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Nitrate and Chloride Ions Have Different Permeation Pathways in Skeletal Muscle Fibers of *Rana pipiens*

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Summary. The effects of pH on the permeability and conductance of the membranes to nitrate and to chloride of semitendinosus and lumbricalis muscle fibers were examined.

Membrane potential responses to quick solution changes were recorded in semitendinosus fibers initially equilibrated in isotonic, high K_2SO_4 solutions. External solutions were first changed to ones in which either Rb^+ or Cs^+ replaced K^+ and then to solutions containing either NO_3^- or Cl^- to replace SO_4^{2-}. The hyperpolarizations produced by Cl^- depend on external pH, being smaller in acid than in alkaline solutions. By contrast, hyperpolarizations produced by NO_3^- were independent of external pH over a pH range from 5.5 to 9.0.

In addition, voltage-clamp measurements were made on short lumbricalis muscle fibers. Initially they were equilibrated in isotonic solutions containing mainly K_2SO_4 plus Na_2SO_4. KCl or KNO_3 were added to the sulfate solutions and the fibers were equilibrated in these new solutions. When finally equilibrated the fibers had the same volume they had in the sulfate solutions before the additions. Constant hyperpolarizing voltage pulses of 0.6-sec duration were applied when all external K^+ was replaced by TEA^+. For these conditions, inward currents flowing during the voltage pulses were largely carried by Cl^- or NO_3^- depending on the final equilibrating solution. Cl^- currents during voltage pulses were both external pH and time dependent. By contrast, NO_3^- currents were independent of both external pH and time.

The voltage dependence of NO_3^- currents could be fit by constant field equations with a P_{NO_3} of $3.7 \cdot 10^{-6}$ cm/sec. The voltage dependence of the initial or "instantaneous" Cl^- currents at pH 7.5 and 9.0 could also be fit by constant field equations with P_{Cl} of $5.8 \cdot 10^{-6}$ and $7.9 \cdot 10^{-6}$ cm/sec, respectively. At pH 5.0, no measurable "instantaneous" Cl^- currents were found.

From these results we conclude that NO_3^- does not pass through the pH, time-dependent Cl^- channels but rather passes through a distinct set of channels. Furthermore, Cl^- ions do not appear to pass through the channels which allow NO_3^- through. Consequently, the measured ratio of P_{Cl}/P_{NO_3} based on membrane potential changes to ionic changes made on intact skeletal muscle fibers is not a measure of the selectivity of a single anion channel but rather is a measure of the relative amounts of different channel types.

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Key Words skeletal muscle · NO_3^- currents · Cl^- currents · pH · muscle membrane · relative anion permeabilities

Introduction

Boyle and Conway (1941) demonstrated that frog skeletal muscle membranes are permeable to K^+ and Cl^- but are impermeable or sparingly permeable to other ions. At rest, in normal physiological solutions at pH 7.2, about two-thirds of the membrane conductance is due to Cl^- and one-third due to K^+ (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960).

At physiological pH, NO_3^- is less permeant than Cl^- (Hutter & Padsha, 1959; Washio & Mashima, 1963) and decreases $^{36}Cl^-$ efflux (Harris, 1958; Adrian, 1961). From the observation that the membrane potential does not change much when extracellular Cl^- is replaced by NO_3^- it has been concluded that NO_3^- reduces the Cl^- permeability to about the NO_3^- permeability (Hutter & Padsha, 1959; Hodgkin & Horowicz, 1959, 1960; Adrian, 1961).

Decreasing external pH decreases the Cl^- conductance of and $^{36}Cl^-$ efflux from frog skeletal muscle (Brooks & Hutter, 1962; Hutter & Warner, 1967*a*, *b*). Furthermore, from experiments in which membrane potential changes in response to ionic concentration changes were measured in frog skeletal muscle, it has been reported that NO_3^- is more permeant than Cl^- in acid solutions and is less permeant than Cl^- in alkaline solutions (Hutter & Warner, 1968). To account for this finding and the more general result that there is an inversion of the permeability sequence to various anions at pH 5.0 as compared with pH 9.8, Hutter, Mello and Warner (1969) have proposed a model in which the selectivity of a single permeation mechanism is altered by pH.

Table 1. Composition of solutions (in mM)

A. Sulfate solutions for experiments on semitendinosus fibers

Ref.	Na_2SO_4	$CaSO_4$	K_2SO_4	Cs_2SO_4	Rb_2SO_4
S1	27	8	68	—	—
S2	27	8	—	68	—
S3	27	8	—	—	68

B. Nonsulfate solutions for experiments on semitendinosus fibers

Ref.	$CaCl_2$	$Ca(NO_3)_2$	CsCl	$CsNO_3$	$RbNO_3$
S4	1.8	—	118	—	—
S5	—	1.8	—	118	—
S6	—	1.8	—	—	118

C. Solutions for voltage-clamp experiments on lumbricalis fibers

Ref.	K_2SO_4	Na_2SO_4	TEA_2SO_4	$CaSO_4$	$MgSO_4$	KCl	TEACl	KNO_3	$TEANO_3$
L1	75	20	—	8	5	—	—	—	—
L2	—	20	75	8	5	—	—	—	—
L3	75	20	—	8	5	60	—	—	—
L4	—	20	75	8	5	—	60	—	—
L5	75	20	—	8	5	—	—	60	—
L6	—	20	75	8	5	—	—	—	60

The Cl^- conductance system is voltage and time dependent. In voltage-clamp experiments at alkaline pHs, Cl^- conductance falls during hyperpolarizing voltage pulses, while at acid pHs Cl^- conductance increases during hyperpolarizing voltage pulses (Hutter & Warner, 1972; Warner, 1972; Loo, McLarnon & Vaughan, 1980; Vaughan, McLarnon & Loo, 1980).

The chloride conductance system in rat diaphragm fibers has many properties qualitatively similar to the chloride conductance system in frog muscles. One significant difference is that the anion conductance sequence does not invert with decreasing pH; the conductances of all permeant anions studied decrease proportionally as pH is lowered (Palade & Barchi, 1977). Although it should be noted that NO_3^- is not included in the anion series studied by Palade and Barchi (1977).

In view of this difference, we examined in greater detail than heretofore the characteristics of the membrane permeability system to NO_3^- in skeletal muscle fibers of *Rana pipiens*. We find that the NO_3^- permeability in these fibers is not pH dependent and that the NO_3^- conductance system does not depend on voltage and time as the Cl^- conductance system does. A preliminary account of these results has been presented at a meeting of the Biophysical Society (Kotsias & Horowicz, 1989).

Materials and Methods

Muscle Preparations and Chambers

In all experiments, muscles were obtained from American leopard frogs, *Rana pipiens*. For experiments where membrane potential changes were recorded in response to sudden changes in extracellular solutions, small bundles containing from 5 to 15 fibers from the semitendinosus muscles were mounted in a recording cell similar to that used by Hodgkin and Horowicz (1959). Semitendinosus fibers were about 16 mm in length. For voltage-clamp experiments, bundles of about 20 fibers were dissected from the lumbricalis muscle of the fourth toe of the hindlimb. These fibers typically had lengths of 1.0 to 1.2 mm. Lumbricalis bundles were mounted in a chamber similar to that used by Lynch (1985) modified to allow solution changes.

Solutions

Table 1 gives the final concentrations of the salts dissolved in the solutions employed for these experiments. Specific reference to these solutions will be given in the figure legends and text. With regard to buffers, for the experiments on semitendinosus fibers 1.5 mM phosphate buffer was used at pH 5.5 and 7.2, 2.5 mM HEPES was used at pH 8.0, and 2.5 mM Tris at pH 9.0. For the voltage-clamp experiments on lumbricalis fibers, 5 mM MES was used at pH 5.0, 5 mM HEPES at pH 7.5, and 5 mM Tris at pH 9.0. Since the ionic strength of the sulfate solutions is higher than that of either the chloride or nitrate solutions, the activity coefficient of monovalent cations is estimated to be only 0.87 times that in

the chloride solutions (Hodgkin & Horowicz, 1959). Thus, a monovalent ion concentration of 136 mM in the sulfate solutions is estimated to have the same activity as 118 mM in the chloride or nitrate solutions ($0.87 \cdot 136 = 118$).

