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Localization of Ionic Conductances in Crayfish Muscle Fibers

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Summary. Analysis of the changes in membrane potential and conductance of isolated crayfish muscle fibers caused by rapid solution changes leads to the following conclusions. First, the extensive invagination system of this fiber presents a barrier for diffusion between bath and sarcolemma that accounts for the time lag of electrical responses to changes in bath chloride concentration. Morphological data regarding these invaginations were used in a model which simulated the fiber response on an analog computer. Second, the potassium conductance is effectively localized on the sarcolemma in direct contact with the bath (superficial sarcolemma), whereas the chloride conductance is restricted to the invaginations. This distribution of conductances is the reverse of that found in frog muscle.

This paper elucidates some relationships between surface morphology and electrical responses of isolated muscle fibers. We present evidence for morphological distribution of conductances in crayfish muscle which is approximately the reverse of that found in frog (Gage & Eisenberg, 1969). Comparison of the K^+ conductance with the Cl^- conductance of crayfish muscle fibers demonstrates the dependence on morphology of electrical responses to ionic changes in the bath as well as some of the difficulties in interpretation of these responses.

In the first section of this paper, we demonstrate that nearly all K^+ conductance, G_K, is located in immediate contact with the bathing saline. We then show that the Cl^- conductance, G_{Cl}, is separated from the bath by a diffusion barrier. (In this paper, the conventional specific conductance, conductance per unit area of the cell's cylindrical envelope, is denoted by G, and the conventional specific flux by J. Nomenclature is given below.) The major portion of the paper relates the electrical response to sudden changes in external Cl^- concentration to the cell morphology and to the path of ionic movement.

Nomenclature

c	concentration	r	equilibrium ratio of fiber Cl^- to ba Cl^-
D	diffusion coefficient	T	absolute temperature
D_f	fiber diameter	t	time
E	membrane potential	v	volume
F	Faraday constant	V	displacement of membrane potential, $E-E_i$
G	conductance	w	cross-sectional area of invagination
H	flux-rate constant		
I	current		
J	total flux		
j	flux/area		
L	length	*Subscripts:*	
N	number of invaginations	f	fiber
n	total invagination volume/fiber volume	i	initial
p	perimeter of invagination	1	invagination
R	gas constant	2	fiber

Table 1. *Structural properties of crayfish tubular system*

Structure	Length (μ)	Diameter (μ)	Number/Sarcomere
Sarcomere	8	200	—
Invagination	200	0.2	10^2
Tubule	10	0.03	10^4

Extensive anatomical studies have been made of muscle fibers isolate from the walking legs of the crayfish (Brandt, Reuben, Girardier & Grunc fest, 1965; Brandt, Reuben & Grundfest, 1968). Table 1 summarizes th relevant structural data from these papers. The sarcolemma is densely ir vaginated and most of the tubules connecting the diadic structures impl cated in excitation-contraction coupling to the bath originate from the dee cylindrical invaginations. Previous physiological and morphological studie have indicated that the diadic-tubular membrane is anion selective (Gira dier, Reuben, Brandt & Grundfest, 1963; Brandt *et al.*, 1968).

Materials and Methods

Isolated fibers were prepared as described by Girardier *et al.* (1963) from the walkin leg muscles of crayfish, mostly of the genus *Orconectes*. Some work was also done wit fibers of *Procambarus*. Equipment and crayfish saline were standard for this laborator A fast-flow chamber was employed, of 1.5-ml capacity, and solution changes were mac by flushing through 10 times this volume in 3 to 4 sec. When Cl^- was removed fro a saline solution, it was replaced by propionate, an impermeant anion (Reuben, Gira dier & Grundfest, 1964), and changes in K^+ were balanced with changes in Na^+.

An intracellular micropipette was used to record membrane potential, and a second micropipette was inserted for those experiments in which conductance was measured. The micropipettes were left in the fibers during solution changes so that continuous records of electrical changes were obtained.

Results

Localization of G_K

The cores of the sarcolemmal invaginations of crayfish fibers are a possible barrier to diffusion interposed between the bath and the major areas of the cell membrane. Invaginations run obliquely into the fibers and are approximately one fiber diameter, about 200 μ, in length (P. Brandt, *personal communication*). A scale factor for time, τ_1, in diffusion processes is given by L^2/D. An estimate of $\tau_1 = 40$ sec follows from the diffusion length $L = 200$ to 250 μ and a diffusion coefficient $D = 2 \times 10^{-5}$ cm^2/sec. Simple one-sided diffusion into a sheet of thickness, L, with $\tau_1 = 40$ sec (Crank, 1956, pp. 55) requires a half-time of $t_{1/2} = 7.6$ sec for equilibration between sheet and bath. A lag of this magnitude in the response of a cell could easily be measured with our system.

A typical response of a fiber to a sudden change in bath K^+ is given in Fig. 1. The responses to K^+ changes are 20 times faster than the diffusion barrier described above would allow. The average $t_{1/2}$ for the transition 3 mM K^+ to 10 mM K^+ (17 mV) was 0.3 sec (seven cells). This value for $t_{1/2}$ was also obtained for the transition 20 mM K^+ to 30 mM K^+ (five cells). The rapid responses imply that the K^+ conductance is localized at the superficial membrane. The rapid blockader of K^+ movement, Ba^{++} (Grundfest, 1966), was used to test this hypothesis.

