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Frog Striated Muscle Is Permeable to Hydroxide and Buffer Anions

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Abstract. Hydroxide, bicarbonate and buffer anion permeabilities in semitendinosus muscle fibers of *Rana pipiens* were measured. In all experiments, the fibers were initially equilibrated in isotonic, high K_2SO_4 solutions at $pH_o = 7.2$ buffered with phosphate. Two different methods were used to estimate permeabilities: (i) membrane potential changes were recorded in response to changes in external ion concentrations, and (ii) intracellular pH changes were recorded in response to changes in external concentrations of ions that alter intracellular pH. Constant field equations were used to calculate relative or absolute permeabilities.

In the first method, to increase the size of the membrane potential change produced by a sudden change in anion entry, external K^+ was replaced by Cs^+ prior to changes of the anion under study. At constant external Cs^+ activity, a hyperpolarization results from increasing external pH from 7.2 to 10.0 or higher, using either CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) or CHES (2-[*N*-cyclohexylamino]-ethanesulfonic acid) as buffer. For each buffer, the protonated form is a zwitterion of zero net charge and the nonprotonated form is an anion. Using reported values of H^+ permeability, calculations show that the reduction in $[H^+]_o$ cannot account for the hyperpolarizations produced by alkaline solutions. Membrane hyperpolarization increases with increasing total external buffer concentration at constant external pH, and with increasing external pH at constant external buffer anion concentration. Taken together, these observations indicate that both OH^- and buffer anions permeate the surface membrane. The following relative permeabilities were obtained at $pH_o = 10.0 \pm 0.3$: $(P_{OH}/P_K) = 890 \pm 150$, $(P_{CAPS}/P_K) = 12 \pm 2$, $(P_{CHES}/P_K) = 5.3 \pm 0.9$, and $(P_{NO_3}/P_K) = 4.7 \pm 0.5$. P_{NO_3}/P_K was independent of pH_o up to 10.75. At $pH_o = 9.6$, $(P_{HCO_3}/P_K) = 0.49 \pm 0.03$; at $pH_o = 8.9$, $(P_{Cl}/P_K) = 18 \pm 2$ and at $pH_o = 7.1$, $(P_{HEPES}/P_K) = 20 \pm 2$.

In the second method, on increasing external pH from 7.2 to 10.0, using 2.5 mM CAPS (total buffer concentration), the internal pH increases linearly with time over the next 10 min. This alkalinization is due to the entry of OH^- and the absorption of internal H^+ by entering $CAPS^-$ anion. The rate of $CAPS^-$ entry was determined in experiments in which the external CAPS concentration was increased at constant external pH. Such increases invariably produced an increase in the rate of internal alkalinization, which was reversed when the CAPS concentration was reduced to its initial value. From the internal buffer power, the diameter of the fiber under study and the rates of change of internal pH, the absolute permeability for both OH^- and $CAPS^-$ were calculated. At external pH = 10.0, the average (± SEM) permeabilities were: $P_{OH} = 1.68 \pm 0.19 \times 10^{-4}$ cm/sec and $P_{CAPS} = 2.10 \pm 0.74 \times 10^{-6}$ cm/sec.

We conclude that OH^- is about 50 times more permeable than Cl^- at alkaline pH and that the anionic forms of commonly used buffers have significant permeabilities.

Key words: Skeletal muscle — Muscle membrane — Internal pH — Hydroxide permeability — Buffer anion permeability — Bicarbonate permeability

Introduction

Under normal resting conditions, Cl^- and K^+ movements account for almost all of the membrane conduc-

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tance in frog skeletal muscle (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960). At rest, the main cation channel that is open is the K^+ inward rectifier (Katz, 1949), which is unaffected by extracellular pH (pH_o) in the range between 5.0 and 9.8 (Hutter & Warner, 1967*a*). Chloride conductance, on the other hand, increases with external pH over a range centered at about $pH_o = 7.0$ (Brooks & Hutter, 1962; Hutter & Warner, 1967*a*). Recent studies have shown that there are at least two anion channels in frog skeletal muscle. One, a pH_o- and voltage-dependent channel, allows passage of Cl^- but not NO_3^-, while the other, a pH_o- and voltage-independent channel, allows passage of NO_3^- but not Cl^- (Kotsias & Horowicz, 1990).

Estimates of H^+ permeability (P_H) and HCO_3^- permeability (P_{HCO_3}) in frog skeletal muscle have been reported in the literature mainly as a part of studies concerned with intracellular pH (pH_i) regulation. On the basis of changes in transmembrane voltage and resistance produced by extracellular changes in H^+ and/or HCO_3^- concentrations, Woodbury (1971) concluded that $P_H/P_{Cl} \approx 500$ and $P_{HCO_3}/P_{Cl} \approx 0.1$. Izutsu (1972), measuring the rate of intracellular acidification when external HCO_3^- was reduced at a constant partial pressure of CO_2, concluded that $P_H \approx 10^{-3}$ cm/sec. Abercrombie, Putnam and Roos (1983), on the basis of the slow component of intracellular acidification during exposure to CO_2 in Na^+-free Ringer fluid, obtained a value of $P_{HCO_3} \approx 7.4 \cdot 10^{-8}$ cm/sec.

Our primary aim in this study was to measure the OH^- permeability, since it had not been measured previously. For comparison, bicarbonate and nitrate permeability were also determined. In the course of our experiments, we discovered that some frequently used buffers, which are usually assumed to have negligible permeabilities, in fact have measurable and not insubstantial permeabilities in their deprotonated form. A preliminary report of our results has been presented at a meeting of the Biophysical Society (Venosa, Kotsias & Horowicz, 1993).

Materials and Methods

Muscle Preparations and Solutions

In all experiments, small bundles containing from 5 to 15 fibers of the semitendinosus muscle were isolated from *R. pipiens*. Each bundle was mounted in a recording cell, similar to that used by Hodgkin and Horowicz (1959), which permitted rapid change of extracellular solutions.

Table 1 gives the final concentrations of dissolved salts in the solutions used in these experiments. Reference to specific solutions is given in the text and figures. The pH of the solutions applied to the fibers was adjusted to the desired value with a calibrated pH meter just prior to use. Since the ionic strength of the sulfate solutions is higher than that of the bicarbonate, chloride or nitrate solutions, the activity coefficient of monovalent cations in sulfate solutions is only about 0.87 times that in the customary Ringer solution (Hodgkin & Horowicz, 1959). Thus, a monovalent cation concentration of 136 mM in the sulfate solutions is estimated to have the same activity as 118 mM in the bicarbonate solution ($0.87 \times 136 = 118$).

Measurement of Membrane Potential and Internal pH

Some general remarks may be helpful to begin with. The goal of this study was to measure the permeabilities of hydroxide and other anions. To attain this aim, two different methods were used. In the first, using standard KCl-filled microelectrodes, membrane potential changes were recorded in response to changes in external ion concentrations. In the second, using both pH-sensitive and KCl-filled microelectrodes, intracellular pH changes were recorded in response to changes in external concentration of ions that alter intracellular pH, which in our studies included OH^- and buffer ions. Constant field equations were used to calculate relative or absolute permeabilities. The details of the various calculations are given in the appendices.

With both methods, isolated bundles from the semitendinosus muscle were initially equilibrated in depolarizing isotonic sulfate solution (external pH = 7.2) to remove internal chloride ions and to inactivate the contractile apparatus. In the first method, to increase the membrane potential change produced by a sudden change in anion entry, external K^+ was replaced by Cs^+ prior to changes in the anion under study. In the absence of external K^+ and the presence of external Cs^+ the membrane has a much lower conductance than when extracellular K^+ is present. Potential changes produced by briefly increasing the concentrations of NO_3^- or Cl^- were used as a comparison reference. In the second method, which measured internal pH changes, external K^+ was kept constant throughout without introducing Cs^+ when other ion concentrations were altered.

At the start of these studies it was clear that, even if OH^- permeability is two orders of magnitude greater than the normal resting K^+ or Cl^- permeability, relatively small changes in external pH would not produce measurable effects with either method. Consequently, external pH had to be changed by more than 2 pH units. Thus, buffers with alkaline pK_a were used: either CAPS (3-[cyclohexylamino]-1-propanesulfonic acid; $pK_a = 10.4$) or CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid; $pK_a = 9.3$). These buffers were chosen because of their reputed negligible permeabilities and their alkaline pK_a. In each case, the protonated form is a zwitterion of zero net charge and the nonprotonated form is an anion. As will be seen, the nonprotonated buffer anions do have measurable permeabilities that have to be taken into account when calculating the permeabilities of other ions when external pH is changed using these buffers. In experiments done near physiological pH_o and at acid pH_o, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; $pK_a = 7.5$) or MES (2-[*N*-morpholino]ethanesulfonic acid; $pK_a = 6.15$) were used.

Microelectrodes used for measuring internal pH (pH_i) were pulled from borosilicate glass capillaries 1.2 mm in diameter (WPI, Kwik-Fil) and were similar, at this stage, to those used for membrane potential measurements. Next, we followed, with slight variations, the procedure used by Peracchia (1990). Briefly, the microelectrodes were put in a metal can and baked in an oven at 260°C for at least 2 hr. They were silanized at that temperature by exposure to vapors of dimethyltrimethylsilanamine (Fluka Chemical, Ronkonoma, NY) by injecting 20 μl of the silanizer through a small hole in the metal can. After 2 min, the can was removed and the microelectrodes were baked for another 20 min. They were then stored in a dessicator for

Table 1. Composition of solutions (in mM)

Ref.	Na_2SO_4	$CaSO_4$	K_2SO_4	Cs_2SO_4	$CsHCO_3$	$NaNO_3$	NaCl	Na_2HPO_4	NaH_2PO_4	HEPES	CHES	CAPS
S1	27	8	68					1.08	0.43			
S2	27	8		68				1.08	0.43			
S3	27	8		68						2.5 to 10		
S4	27	2		68							2.5 to 10	
S5	27	2		68								2.5 to 10
S6		1			118							
S7		1		68		40.5					2.5	
S8		2		68			40.5			2.5		

Buffer abbreviations: CAPS 3-(cyclohexylamino)-1-propanesulfonic acid; $pK_a = 10.4$. CHES 2-(*N*-cyclohexylamino)ethanesulfonic acid; $pK_a = 9.3$. HEPES *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $pK_a = 7.5$. MES 2-(*N*-morpholino)ethanesulfonic acid; $pK_a = 6.15$.

periods of up to one week. On the day of the experiment, they were backfilled by pressure with 3 M KCl at pH 7. After the solution reached the tip (monitored under a compound microscope), the microelectrode was dipped into a H^+ liquid exchanger (WPI, IE010). The exchanger was allowed to enter the tip by capillarity and to form a column of no more than 100 μm, which took about 20 sec.