Electrophysiological Measurements

In the experiments with semitendinosus fibers, membrane potentials were measured by means of glass internal and external microelectrodes connected to a high impedance amplifier system and displayed on a strip-chart recorder. To eliminate junction potentials, the external microelectrode was located downstream from the fibers and had a slow KCl flow coming out of the tip that was driven by pressure. Muscle fibers were slowly perfused with bathing solution even when the external solution was not being changed to prevent the KCl from mixing with the solution adjacent to the fibers. During a solution change the flow was increased to rates of about 3 chamber volumes per sec.

With the short lumbricalis fibers, a two-microelectrode method analogous to those described by Caputo, Bezanilla, and Horowicz (1984) and Lynch (1985) was used. Fiber length and diameter were measured employing a calibrated graticule in a dissecting microscope at 200 ×. The voltage recording electrode was filled with 3 M potassium chloride and the current injecting electrode with 2 M potassium citrate. The fibers were held at their resting potentials in high K^+ solutions (about −5 mV). From the holding potentials, test pulses of 600-msec duration were applied at 15-sec intervals, generating families of current signals. The voltage and current signals were sampled at 1 kHz using an analog-to-digital converter controlled by a computer. Data were stored on floppy disks for subsequent analysis.

Experiments on semitendinosus muscles were performed at room temperature (ca. 23°C) while those on lumbricalis muscles were performed at 15°C.

Results

Membrane Potential Measurements of the Effect of pH on Chloride and Nitrate Permeability

In view of the differences in published reports about whether pH alters the relative membrane permeabilities of anions in skeletal muscle, our first aim was to confirm that altering external pH between 7.2 and 5.5 changes the relative permeabilities of NO_3^- and Cl^- in *Rana pipiens* as it does in *Rana temporaria*.

For this purpose we measured the hyperpolarizations produced in muscle fibers when chloride or nitrate ions rapidly replace impermeable sulfate ions in the external solution.

Membrane potential changes occur in response to solution changes in the following way. At the steady resting potential in any given solution, the net membrane ionic current is zero. If an external solution is suddenly replaced by a solution containing a more permeant anion, the net membrane current will be negative and the membrane will hyperpolarize. With time, the net membrane current returns toward zero as the driving force for each permeant ion is altered by the changing membrane potential. The membrane potential comes to rest at a new steady value such that net ionic current is again zero. Membrane hyperpolarization is indicative of a change to an external solution containing a more permeant anion or a less permeant cation.

The muscle fibers were initially equilibrated in depolarizing isotonic sulfate solution (S1) to remove internal chloride ions and to inactivate the contractile apparatus. Since the magnitude of hyperpolarization required to obtain an equivalent amount of cation entry to balance a sudden increase of anion entry is much larger when Cs^+ than K^+ is the compensating cation, owing to the lower membrane conductance to Cs^+, external K^+ was replaced by Cs^+ (solution S2).

Figure 1 illustrates two experiments in which membrane potential changes in response to external ion changes were measured. Figure 1*A* compares the hyperpolarizations produced by chloride and nitrate at pH 7.2; Fig. 1*B* compares the hyperpolarizations at pH 5.5.

In Fig. 1*A*, at the start in 68 mM K_2SO_4, the membrane potential was −4.5 mV. In this solution the membrane transport number for K^+ is nearly one and the membrane behaves as a pure K^+ electrode; i.e., the membrane potential equals the K^+ equilibrium potential and K^+ influx equals K^+ efflux (Hodgkin & Horowicz, 1959). After about 2 min, external K^+ was replaced by Cs^+ and the membrane potential changed to −34 mV. This is expected since the membrane is less permeable to Cs^+ than K^+. After about another 2 min, the sulfate solution was replaced by isotonic $CsNO_3$ solution (the activity of Cs^+ ions remaining unchanged) and this drove the internal potential to −56 mV. After about another 1 min, NO_3^- ions were replaced by Cl^- ions and this drove the internal potential to a value of −78.5 mV. The clear implication of this is that the membrane is more permeable to Cl^- than to NO_3^- at pH 7.2 since equimolar Cl^- produced an additional inflow of negative charge compared to NO_3^- which necessitated hyperpolarization of the membrane to −78.5 mV. The sag in the potential after the peak is ascribable to accumulation of significant amounts of internal Cl^-.

Figure 1*B* gives the results of a similar experiment on another muscle fiber in which the anion hyperpolarizations were measured at pH 5.5. In this