The experimental procedure and dose-response curve are given in Fig. 2. $BaCl_2$ was added isosmotically to the control solution to make up the solutions shown on the graph. Changes in bath Ba^{++} were made while continuously monitoring membrane resistance by pulsing the fiber as shown in the inset. Hyperpolarizing pulses were used to avoid complications caused by tension or by depolarization-induced membrane changes. Effective conductance, $G_{eff} = I/V$, was used to measure the change in G_K produced by

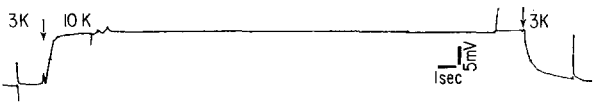

Fig. 1. Response of crayfish fiber membrane potential to the change in K^+ concentration from 3 to 10 mM. The spikes near the beginning and end of the record are start-stop signals from the pump. Ripples in the record are flow artifacts

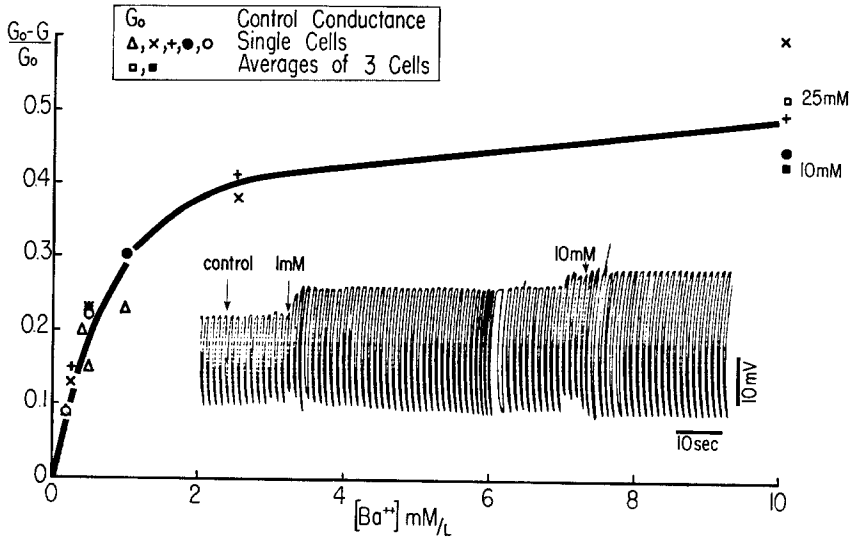

Fig. 2. Effect of $BaCl_2$ on effective conductance of crayfish muscle fibers. Inset show increase in voltage excursion in response to a constant current pulse when $BaCl_2$ i added to the bath

Ba^{++}. Data from different cells could be fit to one dose-response curv when the relative conductance change $\frac{G_{\text{eff},0} - G_{\text{eff}}}{G_{\text{eff},0}}$ was plotted agains $BaCl_2$ dose. The maximum reduction in effective conductance caused b Ba^{++} was about 55%.

The records of Fig. 3 demonstrate the rapidity of the response to Ba^{++} Changes were made from the higher Ba^{++} concentrations (higher resistance to the lower in order to avoid hyperpolarizing Cl^- activation effects. In th linear range of the dose-response curve, the half-time for a Ba^{++} respons was under 1 sec.

A more impressive demonstration of the absence of a diffusion barrie for most of the K^+ conductance is given in Fig. 4. In this case, 25 mM Ba^+

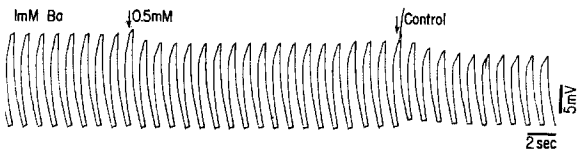

Fig. 3. Time course of effective conductance change in response to low doses of BaCl

Fig. 4. Time course of effective conductance change on washout of high dose of BaCl from bath

was reduced in 5 sec to a concentration of 0.25 mM, estimated from the conductance vs. Ba^{++} concentration curve. If there were a diffusion barrier between the K^+ channels and the bath with $\tau_1 = 40$ sec, this concentration change would have required more than 1 min.

Response of the Crayfish Fiber to Sudden Changes in Cl^- Concentration

The electrical response of crayfish muscle to Cl^- reduction is more complex than that of frog muscle. Rapid withdrawal of Cl^- from frog saline produces a sudden large depolarization followed by a smooth return to baseline (Hodgkin & Horowicz, 1959). Crayfish response is somewhat variable (Reuben *et al.*, 1964). The depolarization rises during 30 to 60 sec to a peak of 10 to 20 mV, and the smooth falling phase is characterized by a time constant of 3 to 15 min. The variation in amplitude and falling phase can be elicited in a single fiber by varying bath pH, as shown in Fig. 5. We have found that lowering pH markedly increases G_{Cl} consistent with previous data on the crayfish (Reuben, Girardier & Grundfest, 1962; De Mello & Hutter, 1966) and on barnacle muscle (Hagiwara, Gruener, Hayashi, Sakata & Grinnell, 1968). This effect is opposite to that of pH on Cl^- permeability in frog (Hutter & Warner, 1967). Enhancement of Cl^- permeability causes both the larger peak and the more rapid decay of the voltage transient at pH 6.8 vs. pH 7.4 observable in Fig. 5.

