparisons between the model of Sandblom *et al.* (1967*a*, *b*) and the present experimental results requires knowledge of the absolute values of all these parameters. Until these values become available quantitative calculations to assess the detailed applicability of this model (Sandblom *et al.* 1967*a*, *b*) do not seem warranted.

The above considerations do not constitute strong evidence that a carrier mechanism is involved in anion permeation in muscle, but they suggest that the liquid ion-exchange model might be worth testing further. Information on the relation between chloride concentration and chloride conductance and the voltage dependence of the membrane conductance in the presence of foreign anions which can interfere with the movement of chloride ions (Adrian, 1961; Harris, 1958; Hutter & Padsha, 1959; Hutter & Warner, 1967*c*) and foreign cations which reduce the anion conductance (Mashima & Washio, 1964; Hutter & Warner, 1967*c*) would clearly be helpful. On present evidence anion permeation in red cells, which shares some of the properties of the anion conductance in muscle, is supposed to take place via a carrier molecule (Dalmark & Wieth, 1972).

The relation between the time-dependent variations in anion conductance described here and those found by Dudel, Peper, Rüdel & Trautwein (1967) in cardiac Purkinje fibres is not clear. These authors found a transient increase in chloride conductance during depolarizing clamp pulses, which reached a peak about 20 msec after the start of the pulse. In the present study the behaviour of the chloride conductance was only examined during hyperpolarizing voltage steps and an increase in chloride current with time was only seen consistently in acid solution. The anion conductance in heart muscle is much less than in skeletal muscle (Hutter & Noble, 1961) and could therefore be more like the anion conductance seen here at pH 5·0. No detailed information about the kinetics of either the present system, or that described by Dudel *et al.* (1967) is available to allow comparison of the two systems.

The functional role of any time-dependent variations in anion conductance is not clear. In heart muscle the action potential is sufficiently long for a varying anion conductance to be implicated in the underlying currents, but in skeletal muscle the action potential lasts for only 2 msec and would be complete long before any significant alterations in anion current could have taken place. This would not necessarily be true during trains of action potentials when variations in anion current might begin to assume some importance.

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