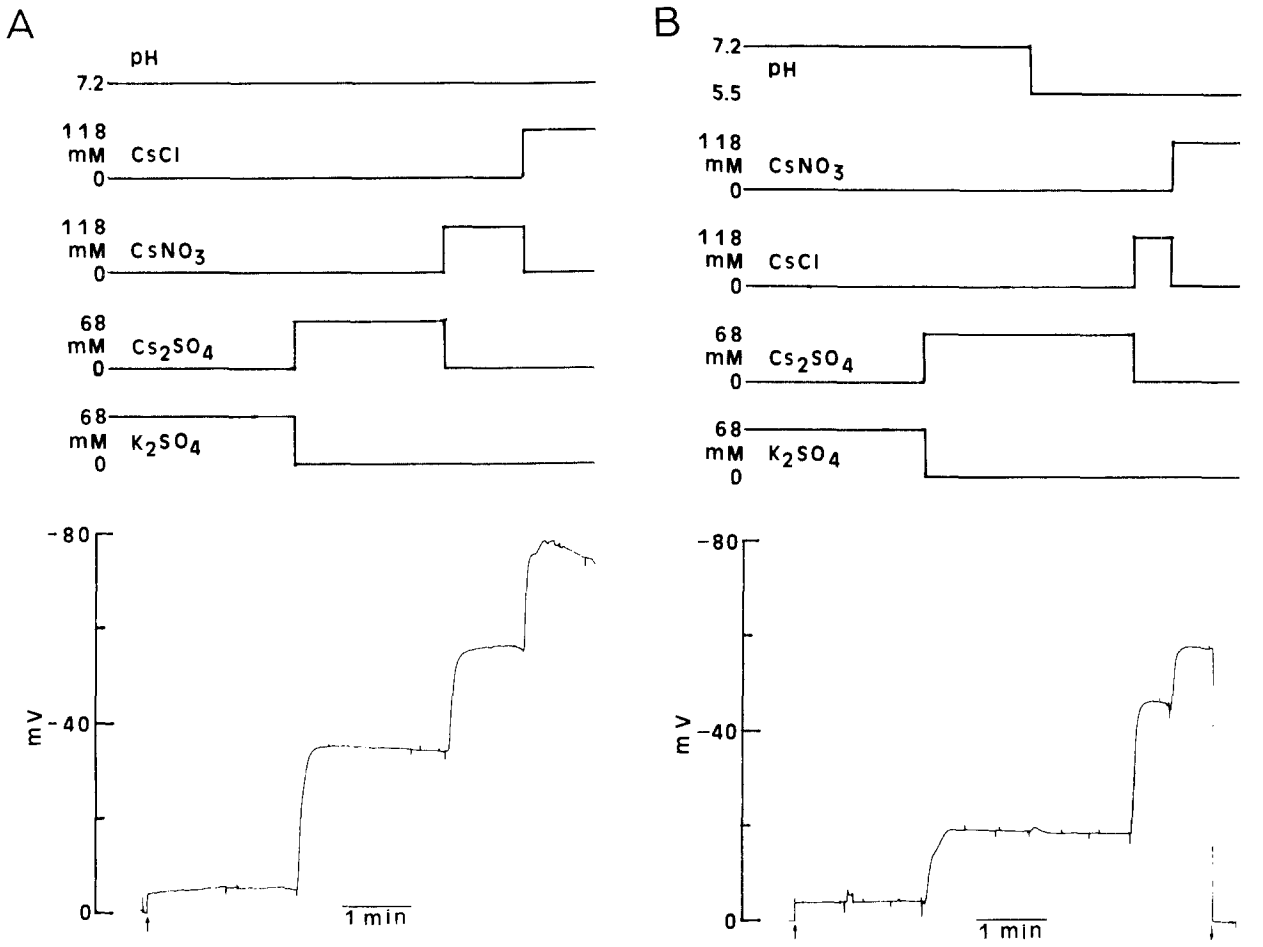

Fig. 1. Effect of external pH on the response of membrane potential to external solutions of different ionic composition. Short vertical spike-like deflections in membrane potential indicate the start (downward) and end (upward) of a flush with rapid flow of solution. Flushes were made both to change solutions and to determine adequacy of an earlier change and stability of recording electrodes. The small step change in record *B* during the period in K_2SO_4 solution was caused by a brief, adventitious change in grounding. Upward arrows indicate insertion of internal microelectrode; downward arrow indicates withdrawal. Composition of cesium containing solutions are given by S2, S4, and S5 in Table 1. (*A*) Comparison of hyperpolarizations produced by NO_3^- and Cl^- at pH 7.2 with external Cs^+ activity kept constant. Exp't ref. 11/24/02. (*B*) Comparison of hyperpolarizations produced by Cl^- and NO_3^- at pH 5.5 with external Cs^+ activity kept constant. Exp't ref. 12/11/07

case the initial membrane potential in the sulfate solution was −4 mV. When Cs^+ replaced K^+ the internal potential hyperpolarized to −19 mV and, apart from a small transient flush artifact, did not change when the external pH was lowered from 7.2 to 5.5. When isotonic CsCl at pH 5.5 replaced the sulfate solution the internal potential stabilized rapidly at −46 mV and when NO_3^- replaced Cl^- the membrane hyperpolarized even further to −58 mV. From this it is clear that at pH 5.5 the membrane is less permeable to Cl^- than to NO_3^-. This is the reverse order of the permeabilities at an external pH of 7.2. Thus, these findings using semitendinosus fibers from *Rana pipiens* confirm those of Hutter and Warner (1968) on *Rana temporaria*.

The question which can be asked at this stage is whether the membrane permeability to both Cl^- and NO_3^- ions change with pH or whether only Cl^- permeability changes. The results shown in Fig. 1 suggest that there is little difference in the membrane potential in isotonic $CsNO_3$ solution between pH 7.2 and 5.5 but substantial difference in the membrane potential in isotonic CsCl solution between these two pHs. Since there is some variation in the hyperpolarizations produced by a given anion between fibers we examined the effect of external pH on the hyperpolarization produced by a given anion in the same fiber.

Figure 2 presents two experiments of this type: one illustrating the effect of pH on Cl^- hyperpolarizations, the other on NO_3^- hyperpolarizations. In Fig. 2*A* the initial membrane potential in the standard sulfate solution at pH 7.2 was −12 mV which then changed to −38.5 mV by the end of the period

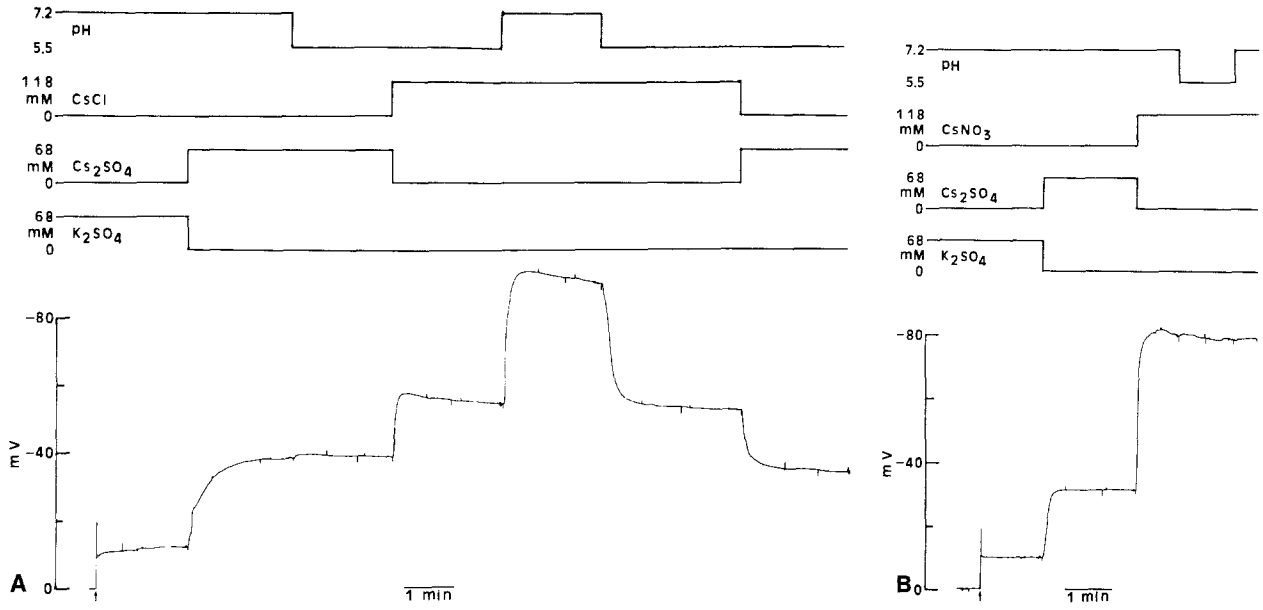

Fig. 2. Comparison of the effects of altering external pH between pH 5.5 and 7.2 on the hyperpolarizations produced by Cl^- (*A*) and NO_3^- (*B*). (*A*) Exp't ref. 11/23/01. (*B*) Exp't ref. 11/24/03. For further details see legend of Fig. 1 and text

when Cs^+ replaced K^+. When the pH was changed to 5.5 there was a small further hyperpolarization to −38.8 mV after an initial transient was over. On switching to CsCl at pH 5.5 the internal potential reached a peak value of −57.2 mV before it began to subside slowly. After a little over 2 min the external pH was raised to 7.2 and the internal potential peaked at −93.5 mV before it began to subside again. The potential changes were largely reversible when the external solutions were flushed past the fiber in reverse order. The generally slow subsidence of internal potential during the exposure to Cl^- solutions was probably due to slow entry of CsCl into the myoplasm. Two points are worth noting with regard to this experiment. The first point is that raising external pH from 5.5 to 7.2 substantially increased Cl^- permeability; the second is that at pH 5.5 the Cl^- permeability is not negligible. The second point was already clear from the results presented in Fig. 1*B*.

The lack of a pH effect on hyperpolarizations produced by NO_3^- is shown in Fig. 2*B*. The initial membrane potential in the standard sulfate solution at pH 7.2 was −10 mV which then went to −31.5 mV when Cs^+ replaced K^+. Keeping pH constant while switching to $CsNO_3$ solution drove the internal potential to a peak value of −82 mV after which it began to subside. The external pH was then lowered to 5.5 for a period lasting about 1.3 min, then returned to 7.2. There was no significant change in the membrane potential during this interval. The potential at pH 7.2 had declined to −80 mV just before the pH was lowered to 5.5 and continued to slowly decline to a value of −79 mV when the pH was returned to 7.2. At first the membrane potential at pH 7.2 did not decline further but then the fiber began to contract with an initial potential drop of about 2 mV and then the microelectrode came out of the fiber as the contraction continued.[1] Thus, over the pH range which produces a major change in the membrane permeability to Cl^- there is no measurable change in permeability to NO_3^-.