Studies of the crayfish Cl^- transient have been conducted in this laboratory over a period of years (Girardier, Reuben & Grundfest, 1961). Reuben and Dunham *(unpublished data)* found that efflux of tagged Cl^- was approximately exponential with a time constant $\tau_{Cl} = 3$ min. Electrical measurements of voltage transients following Cl^- removal were also made at that time. They found a peak depolarization of 10 to 15 mV at 30 sec after the substitution of propionate for Cl^-. The time course of the voltage decay was consistent with a τ_{Cl} of 3 min.

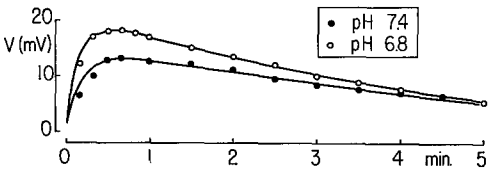

Fig. 5. Chloride transient: Effect of pH on membrane-potential change following substitution of propionate for bath Cl^-. Solid lines are calculated transients and circles are experimental points

Other data were obtained in this laboratory by M. Berman *(unpublished* with a series of fibers with slow-falling Cl^- transients. These fibers had tim constants of 10 to 15 min.

Interpretation of these data necessitated a precise analysis of the Cl transient.

Ionic Fluxes in the Presence of a Diffusion Barrier

A voltage excursion follows a change in the Cl^- concentration of th saline bathing a muscle. This voltage is the driving force required for K^+ t efflux from the cell at the same rate as Cl^-. Since the resting potential i equal to both E_K and E_{Cl}, $E_i = E_K = E_{Cl}$; the relation between voltage an fluxes of K^+ and Cl^- can be expressed:

$$J_{Cl} = J_K = \frac{G_K}{F} V. \qquad (1$$

The voltage excursion V is proportional to the flux. Therefore, analysi of the potential change during the Cl^- transient reduces to analysis of th kinetics of the Cl^- flux. These kinetics are controlled by two processes movement of ions across the plasma membrane and diffusion of ions alon the invaginations. We will first consider the diffusion problem.

Most of the transmembrane Cl^- movement is at the end of a tubul which is connected to an invagination. Since diffusion time varies as th square of the diffusion path length, the contribution of the tubule lume to the diffusion barrier is only $(10/200)^2 = 1/400$ of the contribution of th invagination. For this reason, the invagination is taken as the sole barrie to diffusion. A model of this system is a cylinder, open to the bath at on end, with a uniform permeability to Cl^- distributed over the cylinde surface.

The electro-diffusion process in the invagination involves movement o the impermeant anion, cations, and water as well as Cl^-. In the calculations described in a later section, these complexities were ignored and the process was treated as though it were tracer diffusion in order to use Fick's law. This adequately demonstrates the effect of a diffusion barrier on the response of a fiber to anion substitution, which is the purpose of the computations.

The results of this section can be summarized by Eq. (2). This is the familiar diffusion equation with the term jp added to account for the flux into an invagination across the cell membrane.

$$w \frac{\delta c}{\delta t} = wD \frac{\delta^2 c}{\delta x^2} + jp, \qquad (2)$$

where both c and j are functions of distance from the bath, x; w is the cross-sectional area of the cylinder representing an invagination, p its perimeter, and j the flux per unit surface area between sarcoplasm and invagination.

Flux Across the Plasma Membrane

Although it is not our purpose to construct a physical model of ion permeation through the cell membrane, we require an accurate expression relating membrane flux to the concentration and electrical driving forces. In this section, we describe the deduction of such an expression and some tests of its utility. Most of the test data are on the frog semitendinosus muscle, taken from the work of Hodgkin and Horowicz (1959).

The crayfish data of Reuben and Dunham previously cited indicated an exponential time course for the efflux of cell Cl^- when the external Cl^- is reduced. Similarly, flux data from the frog (Hutter & Warner, 1967) indicate an exponential equilibration of cell Cl^- with that in the bath, this behavior implies that the Cl^- flux is at least approximately proportional to the deviation of the cell Cl^- from the equilibrium value. Stating this algebraically,

$$j = H(c_f - rc),$$

where

$$r = \exp\left(\frac{FE}{RT}\right). \tag{3}$$

Eq. (3) when substituted into Eq. (2) gives the differential equation describing flux in an invagination. This expression is employed in the next section. At this point, Eq. (3) will be used to compute J (the Cl^- flux) and thereby relate H to the more familiar terms P_{Cl} and G_{Cl}. Permeability and conductance data used in evaluation of Eq. (3) are free from diffusion effects. Therefore, the terms j and c of Eq. (3) will be taken as uniform along an invagination for the remainder of this section.

The following considerations regarding the accuracy of Eq. (3) are important, since the interpretation in terms of permeability of a voltage excursion after a bath-composition change depends on the relation assumed between voltage and ionic flux. Implications for G_{Cl} of Eq. (3) will be tested on frog and crayfish data from the literature. Eq. (3) will be used to correlate the data on initial voltage excursions subsequent to changes in bath Cl^-.

