Characteristics of the Chloride Conductance in Muscle Fibers of the Rat Diaphragm

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ABSTRACT In muscle fibers from the rat diaphragm, 85% of the resting membrane ion conductance is attributable to Cl^-. At 37°C and pH 7.0, G_{Cl} averages 2.11 mmho/cm^2 while residual conductance largely due to K^+ averages 0.34 mmho/cm^2. The resting G_{Cl} exhibits a biphasic temperature dependence with a Q_{10} of 1.6 between 6°C and 25°C and a Q_{10} of nearly 1 between 25°C and 40°C. Decreasing external pH reversibly reduced G_{Cl}; the apparent pK for groups mediating this decrease is 5.5. Increasing pH up to 10.0 had no effect on G_{Cl}. Anion conductance sequence and permeability sequence were both determined to be $Cl^- > Br^- \geq I^- > CH_3SO_4^-$. Lowering the pH below 5.5 reduced the magnitude of the measured conductance to all anions but did not alter the conductance sequence. The permeability sequence was likewise unchanged at low pH. Experiments with varying molar ratios of Cl^- and I^- indicated a marked interaction between these ions in their transmembrane movement. Similar but less striking interaction was seen between Cl^- and Br^-. Current-voltage relationships for G_{Cl} measured at early time-points in the presence of Rb^+ were linear, but showed marked rectification with longer hyperpolarizing pulses (>50 ms) due to a slow time- and voltage-dependent change in membrane conductance to Cl^-. This nonlinear behavior appeared to depend on the concentration of Cl^- present but cannot be attributed to tubular ion accumulation. Tubular disruption with glycerol lowers apparent G_{Cl} but not G_K, suggesting that the transverse tubule (T-tubule) system is permeable to Cl^- in this species. Quantitative estimates indicate that up to 80% of G_{Cl} may be associated with the T tubules.

INTRODUCTION

Surface membrane of striated muscle at rest is generally permeable to both Cl^- and K^+. The total conductance attributable to those two resting permeabilities is of major importance in determining the excitability characteristics of the muscle fibers. In many species fibers with abnormally low chloride conductance (G_{Cl}) are hyperexcitable, often exhibiting long trains of action potentials that delay muscle relaxation after contraction (Rudel and Senges, 1972*b*). The congenital myotonias affecting man and goat appear to be naturally occurring examples of this hyperexcitable state, and chemically induced reduction of chloride permea-

bility in skeletal muscle fibers can lead to clinical myotonia (Bryant and Morales-Aguilera, 1971).

Most detailed information on muscle membrane chloride conductance, however, has been obtained from nonmammalian systems. In the majority of those studied, Cl^- conductance represents the dominant resting ion conductance. The ratio of G_{Cl} to G_K at rest in these systems ranges from about 2 in frog muscle (Hutter and Noble, 1960; Adrian and Freygang, 1962) to 10 or more in certain fish muscle (Hagiwara and Takahashi, 1974). Characterization of G_{Cl} under voltage clamp conditions has been undertaken in the frog sartorius (Warner, 1972; Vaughan et al., 1976). Such studies suggest that at least in that system the membrane G_{Cl} exhibits nonlinear behavior as a function of membrane potential.

Since reduced chloride conductance appears to be an important pathophysiological factor in human myotonia, comparable information on G_{Cl} in mammalian systems seems needed. With this in mind, the present studies were undertaken. This paper will describe the characteristics of chloride conductance in the rat diaphragm, including the effects of variations in pH and temperature, anion permeability and conductance sequences, and current-voltage relationships. An overall similarity between G_{Cl} in mammalian and amphibian muscle is noted, although several important differences are described.

MATERIALS AND METHODS

Male Wistar rats of 200–300 g were used. A strip of diaphragm between 0.5 and 1.0 cm wide was removed intact from rib insertion to central tendon, placed in a flow chamber with the thoracic side up, and allowed to equilibrate for approximately 15 min in oxygenated Ringer's solution before the start of physiological recording.

For most studies continuous perfusion with oxygenated Ringer's solution at a rate of 5–10 cm^3/min was routinely used. More rapid perfusion was employed for potential shift measurements in a 3.0 cm^3 volume chamber with flow rates of up to 20 cm^3/min. Temperature was maintained at 35–37°C except as indicated in the temperature-dependence studies.

Normal Ringer's solution had the following composition (mM): Na^+, 147; K^+, 5; Ca^{++}, 2; Mg^{++}, 1; Cl^-, 146; $CH_3SO_4^-$, 12; glucose, 11; Tris-maleate, 1; glycylglycine, 1; (pH 7.4 at indicated temperature unless otherwise specified). Chloride-free Ringer's was the same as the normal Ringer's but with the 146 mM chloride replaced by 140 mM $CH_3SO_4^-$ (methylsulfate) and 6 mM NO_3^-. All solutions with further additions of more than 5 mosmol had equal concentrations of either NaCl or $NaCH_3SO_4$ removed, depending upon whether the solution was normal or chloride-free Ringer's. Chloride-free Ringer's with anions other than methylsulfate had all chloride replaced by equimolar amounts of test anion. When sulfate was used in place of chloride, total Ca^{++} was increased to 8 mM in order to maintain a constant concentration of ionized Ca^{++} (Hodgkin and Horowicz, 1960), and sucrose (75.3 mM) and less than equimolar sulfate (85.3 mM) was added, based on the sulfate-substituted goat Ringer's of Adrian and Bryant (1974).