The next point to be examined is whether a dependence of NO_3^- permeability on external pH may in fact exist but is shifted in the alkaline direction. Figure 3 gives the results of two experiments testing the effects of pH 8 on NO_3^- hyperpolarizations. In Fig. 3*A* the usual $CsNO_3$ type of experiment was performed; the membrane potential in the starting sulfate solution was −8 mV, Cs^+ replacement for K^+ drove the internal potential to −28 mV, switching to $CsNO_3$ at pH 7.2 brings the internal potential to −61 mV and there is no further change on raising the external pH to 8. The potential recording is terminated when a contraction developed simultaneously with raising the external pH to 9 and the

[1] In general, the time available for membrane potential measurement in nitrate solutions was limited by the repriming time of the calcium release mechanism since the membrane potentials in these solutions was in the contracture producing range.

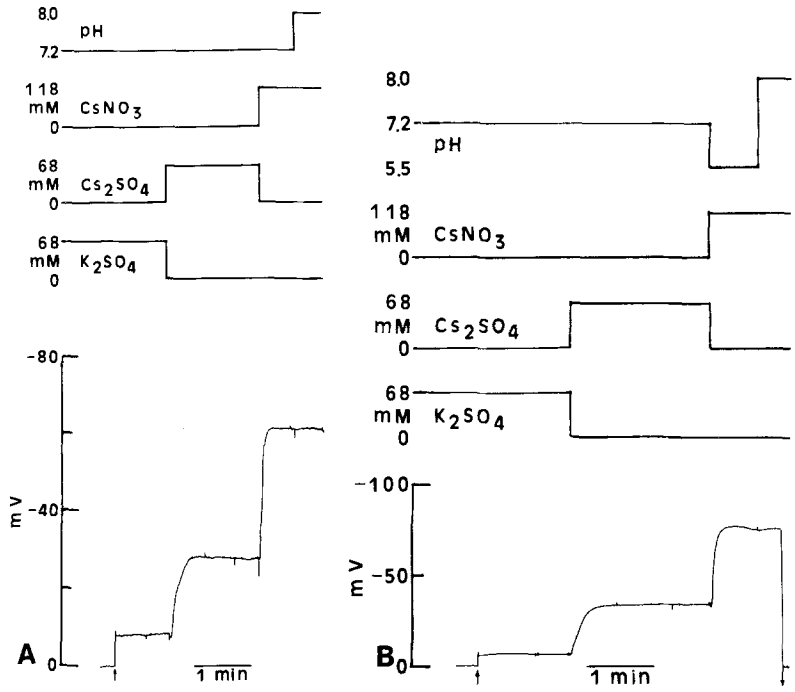

Fig. 3. Lack of effect of altering external pH between 7.2 and 8.0 (*A*) and between 5.5 and 8.0 (*B*) on the hyperpolarizations produced by NO_3^-. (*A*) Exp't ref. 12/11/01. (*B*) Exp't ref. 11/24/06. For further details see legend of Fig. 1 and text

microelectrode came out of the fiber. In Fig. 3*B* the initial potential in the equilibrating solution was −6.3 mV (note scale change), on replacing Cs^+ for K^+ the potential went to −33.8 mV, and on switching to $CsNO_3$ at pH 5.5 to −75.8 mV. The potential did not change appreciably when the pH was raised to 8.0 before the microelectrode came out with the start of a contracture.

In order to obtain potential recordings less vulnerable to contractures at pH 9.0 we did a few experiments in which Rb^+ solutions instead of Cs^+ solutions were used. In Rb^+ solutions hyperpolarizations are sufficiently diminished to reduce repriming of the calcium release mechanism. The results are summarized in Table 4. The first three rows show that there was no difference in the internal potential in $RbNO_3$ solutions between pH 7.2 and pH 9.0.

The conclusion we draw from the above results is that the NO_3^- permeability of frog skeletal muscle membranes is not altered by external pH over the range 5.5 to 9.0.

Estimates of Relative Permeabilities Based on Membrane Potential Measurements

In the initial equilibrating sulfate solution containing high K^+ concentrations the membrane potential in frog skeletal muscle is equal to the K^+ equilibrium potential which is given by the relation

$$V_K = \frac{RT}{F} \ln \frac{f_{K_o}[K^+]_o}{f_{K_i}[K^+]_i} \tag{1}$$

where R, T, F, $[K^+]_o$, and $[K^+]_i$ have the usual significance and f is the activity coefficient with the subscript identifying the ion and space. Although Na^+ is present and permeant, its contribution to the membrane potential can be neglected because its permeability and concentration relative to K^+ are low (Hodgkin & Horowicz, 1959). If we let r equal the ratio of the K^+ activity coefficient in the external space to that in the internal space and equate the measured membrane potential in the initial potassium plus sodium sulfate solution, V_0, to V_K then Eq. (1) can be transformed to give

$$r = \frac{[K^+]_i}{[K^+]_o} \cdot \exp(V_0 F/RT). \tag{2}$$

Neglecting the contribution of Na^+ ions when external K^+ is replaced by Cs^+ the membrane potential takes on a new value, V_1, which is assumed to be given by the constant field relation

$$V_1 = \frac{RT}{F} \ln \frac{P_{Cs} f_{Cs_o}[Cs^+]_o}{P_K f_{K_i}[K^+]_i} \tag{3}$$

where P_{Cs}, and P_K represent the membrane permeability to Cs^+ and K^+, respectively. If we assume that the activity coefficient for Cs^+ in the external

sulfate solution equals that of the K^+ in the external sulfate solution then Eq. (3) can be transformed to give

$$\frac{P_{Cs}}{P_K} = \frac{[K^+]_i}{r[Cs^+]_o} \cdot \exp(V_1 F/RT). \quad (4)$$

Equation (4) permits the calculation of the ratio of Cs^+ permeability to K^+ permeability from V_1 when the external solution is K^+-free and contains 136 mM Cs^+. The value of r is calculated from V_0 using Eq. (2). In all cases it is assumed that $[K^+]_i$ = 140 mM (*see* Venosa, 1979).

When a monovalent anion such as NO_3^- or Cl^- replaces sulfate at constant external Cs^+ activity the membrane potential takes on a new steady value, V_2, which is, by the constant field relation

$$V_2 = \frac{RT}{F} \ln \frac{P_{Cs} f_{Cs_o} [Cs^+]_o}{P_K f_{K_i} [K^+]_i + P_{Cl} f_{Cl_o} [Cl^-]_o} \quad (5)$$

where P_{Cl} and f_{Cl_o} are, respectively, the Cl^- permeability and Cl^- activity coefficient in the external solution. A similar equation applies when NO_3^- is the external permeant anion. On transforming Eq. (5) and again assuming that the activity coefficient for Cs^+ equals that of K^+ in the external sulfate solution one obtains the relation

$$\frac{P_{Cl} f_{Cl_o}}{P_K f_{K_i}} = \frac{(P_{Cs}/P_K) r[Cs^+]_o \exp(-V_2 F/RT) - [K^+]_i}{[Cl^-]_o}. \quad (6)$$

For convenience the following definitions for relative permeabilities are made

$$\frac{P_{Cl} f_{Cl_o}}{P_K f_{K_i}} = R_{Cl} \quad \text{and} \quad \frac{P_{NO_3} f_{NO_{3o}}}{P_K f_{K_i}} = R_{NO_3}. \quad (7)$$

With these definitions and the assumption that the Cl^- and NO_3^- external activity coefficients are equal for solutions of equal ionic strength, it follows that

$$R_{Cl}/R_{NO_3} = P_{Cl}/P_{NO_3}. \quad (8)$$

Furthermore, if one takes the ratio of R_{Cl} for two different pHs then this ratio equals the ratio of P_{Cl} for the two pHs. The same holds true for similar calculations based on NO_3^- measurements.