The response (in mV) of these electrodes to pH changes ranging from 5 to 11 was quite linear. We used those whose slope was in the range between 50 and 59 mV/pH-unit.

The H^+-sensitive and the KCl-reference microelectrodes were connected to a high input impedance electrometer (WPI, FD 223). The value of pH_i was obtained by electronically subtracting the membrane potential (V_m) measured with the KCl-reference microelectrode from the voltage obtained with the H^+-sensitive microelectrode (V_H). The output signals from the electrometer were displayed on an oscilloscope and a pen recorder. In addition, after filtering at 3 Hz, the signals were sampled at 1 Hz using an analog-to-digital converter controlled by a computer. Data were stored on floppy disks for subsequent analysis. A similar approach was used when only membrane potential changes were measured.

Fiber diameters were measured using a calibrated graticule in a dissecting microscope at 100×. All experiments were performed at room temperature (*ca.* 23°C).

Background of Experimental Measurements

General Considerations

In evaluating the permeability of OH^- and other ions that affect intracellular pH, two methods were used: membrane potential changes or intracellular pH changes were recorded when the extracellular concentrations of ions were altered. Before considering the experimental results, however, the possible effects of H^+ and protonated buffer permeabilities will be examined.

H^+ Permeability Has No Effect at Alkaline pH

As will be seen, hyperpolarization occurs in K^+-free, Cs^+-containing sulfate solutions when pH_o is raised from 7.2 to a value between 9.5 and 11. Although the hyperpolarization is explained, in part, by inflow of OH^- at high pH_o due to increased $[OH^-]_o$, hypothetically it also might be explained by a net outflow of H^+ due to decreased $[H^+]_o$. To resolve this point, we quantitatively estimated the effect of external pH on membrane potential, V, using the constant field equation and permeabilities found in the literature, corrected for the experimental conditions used (*see* Appendix A). Buffer effects are considered separately; here, both acid and base forms of buffer are assumed to be impermeant.

Figure 1 shows the calculated dependence of V on pH_o assuming that the hydrogen ion permeability is 10^{-3} cm/sec (Izutsu, 1972) and that the only other ions contributing significantly to V are external Cs^+ and internal K^+. It is clear that V is practically constant for pH_o above 7.2 and that a pH_o increase will cause a hyperpolarization only if the initial pH_o is acid. The calculations indicate that increased H^+ outflow on changing pH_o from 7.2 to 11.0 can at most account for a hyperpolarization of 0.14 mV, which is small compared to the values measured. Consequently, net H^+ outflow plays an insignificant role in hyperpolarizations produced by alkaline solutions. In what follows, therefore, the H^+ terms in the constant field equations are omitted.

Protonated Buffer ($pK_a > 9.2$) Permeability Has No Effect at Alkaline pH

Although transmembrane movement of neutral buffer zwitterion does not modify membrane potential directly, it can affect membrane potential, as well as internal pH, if the zwitterion moves rapidly into the myoplasm and dissociates into (permeant) buffer anion and hydrogen ion. As the internal buffer anion concentration increases owing to speedy dissociation of myoplasmic $HB^{\pm}$, internal buffer anion will exit through the membrane and offset the hyperpolarization produced by external buffer anion entry. Appendix B presents calculations which show that even if external zwitterion equilibrates rapidly with the myoplasm, buffers with pK_as in the alkaline range produce effects that are undetectably small on internal pH and membrane potential due to the very limited dissociation of the zwitterions in the myoplasm where the pH is two to three pH-units acid to their pK_as and because the effect of released H^+ is attenuated by the high buffer capacity of the myoplasm.

The indirect effect of neutral buffer entry on membrane potential, and how it depends on the pK_a of the buffer relative to pH_i, is revealed by considering a hypothetical situation where buffers are available having neutral protonated forms with any pK_a between 7 and 11. In this situation, for any desired pH_o a buffer can be chosen such that $pK_a = pH_o$. For the calculations, total buffer concentration is set at a constant value of 10 mM, throughout. Two cases will be exam-

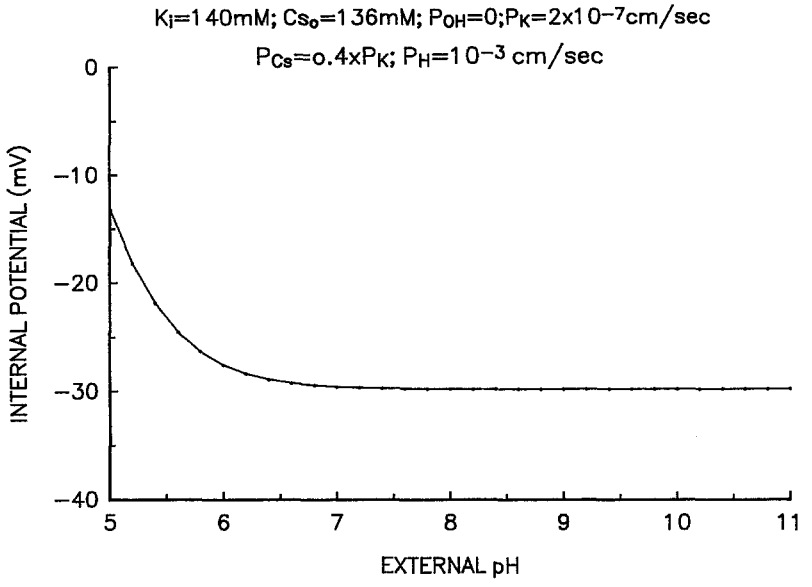


Fig. 1. Calculated dependence of the membrane potential on external pH in K^+-free, Cs^+-containing sulfate solutions. The curve plotted is based on the constant field equation using the ionic concentrations and permeabilities indicated; *see* Appendix A for details.

ined: in one case the protonated buffer form is taken to be impermeant and in the other it is taken to be so permeant as to equilibrate instantaneously with the myoplasm. For both cases, it is assumed that $P_B = P_{Cl}$ and $P_{OH} = 50 \cdot P_{Cl}$, where P_{Cl} is the value of chloride permeability in normal Ringer fluid. As will be seen, these permeabilities are close to those found.

Figure 2*A* shows how pH_i would vary with pK_a (=pH_o) of the rapidly equilibrating buffers when the initial $pH_i = 7.2$. It is clear that above a pK_a of 9 the internal pH is negligibly changed from its initial value after equilibration. Only when the buffer has a pK_a less than 9 does pH_i decrease appreciably. As an example, consider the effect of a buffer with a pK_a of 10.2. With a total external buffer concentration of 10 mM and pH_o of 10.2, the internal concentration of the protonated form will be 5 mM when equilibrated. Since pK_a is 3 pH-units higher than the initial pH_i, only 5 μmol of H^+ per liter will be released inside. Since the average buffer capacity for the fibers is about 50 mM/pH-unit (v.i.), the pH_i will decrease by about 0.0001 pH-unit; this decrease is negligible and, in fact, would be difficult to measure.

Figure 2*B* shows how the membrane potential would vary with pK_a of the buffer when the protonated buffer (i) rapidly equilibrates across the membrane (circles), or (ii) is impermeant (squares). Also shown is the case where neither hydroxide nor the buffer anion is permeant and the relative permeability of Cs^+ with respect to K^+ is unaltered with pH_o (crosses); this merely displays the reference potential from which either hyperpolarizations or depolarizations are measured. The calculations show that for pK_as above 9.2 the membrane potential would have the same value whether or not the protonated buffer form is permeant. The reason for this is that for buffers with pK_as in this range the dissociation of the protonated form in the myoplasm is so low that the concentration of buffer anion released is too small to affect the membrane potential, given the relative permeabilities of the ions contributing to the potential.

For buffers with pK_as below 9.2, this is not the case and the membrane potential is less negative when the protonated form equilibrates than when it is impermeant. For pK_as less than 7.7, changes to a highly permeable buffer produce sufficient exit of anionic buffer to result in a depolarization from the reference value at pH_o 7.2. This will be considered further when the results on HEPES (pK_a = 7.5) anion effects are presented.

Results

Alkaline Solutions Hyperpolarize Muscle Membranes

When increasing pH_o to raise external OH^- concentration, the buffer chosen should ideally be impermeant. For the pH range between 9.5 and 11.0, a cyclohexylamino-sulfonic acid such as CHES or CAPS is commonly selected because of its pK_a and reputed membrane impermeability. The protonated acid form of these buffers are zwitterions of zero net charge while the nonprotonated base forms are anions. If the protonated acid form permeates, it will not directly affect the membrane potential due to its zero net charge; on the other hand, if the nonprotonated base form permeates, it will hyperpolarize the membrane due to its negative charge. In general, therefore, hyperpolarizations produced by buffered alkaline solutions could be due to both OH^- and buffer anion, B^-, penetration, and the relative contribution of each needs to be estimated.