Standard cable analysis procedures were employed for conductance measurements (Hodgkin and Rushton, 1946; Boyd and Martin, 1959). A 3 M KCl-filled microelectrode of 10–20 MΩ resistance was used for potential measurements. Microelectrodes of 5–15 MΩ resistance filled with 2 M potassium citrate were used for current injection. Hyperpolarizing pulses were applied and maximum membrane potential change limited to no more than 15 mV at the recording location closest to the current electrode. Current was monitored by a current-to-voltage converter placed between the system ground and the

recording chamber (Cole and Moore, 1960). Potential measurements were made at three separate points between 0.1 and 1.0 mm interelectrode separation. Data collected from a given fiber was rejected if the resting membrane potential (RP) depolarized more than 10 mV during the course of the measurements or ever dropped below −55 mV. Pulses were generally of 125 ms duration except for current-voltage measurements (700–1,000 ms) or for measurements requiring analysis of the specific membrane capacitance (4–10 ms).

For calculation of specific membrane parameters, an internal resistivity of 185 Ωcm at 35°C was assumed (Farnbach and Barchi, 1977). In studies at temperatures other than 35°C, internal resistivity was assumed to vary with a Q_{10} of 1.2 and appropriate values were calculated for each temperature. This Q_{10} represents an average of those reported in the literature for the variation of internal resistivity with temperature in mammalian, amphibian, and fish muscle (Boyd and Martin, 1959; Del Castello and Machne, 1953; Hagiwara and Takahashi, 1974; Lipicky and Bryant, 1972).

The space constant (λ), diameter (d), specific membrane resistance (Rm), and specific membrane conductance ($Gm = 1/Rm$) were determined in the usual manner. Specific membrane capacitance (Cm) was calculated from the half-rise times of the electrotonic potential after the method of Hodgkin and Rushton (1946) and Gage and Eisenberg (1969). Current-voltage measurements were made with a single insertion of the recording electrode at a distance of 0.10–0.15 mm from the current electrode.

For determinations of transient membrane potential changes (Adrian, 1956; Hodgkin and Horowicz, 1960) two KCl-filled microelectrodes were used in differential mode, one inserted into the fiber, the other outside the fiber and close to the first electrode. The internal electrode was left in the fiber for periods of up to 30 min provided there was little spontaneous shift in the RP. For estimates of permeability sequences several different test anion solutions were applied to each fiber at different times, and data was used only when symmetrical deflections were encountered upon switching to test solution and back again. Furthermore, solution changes were repeated in each fiber to ensure that slow, time-dependent permeability changes occasionally seen were not responsible for any individual response. Single fibers were not dissected from the diaphragm.

In several preparations diaphragms equilibrated in different solutions were quickly frozen in isopentane and sectioned perpendicular to the fiber axis. Photomicrographs were prepared and average fiber cross-sectional areas were determined with a planimeter.

RESULTS

Resting Membrane Conductance

The resting cable parameters from 832 fibers in 125 preparations are detailed in Table I. In the set of all fibers meeting the criteria for membrane potential and stability outlined in the methods, the average membrane resistance was 445 Ωcm^2. A subset of these fibers was analyzed in which the resting potential at all times exceeded 75 mV. For these fibers the average resting membrane resistance was 472 Ωcm^2, a value not significantly different from that obtained for the entire population. This suggests that the rejection criteria established were adequate and that the population of fibers selected by these criteria were homogeneous with respect to the parameters studied.

Measurements were made in this initial series on a total of 75 fibers in chloride-free Ringer's solution ($CH_3SO_4^-$ substitution). In these fibers the average membrane resistance increased nearly 10-fold to 3,890 Ωcm^2. Clearly, the

major part of the resting membrane current in the rat sarcolemma is carried by Cl^- ions as has been reported for other muscle surface membranes.

These values for specific membrane resistance may be re-expressed in terms of membrane conductance (G_m). Average G_m for all fibers in normal Ringer's solution at pH 7.0 and 35–37°C was 2.45 mmho/cm² and for the subgroup of fibers with the highest resting potentials 2.22 mmho/cm². The average conductance of fibers in chloride-free Ringer's was 0.34 mmho/cm². Since the measured membrane conductances are additive, G_{Cl} can be calculated to average 2.11 mmho/cm² in this fiber population. If the conductance in the absence of Cl^- is assumed to be attributable largely to K^+ ions, the ratio of G_{Cl}/G_K is approximately 6.2.

Muscle fiber diameters varied as a function of pH and ionic composition of the bathing medium. As an estimate of the accuracy of our data, calculated fiber diameters were abstracted from the electrical measurements of preparations in normal and Cl^--free Ringer's at three pH values and compared to average

TABLE I

CABLE PARAMETERS OF RAT DIAPHRAGM FIBERS

No. exp.	No. fibers	RP	R_m	G_m	λ	d
			Ω-cm²	mmho/cm²	mm	μm
Cl^- containing solution						
125	832	70.3±6.2	445±131	2.45±0.62	0.57±0.17	54.8±13.4
125*	201	78.0±2.8	472±113	2.22±0.49	0.60±0.09	57.9±10.7
Cl^- free solution						
17	74	69.2±7.3	3,890±2,626	0.34±0.16	1.93±0.74	75.5±20.7

All experiments performed at 35°C, pH 7.0.
All results expressed as mean ± sd.
* Subgroup of fibers with RP > 75 mV.

diameters measured from paired preparations equilibrated in the same solutions and then frozen, sectioned, and photomicrographed. Calculated and measured diameters agree well, as shown in Table II, indicating that the assumed internal resistivity of 185 Ωcm at 25°C is reasonable for these fibers. This supports a similar conclusion arrived at by using a different measurement technique in the same fiber type (Farnbach and Barchi, 1977).