A summary of the membrane potential measurements in Cs^+ solutions and the calculated relative permeabilities based on the above equations is given in Tables 2 and 3. The first seven rows summarize the measurements and calculations in which NO_3^- hyperpolarizations were measured in single fibers at two pHs. It is clear from these results in which the fiber-to-fiber variation was obviated that there was no measurable change in NO_3^- permeability over the range of pHs between 5.5 and 8.0 (*see* columns 10 and 11 of Table 3). Table 4 summarizes the results and calculations of measurements made in Rb^+ solutions. The first three rows give the comparison of NO_3^- hyperpolarizations in single fibers at pH 7.2 and 9.0. In these experiments in Rb^+ solutions, there was no change in NO_3^- permeability between pH 7.2 and 9.0. A further basis for the conclusion that NO_3^- permeability does not vary with pH will be given below.

Taking all the data in Cs^+ solutions into account (Tables 2 and 3), the ratio of Cl^- permeability at pH 7.2, P_{Cl} (7.2), to Cl^- permeability at pH 5.5, P_{Cl} (5.5), is equal to 9.50/1.99 or 4.8. Using the average R_{NO_3} for all measurements in Cs^+ solutions between pH 5.5 and 8.0 (column 9, Table 3), then at pH 5.5 P_{NO_3}/P_{Cl} (5.5) = 4.07/1.99 = 2.05 and at pH 7.2 P_{NO_3}/P_{Cl} (7.2) = 4.07/9.50 = 0.43. The decrease in the relative permeability of NO_3^- with respect to Cl^- on raising external pH from 5.5 to 7.2 is entirely due to the nearly fivefold increase in Cl^- permeability produced by raising pH.

It is interesting to note that R_{NO_3} = 4.07 in external K^+-free, Cs^+ solution (column 9, Table 3) while R_{NO_3} = 0.52 in external K^+-free, Rb^+ solution (column 8, Table 4). If one assumes that the NO_3^- permeability is the same in both situations this implies that the K^+ permeability in the K^+-free, Rb^+ solution is 7.8 (4.07/0.52) times larger than it is in K^+-free, Cs^+ solution. This is very likely due to the stimulation of K^+ efflux produced by external Rb^+ in K^+-free solutions (Adrian, 1962; Spalding et al., 1982). Quantitatively this effect is much smaller with Cs^+ and at the concentrations used in this study Cs^+ slightly inhibits K^+ efflux (B. Spalding, P. Taber & P. Horowicz; *unpublished observations*).

Anion Currents in Voltage-Clamped Short Fibers

In addition to the experiments described above, we also measured currents during voltage-clamp pulses using short fibers of the lumbricalis muscle.

Fibers were first exposed to solutions that contained 60 mM of either KNO_3 or KCl plus 75 mM K_2SO_4 and 20 mM Na_2SO_4 as the major salts (L3 or L5). When equilibrated in these solutions, fibers recovered the volume they had in Ringer's solution and had a resting membrane potential of about −5 mV. The fibers were impaled with a voltage recording and a current passing microelectrode and the holding potential was set at the resting potential in the high K^+ solutions. Current responses to a

Table 2. Internal potential of fibers in various Cs^+ solutions (in mV)

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
No.	pH Solution Exp't	7.2 K_2SO_4 V_i	7.2 Cs_2SO_4 V_i	7.2 CsCl V_i	5.5 CsCl V_i	7.2 $CsNO_3$ V_i	5.5 $CsNO_3$ V_i	8.0 $CsNO_3$ V_i	$CsNO_3$ avg. (V_i)
1	11/24/03	−10.0	−31.5			−80.3	−79.0		−79.7
2	11/24/06	−6.3	−33.8				−75.8	−75.3	−75.6
3	12/11/02	−6.0	−31.5			−69.0	−69.0		−69.0
4	01/08/05	−15.0	−37.0			−56.0	−56.5		−56.3
5	11/24/04	−5.0	−32.5			−75.8		−75.3	−75.6
6	11/24/05	−5.0	−30.0			−83.8		−84.3	−84.1
7	12/11/01	−8.0	−28.0			−61.0		−61.0	−61.0
8	12/04/01	−5.0	−28.0			−48.5			−48.5
9	12/04/02	−5.5	−31.0			−64.5			−64.5
10	12/11/03	−5.0	−23.5			−59.0			−59.0
11	01/08/01	−7.5	−41.0				−84.5		−84.5
12	01/11/03	−6.0	−25.0				−69.0		−69.0
13	11/24/01	−6.0	−38.0		−55.0		−73.0		−73.0
14	12/11/07	−4.0	−19.0		−46.0		−58.0		−58.0
15	01/11/02	−7.0	−30.0		−42.5		−67.0		−67.0
16	01/08/02	−5.0	−33.0		−49.0		−65.0		−65.0
17	11/24/02	−4.5	−34.0	−78.5		−56.0			−56.0
18	12/11/04	−13.0	−33.5	−100.0		−71.0			−71.0
19	11/23/01	−12.0	−38.7	−93.5	−57.2				
20	01/08/04	−3.0	−30.0	−87.0	−59.5				
21	01/11/01	−6.5	−25.5	−88.0	−67.5				
22	11/23/02	−3.5	−32.5	−86.0					
23	12/11/05	−6.0	−25.0	−81.0					
24	12/11/06	−4.0	−21.0	−67.5					
Avg.		−6.6	−30.5	−85.2	−53.8	−65.9	−69.7	−74.0	−67.6
Std.		3.0	5.4	9.2	8.0	10.6	8.3	8.3	9.8
n		24	24	8	7	11	10	4	18
SEM		0.6	1.1	3.2	3.0	3.2	2.6	4.2	2.3

Column (10) gives the average of columns (7), (8) and (9). Solution designations with respect to Table 1 are as follows: K_2SO_4 denotes S1, Cs_2SO_4 denotes S2, CsCl denotes S4, and $CsNO_3$ denotes S5.

series of voltage pulses were then recorded. With the microelectrodes remaining in the fiber, the external solution was changed and, after a delay of a few minutes to allow for equilibration of the extracellular space, another set of voltage clamp records were taken. In many instances a second solution change could be made and a voltage clamp series recorded before the fiber became excessively leaky.

Figure 4 shows the current responses in a fiber to three voltage pulses when TEA^+ replaced external K^+ and the permeant anion was NO_3^-. For each voltage pulse, two current traces are shown; one when the external pH was 5.0 and the other when the pH was 7.5. Each trace for pH 7.5 is the average of two runs; one preceding and the other following the run at pH 5.0. Since SO_4^{2-} is impermeant and frog twitch fiber membranes are relatively impermeable to both TEA^+ and Na^+, most of the inward current was carried by NO_3^- exiting from the fibers. It is clear that the NO_3^- currents were nearly the same at the two pHs and had very little time dependence. Similar results were obtained when the currents in NO_3^- solutions at pH 5.0 were compared with those at pH 9.0.

When the permeant anion is Cl^-, the current responses to hyperpolarizing pulses are very different. Figure 5 shows the results of an experiment on a fiber which had been initially equilibrated in solutions with added KCl rather than KNO_3. As in Fig. 4, the traces for pH 7.5 are the average of two runs bracketing the run at pH 5.0. At pH 5.0, after the capacitive surge, the currents were initially small and grew with time at rates that depended on membrane potential. At pH 7.5 the currents were initially large and fell with time at membrane potential dependent rates. The currents had not reached a steady level at the end of the 600-msec pulses. This kind of Cl^- current behavior has been extensively studied by several investigators in other muscle preparations (Warner, 1972; Loo et al., 1980; Vaughan et al., 1980), and our results demonstrate that it occurs in lumbricalis fibers as well.