Multiplication of Eq. (3) by the invagination area per unit area of fiber envelope gives the equation for J:

$$J = \frac{HNLp}{\pi D_f L_f}(c_f - rc). \tag{4}$$

From this it follows that

$$P_{Cl}=\frac{HNLp}{\pi D_f L_f},$$

and (5

$$I_{Cl}=-FP_{Cl}(c_f-rc).$$

Differentiation of Eq. (5) gives the slope conductance for Cl^-. Evalua tion of this derivative at E_i yields the resting Cl^- conductance, G_{Cl}.

$$\frac{dI_{Cl}}{dE}=FP_{Cl}\left(\frac{F}{RT}rc-(c_f-rc)\frac{d\ln P_{Cl}}{dE}\right)$$

$$G_{Cl}=P_{Cl}\frac{F^2}{RT}c_f. \quad (6$$

Hodgkin and Horowicz (1959) employed an equation for the equilibrium value of G_{Cl} from constant-field theory which can be written in our ter minology as

$$G_{Cl}=P_{Cl}\frac{F^2}{RT}c_f\frac{FE/RT}{(e^{FE/RT}-1)}. \quad (6a$$

Eqs. (6) and (6a) agree only to first order in FE/RT. That there is not em pirical justification for the selection of the more complex Eq. (6a) ove Eq. (6) is demonstrated in Fig. 6 and Table 2. Neither equation gives a precise fit to the G_{Cl} values given by Hodgkin and Horowicz (1959) for frog but they both do reasonably well over a range from the physiological c_f o

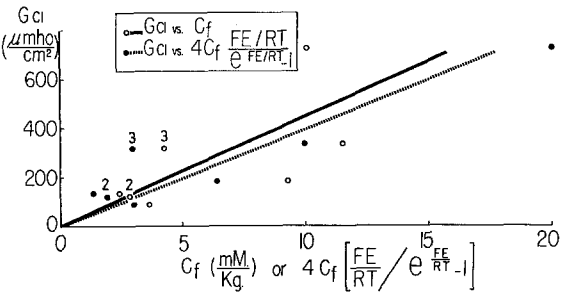

Fig. 6

Fig. 6. Correlation of Cl^- conductance, G_{Cl}, with fiber Cl^-, c_f (from frog muscle data of Hodgkin & Horowicz, 1959). Where points clustered, averaged data was plotted for clarity. These points are labeled with the number of data points used in the average. —o— G_{Cl} vs. C_f; ····•···· G_{Cl} vs. $4C_f \frac{FE/RT}{e^{FE/RT}-1}$

Fig. 7. Change in membrane potential when bath Cl^- is reduced from c_i to c.

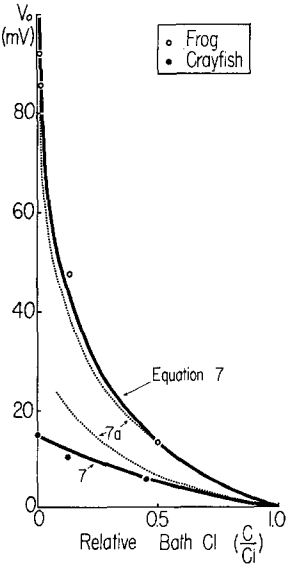

Fig. 7

Table 2. *Statistical tests of equations relating Cl^- conductance (G_{Cl}) to cell Cl^- (c_f)*

Test	$G_{Cl}=m_1 c_f$	$G_{Cl}=m_2 c_f \frac{FE/RT}{(e^{FE/RT}-1)}$	Significance at 95% level, $df=9$
Slope (m)	44.0	38.9	
Correlation coefficient (r)	0.60	0.72	$r \geqq r_{0.95}=0.60$
Residual variance (s^2)	25,500	18,800	$F=\frac{s_1^2}{s_2^2}=1.36$ $<F_{0.05}=3.18$

2.4 mmoles/kg cell water to five times this value. This variation in c_f corresponded to a membrane potential variation from -77 to -32 mV.

The lines in Fig. 6 are least squares fits of Eqs. (6) and (6a) to the frog G_{Cl} data.[1] The correlations given by these equations are both significant at the 95% level, and the difference between the residual variances for the two equations is not statistically significant at this confidence level (Table 2). A value of $P_{Cl}=1.2\times10^{-6}$ cm/sec is deduced from Eq. (6); Eq. (6a) gives $P_{Cl}=1.1\times10^{-6}$ cm/sec. These values are consistent with values of P_{Cl} for frog muscle deduced from tracer studies (Harris, 1965; Hutter & Warner, 1967) and conductance measurements (Adrian & Freygang, 1962).

We do not have data on G_{Cl} vs. c_f for the crayfish, but a check on the applicability of Eqs. (4) and (6) to crayfish muscle can be made from computation of τ_{Cl}. The time constant for Cl^- efflux is simply the cell volume $\frac{\pi}{4}D_f^2 L_f$ divided by the product of P_{Cl} and cell surface,

$$\tau_{Cl}=\frac{D_f}{4P_{Cl}}.$$

If we multiply Eq. (6) by τ_{Cl}, we can relate two experimentally accessible quantities τ_{Cl} and G_{Cl}:

$$G_{Cl}\tau_{Cl}=\frac{F^2}{RT}\frac{D_f}{4}c_f. \tag{6b}$$

For the 200 μ crayfish fiber equilibrated in control saline, $c_f=6-8\times10^{-6}$ equiv/ml, and the right-hand side of Eq. (6b) is 0.15 coul/V cm^2. These fibers have less than half of their conductance of $\frac{10^{-3}}{2}$ $(\Omega\,\text{cm}^2)^{-1}$ allocated

1 Data from Table 7 of Hodgkin and Horowicz (1959). The 10 points with c_f of 2.4 to 12 mmoles/kg were correlated. Hodgkin and Horowicz (1959) calculated a P_{Cl} for each of their experimental points and found an average $P_{Cl}=4\times10^{-6}$ cm/sec.

to Cl^-, and time constants are given within a factor of 2 by 6 min[2]. There fore, $G_{Cl}\,\tau_{Cl}=\frac{10^{-3}}{4}\times 360=0.09$ coul/V cm^2. There is as good agreement a one could expect from estimated values.