Effects of Temperature on Component Conductances

The temperature dependence of G_{Cl} and G_K was examined over the range between 5°C and 40°C. In each experiment a preparation was studied in normal Ringer's solution at three temperatures and then in Cl^--free Ringer's at the same three temperatures in reverse sequence. Average values of G_K and G_{Cl} were calculated for each point from a number of experiments, by assuming a standard value for internal resistivity of 185 Ωcm at 35°C (Farnbach and Barchi, 1977) and a Q_{10} of 1.2 for this parameter (Boyd and Martin, 1959; Del Castello and Machne, 1953; Hagiwara and Takahashi, 1974; Lipicky and Bryant, 1972). The averages for all preparations over the entire temperature range are shown in Fig. 1. The temperature dependency of G_{Cl} appears to have two separable

phases. Between 25°C and 40°C there is little detectable change in conductance with temperature and the calculated Q_{10} in this range is not significantly different from 1.0. Between 25°C and 5°C, however, G_{Cl} declines with a Q_{10} of 1.6. G_K on the other hand demonstrates a slight but constant decline with temperature over the entire range studied with calculated Q_{10} of 1.1.

The resting potentials of the fibers studied at each temperature showed no significant variation in normal Ringer's at temperatures between 10°C and 35°C,

TABLE II

COMPARISON OF EXPERIMENTALLY CALCULATED AND HISTOLOGICALLY MEASURED DIAMETERS

Chloride-containing			Chloride-free		
pH	Average diameter (calculated)	Average diameter (measured)	pH	Average diameter (calculated)	Average diameter (measured)
	μm	μm		μm	μm
4	61.6 (13)	57.9 (50)	4	46.5 (5)	54.3 (50)
7	56.3 (32)	52.4 (77)	7	76.3 (11)	72.5 (50)
10	64.7 (18)	63.6 (50)	10	84.7 (11)	70.8 (50)

Numbers within parentheses indicate numbers of fibers examined.

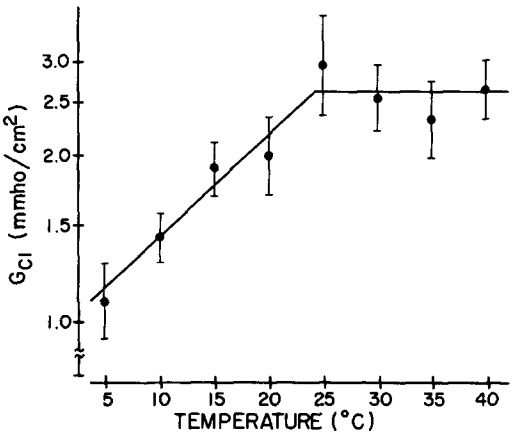

FIGURE 1. The dependence of G_{Cl} on temperature. Each point represents the mean ± SEM of values determined from fibers in several different preparations.

all averaging between 68 and 72 mV. At 5°C the average RMP declined to 63 mV while at 40°C it was found to be 66 mV. Slightly more variability was noted in Cl^--free Ringers, but in no case was the average RMP below 60 mV. Since it will be shown below that G_{Cl} appears to remain constant at least over the 55–75 mV range of RMP, it is felt that the calculated changes in G_{Cl} reported here reflect true changes in membrane conductance as a function of temperature rather than secondary changes due to variations in RMP. However, depolarization in methylsulfate at low temperatures could lead to an underestimation of residual G_K in chloride-containing solutions due to reduction in this parameter associated with anomalous rectification. If the maximal potential error from this source,

which is significant only at the lowest temperatures, is considered in the calculation of G_{Cl}, the apparent Q_{10} over the range between 5°C and 25°C increases to approximately 1.8. This may be considered to be the upper limit for this value with the true value lying between 1.6 and 1.8.

Effects of pH on Component Conductances

In all nonmammalian systems that have been studied a marked dependence of G_{Cl} on pH has been demonstrated. The effects of pH on G_{Cl} and G_K in the rat diaphragm are shown in Fig. 2. In these experiments measurements were made in normal Ringer's at pH 7.0, normal Ringer's at a test pH, and finally chloride-

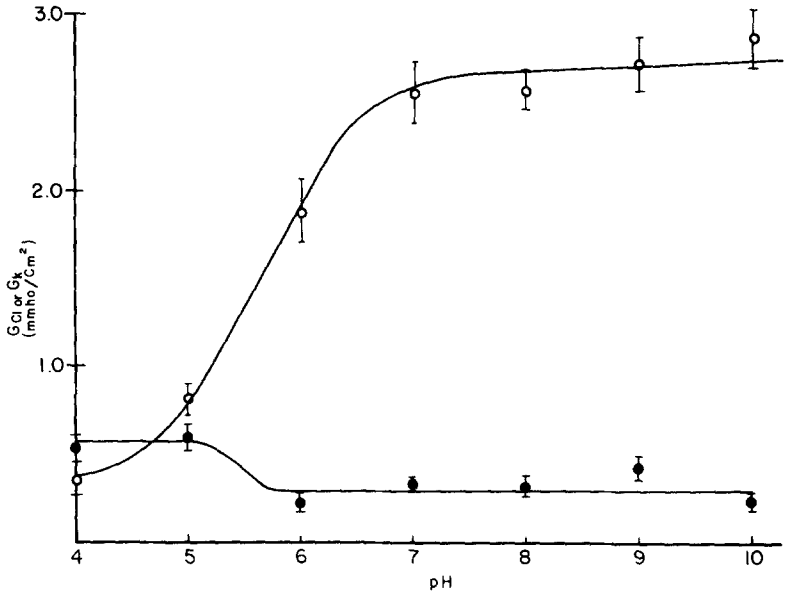

FIGURE 2. Variation in membrane G_{Cl} and G_K as a function of external pH. In all cases an equilibration period of 20 min was allowed when changing from one pH value to another. Data represent values accumulated from several diaphragms ± SEM.

free Ringer's at the same test pH. Each point on the graph represents the average G_{Cl} or G_K value from several preparations each with a sampling of four to seven fibers in each solution. It may be seen that increasing [OH^-] up to pH 10.0 has negligible effect on either G_{Cl} or G_K, but that with decreasing pH, G_{Cl} decreases markedly while G_K increases slightly. The curves through the experimental points approximate the titration curve of a functional group with apparent pK of 5.5. The pH effect on G_{Cl} requires 15–20 min to develop fully. This effect is reversible, however, although resting potentials tend to fall slightly upon return to pH 7 solution. The significance of this prolonged equilibration time is unclear but may indicate that the functional group involved is located either within the membrane or near its inner surface. Under these circumstances cytoplasmic buffering and transmembrane potential may create a hydrogen ion

gradient across the membrane, and the true pK of the group being titrated may well be significantly higher than the observed value of 5.5.