Table 3. Calculated permeability ratios from internal potentials

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
pH	7.2	7.2	7.2	5.5	7.2	5.5	8.0			
No.	r	P_{Cs}/P_K	R_{Cl}	R_{Cl}	R_{NO_3}	R_{NO_3}	R_{NO_3}	R_{NO_3} Avg.	(6)/(7) or (8)/(7)	(8)/(6)
1	0.70	0.43			6.84	6.44		6.64	1.06	
2	0.80	0.34				4.96	4.85	4.91	0.98	
3	0.81	0.37			3.97	3.97		3.97	1.00	
4	0.57	0.42			1.31	1.36		1.34	0.96	
5	0.85	0.34			5.29		5.16	5.22		0.98
6	0.85	0.38			8.58		8.78	8.68		1.02
7	0.75	0.46			3.14		3.14	3.14		1.00
8	0.85	0.41			1.46			1.46		
9	0.83	0.37			3.22			3.22		
10	0.85	0.48			3.58			3.58		
11	0.77	0.27				5.34		5.34		
12	0.81	0.48				5.47		5.47		
13	0.81	0.28		1.12		3.49		3.49		
14	0.88	0.56		2.23		4.28		4.28		
15	0.78	0.41		0.75		3.87		3.87		
16	0.85	0.33		1.03		2.97		2.97		
17	0.86	0.32	5.60		1.62			1.62		
18	0.62	0.45	14.88		3.97			3.97		
19	0.64	0.35	8.97	1.26						
20	0.92	0.35	9.89	2.58						
21	0.80	0.48	12.55	4.97						
22	0.90	0.32	8.47							
23	0.81	0.48	9.46							
24	0.88	0.51	6.15							
Avg.	0.80	0.40	9.50	1.99	3.91	4.22	5.48	4.07	1.00	1.00
Std.	0.09	0.07	2.88	1.36	2.16	1.37	2.05	2.78	0.04	0.02
SEM	0.02	0.02	1.02	0.52	0.65	0.43	1.03	0.42	0.02	0.01

Row No. is the same as in Table 2. r is the ratio of the K^+ activity coefficient in the external solution to that in the myoplasm. Column (9) gives the average P_{NO_3}/P_K of columns (6), (7) and (8). Column (10) gives the ratio of value in column (6) or column (8) to value in column (7). Column (11) gives the ratio of value in column (8) to value in column (6). All relative permeabilities are based on constant field equations; for details *see* text Eq. (4), (6), and (7).

Table 4. Internal potential (mV) and permeability ratio in Rb^+ solutions

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
pH	7.2	7.2	7.2	9.0		7.2	7.2	9.0	
Solution	K_2SO_4	Rb_2SO_4	$RbNO_3$	$RbNO_3$	r	P_{Rb}/P_K	R_{NO_3}	R_{NO_3}	(9)/(8)
Exp't	V_i	V_i	V_i	V_i					
12/18/01	−2.0	−9.5	−20.0	−20.0	0.95	0.75	0.60	0.60	1.00
12/18/02	−2.0	−13.5	−22.5	−22.5	0.95	0.64	0.50	0.50	1.00
12/22/01	−3.0	−21.0	−30.5	−30.5	0.92	0.49	0.54	0.54	1.00
12/18/03	−5.0	−8.5	−18.5		0.85	0.87	0.57		
12/18/04	−8.0	−18.8	−27.7		0.75	0.66	0.50		
12/18/05	−5.5	−17.5	−26.0		0.83	0.63	0.47		
12/18/06	−8.0	−23.0	−31.0		0.75	0.56	0.44		
12/22/02	−4.0	−17.0			0.88	0.60			
12/01/04	−7.0	−16.0			0.78	0.70			
12/01/05	−6.0	−19.8			0.81	0.58			
Avg.	−5.1	−16.5	−25.2	−24.3	0.85	0.65	0.52	0.55	1.00
Std.	2.2	4.5	4.6	4.5	0.07	0.10	0.05	0.04	0.00
n	10	10	7	3	10	10	7	3	3
SEM	0.7	1.4	1.7	2.6	0.02	0.03	0.02	0.03	0.00

Column (10) gives the ratio of the value in column (9) to value in column (8). Solution designations with respect to Table 1 are as follows: K_2SO_4 denotes S1, Rb_2SO_4 denotes S3, and $RbNO_3$ denotes S6. All permeability ratios are based on constant field equations; for details *see* text Eq. (4), (6), and (7).

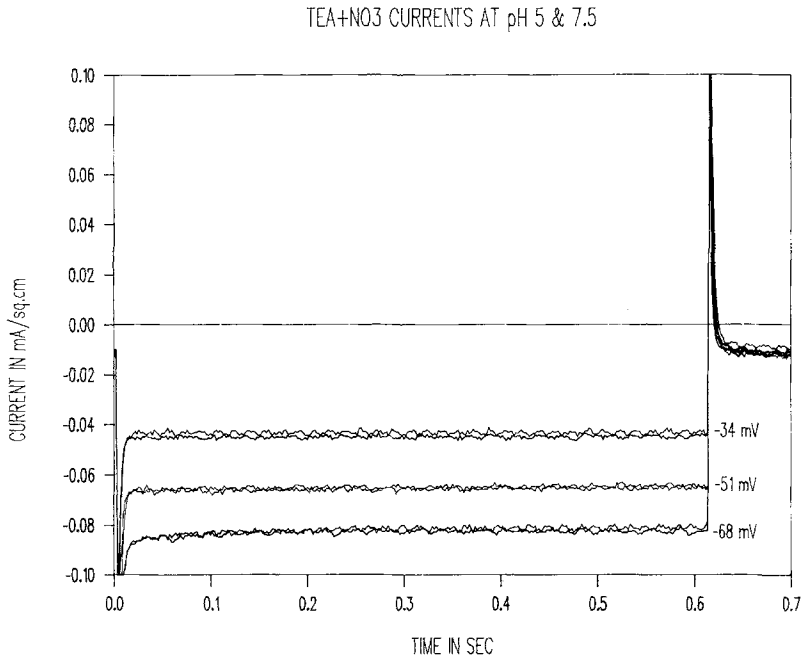

Fig. 4. Current responses to three voltage pulses in a fiber initially equilibrated in a solution containing 60 mM KNO_3 and 75 mM K_2SO_4 (L5 in Table 1) and then exposed to a solution in which all the external K^+ was replaced by TEA^+ (L6 in Table 1), first at pH 5.0 and then at 7.5. Holding potential −5 mV; fiber diameter 47 μm; fiber length 1.13 mm; Exp't ref. 09/02/F2. For details see text

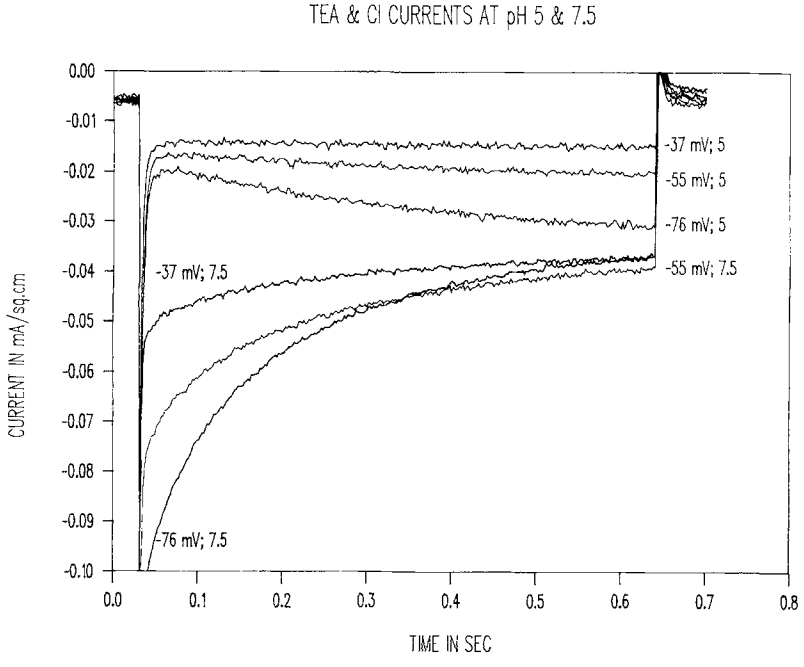

Fig. 5. Current responses to three voltage pulses in a fiber initially equilibrated in a solution containing 60 mM KCl and 75 mM K_2SO_4 (L3 in Table 1) and then exposed to a solution in which all the external K^+ was replaced by TEA^+ (L4 in Table 1) at pH 5.0 and at 7.5. Holding potential −5 mV; fiber diameter 66 μm; fiber length 1.32 mm; Exp't ref. 08/29/F2. For details see text

The new and significant point of this comparison is that the behavior of NO_3^- currents is quite unlike that of Cl^- currents. NO_3^- currents are relatively constant in time and independent of pH.