Figure 3 illustrates an experiment demonstrating these effects and the procedure used to separate the relative contributions of each anion. At the start in 68 mM K_2SO_4 at pH_o 7.2, the resting membrane potential was −5 mV. In this solution the membrane behaves as a pure K^+ electrode (Hodgkin & Horowicz, 1959). After about 2 min, external K^+ was replaced by Cs^+ and the potential changed to −34.5 mV; this is expected since Cs^+ is less permeant than K^+ (Kotsias & Horowicz, 1990). The pH_o was then increased to 10 using a total concentration of CAPS, [$CAPS_t$], of 2.5 mM, and the potential changed to −46 mV. After about 1.5

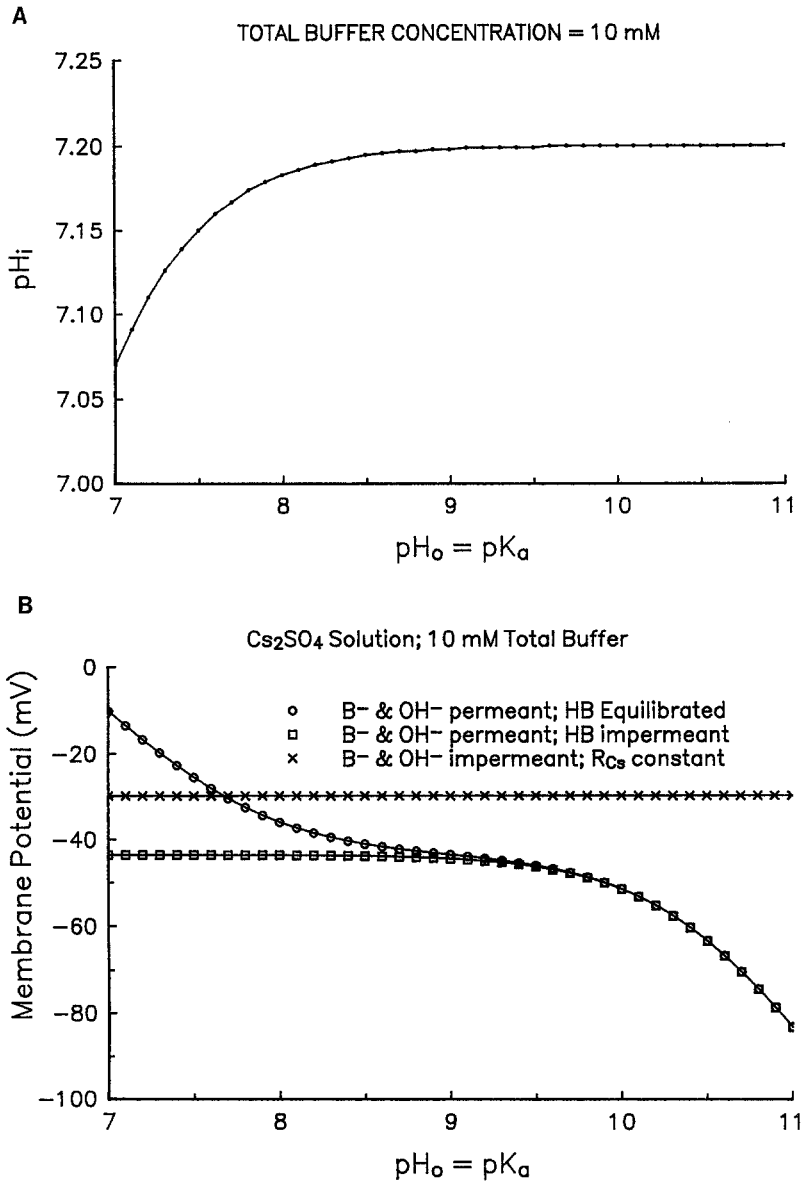


Fig. 2. Calculated effects of buffer pK_a on internal pH (*A*) and membrane potential (*B*) of fibers in K^+-free, Cs^+-containing sulfate solutions. Panel *A* shows the value of internal pH after equilibration of protonated, neutral buffers whose pK_a equals the external pH and where the total buffer concentration applied is 10 mM and where the initial internal pH before equilibration is 7.2. The curve plotted is the numerical solution of Eq. (B10) of Appendix B in which the value of myoplasmic buffer capacity is the average found (i.e., 47 slykes; *see* text and Fig. 10). Panel *B* shows the hypothetical dependence of the membrane potential, calculated from the constant field equation, on the pK_a of buffer for three circumstances. The line through the crosses gives the potential for the case where neither OH^- nor buffer anion is permeable; it provides the reference level for the other curves. The curve through the squares gives the potential for the case where both OH^- and buffer anion are permeable but the protonated buffer form is impermeable. The curve through the circles gives the potential for the case where the protonated buffer form equilibrates and where both OH^- and buffer anion are permeable. The relative permeabilities used for the calculations are about equal to the averages found in this study; *see* Appendix B for details.

min, the $[CAPS_t]$ was increased to 10 mM keeping the pH_o constant at 10 and this drove the potential even more negative to about −51 mV. After a brief period in this solution, the fiber was returned to pH_o 7.2 and the membrane potential depolarized back to a level slightly less negative than initially found in the Cs^+ solution at this pH_o. Following a brief recovery at pH_o 7.2, a similar sequence at pH_o 10 was repeated using CHES as the buffer and similar results were obtained. It is clear that both OH^- and the buffer anions, $CAPS^-$ and $CHES^-$, produce hyperpolarization of the membrane; in both cases, a 7.5 mM increase in buffer concentration at pH_o 10 produces a smaller hyperpolarization than increasing the pH_o to 10 with 2.5 mM buffer.

Figure 4 illustrates another kind of experiment in which hyperpolarization occurs when pH_o was raised while keeping constant the buffer anion concentration. The resting potential of the fiber was −9 mV in 68 mM K_2SO_4 at pH_o 7.2. As before, Cs^+ replacement of K^+ produced a hyperpolarization. The membrane potential reached an initial level of −34 mV in 136 mM Cs^+ at pH_o 7.2 and then declined somewhat. Changing pH_o to 9.7 with $[CAPS_t]$ at 2.5 mM further hyperpolarized the membrane to −45 mV (i.e., a hyperpolarization of over 10 mV). Increasing $[CAPS_t]$ to 10 mM at constant pH_o produced an additional 2 mV of hyperpolarization. (For this fiber, $CAPS^-$ was less permeant than for the fiber shown in Fig. 3.) Next, pH_o was raised from 9.7 to 10.3

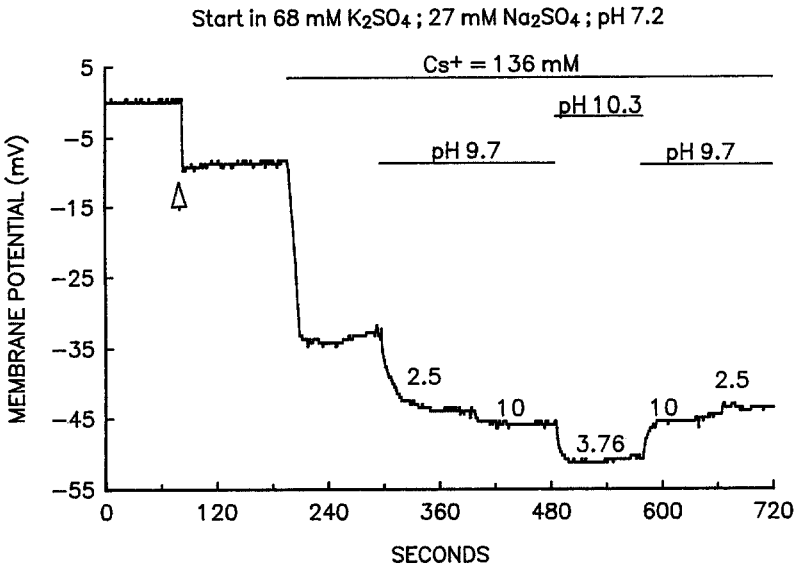


Fig. 3. Effect of increased external pH and buffer anions on membrane potential in cesium sulfate solutions. External pH was increased from 7.2 (total phosphate of 1.5 mM) to 10.0 using either CAPS or CHES at concentrations of 2.5 and 10.0 mM as indicated. Arrowheads indicate when microelectrode was first introduced into and then removed from fiber. Exp. ref. 1107v3.

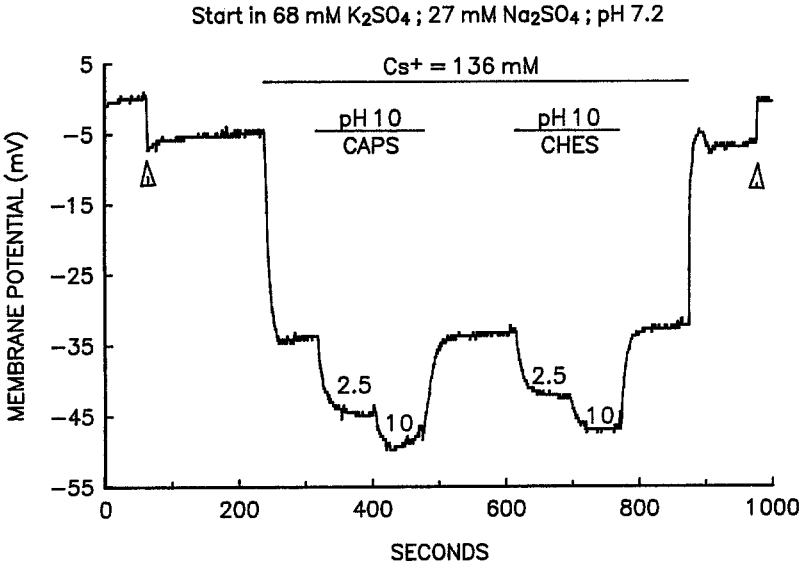


Fig. 4. Separate effects of raising $CAPS^-$ anion concentration at constant pH_o and raising OH^- concentration at constant $CAPS^-$ concentration on membrane potential. Arrowhead shows when microelectrode was introduced into fiber. Total CAPS buffer concentration ($[CAPS_t]$ in mM) is indicated by figures near voltage trace. For both $[CAPS_t]$ = 10 mM at pH_o = 9.7 and $[CAPS_t]$ = 3.76 mM at pH_o = 10.3, $[CAPS^-]_o$ = 1.66 mM. Exp. ref. 1104v1.

and simultaneously $[CAPS_t]$ was lowered to 3.76 mM so as to keep $[CAPS^-]_o$ constant at 1.66 mM while increasing $[OH^-]_o$. The membrane potential changed to about −51.5 mV demonstrating that increasing $[OH^-]_o$ alone produces a hyperpolarization.