Combination of Eq. (5) with Eq. (1) allows the calculation of the initia voltage excursion in the absence of a diffusion barrier, Eq. (7). Eq. (7) reduce to the same differential form (7b) used by Hodgkin and Horowicz (1959 for small changes in bath Cl^-, c; but this equation differs significantly fron the commonly used form, Eq. (7a), in that finite voltages are predicted fo all values (including 0) of c. No *ad hoc* membrane processes are require to account for deviations from a Nernst-type relation at low values of c since the saturation of J_{Cl} predicted by Eq. (3) implies a limiting voltag excursion at $c=0$ in Eq. (7).

$$\frac{FV}{RT}=\frac{G_{Cl}}{G_K}\left(1-\frac{rc}{c_f}\right), \tag{7}$$

$$\frac{FV}{RT}=\frac{G_{Cl}}{G_K+G_{Cl}}\ln\left(\frac{c}{c_i}\right), \tag{7a}$$

$$\operatorname*{Lim}_{rc\to c_f}\left(\frac{\delta V}{\delta \ln c}\right)_{c_f}=-\frac{G_{Cl}}{G_K+G_{Cl}}\,\frac{RT}{F}. \tag{7b}$$

Eqs. (7) and (7a) are compared with experimental data in Fig. 7. The slow-falling transients recorded by Berman (two cells) were used to test the applicability of Eq. (7) to crayfish, since the diffusion-barrier effect was small under these conditions. In contrast to this preparation where G_{Cl} and G_K are comparable, we have the data of Hodgkin and Horowicz (1959) on frog muscle equilibrated in high K^+ (five cells) where $G_K \ll G_{Cl}$. For both sets of data, the value of G_{Cl}/G_K was obtained from the $c=0$ point; Eq. (7) was solved with the aid of an analog computer for this value of G_{Cl}/G_K. Since Eqs. (7) and (7a) both reduce to Eq. (7b) for small changes in c, the same value of G_{Cl}/G_K was used to compute V from Eq. (7a).

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2 The values given for c_f and the specific conductance require justification. The value for c_f was derived from tracer studies (Dunham, Reuben & Brandt, 1964; and Dunham; *unpublished*). The methods for determination of crayfish Cl^- were those described in a study of lobster muscle (Dunham & Gainer, 1968), where a SE of less than $\pm 10\%$ was obtained. The effective resistance of crayfish muscle has been previously reported from this laboratory to be about $10^5\ \Omega$ (Girardier, Reuben & Grundfest, 1962; Ozeki, Freeman & Grundfest, 1966). A typical 200-μ fiber of 0.5 cm length has a cylindrical envelope of 3×10^{-2} cm^2 area. The specific effective resistance is therefore about $3\times 10^3\ \Omega$cm^2. Since the length constant is about 1.5 mm, the specific resistance is only 0.6 of the effective value (Falk & Fatt, 1964) or $2\times 10^3\ \Omega$cm^2, which is the value used in the text.

It is clear from Fig. 7 that when the fiber is nearly a single-ion electrode, as in the frog case ($G_{Cl}/G_K = 4.35$), the two equations are nearly identical for any finite value of c. However, when the fiber is not so selective, serious differences appear. For the crayfish $G_{Cl}/G_K = 0.65$, and Eq. (7a) overestimates V by 25% when c has been reduced by 50%, and by 100% when c is reduced by 90%. This could result in a serious underestimation of the Cl^- conductance in this preparation.

Time Course of the Cl^- Transient

The previous section indicates that Eq. (3) is a reasonable expression for the relation between flux and driving force across the sarcolemma. Combination of this equation with that for diffusion [Eq. (2)] gives the following equation which governs movement of Cl^- in the invaginations:

$$\frac{\delta c}{\delta t} = D\frac{\delta^2 c}{\delta x^2} + \frac{Hp}{w}(c_f - rc). \tag{8}$$

While the invagination concentrations are changing, the fiber Cl^- is also changing. This requires that another differential equation be solved with Eq. (8). The time course of the change in c_f is given by Eq. (9); i.e., cell Cl^- loss equals total Cl^- efflux:

$$-v_f\frac{dc_f}{dt} = NHp\int_0^L (c_f - rc)\,dx. \tag{9}$$

This pair of equations was put into dimensionless form for solution on an analog computer (Appendix I). The solutions depended on three dimensionless parameters. Two were morphological parameters: the ratio of invagination volume to fiber volume $\left(n = \frac{NLw}{v_f}\right)$ and the time scale for invagination diffusion $\left(\tau_1 = \frac{L^2}{D}\right)$. The diffusion coefficient D is that for Cl^- in bath saline, approximately 2×10^{-5} cm^2/sec. The third parameter, $\tau_2 = w/Hp$, is physiological since it involves the invagination permeability H.