Average resting potentials were essentially constant over the pH range in which maximal changes in G_{Cl} and G_K were noted (pH 4–7) varying between 66 and 71 mV. RMP declined to 62–64 mV at pH 9 and 10 in normal Ringer's solutions. No concomitant change in either G_{Cl} or G_K was noted at these points. Since the average resting membrane potential was constant over the range where maximal conductance changes were seen, these changes cannot be secondary to variations in membrane potential and most are likely to represent a change in the charge of a site or group of sites within the membrane which affect ion movement. Measurement errors introduced by variations in the residual K^+ conductance with membrane potential (anomalous rectification) would be a consideration only in the pH range above 8, and in this range no variation in measured G_{Cl} or G_K is observed.

Interaction between Anions

The interaction of Cl^- with other anions in their movement through the membrane was assessed by determining membrane conductance as a function of mole fraction replacement of Cl^- by the test anion. In each case 15 or more min were allowed for equilibration of the new Cl^- concentration across the membrane. Fig. 3 A demonstrates that there is a nearly linear relationship between apparent anion conductance and mole fraction of chloride when the substituting ion is methanesulfonate, an ion which is presumably impermeant to the membrane. Slight deviations are noted with sulfate and methylsulfate as replacement ions, suggesting some inhibitory effect of these anions on Cl^- movement. These are, however minimal. The average residual conductance after complete replacement of Cl^- with any of these three anions is the same.

When Cl^- is partially replaced with I^-, however, a marked deviation from linearity is noted (Fig. 3 B). Membrane conductance falls off rapidly and actually appears to approach a minimum in the presence of low concentrations of I^-. Conductance with complete I^- substitution is often 15–20% higher than this apparent minimum. Replacement of Cl^- with Br^- also produces a significant but less marked degree of nonlinearity, indicating interaction between this anion and Cl^-. It would appear from these data that Cl^-, Br^-, and I^- do not move independently through the membrane, but most probably share a common permeation pathway. Further, the presence of one ion in this pathway significantly affects the movement of others. Similar interaction between Cl^- and I^- movement has recently been described in avian muscle (Morgan et al., 1975).

Anion Conductance Sequence

The conductance sequence for Cl^-, Br^-, I^-, and $CH_3SO_4^-$ was determined by measuring G_m in a given preparation after incubation for 20–30 min in each of several different solutions in which a test ion completely replaced chloride. Membrane conductance parameters were found to reach new steady-state values well within this time period. Control measurements in normal Ringer's solution were also made with each preparation. The conductance sequence determined from these experiments was found reproducibly to be $Cl^- > Br^- \geq I^- >$

$CH_3SO_4^-$. Chloride conductance was markedly higher (five to eight fold) than that of either I^- or Br^-, while values for the latter two anions were often quite similar in the same preparation (Table III).

The anion conductance sequence was also determined after equilibration of the muscle at lowered pH (5.0) and 37°C. Under these conditions conductance to

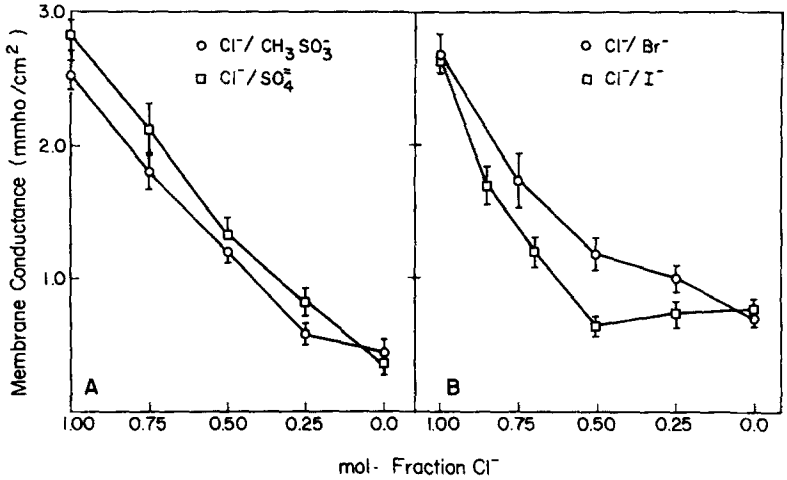

FIGURE 3. Total membrane conductance determined after equilibration of diaphragm fibers in solutions containing variable mole fractions of Cl^- made isomolar with test anions. Methane-sulfonate and sulfate, presumably impermeant ions, yield nearly straight lines. Partial replacement with I^- or Br^- produce marked deviations from linearity. Data presented as mean ± SEM.

TABLE III

MEMBRANE CONDUCTANCE IN RINGER'S SOLUTIONS WITH TOTAL ANION SUBSTITUTION

Anion	Cl^-	Br^-	I^-	$CH_3SO_4^-$
No. fibers	30	28	24	25
Total G_m ± SEM (mmho/cm²)	2.64±0.07	0.79±0.05	0.68±0.06	0.34±0.04
Calculated conductance ratio*		$\frac{G_{Br^-}}{G_{Cl^-}}=0.20$	$\frac{G_{I^-}}{G_{Cl^-}}=0.15$	

* Calculated after correction for residual conductance (G_K) in methylsulfate for this group of fibers.