Comparison of Bicarbonate and Nitrate Ion Permeabilities

Since membrane movement of bicarbonate can be expected to play some role in the acid-base status of muscle fibers, the relative permeability of HCO_3^- was examined. Since bicarbonate permeability in Cl^--free solutions is low, it was possible to obtain reliable estimates for the relative permeability of HCO_3^- only when bicarbonate completely replaced sulfate. To keep Cs^+ activity and osmotic strength of the replacement solution equal to the sulfate solution, 118 mM $CsHCO_3$ is required as a replacement. When exposed for long periods to atmospheric P_{CO_2}, 118 mM $CsHCO_3$ solution was measured to have a pH of 9.6. Consequently, the membrane potential response to HCO_3^- was determined at this external pH. For comparison, the potential response to 40 mM NO_3^- at pH 9.6 (in the absence of HCO_3^-) was also obtained in each fiber tested with HCO_3^-.

Figure 5 illustrates the results of one such experi-

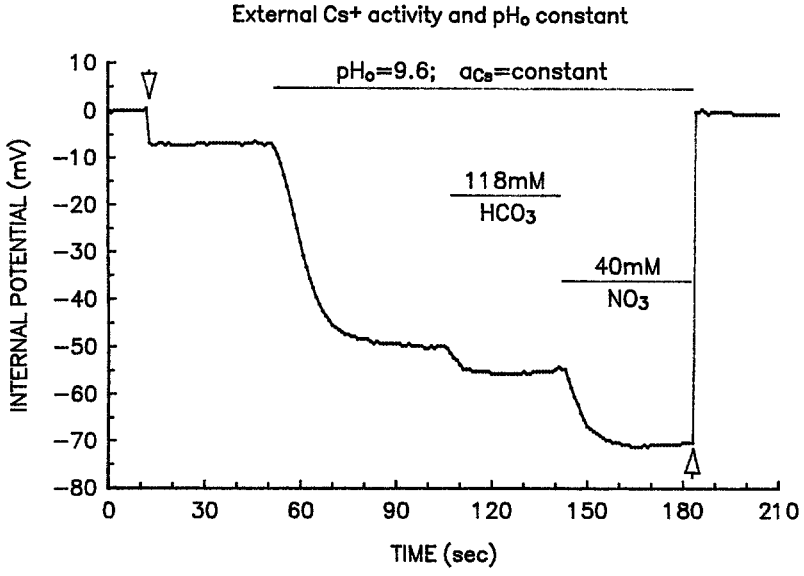


Fig. 5. Effect on membrane potential of bicarbonate (118 mM) or nitrate (40 mM) at pH_o = 9.6. CHES at 2.5 mM was used to obtain pH_o = 9.6 except when bicarbonate was present. No other buffer was used when bicarbonate present (*see text*). Arrowheads indicate entry and exit of microelectrode. Initial pH_o = 7.2 (1.5 mM phosphate). Solutions used were: S4 before bicarbonate, S6 during bicarbonate and S7 during nitrate exposure. Exp. ref. 0211v6.

ment. After obtaining the resting potential (−7.2 mV) in the high potassium sulfate solution at pH_o 7.2, the solution was changed to one in which Cs^+ replaced K^+ and pH_o was 9.6 using 2.5 mM CHES. In response, the membrane hyperpolarized to −51 mV. When 118 mM $CsHCO_3$ at pH_o 9.6 (no CHES) replaced the sulfate solution with Cs^+ activity kept constant, the membrane hyperpolarized by an additional 5.5 mV, despite the elimination of inward $CHES^-$ movement. This result demonstrates that HCO_3^- has a finite, measurable permeability. In the final solution change, the $CsHCO_3$ solution was replaced by a sulfate/nitrate solution containing 40 mM $NaNO_3$ at constant Cs^+ activity and at pH_o 9.6 (2.5 mM CHES). In response, the membrane potential reached a level of −72 mV before it began to slowly decline and the internal microelectrode was removed.

Effect of HEPES Buffer on Membrane Potential

Up to this point, we have considered the membrane potential effects of buffers whose pK_as are above 9.2. In the background section, it was noted that when buffers with pK_as in the range between 7 and 8 are introduced externally, depolarization would ensue if the protonated form equilibrates rapidly, whereas hyperpolarization would occur if the protonated form is impermeant (*see* Fig. 2*B*). Since the pK_a of HEPES is 7.5, the membrane potential effects of this buffer can provide information about the permeability of its protonated, zwitterion form.

In fact, when HEPES (pH_o = 7.1) replaces phosphate (pH_o = 7.2) in K^+-free, Cs^+-containing sulfate solutions, the membrane hyperpolarizes and does so monotonically as $[HEPES]_o$ increases. Figure 6 shows the changes in membrane potential produced by the exposure of two fibers to HEPES at two concentrations. The curve through the points gives calculated hyperpolarizations on the assumption that $HEPES^{\pm}$ is impermeant and that the permeability of $HEPES^-$ relative to K^+ is the average value obtained when the pH_o was 8.5. At pH_o 8.5, there is only a small difference in the membrane potential when $HEPES^{\pm}$ is impermeant as compared with when it equilibrates (i.e., in both cases, hyperpolarizations of almost equal magnitude are produced). Consequently, the relative permeability of $HEPES^-$ obtained at pH_o 8.5 is practically independent of the magnitude of $HEPES^{\pm}$ permeability. The curve in the upper portion of Fig. 6 gives the depolarization calculated on the assumption that the $HEPES^{\pm}$ zwitterion equilibrates using the same relative $HEPES^-$ to K^+ permeability as in the lower curve. The comparison in Fig. 6 suggests that very little of the zwitterion enters the fibers during the usual few minutes of exposure to the altered solutions. It is very likely that the protonated, zwitterion forms of HEPES and the other standard cyclohexylaminosulfonic acid buffers are for practical purposes impermeant (*see* Discussion).

Hydroxide and Other Anion Permeabilities from Membrane Potentials

From membrane potential measurements like those shown in Figs. 3 and 4, the hydroxide and buffer anion

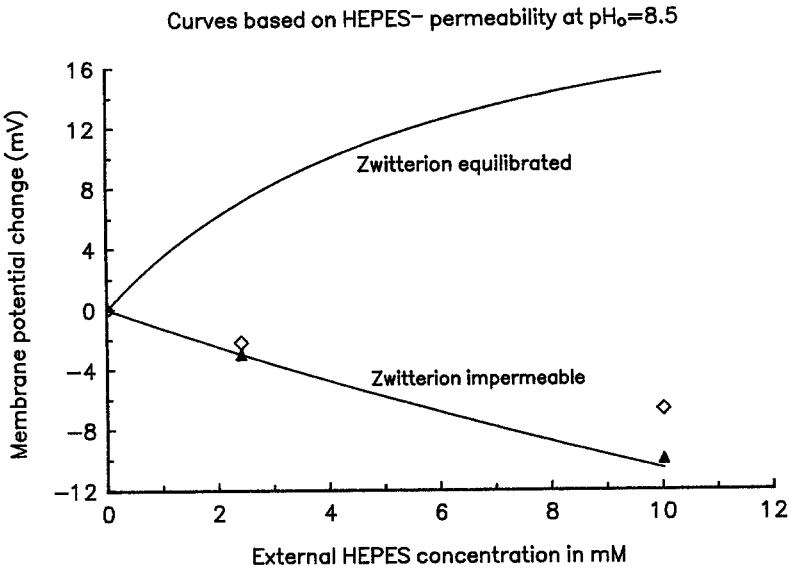


Fig. 6. Dependence of membrane potential on external HEPES concentration at pH_o = 7.1. Points are observations from two experiments (diamonds—exp. ref. 1120khv1; triangles—exp. ref. 1120khv2). Curves are from constant field equation calculations with the permeability of $HEPES^-$ relative to K^+ equal to 25.4. Lower curve based on the assumption that $HEPES^{\pm}$ is impermeable; upper curve based on assumption that $HEPES^{\pm}$ equilibrates across the fiber membrane. *See* Appendix B and text for details.

Table 2. Relative anion permeabilities in 68 mM Cs_2SO_4 from membrane potentials and constant field equation

Anion	pH_o	R_{anion} [a]	±	SEM	(*n*)
OH^-	9.7	986.0	±	218.0	(3)
OH^-	10.0	979.0	±	248.0	(5)
OH^-	10.3	702.0	±	116.0	(3)
OH^-	10.5	419.0	±	24.0	(11)
OH^-	10.75	530.0	±	43.0	(10)
OH^-	11.0	356.0	±	31.0	(6)
$HEPES^-$	7.1	20.2	±	2.0	(4)
$HEPES^-$	8.5	25.4	±	3.8	(27)
$CAPS^-$	10.0	11.7	±	1.8	(11)
$CHES^-$	10.0	5.3	±	0.9	(3)
Cl^-	7.2[b]	9.5	±	1.0	(8)
Cl^-	8.9	18.2	±	2.0	(9)
NO_3^-	8.9	4.6	±	0.5	(11)
NO_3^-	9.6	4.7	±	0.5	(6)
NO_3^-	10.75	4.8	±	0.8	(3)
HCO_3^-	9.6	0.49	±	0.03	(6)

[a] $R_{anion} = \frac{P_{anion} f_{anion}}{P_K f_{K_i}}$, (*see definition of* Eq. D7 of Appendix D).