When these equations are combined with Eq. (1), it is possible to compute the voltage excursion following a change in bath Cl^-. This computation was performed on an analog computer for the case of total Cl^- withdrawal. It was necessary to neglect the negative-feedback effect of changes in the membrane potential on the flux in order to perform the computation with the available machine capacity. The system of equations solved is

given below, and a description of the computation and the effects of th neglect of voltage feedback on flux are given in Appendix II.

$$\gamma_1 = \frac{c}{c_i} \qquad \gamma_2 = \frac{c_f}{c_{f,i}}.$$

Invagination Diffusion:

$$\frac{\delta \gamma_1}{\delta t} = \frac{1}{\tau_1} \frac{\delta^2 \gamma_1}{\delta y^2} - \frac{r_i}{\tau_2}(\gamma_1 - \gamma_2). \tag{10}$$

$$\text{I.C. } \gamma_1 = 1, \quad \text{B.C. } y = 0, \quad \gamma_1 = 0, \quad y = 1, \quad \frac{\delta \gamma_1}{\delta y} = 0.$$

Change in Cell Cl^-:

$$\frac{\delta \gamma_2}{\delta t} = \frac{n}{\tau_2}\left(\int_0^1 \gamma_1 \, dy - \gamma_2\right). \tag{11}$$

$$\text{I.C. } \gamma_2 = 1.$$

Voltage Transient:

$$V = \frac{RT}{F} \frac{G_{Cl}}{G_K}\left(\gamma_2 - \int_0^1 \gamma_1 \, dy\right). \tag{12}$$

Results of Cl^- Transient Computations

The preceding set of equations contains four parameters. The peak voltage excursion is fit with G_{Cl}/G_K, leaving the three parameters τ_1, τ_2 and n to describe the shape. This overabundance of parameters was handled as follows. The data of Reuben and Dunham *(unpublished)* on tracer efflux as well as voltage transients was used to fit τ_1 and n. The shapes of other voltage transients were fit with these same values of τ_1 and n, leaving only τ_2 as a free parameter.

Over the range $n = 0.4$ to 2.0×10^{-2}, the values of $\tau_1 = 40$ sec and $G_{Cl}/G_K = 5/6$ gave a peak of 10 to 15 mV occurring 30 sec after bath Cl^- was dropped to zero, as required by the results of Reuben and Dunham *(unpublished)*. A value of $n = 1 \times 10^{-2}$ (i.e., midrange) was selected. This value of n corresponds to $\tau_2 = 1.4$ sec. The numerical values of τ_2 and n are not quantitatively significant since they can be varied (with $\tau_2/n = 3$ min) over more than a factor of two up or down with no effect on the fit.

In order to fit the data of Berman *(unpublished observations)* on the time course of a voltage transient after Cl^- withdrawal, values of $G_{Cl}/G_K = 2/3$ and $\tau_2 = 8.4$ min were required. With $\tau_1 = 40$ sec, the increase in τ_2 shifted the peak from 30 to 60 sec. Similarly, the curves of Fig. 5 were fit with the fixed values of τ_1 and n to yield the parameters given in Table 3. These results show that a model 200 μ diameter fiber with Cl^- permeability distrib-

Table 3. *Parameters to fit experimental Cl^- transients*

Data source	Parameters			
	n	τ_1 (sec)	τ_2 (sec)	G_{Cl}/G_K
Reuben and Dunham *(unpublished)*	0.01	40	1.4	0.83
Berman *(unpublished)*	0.01	40	8.4	0.67
This work, pH 7.4	0.01	40	2.6	0.70
This work, pH 6.8	0.01	40	1.9	1.04

uted along $L=(D\tau_1)^{1/2}=250$ μ invaginations, which comprise 1% of its volume, will duplicate the observed time course of the Cl^- transient in crayfish muscle.

Summary of Estimates of T_K

From each of the three types of experiments reported above, an estimate of $T_K=G_K/(G_K+G_{Cl})$ was made. The method for obtaining conductance ratios from the Cl^- transient was just described. A value for T_K was also calculated from the Nernst relation and the K^+ responses presented earlier, $V=T_K\Delta E_K$. An estimate of T_K could also be made from the maximum conductance block produced by $BaCl_2$ (Fig. 2), but this requires some approximation to obtain membrane conductance from G_{eff}. Resting crayfish fibers are neither ideal cables nor equipotential volumes, since λ/L is about 1/3 (Girardier *et al.*, 1962). In the high-resistance Ba^{++}-treated fiber, $\lambda/L\sim0.7$, and the latter model gives the smaller error (Falk & Fatt, 1964). Therefore, T_K was estimated as $\frac{G_{eff,0}-G_{eff,Ba^{++}}}{G_{eff,0}}$, a low estimate of T_K. The results in Table 4 give $T_K=0.6$, which is consistent with the resistance change produced by Cl^- depletion (Grundfest, 1966, Fig. 27).

Table 4. *T_K from various measures*

Measurement	T_K
Peak of Cl^- transient	0.60
Change in E, 3 to 10 mM K^+	0.60
Conductance block by Ba^{++}	0.55

Discussion

Our research has yielded certain conclusions specific to the crayfish muscle and other conclusions of a more general nature. The success of the model presented in this paper in correlating voltage responses of crayfish

fibers with their known morphology supports the conclusion of Girardie *et al.* (1963) that the tubules are permselective to Cl^-. In addition, Figs.2– demonstrate that G_K is localized in the superficial sarcolemma of the cray fish muscle. An analogous but reverse localization exists in the frog sa torium muscle. Gage and Eisenberg (1969) found that the K^+ conductanc in frog is localized in the tubular system; this had been suggested b Hodgkin and Horowicz (1960) and Adrian and Freygang (1962).