Cl^-, Br^-, and I^- all appeared reduced although the permeability sequence remained $Cl^- > Br^- \geq I^- > CH_3SO_4^-$. The residual conductance (that measured with all Cl^- replaced by methylsulfate) did not change significantly in this experiment, although a trend towards higher values which was not statistically significant was noted. Thus it appears that the conductance sequence in rat diaphragm does not invert with increasing hydrogen ion concentration, but rather that the conductances to all permeant anions are decreased proportionately under these circumstances.

As outlined in Materials and Methods, shifts in membrane potential observed after rapid solution changes from chloride Ringer's to Ringer's in which chloride

is replaced by a test anion were used to determine the relative permeabilities of the sarcolemma to various anions. A reproducible permeability sequence is easily obtained and falls in the order $Cl^- > Br^- \geq I^- > CH_3SO_4^-$, although there is sufficient variability in the data to make quantitation difficult. This sequence is the same as that observed for relative membrane conductance to these anions after equilibration in the substituted Ringer's solutions. The amplitude of the potential changes observed when Br^- or I^- was rapidly substituted for Cl^- was usually rather small (5–10 mV), suggesting that the permeabilities of these ions relative to Cl^- differed by a smaller factor than did their relative conductances. Anion permeability sequence was determined in two preparations at pH 4.0 and once again was found to be unchanged from that seen at pH 7.0. In frog muscle, the permeability sequence has been reported to invert at low pH (Hutter et al., 1969).

Current-Voltage Relationships for G_{Cl}

Determination of membrane current-voltage relationships in the presence and absence of Cl^- were carried out over the membrane potential range of −50 to −160 mV. Potassium was replaced by Rb^+ in order to reduce the contribution of cation currents to the total measured membrane current (Adrian, 1964). In the presence of normal $[Cl]_0$, steady-state current-voltage relationships in the hyperpolarizing direction are markedly nonlinear, and they deviate from linearity in a direction opposite to that usually associated with the anomalous rectification of the K^+ system. Membrane voltage responses to square current pulses producing hyperpolarization in excess of 10 mV show time-dependent changes suggesting an increase in membrane resistance. These changes display an apparent time constant between 100 and 300 ms (Fig. 4).

Data from seven fibers in Rb^+ Ringer's and seven fibers in Cl^--free Rb^+ Ringer's are shown in Fig. 5. Voltage responses determined 20 ms after the onset of a 700-ms current pulse are linear with respect to the magnitude of the input current over a 90 mV range in the hyperpolarizing direction and in separate experiments over at least a 15 mV range in the depolarizing direction. Similar measurements made at 700 ms were again linear in the depolarizing direction over the limited range tested, but showed a marked deviation from linearity in the hyperpolarizing direction. The membrane response at both 20 and 700 ms in the absence of chloride was linear in both hyperpolarizing and depolarizing directions.

From these data the current carried by Cl^- as a function of membrane potential can be calculated by using Cole's theorem (for a discussion, see Jack et al., 1976). An estimate of true membrane current can then be made and the experimental data transformed into membrane I–V relationships. Such a conversion suggests that the membrane exhibits a region of nonlinear behavior with respect to hyperpolarizing current in such a manner that the effective membrane resistance appears to increase. A similar observation has been made previously in frog muscle (Hutter and Warner, 1969). In the rat, however, membrane G_{Cl} appears to decrease progressively with hyperpolarization in such a way that membrane current passes through a maximum and then decreases,

rather than asymptotically approaching a limiting current as reported in the frog (Fig. 6).

Determinations of current-voltage relationships were made after equilibration in solutions containing 75%, 50%, or 25% of the normal concentration of Cl^- in the presence of Rb^+. The amount of nonlinearity observed in the hyperpolarizing direction decreased markedly with decreasing $[Cl^-]_0$ (Fig. 7). At 25% of normal $[Cl^-]_0$, deviations from linearity were hardly detectable, suggesting that the time-dependent changes to chloride are in some way dependent on the density of chloride current moving through the membrane.

At pH 10 the I-V relationship is essentially the same as that observed at pH 7. At pH 4, where the measured G_{Cl} at membrane potentials near the resting

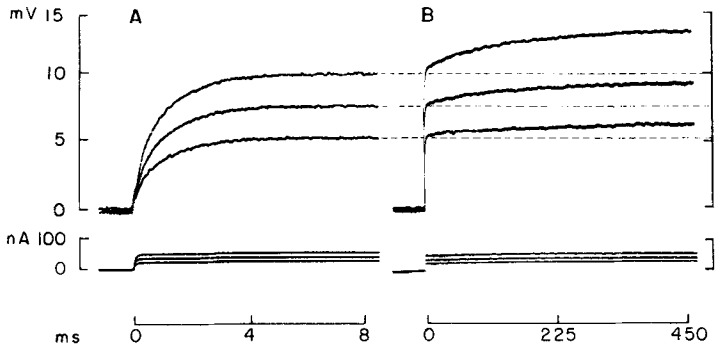

FIGURE 4. A, Early hyperpolarizing membrane voltage responses in a rat diaphragm fiber to square current pulses of varying amplitude. B, The same response as recorded in A, but at a much slower time scale. The early voltage response (<20 ms) to current pulses in this low range is linear and approximates that expected from a passive cable. Even at these low potentials, however, a voltage- and time-dependent drift in potential is clearly seen with long current pulses (>50 ms). For larger hyperpolarizing pulses this nonlinear behavior becomes increasingly apparent. Rb^+-Ringer's, pH 7.4. Interelectrode distance is 175 μm and resting V_m 78 mV.

potential is markedly reduced, rectification with hyperpolarization appears to take place in the opposite direction from that observed at pH 7, suggesting a time-dependent decrease in membrane resistance in this region. This would indicate a net increase in membrane G_{Cl}. Under these conditions steady-state membrane chloride currents at 50–80 mV hyperpolarization are always larger at pH 4.0 than at either pH 7.0 or pH 10.0.