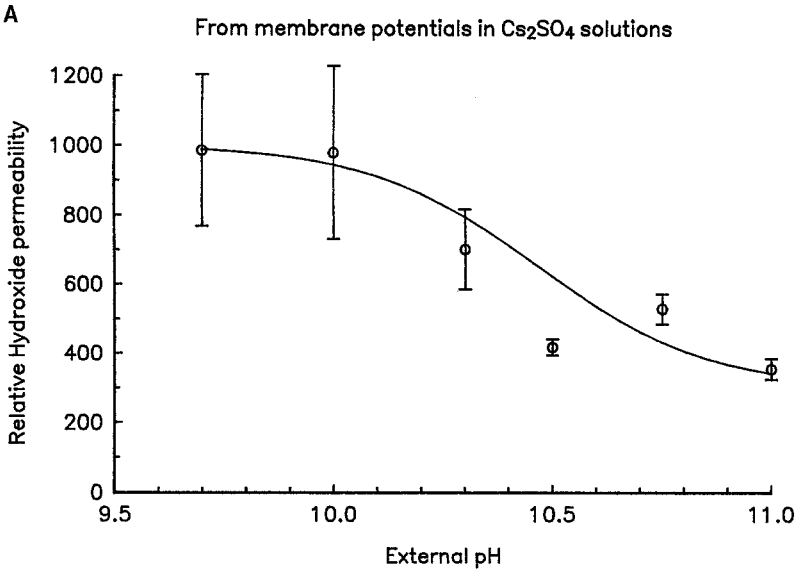
[b] From Kotsias and Horowicz (1990).

permeabilities relative to potassium permeability in cesium-containing sulfate solutions can be calculated using constant field equations. Similarly, experiments like Fig. 5 can be used to obtain the relative permeabilities to bicarbonate and nitrate ions. The equations used are detailed in Appendix D. A summary of the average relative permeabilities for various anions is given in Table 2.

Two noteworthy features of the results are the pH_o dependence of hydroxide and nitrate permeabilities. For convenience, the relative permeabilities for OH^- and NO_3^- are plotted *vs.* pH_o in Fig. 7 where the nitrate permeabilities obtained by Kotsias and Horowicz (1990) at lower pH_os are also shown. Figure 7*A* shows that hydroxide permeability declines as pH_o increases from 10 to 11; the hydroxide permeability at pH_o 11 is about one-third of what it is at pH_o 10. By contrast, Fig. 7*B* shows that nitrate permeability remains constant over this range of alkaline pH and is, in fact, constant for pH_o between 5.5 and 10.75. This difference in pH_o dependence of the respective permeabilities in alkaline solutions suggests that OH^- and NO_3^- cross the surface membrane by different pathways.

The range of permeabilities for permeant anions in alkaline solutions is large; at pH_o mean 9.7, P_{OH} is over three orders of magnitude greater than P_{HCO_3} (*see* Table 2). This particular comparison may be somewhat misleading since OH^- and HCO_3^- probably move through the membrane by means of different channels: OH^- passing through the same channel as Cl^-, and HCO_3^- through the same channel as NO_3^- (*see* Discussion). The more meaningful comparisons are that hydroxide permeability at pH_o = 9.7 is about 50 times greater than chloride permeability at pH_o = 8.9 and that bicarbonate permeability at pH_o = 9.6 is about one-tenth the nitrate permeability.

One other point worth noting is that the permeabilities of the buffer anions used are of the same order of magnitude as that of chloride, with $CHES^-$ being the least permeable and $HEPES^-$ being the most permeable. At present, the membrane pathway used by these anions is uncertain.



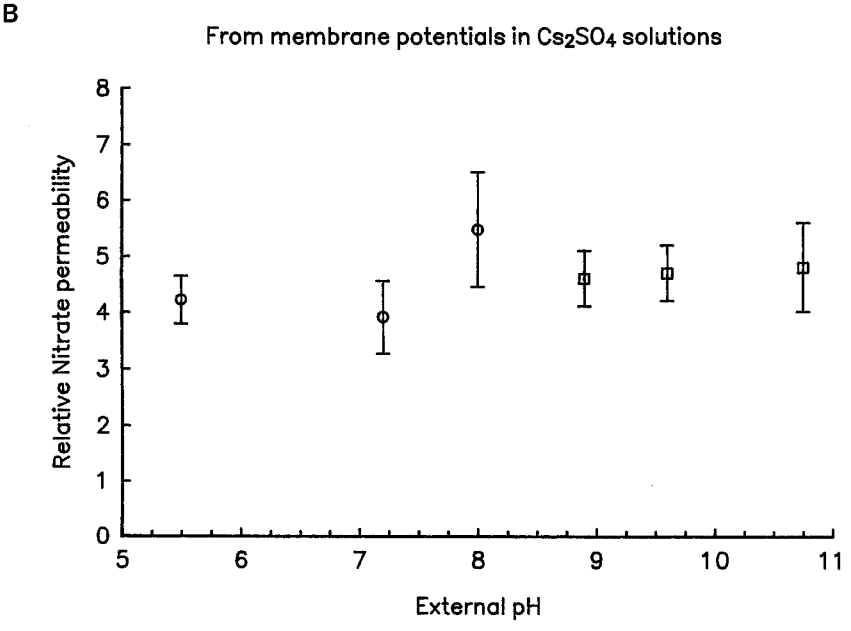


Fig. 7. Dependence of relative hydroxide (*A*) and nitrate (*B*) permeabilities on external pH. Relative permeabilities derived from membrane potentials and constant field equations as outlined in Appendix D. Data from experiments of this report (*see* Table 2) and those in Kotsias and Horowicz (1990). The curve in *A* was obtained by fitting the data points to a function whose final form is

$$R_{OH} = \frac{700}{1 + 10^{-2.25(10.47 - pH_o)}} + 300.$$

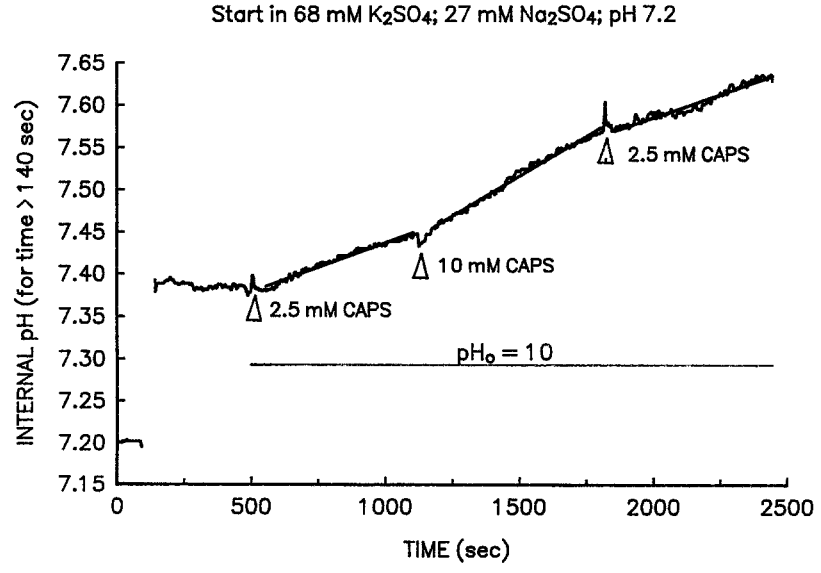


Fig. 8. Effect on internal pH of increasing pH_o from 7.2 to 10.0 ($[CAPS_i] = 2.5$ mM) and increasing $[CAPS_i]$ from 2.5 to 10.0 mM at constant $pH_o = 10.0$. At the start, both KCl and H^+-sensitive microelectrodes were in the external solution at $pH_o = 7.2$. Both electrodes were introduced into the fiber during gap in record. Initial pH_i was about 7.39. Arrowheads indicate when solutions were changed. Lines are least-square fits to data points (1 per sec) obtained in each solution, except for initial transients. The slopes of these lines provide estimates of the time rate of change of pH_i in each solution for use in equations of Appendix C to calculate OH^- and $CAPS^-$ permeabilities. Fiber diameter was 56.4 μ. Exp. ref. 1024v1.

Table 3. Hydroxide permeability from time rate of change of internal pH

Row	Fiber Reference	Fiber Radius (μ)	External pH	V(mV)	Buffer (mM)-Name
1	0927V1	28.2	9.7	−6.0	5.0 CAPS
2	0927V3	30.6	9.7	−6.8	5.0 CAPS
3	0930V1	42.3	9.7	−13.0	5.0 CAPS
Mean ± SEM					
4	0912V1	28.2	10.0	−7.9	5.0 CAPS
5	0912V3	23. 5	10.0	−13.0	5.0 CAPS
6	0913V2	42.3	10.0	−9.3	5.0 CHES
7	0917V1	37.6	10.0	−13.0	2.5 CAPS
8	0917V3	44.7	10.0	−5.3	2.5 CAPS
9	0919V1	42.3	10.0	−5.9	2.5 CHES
10	0919V3	44.7	10.0	−4.3	5.0 CAPS
11	0919V3	44.7	10.0	−4.3	5.0 CHES
12	1024V1	28.2	10.0	−7.8	2.5 CAPS
Mean ± SEM					
13	0927V3	30.6	10.3	−6.8	5.0 CAPS
14	0930V1	42.3	10.3	−13.0	5.0 CAPS
15	1018V2	23.5	10.3	−5.6	5.0 CAPS
16	1018V4	32.9	10.3	−5.3	5.0 CAPS
17	1025V1	23.5	10.3	−4.5	2.5 CAPS
18	1227V1	32.9	10.3	−8.3	2.5 CAPS
19	1227V4	28.2	10.3	−5.6	2.5 CAPS
Mean ± SEM					
Mean ± SEM (all measurements)					

[a] Time rate of change of internal pH due to buffer anion entry was experimentally determined for the fiber as in Fig. 7. For other fibers, this value was obtained by using the average value for P_{CAPS} of 2.10×10^{-3} cm/sec and P_{CHES} of 1.09×10^{-6} cm/sec. *See* Appendix C for equations.