In order to obtain an estimate of the net current carried by NO_3^-, the current *vs.* voltage relation was measured in several fibers in a solution containing TEA_2SO_4 and Na_2SO_4 but no nitrate. The currents obtained in response to voltage pulses were relatively small and constant. Figure 6 gives the average inward currents from fibers in the absence and presence of NO_3^-. For both situations, the currents at the beginning of the pulses (after the capacitive surges) and at the end of 600 msec are plotted. The line is drawn according to a linear fit to the initial currents in the sulfate solutions with a slope of 0.29 mS/cm^2 and a SE (*not shown*) of 0.05 mS/cm^2. It is clear that in the presence of nitrate about 70% of the inward current is carried by NO_3^-.

A similar comparison of the average initial cur-

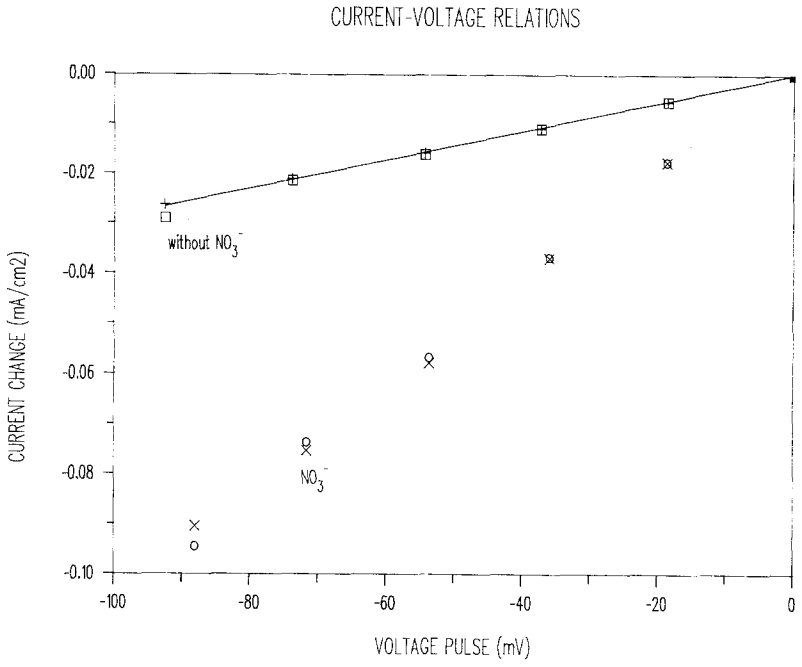

Fig. 6. Average current-voltage relations for fibers exposed to solutions in the absence and presence of 60 mM NO_3^- when all external K^+ was replaced by TEA^+. For the fibers exposed to nitrate-free solution (L2 of Table 1), the average currents at the beginning of the voltage pulses are given by plus symbols and at the end of the 600-msec pulses by open squares. The plotted line has been fitted to the currents at the beginning of the voltage pulses and has a slope of 0.29 mS/cm² and a SE of 0.05 mS/cm². For the set of fibers exposed to nitrate-containing solution (L6 of Table 1), the average currents at the beginning of the voltage pulses are given by cross symbols and at the end of the 600-msec pulses by open circles. The slope of the line (*not shown*) fitted to the currents at the beginning of the pulses in the NO_3^- solutions is 1.03 mS/cm² with a SE of 0.02 mS/cm². For details see text

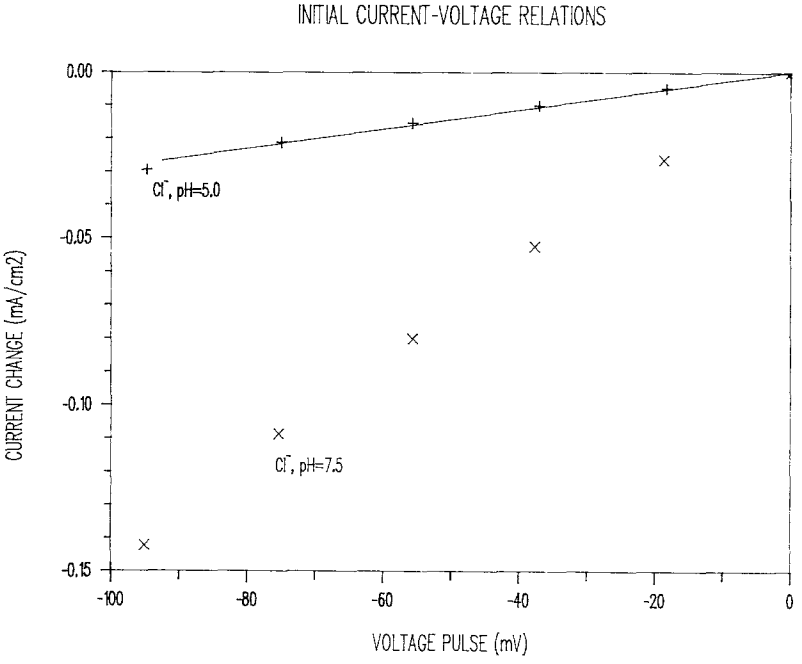

Fig. 7. Average initial current-voltage relations for fibers exposed to solutions containing 60 mM Cl^- with all external K^+ replaced by TEA^+ at pH 5.0 and 7.5 (solution L4 of Table 1). See Fig. 5 for a typical experiment. The line plotted in this figure is the same one plotted in Fig. 6 for average currents obtained with solution L2 of Table 1. The plus symbols give the averages at pH 5.0 with solution L4; the crosses give the averages at pH 7.5. For details see text

rent *vs.* voltage relations for several fibers in Cl^--containing solutions with TEA^+ replacing K^+ at pH 5.0 and 7.5 is given in Fig. 7. The plus symbols give the initial current, after the capacitive surges, when the external pH was 5.0 before the time-dependent component developed. The line is the same as that drawn in fig. 6 for current responses in the solution containing only TEA_2SO_4 and Na_2SO_4. It is clear that at pH 5.0 there is very little measurable initial current ascribable to Cl^- when the holding potential is at −5 mV; with time Cl^- currents develop at more negative internal potentials. At pH 7.5 the initial currents are significantly larger and then the currents decline with time. About 80% of the initial currents in Cl^--containing solutions at pH 7.5 is carried by Cl^- when TEA^+ is the major external cation and the holding potential is −5 mV.