Localization of Chloride Conductance

Component conductances can be partially localized by means of treatments affecting the transverse tubular system. By far the most widely documented of these is the glycerol shock treatment (Eisenberg and Gage, 1969). After equilibration of a muscle fiber in a solution containing hypertonic glycerol the transverse tubular system elements can be disrupted by returning the preparation to an isotonic Ringer's solution. The physiological manifestations of such detubulation are a decrease in the specific membrane capacitance (C_m) and dis-

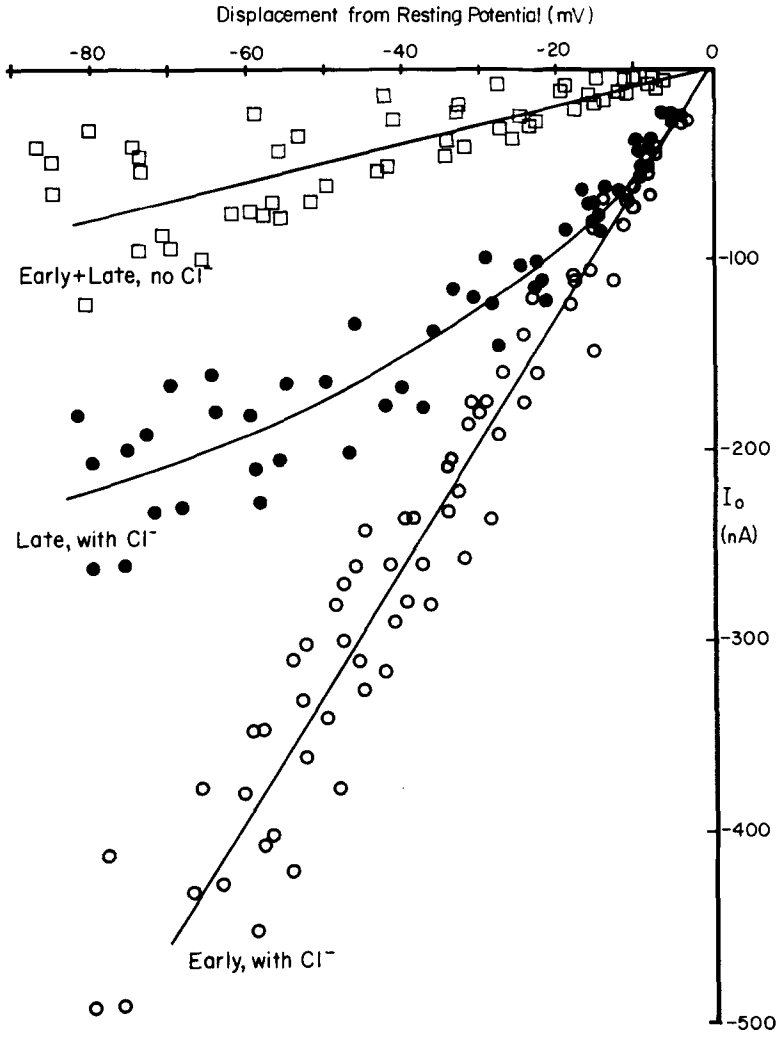

FIGURE 5. Current-voltage data from seven fibers of approximately equal diameter in Cl^--containing and Cl^--free Rb^+ Ringer's. In the presence of Cl^-, data points measured at 20 ms (○) show a linear relationship between input current and membrane potential, while points determined 700 ms (●) after the onset of the current pulse indicate significant rectification. Data from both 20 ms and 700 ms in the absence of Cl^- are linear (□). Solid lines represent average values of data distribution for each class. Average R_m of fibers studied was 67 mV.

ruption of excitation-contraction coupling. In the present experiments the protocol followed was that used by Eisenberg et al. (1971). Control measurements of C_m were performed as delineated in the Materials and Methods and are in reasonable agreement with values reported by Zolovick et al. (1970) and Rudel and Senges (1972) for the rat diaphragm.

After glycerol treatment fibers may be found in the preparation with de-

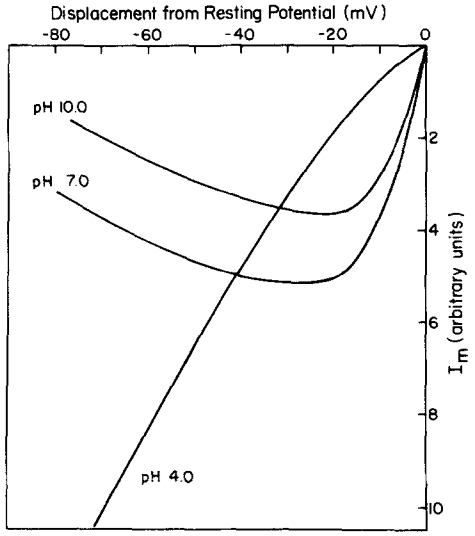

FIGURE 6. Calculated membrane current as a function of displacement from resting potential by a hyperpolarizing constant current pulse at various $[H^+]$. Values of V_m were those measured 700 ms after the onset of the current pulse. At pH 7 and 10, current values pass through a maximum and then decline. Slight rectification in the opposite direction is noted at pH 4. All experiments were performed in Rb^+-Ringer's solution with normal $[Cl^-]$.