[b] Buffer power was determined experimentally using 10 mM sodium butyrate for the given fiber. For other fibers, and average β value of 47.1 mEq/pH-unit was used (*see* Fig. 9).

Influence of Alkaline Solutions on Internal pH

Figure 8 illustrates the measurement of hydroxide and buffer anion permeabilities from the rates of change of internal pH. At the start, both the KCl-filled microelectrode and the H^+-sensitive ion exchanger filled microelectrode were in the external sulfate solution at pH 7.2 (buffered with phosphate). Shortly after the start, both microelectrodes were introduced into the myoplasm, and the internal pH was about 7.39. The record is blanked out during the penetrations. At about 500 sec, the external solution was changed to one in which the pH was 10 using CAPS at a total concentration of 2.5 mM. For the next 10 min, the internal pH steadily increased. When $[CAPS_t]$ was increased to 10 mM keeping pH_o at 10, internal pH increased at a faster rate, slowing again when $[CAPS_t]$ was changed back to 2.5 mM. The straight lines shown are least-square fits to the measurements made during each exposure period, and the slopes give the rate of change of internal pH used to estimate the hydroxide and buffer anion permeability. We took the difference in the slope between 10 mM CAPS and 2.5 mM CAPS solutions to be a measure of the increment in the rate of entry of buffer anion in response to the increment in total buffer concentration.

Using constant field equations, the permeabilities of both hydroxide and buffer anions can be calculated from the rates of internal pH change, the myoplasmic buffer capacity (measured with butyrate, as shown in the next section), fiber radius, external CAPS concentrations and external pH (*see* Appendix C). Tables 3 and 4 give summaries of data from individual fibers and average values of P_{OH} as a function of external pH. The average P_{OH} for pH_o between 9.7 and 10.3 is 1.68×10^{-4} cm/sec and the average P_{CAPS} is 2.14×10^{-6} cm/sec.

Buffer Power of Myoplasm, β

In calculating absolute permeabilities from the time rate of change of pH_i, a value for the buffering capaci-

Table 3. *Continued.*

D_tpH (Tot) (10^{-5} pH-unit/sec)	D_tpH (buf) (10^{-5} pH-unit/sec)	$D_tpH(OH^-)$ (10^{-5} pH-unit/sec)	Buffer Power (mEq/pH-unit)	P_{OH} (10^{-4} cm/sec)
14.81	2.33	12.48	47.1	1.88
8.53	2.11	6.42	47.1	1.07
7.80	1.34	7.67	47.1	2.02
				1.65 ± 0.24
26.0	3.84	22.16	47.1	1.74
21.33	7.68	13.65	25.4[b]	0.54
46.99	4.86	42.13	36.6[b]	3.96
24.85	0.65[a]	24.20	47.1	2.81
18.82	1.28	17.54	47.1	2.06
20.59	2.03	18.57	47.1	2.09
12.39	2.61	9.78	47.1	1.13
17.27	3.96	13.31	47.1	1.53
14.08	2.47[a]	11.61	83.0[b]	1.60
				1.94 ± 0.31
29.68	5.63	24.05	47.1	1.00
22.9	3.58	19.3	47.1	1.26
67.9	9.83	58.1	36.0[b]	1.38
68.8	4.38	64.4	58.0[b]	2.45
19.6	2.42[a]	17.2	47.1	0.52
76.82	3.28	73.54	36.5[b]	2.25
12.09	1.23[a]	10.85	73.0[b]	0.63
				1.35 ± 0.26
				1.68 ± 0.19

Table 4. CAPS anion permeability from changes in time rate of change in pH_i

Row	Fiber reference	Fiber radius (μ)	External pH	V (mV)	Changes in total concentration (mM)	$\Delta D_t pH$ (10^{-5} pH-units/sec)	Buffer power (mEq/pH-unit)	P_{CAPS} (10^{-6} cm/sec)
1	0917V1	37.6	10.0	−13.0	7.5	2.03	47.1	1.10
2	1024V1	28.2	10.0	− 7.8	7.5	7.27	83.0[a]	4.66
3	1025V1	23.5	10.3	− 4.5	7.5	8.2	47.1	1.50
4	1227V4	28.2	10.3	− 5.6	7.5	3.69	73.0[a]	1.28
Mean ± SEM								2.14 ± 1.07

[a] Buffer power determined experimentally using 10 mM sodium butyrate on fiber. In other fibers, average buffer power used (*see* Fig. 9). ΔD_t pH column represents the change in the slope of the pH_i *vs* time relation when external total buffer concentration was increased from 2.5 to 10 mM. P_{CAPS} calculated from Eq. (C9) in Appendix C.

ty of the myoplasm is needed. This was obtained in nine fibers from the effects of 10 mM sodium butyrate on pH_i. Figure 9 shows the response of pH_i in a fiber exposed to 10 mM butyrate while recovering in high potassium sulfate solution after an extended period in alkaline solutions. Upon introduction of external butyrate, pH_i falls promptly and then, when butyrate is removed, returns to the initial curve of slow acidification during recovery at pH_o 7.2. At about 1,200 sec, both internal microelectrodes were taken out of the fiber. In this fiber, using the equations given in Appendix B, the estimated value of β was 83 mEq/pH-unit.

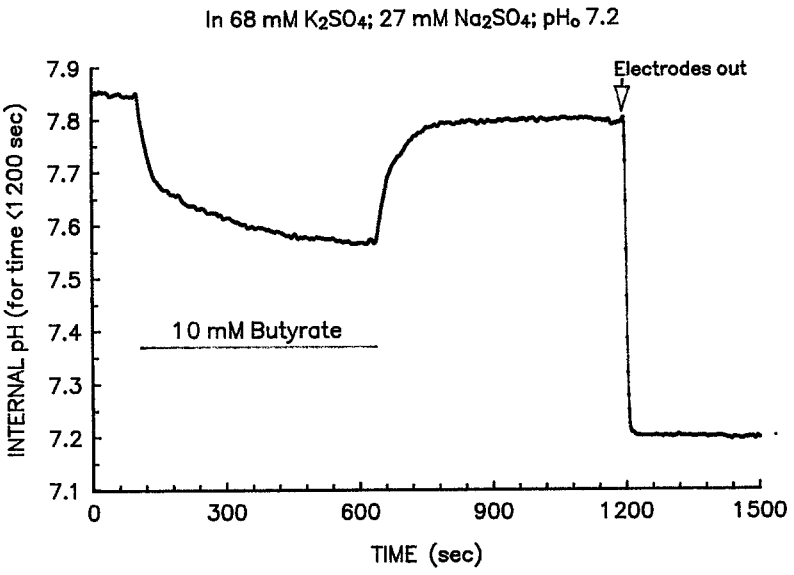


Fig. 9. Effect of short exposure to 10 mM sodium butyrate on internal pH on a fiber recovering from an extended period in alkaline solutions. Exp. ref. 1024v2.

Figure 10 shows a scatter plot of β *vs.* initial pH_i. Most of the determinations of β were made after the fibers had been exposed to alkaline solutions for various periods and the pH_is shown cover the range over which the measurements were made. The range of values observed for β is similar to that reported by Curtin (1986). The average value of β for the fibers in Fig. 10, taken as a group, is 47.1 ± 6.3 (SEM). In permeability calculations on fibers for which β was not measured, this average value was used.

Discussion

Comparison of Permeabilities Obtained by the Two Methods

Before discussing the significance of the various permeabilities obtained, we shall commence with some comparisons.

The first method used, which measures membrane potential changes in response to permeant anions, yields anion permeabilities relative to K^+ permeability. The second method, which measures internal pH changes in response to permeant anions that alter pH, yields absolute permeabilities. Since these derived permeabilities are not directly comparable, one approach is to factor out the K^+ permeability by taking the ratio of the relative permeabilities of two anions obtained from membrane potentials and comparing it to the ratio of permeabilities for the same anions obtained from the pH_i measurements. The ratio P_{OH}/P_{CAPS} at $pH_o = 10$ from the first method is 84 (i.e., 979/11.7; Table 2) while the same ratio from the second method is 79 (i.e., $1.68 \times 10^{-4}/2.14 \times 10^{-6}$; Tables 3 and 4). The agreement is not unreasonable, considering the standard errors.

Another useful comparison is to use the derived relative and absolute permeabilities from the two methods to calculate a permeability that is available from previous reports. For example, chloride permeability, P_{Cl}, can be estimated from the relation $P_{Cl} = (R_{Cl}/R_{OH})P_{OH}$; using the values in Tables 2 and 3, this relation yields an estimate for P_{Cl} of 3.6×10^{-6} cm/sec at $pH_o = 8.9$ (i.e., $(18.2/979) \times 1.94 \times 10^{-4}$ cm/sec) and 1.9×10^{-6} cm/sec at $pH_o = 7.1$ (i.e., $9.5/979) \times 1.94 \times 10^{-4}$ cm/sec). In single fibers from semitendinosus muscles of *Rana temporaria,* Hodgkin and Horowicz (1959) found values of P_{Cl} in Ringer fluid ($pH_o \approx 7.3$) that varied between 1.9×10^{-6} and 6.6×10^{-6} cm/sec. Using sartorius muscles from *R. temporaria,* Adrian and Freygang (1962) obtained estimates that varied between 0.9×10^{-6} and 2.4×10^{-6} cm/sec. The difference in the average P_{Cl} estimated at $pH_o = 8.9$ and that at $pH_o = 7.1$ from the data in Table 2 is consistent with the reported dependence of chloride conductance and fluxes on external pH (Hutter & Warner, 1967*a,b*). The general agreement in these various comparisons gives some confidence to the results obtained by the methods used in this report.