Calculated currents using constant field equations were fit to the net NO_3^- currents and the net initial Cl^- currents at pH 7.5 and 9.0. The compari-

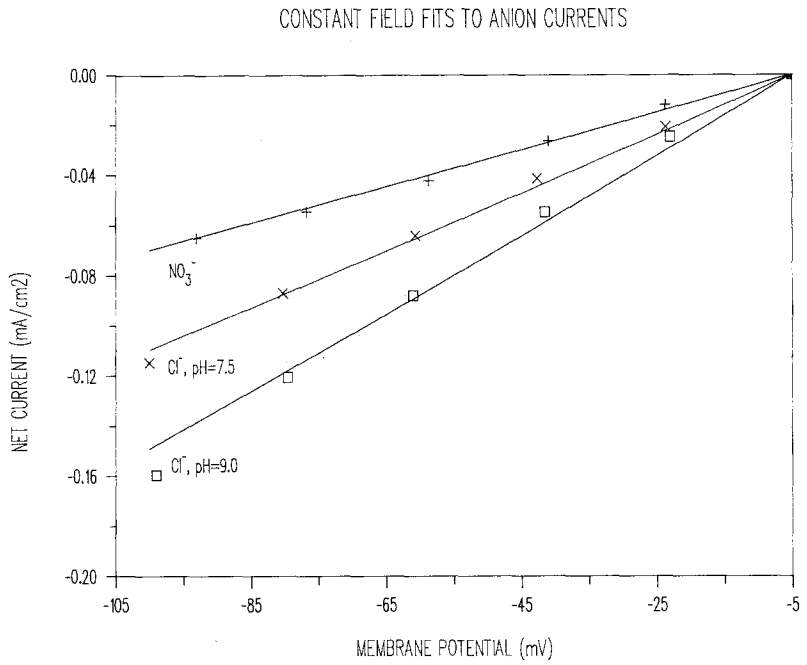

Fig. 8. Fits of constant field equation to net NO_3^- and net initial Cl^- current-voltage measurements. The plotted points are the difference between the total currents in NO_3^- solutions or the total initial currents in Cl^- solutions at pH 7.5 or 9.0 and the currents calculated from the linear fit to the average currents obtained with solution L2 of Table 1 (solid line in Figs. 6 and 7). The curves are calculated from the equation

$$I_A = \frac{P_A V_i F^2([A^-]_o - [A^-]_i \exp(-V_i F/RT))}{RT(1 - \exp(-V_i F/RT))}$$

where A^- represents either NO_3^- or Cl^-, V_i the internal potential and the other symbols have their customary significance. $[A^-]_i$ is calculated on the assumption that it is at electrochemical equilibrium with $[A^-]_o$ at the holding potential of -5 mV which was set at the average resting potential for fibers equilibrated in solutions L3 and L5 of Table 1. For the NO_3^- currents, the curve assumes $P_{NO_3} = 3.7 \cdot 10^{-6}$ cm/sec. For the Cl^- currents at pH 7.5, the curve assumes $P_{Cl} = 5.8 \cdot 10^{-6}$ cm/sec and at pH 9.0, $P_{Cl} = 7.9 \cdot 10^{-6}$ cm/sec

son between the calculated and measured currents are shown in Fig. 8. The value used for P_{NO_3} was $3.7 \cdot 10^{-6}$ cm/sec, for P_{Cl} (pH = 7.5) was $5.8 \cdot 10^{-6}$ cm/sec and for P_{Cl} (pH = 9.0) was $7.9 \cdot 10^{-6}$ cm/sec. Overall the calculated currents approximate fairly well the measured currents over the range of membrane potentials shown.

Discussion

The main new finding of these studies is that skeletal muscle membranes in *Rana pipiens* have a NO_3^- permeability that is independent of external pH, membrane potential and time. By contrast, their Cl^- permeability depends on all three variables. These findings suggest that NO_3^- and Cl^- move through separate and distinct channels. The fact that NO_3^- currents do not exhibit any of the pH and time dependence that Cl^- currents do strongly indicates that NO_3^- ions do not measurably move through the pH-, voltage-dependent Cl^- channels. The observations do not imply, however, that NO_3^- cannot inhibit these Cl^- channels. There is clear evidence that NO_3^- reduces Cl^- efflux at physiological pHs (Harris, 1958; Adrian, 1961). This inhibition occurs, therefore, without NO_3^- penetrating through the Cl^- channel.

It is not clear what physiological function the NO_3^- passing channels have. The fact that there is no measurable initial Cl^- current at pH 5.0 when the membrane is hyperpolarized from a holding potential of -5 mV while there is a substantial initial NO_3^- current under these conditions indicates that very little Cl^- passes through these channels. The subsequent increase in Cl^- current at pH 5.0 during the hyperpolarizing pulses is presumably due to the 'activation' of the voltage-dependent Cl^- channels. Apart from their very low permeability to Cl^-, little can be said about the channels that let NO_3^- through.

An important consequence is that the measured ratio of P_{Cl}/P_{NO_3} based on membrane potential changes to ionic changes made on intact skeletal

muscle fibers is not a measure of the selectivity of a single anion channel but rather is a measure of the relative amounts of different channel types. Another consequence is that there is now no indisputable evidence for the notion that the pH effect on the Cl^- channel is due to a change in the selectivity mechanism of the chloride channel. The macroscopic change in the P_{Cl}/P_{NO_3} ratio when external pH is altered occurs because the channel letting the Cl^- through is pH dependent but the channel letting NO_3^- through is not.

Measurements of the P_{NO_3}/P_{Cl} ratio of single chloride channels using either patch-clamp or bilayer-incorporation methods yield a wide range of values, depending on the cell type. P_{NO_3}/P_{Cl} for chloride channels is 2.35 in rat hippocampal neurons (Franciolini & Nonner, 1987), 0.9 in cultured pulmonary alveolar (type II) cells (Schneider et al., 1985), and <0.04 in *Torpedo* electroplax (Miller & White, 1980). With regard to selectivity for NO_3^-, therefore, the Cl^- channel in frog skeletal muscle seems most analogous to the channel isolated from *Torpedo* electroplax membranes.

The absence of measurable initial Cl^- currents at pH 5.0 in response to hyperpolarizing voltage pulses from a holding potential of −5 mV also indicates that the pH-, voltage-dependent Cl^- channels are largely closed at pH 5.0 at an internal potential of −5 mV. The reason one obtains substantial hyperpolarizations at pH 5.0 when Cl^- replaces SO_4^{2-} at constant Cs^+ activity is that the initial membrane potential in Cs_2SO_4 solution is about −30 mV and at this potential there are probably some open Cl^- in the steady state at pH 5.0. Furthermore, additional channels open as the internal potential becomes more negative; at the final membrane potential of about −54 mV a substantial number of Cl^- channels have been 'activated' in the steady state at an external pH of 5.0 (*see* Fig. 5). One final comment on this point is that even for a holding potential of −5 mV at pH 5.0 there may in fact be a few open Cl^- channels since the SE of membrane conductance in TEA_2SO_4 plus Na_2SO_4 solutions is 0.05 mS/cm^2 and this limits the precision with which net initial currents can be ascribed to Cl^- (or NO_3^- for that matter) in response to voltage-clamp pulses.

Previously published estimates of the average P_{Cl} at pH 7.2 in frog skeletal muscle fibers range from 0.9 to 4.0 · 10^{-6} cm/sec, with individual fibers having values as high as 6.6 · 10^{-6} cm/sec (Hodgkin & Horowicz, 1959; Adrian & Freygang, 1962; Harris, 1965). The value obtained above for P_{Cl} at pH 7.5 of 5.8 · 10^{-6} cm/sec using the net initial Cl^- currents in response to voltage pulses is at the upper end of these published estimates; the value of P_{Cl} estimated at pH 9.0 (7.9 · 10^{-6} cm/sec) is higher than the published estimates. In this regard, it is important to keep in mind that for hyperpolarizing pulses at pH 7.5 and 9.0 Cl^- currents 'inactivate' and therefore the steady-state Cl^- currents are smaller than initial currents for these membrane potentials (Warner, 1972; Vaughan et al., 1980; *see also* Fig. 5).

In general, as was first noted by Hutter and Warner (1972), steady-state Cl^- current *vs.* voltage curves at alkaline pHs cannot be fit by constant field equations. It is of significance, therefore, that the initial Cl^- current *vs.* voltage relations at alkaline pHs are well fit by the constant field equation.

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