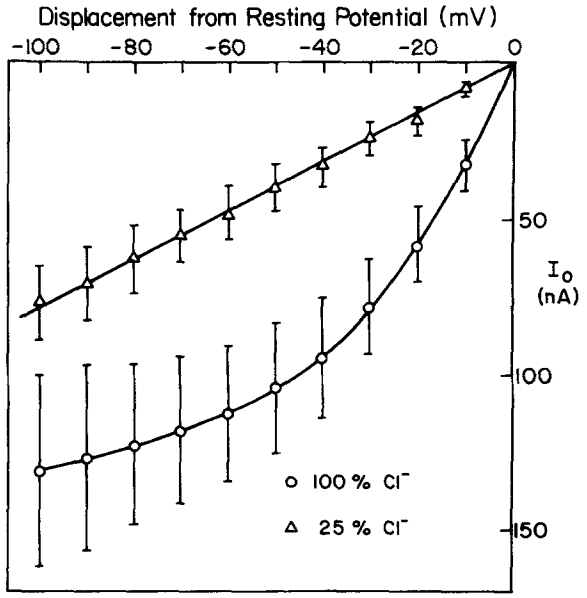

FIGURE 7. Membrane potential vs. input current for several fibers of similar diameter either in 100% Cl^- (O) or after equilibration in 25% Cl^- (Δ) Rb^+ Ringer's. Linear residual currents measured in Cl^--free Rb^+ Ringer's have been subtracted from all values. Data represents mean ± SEM.

creased C_m, though the population of such fibers is no more than 50% of the total sampled in the experiments reported here. Furthermore, there is a general tendency for the fiber capacitance to recover from such treatment with time, so that there is only a limited recording period available for data collection. Despite the technical limitations of such experiments it is clear that those fibers with a lowered C_m also have a greatly increased Rm (greatly reduced G_m) and that this increase in Rm is associated with the shock of return to normal Ringer's rather than an effect of the glycerol itself. The reduction in G_m is too great to be accounted for even by a complete abolition of G_K. Indeed, G_K appears itself not to be reduced by such treatment, indicating that it may be confined to the surface sarcolemma, while chloride conductance must be present to a significant extent in the T-tubule membrane. I–V relationships in detubulated fibers show persistent rectification of Cl^- currents in the hyperpolarizing direction, suggesting that this phenomenon is not a function of tubular ion accumulation. The slight increase in G_K found in the chloride-free experiment may be due to the noted leakiness of glycerol-treated fibers which have depolarized (Eisenberg and Gage, 1969); this would tend to increase G_m values artifactually, and slightly raise estimates of G_{Cl} remaining after glycerol treatment.

Since our success in obtaining detubulated fibers was only fair, it is possible to obtain only an estimate for the minimum percentage of G_{Cl} associated with the tubules. From the data summarized in Table IV gathered from three experiments in chloride-containing solution and one in chloride-free Ringer's, the average G_m of 19 control fibers from measurements made before exposure to hypertonic solution and in the presence of chloride was 2.81 mmho/cm^2, of which approximately 0.34 mmho/cm^2 is G_K. Thus G_{Cl} would be calculated to be 2.50 mmho/cm^2. After tubular disruption 14 fibers from these same preparations were sampled with substantially reduced capacitance (Cm < 2.0 μF/cm^2). These same fibers had an average G_m of 1.34 mmho/cm^2, indicating that G_{Cl} is approximately 1.04 mmho/cm^2. Thus at least 60% of the chloride conductance is localized in the T-tubule system.

Effects of Other Agents on Chloride Conductance

Many other divalent cations have profound effects on excitable membranes. It had been hoped that among this group a useful blocker of G_{Cl} could be found. As the results in Table V indicate, only UO_2^{++} and Cu^{++} reduce G_{Cl}. Cu^{++} also increased resting membrane cation conductance causing rapid depolarization, and UO_2^{++} was active only at relatively high concentrations. Cobalt and zinc had no effect on G_{Cl} but increased G_K, and Mn^{++} increased G_{Cl} and reduced G_K. Finally, picrotoxin, believed effective in reducing G_{Cl} in arthropod muscles (Takeuchi and Takeuchi, 1969) was ineffective in reducing G_{Cl} in rat fibers at 1.5 mM concentration.

DISCUSSION

Rat diaphragm differs from nerve and resembles muscle of most other species studied in having a high resting ratio of G_{Cl} to G_K. The absolute magnitude of the resting G_{Cl} is considerably larger than that reported for frog muscle but is similar to that described for stingray skeletal muscle (Hagiwara and Takahashi, 1974).

To date, muscle chloride conductance has been most thoroughly studied in the frog. The observed G_{Cl} in rat diaphragm resembles that in frog muscle in several respects. In both cases G_{Cl} is reduced markedly by lowering the pH below the normal physiological hydrogen ion concentration of the preparation. This

TABLE I V

EFFECTS OF GLYCEROL DETUBULATION ON RESTING MEMBRANE CONDUCTANCE

Conditions	No. fibers	RP	C_m	G_m
		mV	*μF/cm²*	*mmho/cm²*
Cl^- containing solution				
Control	8	76.2±1.5	2.90±0.15	2.88±0.16
Postglycerol	5	59.8±1.9	1.07±0.09	1.25±0.12
Control	7	71.8±1.1	3.64±0.08	2.67±0.17
Postglycerol	3	65.0±1.2	1.30±0.03	1.58±0.05
Control	4	72.5±0.9	3.58±0.30	2.94±0.14
Postglycerol	6	63.3±2.1	1.26±0.09	1.27±0.10
TOTALS				
Control	19	73.8±0.9	3.31±0.12	2.81±0.09
Postglycerol	14	62.3±1.3	1.21±0.06	1.34±0.07
Cl^- free solution				
Control	7	81.3±1.8	4.05±0.20	0.34±0.04
Postglycerol	4	69.2±0.8	1.98±0.09	0.48±0.04

All results expressed as mean ± SEM.