Bicarbonate Permeability

The bicarbonate permeability can also be estimated from the values in Tables 2 and 3. Using the relation $P_{HCO_3} = (R_{HCO_3}/R_{OH})P_{OH}$, a value of 9.7×10^{-8} cm/sec is obtained for P_{HCO_3} at $pH_o = 9.6$ (i.e., $(0.49/979) \times 1.94 \times 10^{-4}$ cm/sec). This can be compared with an estimated HCO_3^- permeability of 7.4×10^{-8} cm/sec at $pH_o = 7.35$ based on the slow component of acidifica-

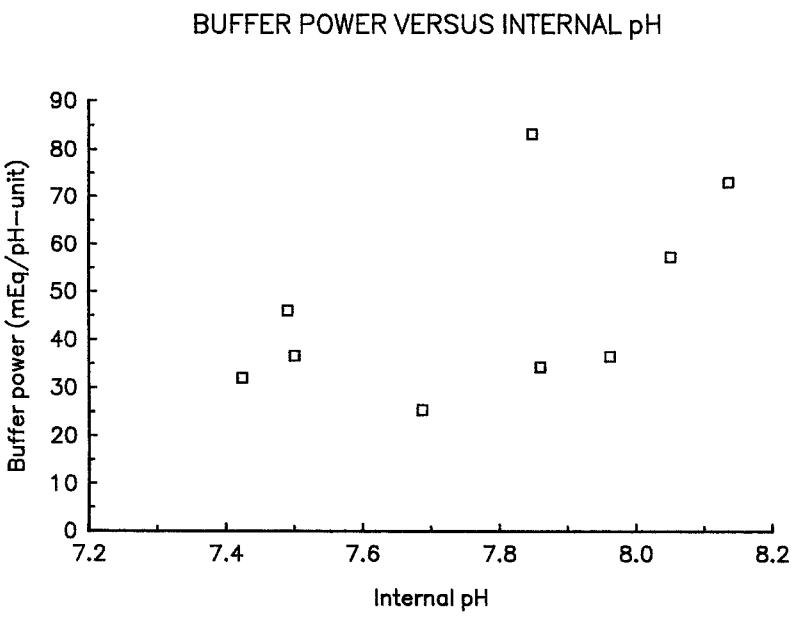


Fig. 10. Scatter plot of myoplasmic buffer power, β, *vs.* pH_i. Results from experiments similar to the one shown in Fig. 9. The average value of β for all fibers is 47.1 ± 6.3 (SEM) mEq/pH-unit.

tion obtained during CO_2 exposure in Na^+-free Ringer fluid in semitendinosus muscles of *R. pipiens* reported by Abercrombie et al. (1983).

With these estimates for P_{HCO_3} in hand, a re-examination of the results of Izutsu (1972) used to estimate H^+ permeability, P_H, in bullfrog toe muscles can be made. The reported value of 10^{-3} cm/sec for P_H is based on the observed initial rate of myoplasmic acidification when external $[HCO_3^-]$ is reduced from 24 to 5 mM at a constant P_{CO_2} of 5%. The observed acidification can clearly be due to exit of HCO_3^- from the myoplasm and on this basis Izutsu (1972) estimated P_{HCO_3} to be 3×10^{-8} cm/sec, assuming a myoplasmic buffer value of 10 slykes. If one uses a buffer value in the range of 20 to 30 slykes (Amorena et al., 1990), one obtains a larger estimate for P_{HCO_3} (in the range of 6×10^{-8} to 9×10^{-8} cm/sec). However, Izutsu discarded the possibility of a finite bicarbonate permeability of about this magnitude for the alternate view that H^+ entry accounts for all of the acidification when external bicarbonate concentration is reduced and pH_o falls from 7.30 to 6.64, and on this basis derived a P_H of 10^{-3} cm/sec. It is now clear, however, that the acidification in these experiments can be largely if not entirely accounted for by HCO_3^- permeability and that, at most, only a minor fraction of the acidification can be due to H^+ entry.

Although the information on the dependence of HCO_3^- permeability on pH_o is not adequate to determine if HCO_3^- passes through the pH_o- and voltage-independent pathways that allow passage of NO_3^-, structural considerations suggest that this may be the case. Both anions are planar with carbon or nitrogen being at the center and the surrounding bonds to oxygen making angles that are 120° (Cotton & Wilkinson, 1972).

Hydroxide Permeability

The results summarized in Table 2 show that in alkaline solutions P_{OH} is about 50 times greater than P_{Cl}. The fact that R_{OH} at $pH_o = 11$ is about 0.36 times R_{OH} at $pH_o = 10$, while P_{NO_3} at $pH_o = 10.75$ is the same as it is at lower pH_os down to 5.0 (Table 2 and Fig. 7), indicates that membrane passage of OH^- does not use the pathways used by NO_3^-. In addition, the fact that R_{NO_3} remains constant for all pH_os up to 10.75 rules out the possibility that the drop in R_{OH} between pH_o 10 and 10.75 or 11 is due to an alteration of the cation movements over this range of pH_o. Hence, P_{OH} must fall over this range, and it is interesting that this behavior parallels the decrease of chloride fluxes over this same range of pH_o observed by Skydsgaard (1987). This suggests that OH^- ions move through the same channels that Cl^- ions use.

One final point worth noting is that the calculated OH^- movement for the experiments of Izutsu (1972) discussed above using the apparent P_{OH}/P_{Cl} ratio from Table 2 is insufficient to contribute significantly to the pH_i changes observed in that study.

Observations in Acid Solutions and H^+ Permeability

The calculations shown in Fig. 1, based on the assumption that the H^+ permeability is 10^{-3} cm/sec, predict a 16 mV depolarization on changing pH_o from 7.2 to 5.0. A few experiments in K^+-free, Cs^+-containing sulfate solutions using the buffer MES at $pH_o = 5.0$ gave depolarizations of about 5 mV. At constant pH_o

less than 5, steadily increasing depolarizations were obtained that were only partially reversible even with short exposure times. Assuming that the depolarization obtained at $pH_o = 5.0$ is due to H^+ permeability, then the calculated H^+ permeability relative to K^+ is about 800; a value that is not significantly different from that obtained for the relative permeability of OH^- (*see* Table 2). Assuming MES^- has a permeability comparable to that of the other buffer anions examined, the offsetting hyperpolarization due to MES^- entry calculated on going to $pH_o = 5$ is less than 1 mV.

A few experiments were also done measuring time rates of change of pH_i in high K^+ sulfate solutions at $pH_o = 5.0$ with 5 mM MES. After a 10 min exposure, there was at most a just barely detectable acidification in some instances. With the stability of the H^+-sensitive electrodes we used, a just barely detectable acidification rate is about 0.001 pH-unit/min (i.e., about 0.5 mV change in the H^+-sensitive electrode over a 10 min period). For a fiber with a diameter of 60 μ and an average buffer capacity, this amounts to a P_H of about 10^{-4} cm/sec. A P_H of 10^{-3} cm/sec in such a fiber would have shown an acidification rate of 0.01 pH-unit/min; a rate of change easily measured but not observed. Our tentative conclusion is that P_H is, at most, about equal to P_{OH} in frog skeletal muscle.

This estimate of P_H (based on the just discernable acidification rate when $pH_o = 5$) assumes that the protonated, zwitterion form of MES is impermeant. On the other hand, if the acidification were due to entry of the protonated form of MES ($pK_a = 6.2$) then an estimate of the maximum $MES^{\pm}$ permeability can be made. Since pH_i is about 7.2 and the $pK_a = 6.2$, most of the entering $MES^{\pm}$ would dissociate to yield H^+. Using an acidification rate of 0.001 pH-unit/min with the same fiber diameter and buffer capacity used above gives a maximum possible $P_{MES^{\pm}}$ of about 2.4×10^{-7} cm/sec. On these assumptions, the H^+ permeability would be less than the maximum estimated above. However, since the membrane depolarization produced by lowering pH_o to 5 gives an estimated P_H which accounts for the barely detectable acidification rate observed, it seems likely that $P_{MES^{\pm}}$ is even lower than the above estimate. Our conclusion is that we have no evidence to indicate that $MES^{\pm}$ is permeant, and this conclusion probably also applies to the other buffers used in this study.

Buffer Anion Permeability

The results show clearly that many of the commonly used cyclohexylamino-sulfonic acid buffers are permeant in their unprotonated anionic forms. In frog skeletal muscle, they have permeabilities comparable to that of Cl^- ions. On entering the fibers these anions combine with H^+ and increase pH_i. The extent to which they increase pH_i depends on the external concentration of the buffer, the membrane potential and the buffer capacity of the interior. When measuring pH_i in the presence of these buffers, these factors need to be taken into account.

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Appendix A

Effect of External H^+ Concentration on Membrane Potential

In this Appendix the calculations shown in Fig. 1*A* are described. The aim is to determine the effects of external H^+ concentration, $[H^+]_o$, on the membrane potential in K^+-free, Cs^+-containing solutions. The calculations are based on the constant field equation where permeabilities of ions other than H^+, K^+, or Cs^+ are assumed to be negligibly small. This allows an estimate to be obtained of the contribution that lowering $[H^+]_o$ makes to the hyperpolarization of the membrane when the pH of the external solution is raised.

For the conditions described, when external K^+ is replaced by Cs^+ the internal potential, V, can be approximated by the relation

$$V = \frac{RT}{F} \ln \frac{P_{Cs} f_{Cs_o} [Cs^+]_o + P_H f_{H_o} [H^+]_o}{P_K f_{K_i} [K^+]_i + P_H f_{H_i} [H^+]_i} \tag{A1}$$

where R, T, F, $[\ldots]_o$, and $[\ldots]_i$ have the usual significance; f is the activity coefficient with the subscript identifying the ion and space; and P is the membrane permeability to the ion denoted in the subscript. It is important to note that for depolarized fibers in K^+-free solutions the K^+ permeability is approximately one order of magnitude smaller than P_K when K^+ is present (Adrian, 1962; Spalding et al., 1981). Since the value of P_K is 2×10^{-6} cm/sec with external K^+ present (Hodgkin & Horowicz, 1959), then to a first order of approximation $P_K = 2 \times 10^{-7}$ cm/sec for the solutions in this study. In earlier experiments using similar solutions Kotsias and Horowicz (1990) found that $P_{Cs} = 0.4 \times P_K$, which makes $P_{Cs} = 0.8 \times 10^{-7}$ cm/sec. With regard to H^+, Izutsu (1972) estimates that $P_H = 10^{-3}$ cm/sec. Although the methods used to obtain this estimate have been criticized by Boron (1983) we use this value of P_H as an approximate upper limit. Assuming that the activity coefficient of any monovalent cation is mainly determined by the ionic strength of the space in which it is located, then $f_{K_i} = f_{H_i}$ and $f_{K_o} = f_{H_o} = f_{Cs_o}$. Kotsias and Horowicz (1990) also determined that the ratio $r = f_{K_o}/f_{K_i} = 0.8$. Since concentrations are conventionally given in mM units, then $[H^+]_o = 10^{(3-pH_o)}$ and $[H^+]_i = 10^{(3-pH_i)}$. For the conditions given, $[Cs^+]_o = 136$ mM and $[K^+]_i = 140$ mM (*see* Venosa, 1979). Finally, fibers equilibrated at normal pH_o have a pH_i of about 7.2 (Abercrombie et al., 1983).