Both crayfish muscle (Brandt *et al.*, 1965) and frog muscle (Peachey 1965) contain transverse tubular systems (TTS) which apparently electricall couple the fiber interior to its surface membrane. In the crayfish, this i accomplished by the sarcolemmal invaginations of about 0.2-μ diamete connected via short tubules to diadic junctions with the sarcoplasmic reticu lum (SR). Frog muscle achieves the same effect with long tubules of 0.05-μ effective diameter connecting the surface to triadic junctions with the SR. I either case, the cylindrical model used in the preceding analysis is appropriate

From his morphological study of the frog muscle fiber, Peachey (1965 concluded that the TTS composed 0.3% of the fiber volume. This figur concurred with the estimates made by Hodgkin and Horowicz (1960). A similar proportion of the volume is indicated by the diffusion properties o the crayfish muscle fiber invaginations. Frog and crayfish are also simila in the apparent localization of permselective membrane at the tubule-SR junctions. These morphological similarities seem to explain the time lag in the responses of these fibers to ionic changes in their baths (Hodgkin & Horowicz, 1960) and some of their slow responses to sustained current (Freygang, Goldstein, Hellam & Peachey, 1964). The similarities in construc tion of the communication systems in these widely different animals may indi cate a common mode of excitation of the contractile system.

A concluding point concerns interpretation of electrical responses tc ionic changes. A small voltage change which occurs after a large variation in an ionic concentration can be caused by a number of things other than a low permeability. Two possible causes, an extra-membrane diffusion bar rier and inapplicability of a Nernst relation far from equilibrium, have been demonstrated in this work. Diffusion barriers generally cause slow cellular response to solution changes as well as lowered response amplitude. The inapplicability of the Nernst relation when it predicts an infinite response (infinite battery) to an ionic substitution should be obvious. A semi-thermo dynamic analysis is logically consistent only with the method employed by Hodgkin and Horowicz (1959), who used the differential form, Eq. (7b), to calculate transport numbers from the voltage changes produced by varia tions in the composition.

Diffusion-barrier effects on apparent transport numbers have been described in lobster and squid axon in a previous paper from this laboratory (Freeman *et al.*, 1966). Salt accumulation was visualized by the swelling produced by inward currents or osmotic challenges in the sheath surrounding the axolemma. Similarly, swelling of the TTS was observed during outward movement of Cl^- in the crayfish fiber (Girardier *et al.*, 1963; Reuben *et al.*, 1964; Brandt *et al.*, 1968). Difficulties introduced by such diffusion barriers, or by non-linear transport processes such as we have described, require a critical approach on the part of investigators who wish to relate electrophysiological data to transport numbers, even in passive systems where only electrical and diffusional forces need be considered.

Appendix I

Computation of Time Course of Cl^- Movement

Chloride movement occurs across the cell membrane and by diffusion through the invaginations. Flux crossing unit area of an invagination is $H(c_f - rc)$. Invaginations are represented as straight, circular cylinders of length L.

Invagination Diffusion

Surface area/length = perimeter = p.

Invagination volume/length = cross-section area = w.

Rate of accumulation of Cl^- = net flow across boundaries.

$$w\frac{\delta c}{\delta t} = wD\frac{\delta^2 c}{\delta x^2} + Hp(c_f - rc).$$

To put this in reduced form, we define two time constants:

$$\frac{\delta \gamma_1}{\delta t} = \frac{1}{\tau_1}\frac{\delta^2 \gamma_1}{\delta y^2} + \frac{r}{\tau_2}\left(\gamma_2\left(\frac{c_{f,i}}{rc_i}\right) - \gamma_1\right); \tag{I-1}$$

$$\frac{c}{c_i} = \gamma_1; \quad \frac{c_f}{c_{f,i}} = \gamma_2; \quad y = \frac{x}{L}; \quad \tau_1 = \frac{L^2}{D}; \quad \tau_2 = \frac{w}{Hp}.$$

Change in Cell Cl^- Concentration

The cell volume v_f exchanges Cl^- through N invaginations:

$$-v_f\frac{dc_f}{dt} = NHp\int_0^L (c_f - rc)\,\delta x.$$

This equation is put into dimensionless form by introducing the parameter n, the ratio of total invagination volume to cell volume:

$$\frac{d\gamma_2}{dt}=\frac{n}{\tau_2}\left(\int_0^1\left(\frac{rc_i}{c_{f,i}}\right)\gamma_1\,\delta y-\gamma_2\right). \tag{I-2}$$

Appendix II

Analog Computation of Cl^- Transient

A TR-48 computer was programmed to solve the simultaneous differential equations given as Eqs. (10)–(12) of the text. The diffusion Eq. (10) was set up as a difference equation, with the invagination divided into m parts, giving $m+1$ stations along its length. Station 0 was at the mouth and station m at the end of the invagination.

Finite difference representations were made for $\delta^2\gamma_1/\delta y^2$ and $\int_0^1\gamma_1 dy$.

$$\left(\frac{\delta^2\gamma_1}{\delta y^2}\right)_j=m^2\left((\gamma_{1,j+1}-\gamma_{1,j})+(\gamma_{1,j-1}-\gamma_{1,j})\right)$$

$$m\int_0^1\gamma_1\,dy=\sum_{j=1}^{m-1}\gamma_{1,j}+\frac{\gamma_{1,0}+\gamma_{1,m}}{2}.$$

When m was increased from 4 to 5, the maximum change produced in γ_2 was 0.01 and the largest change in V was 0.2 mV at the values of τ_1, τ_2 and n that simulated experimental results. All results presented in the text are for $m=5$.