TABLE V

EFFECTS OF FOREIGN DIVALENT CATIONS AND PICROTOXIN ON RAT DIAPHRAGM RESTING CONDUCTANCES

	Control			
Species	Concn	G_m	Test G_m	Test G_K
	mM			
Zn^{++}	1.0	2.68	3.11	0.98
Mn^{++}	1.0	2.10	2.81	0.16
Co^{++}	0.2	3.52	4.58	1.25
UO_2^{++}	0.2	2.94	2.11	0.31
Cu^{++}	0.2	2.15	1.85	depol.*
Cu^{++}	0.2	3.58	1.87	depol.*
Cu^{++}	0.05	2.15	1.25	depol.*
Ca^{++}	0.01	2.20	1.94	0.43
Picrotoxin	1.0	2.40	2.55	0.46
Picrotoxin	1.5	1.77	1.91	0.17

* Depolarization left no fibers measurable according to rejection criteria.

reduction in G_{Cl} is completed over a narrow range, usually 90% within 2 pH units, suggesting the involvement of a single class of titratable groups. The apparent pK's for the functional groups controlling G_{Cl} in these two species are, however, different, being about 7.0 for frog (Hutter and Warner, 1967, 1972)

and 5.5 in the present study. This may represent a true difference in the nature of the charged groups associated with ion translocation or may merely represent differences in the local environment for these functional groups within the membrane. The relatively long time required for equilibration of G_{Cl} at low pH suggests that the involved sites are either well within or at the inner surface of the membrane and that the efficiency of cytoplasmic buffer systems may play an important role.

Steady-state current-voltage relationships in the rat are qualitatively similar to those reported in *Rana* (Hutter and Warner, 1967, 1972) and *Xenopus* (Vaughan et al., 1976). Strong rectification is observed in the hyperpolarizing direction of a form suggesting a decrease in G_{Cl} with increasing transmembrane potential. These changes occur with an approximate time-constant (100–300 ms) relatively long with respect to the passive time constant of the membrane. Rectification in the opposite direction is obtained with hyperpolarizing pulses when the muscle has been previously equilibrated at pH 4.0. In each case I–V relationships measured at early time points (10–15 ms), well beyond the passive charging time of the membrane but before significant delayed changes have occurred, are linear. The observed rectification in the steady state cannot be ascribed to tubular accumulation of ions since it could be detected in cells detubulated as completely as possible by glycerol treatment. The strong dependence of rectification on $[Cl^-]_0$ suggests that current density within the channel might be an important factor in determining channel conductance.

Warner (1972) observed that chloride current in frog muscle appeared to reach a limiting value with large hyperpolarizations and used this observation as an argument in favor of a carrier mechanism for chloride movement. In rat muscle we find that membrane chloride current at large hyperpolarization declines below its peak value rather than maintaining a limiting maximal current. A similar observation has been reported in *Xenopus* muscle with voltage clamp techniques (Vaughan et al., 1976). These observations are difficult to reconcile with a simple diffusible carrier model.

The temperature dependency of G_{Cl} in the rat diaphragm indicates a decreasing conductance with decreasing temperature. This seems to be at variance with results reported for the goat (Lipicky and Bryant, 1972) where a slight negative temperature dependence for G_{Cl} between 15°C and 40°C was observed. The Q_{10} reported there (0.88), however, is not very different from the value (~1.0) which we have determined over the higher temperature range of 25°C to 35°C. The overall temperature dependence in rat is qualitatively similar to that seen in frog (Hutter and Noble, 1960; Adrian and Freygang, 1962) where a value near 1.3 has been reported. The observed Q_{10} of 1.6 is somewhat higher than that anticipated from free diffusion alone but seems much lower than the anticipated Q_{10} for an ion-carrier complex moving through a lipid matrix, the majority of whose fatty acyl chains will have transition temperatures within the region studied. This again suggests that a diffusible carrier operating within the membrane is unlikely.

The sequence of $Cl^- > Br^- \geq I^- > CH_3SO_4^-$ obtained from both permeability and conductance measurements in the rat in the steady state is the same as that reported for frog muscle at pH 7.0. These sequences differ, however, from

those seen in stingray muscle (Hagiwara and Takahashi, 1967, 1974) and barnacle muscle (Hagiwara et al., 1969). In the latter two species the conductance sequence was found to be the reverse of that determined for relative permeability. The permeability sequence in frog muscle inverts at low pH; we have been unable to demonstrate such an inversion in rat muscle.

The observation that both permeability and conductance sequences in the rat proceed in the same order suggests that factors relating to intramembrane mobility rather than site-specific binding may be of dominant importance in determining selectivity in this system.

We feel that the conductance pathway for chloride in rat diaphragm is best described as an aqueous "channel" rather than a carrier in light of the temperature dependence and I-V relationships described. Our data suggest that the halides tested traverse membrane via the same channels and that the presence of one ion within the channel significantly affects the ability of other ions to move through the same channel. The data available on permeability sequences in the rat under various physical conditions is at present too limited to permit conclusions to be drawn concerning the dominant mechanism of ion selection in this membrane.

In many of the aspects discussed above, there is considerable homology between the amphibian and mammalian chloride conductance systems. A major difference, however, seems to lie in the distribution of sites mediating ion movement among the surface membranes. Chloride conductance in the frog has been reported to be confined almost exclusively to the sarcolemmal surface with little or no detectable conductance in the T-tubule system (Eisenberg and Gage, 1969). In rat diaphragm the majority of the G_{Cl} is found in the T-tubule system although a sarcolemmal component does appear to be present. The estimated distribution (60–80% of G_{Cl} associated with the T-tubule system) could be compatible with an even distribution of conductance sites per unit of membrane area in the T-tubule and surface membrane when the relative areas of the two membrane systems are considered. With respect to other muscles studied, this association of G_{Cl} with the T-tubule system resembles results described for crayfish (Brandt et al., 1968) but differs from the results of Bryant (1970) in goat muscle.

Further characterization of the macromolecules mediating chloride conductance in the membrane is required to determine whether the interspecies similarities and differences observed at the cellular level extend to the level of the individual channel unit.

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