In order to make this computation with limited facilities, it was necessary to substitute $(\gamma_2-\gamma_1)$ for $(\gamma_2-\gamma_1 e^{FV/RT})$. A first-order correction for finite V can be made by expanding exp (FV/RT) to first order in Eq. (1a) and solving for V. The average value of γ_1 is $\bar{\gamma}_1=\int_0^1\gamma_1\,dy$.

$$V=\left(\frac{RT}{F}\,\frac{G_{Cl}}{G_K}(\gamma_2-\bar{\gamma}_1)\right)\Big/\left(1+\frac{G_{Cl}\bar{\gamma}_1}{G_K}\right). \tag{II-1}$$

The numerator of the right-hand side is the approximate form used in the machine computation, (V). The relative correction is given by:

$$\frac{(V)-V}{V}=\frac{G_{Cl}}{G_K}\bar{\gamma}_1. \tag{II-2}$$

Note that the fractional overestimate of the voltage excursion is first order in $\bar{\gamma}_1$, and proportional to G_{Cl}/G_K. The fraction errors at the peak voltage excursion for a total anion substitution are tabulated for the two extreme values of τ_2 employed in the text. An overestimate of $G_{Cl}/G_K=1$ was used to get an upper limit for the error.

τ_2(sec)	$\frac{(V)-V}{V}$ at peak V
1.4	0.15
8.4	0.02

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References

Adrian, R. H., Freygang, W. H. 1962. The potassium and chloride conductance of frog muscle fibers. *J. Physiol.* **163**:61.

Brandt, P. W., Reuben, J. P., Girardier, L., Grundfest, H. 1965. Correlated morphological and physiological studies on isolated single muscle fibers. I. Fine structure of the crayfish muscle fiber. *J. Cell Biol.* **25**:233.

— — Grundfest, H. 1968. Correlated morphological and physiological studies on isolated single muscle fibers. II. The properties of the crayfish transverse tubular system: Localization of the sites of reversible swelling. *J. Cell Biol.* **38**:115.

Crank, J. 1956. The Mathematics of Diffusion. Oxford University Press, London.

De Mello, W., Hutter, O. F. 1966. The anion conductance of crustacean muscle. *J. Physiol.* **183**:11 P.

Dunham, P. B., Gainer, H. 1968. The distribution of inorganic ions in lobster muscle. *Biochim. Biophys. Acta* **150**:488.

— Reuben, J. P., Brandt, P. W. 1964. Chloride fluxes in crayfish muscle fibers after vesiculation of the transverse tubular system and after treatment with procaine. *Biol. Bull.* **127**:368.

Falk, G., Fatt, P. 1964. Linear electrical properties of striated muscle fibers observed with intracellular electrodes. *Proc. Roy. Soc. (London) B* **160**:69.

Freeman, A. R., Reuben, J. P., Brandt, P. W., Grundfest, H. 1966. Osmometrically determined characteristics of the cell membrane of squid and lobster giant axons. *J. Gen. Physiol.* **50**:423.

Freygang, W. H., Goldstein, D. A., Hellam, D. C., Peachey, L. D. 1964. The relation between the later after-potential and the size of the transverse tubular system of frog muscle. *J. Gen. Physiol.* **48**:235.

Gage, P. W., Eisenberg, R. S. 1969. Ionic conductances of the surface and transverse tubular membranes of frog sartorius muscle fibers. *J. Gen. Physiol.* **53**:279.

Girardier, L., Reuben, J. P., Brandt, P. W., Grundfest, H. 1963. Evidence for anion-permselective membrane in crayfish muscle fibers and its role in excitation-contraction coupling. *J. Gen. Physiol.* **47**:189.

— — Grundfest, H. 1961. The components of the resting potential in crayfish and lobster muscle fibers. *Biol. Bull.* **121**:366.

— — — 1962. Effects of isolation and denervation of crayfish muscle fibers on their membrane resistance. *Biol. Bull.* **123**:496.

Grundfest, H. 1966. Comparative electrobiology of excitable membranes. *In:* Advances in Comparative Physiology and Biochemistry, vol. 2. p. 1. Academic Press, New York.

Hagiwara, W., Gruener, R., Hayashi, H., Sakata, K., Grinnell, A. D. 1968. Effects of external and internal pH changes on K and Cl conductances in the muscle fiber membrane of a giant barnacle. *J. Gen. Physiol.* **52**:773.

Harris, E. J. 1965. The chloride permeability of frog sartorius. *J Physiol.* **176**:123.

Hodgkin, A. L., Horowicz, P. 1959. The influence of potassium and chloride ions o the membrane potential of single muscle fibres. *J. Physiol.* **148**:127.

— — 1960. Effect of sudden changes in ionic concentration on the membrane potenti of single muscle fibres. *J. Physiol.* **153**:370.

Hutter, O. F., Warner, A. E. 1967. The effect of pH on the ^{36}Cl efflux from fro skeletal muscle. *J. Physiol.* **189**:427.

Ozeki, M., Freeman, A. R., Grundfest, H. 1966. The membrane components of crust cean neuromuscular systems. II. Analysis of interactions among the electrogeni components. *J. Gen. Physiol.* **49**:1335.

Peachey, L. D. 1965. The sarcoplasmic reticulum and transverse tubules of the frog sartorius. *J. Cell Biol.* **25**:209.

Reuben, J. P., Girardier, L., Grundfest, H. 1962. The chloride permeability of crayfis muscle fibers. *Biol. Bull.* **123**:509.

— — — 1964. Water transfer and cell structure in isolated crayfish muscle fiber *J. Gen. Physiol.* **47**:1141.