With these various values in hand and the above equation, V can be calculated for pH_o between 5 and 11. The results are shown in Fig. 1*A*. With regard to experiments in this report, switching from the pH_o of 7.2 to a pH_o of 11 changes the calculated value of V from −29.71 to −29.85 mV. Consequently, increased H^+ outflow, at most, can account for a hyperpolarization of 0.14 mV, which is small compared to the actual measured values. The meager hyperpolarization due to H^+ stems from the H^+ terms being numerically small compared to the Cs^+ and K^+ terms in the above equation. In the analysis of the results, therefore, the H^+ terms in the constant field equations are not included.

Appendix B

1. Possible Effects of Equilibration of Protonated Buffer Zwitterions on Internal pH and Membrane Potential

In this subsection, the calculations shown in Fig. 2*A* and *B* are described. The aim is to determine the effect of rapidly permeating weak acids on the internal pH (pH_i) and membrane potential (V) in muscle fibers exposed to K^+-free, Cs^+-containing sulfate solutions and to compare this with the case where the weak acids are impermeant. For the buffers used in this study, the protonated forms are zwitterions of zero net charge ($HB^\pm$) and, hence, do not directly affect the membrane potential. The nonprotonated forms, however, do affect the membrane potential since they are anions (B^-) and permeate the membrane.

Substantial alteration of pH_o entails the replacement of the initial external buffer (orthophosphate) with a new buffer having a suitable pK_a. If $HB^\pm$ is impermeable and B^- is permeable, entry of B^- through the membrane will produce a hyperpolarization of a magnitude which depends on the anion concentration, $[B^-]_o$. Orthophosphate does not contribute to the initial resting membrane potential (*unpublished observations*). On the other hand, if the membrane is permeable to $HB^\pm$ and permits its equilibration with the myoplasm, then the internal concentration of B^-, $[B^-]_i$, will rapidly increase owing to the dissociation of myoplasmic $HB^\pm$. Internal B^- will exit through the membrane and offset the hyperpolarization produced by B^- entry. Depending on the pK_a of the buffer and pH_i, $[B^-]_i$ may be large enough to produce membrane depolarization owing to net exit of B^-. An estimate of these effects can be based on the following considerations.

The equilibrium condition for buffer in the external solution is given by

$$[HB^\pm]_o = K_a^{-1} [B^-]_o [H^+]_o, \tag{B1}$$

or, using the conventional definitions for pK_a and pH,

$$[HB^\pm]_o = [B^-]_o \cdot 10^{(pK_a - pH_o)}. \tag{B2}$$

The total buffer concentration, T, is given

$$[HB^\pm]_o + [B^-]_o = T. \tag{B3}$$

On substituting Eq. (B2) into (B3) and solving for $[B^-]_o$ one gets

$$[B^-]_o = \frac{T}{1 + 10^{(pK_a - pH_o)}}. \tag{B4}$$

and finally substituting Eq. (B4) into (B3) one has

$$[HB^\pm]_o = \frac{T \cdot 10^{(pK_o - pH_o)}}{1 + 10^{(pK_a - pH_o)}}. \tag{B5}$$

When $HB^\pm$ in the myoplasm is in equilibrium with the external solution then

$$[HB^\pm]_i = [HB^\pm]_o. \tag{B6}$$

On entry into the myoplasm, $HB^{\pm}$ dissociates to yield H^+ and B^-. The released H^+ combines with the internal buffers and pH_i is reduced. The pH_i after equilibration of the protonated form is given by the relation

$$pH_i = pH_{i0} - [B^-]_i/\beta, \tag{B7}$$

where pH_{i0} is the initial pH_i before application of new buffer and β is the buffer capacity of the myoplasm. The reaction equilibrium within the myoplasm is characterized by a relation analogous to Eq. (B2), or

$$[B^-]_i = [HB^{\pm}]_i 10^{(pH_i - pK_a)}. \tag{B8}$$

Substituting Eq. (B6) into (B8) gives

$$[B^-]_i = [HB^{\pm}]_o 10^{(pH_i - pK_a)}. \tag{B9}$$

Finally, substituting Eq. (B9) into (B7) and rearranging, one obtains

$$pH_i + \beta^{-1}\{[HB^{\pm}]_o 10^{(pH_i - pK_a)}\} - pH_{i0} = 0. \tag{B10}$$

Equation (B10) relates the internal pH to the myoplasmic buffer capacity and the external concentration of $HB^{\pm}$, which in turn is related to the total external buffer concentration, pK_a and pH_o by Eq. (B5). Once pH_i is determined then $[B^-]_i$ can be calculated using Eq. (B9). Equation (B10) has to be solved numerically.

With a method for calculating $[B^-]_i$ in hand, the effect of $[B^-]_i$ on the membrane potential in K^+-free, Cs^+-containing sulfate solutions can be estimated as a function of both pK_a and $HB^{\pm}$ equilibration. Since H^+ permeability does not play a role (*see* Appendix A), one can consider mainly the combined effects of OH^- permeability, P_{OH}, and B^- permeability, P_B, when the pH_o is changed using a buffer whose pK_a is equal to pH_o. In this situation the membrane potential is given by the relation

$$V = \frac{RT}{F} \ln \frac{P_{Cs} f_{Cs_o} [Cs^+]_o + P_{OH} f_{OH_o} [OH^-]_i + P_B f_{B_i} [B^-]_i}{P_K f_{K_i} [K^+]_i + P_{OH} f_{OH_o} [OH^-]_o + P_B f_{B_o} [B^-]_o} \tag{B11}$$

where R, T, F, $[\ldots]_o$, and $[\ldots]_i$ have the usual significance; f is the activity coefficient with the subscript identifying ion and space; and P is the membrane permeability to ion denoted in subscript. In the calculations, the term involving $[OH^-]_i$ in the numerator of the logarithmic function of Eq. (B11) is omitted owing to its relative smallness.

For the curves shown in Fig. 2*A* and *B*, the following assumptions were made or values used. The activity coefficients of monovalent ions in the same space were assumed to be equal; i.e., $f_{K_i} = f_{OH_i} = f_{B_i}$ and $f_{Cs_o} = f_{OH_o} = f_{B_o}$. The ratio f_{Cs_o}/f_{K_i} was set equal to 0.8 (Kotsias & Horowicz, 1990). For the experimental conditions used, $[Cs^+]_o$ = 136 mM and $[K^+]_i$ = 140 mM. With regard to relative permeabilities, P_{Cs}/P_K = 0.4, P_B/P_K = 25, and P_{OH}/P_K = 1,000. When $HB^{\pm}$ was taken to be impermeable, then $[B^-]_i = 0$; when $HB^{\pm}$ was taken to be equilibrated across the membrane, then $[B^-]_i$ was calculated using Eqs. (B9) and (B5). For the reference curve, $P_{OH} = 0 = P_B$.

2. Buffering Power, β, from pH_i Change Induced by Butyrate

The myoplasmic buffering power, β, was obtained in several fibers from the change in pH_i when 10 mM sodium butyrate (pK_a = 4.82) was added to the external solution. The calculations assume that the butyric acid is freely permeant, rapidly equilibrates across the plasma membrane, and that the pK_a remains unchanged in the myoplasm (Szatkowski & Thomas, 1986). With these assumptions, using equations analogous to (B9), (B2) and (B4) for butyrate, one can show that the internal concentration of the butyrate anion, $[B^-]_i$, after equilibration is given by the relation

$$[B^-]_i = \frac{T_o \cdot 10^{({}^fpH_i - pH_o)}}{(1 + 10^{(pK_a - pH_o)})}$$

where T_o is the concentration (mM) of sodium butyrate added to the external solution, pH_o is the external pH, and ${}^fpH_i$ is the final equilibrated pH_i in the presence of butyrate. The buffering power is calculated by dividing $[B^-]_i$ by the absolute value of the change in pH_i produced by butyrate, i.e.,

$$\beta = \frac{[B^-]_i}{|{}^fpH_i - {}^ipH_i|}$$

where ${}^ipH_i$ is the initial pH_i when butyrate is absent.

Appendix C

Hydroxide and Buffer Anion Permeabilities Based on Rates of Change of Internal pH

We develop here relations that permit calculation of membrane permeability to hydroxide ions, P_{OH}, from the observed rate of change of pH_i consequent to raising $[OH^-]_o$. The pH_i change produced by net entry of OH^- is determined by the myoplasmic buffer capacity according to the relation

$$DpH_i = D[OH^-]/\beta, \tag{C1}$$

where DpH_i is the rate of pH_i change expressed as pH-unit/sec, $D[OH^-]$ is the rate of net OH^- entry expressed in concentration units per unit of time or (millimol/liter)/sec and β is the myoplasmic buffer capacity expressed as (millimol/liter)/pH-unit. $D[OH^-]$ is determined by the product of two factors: (i) the net influx of OH^-, M_{OH}, and (ii) the surface area to cell volume ratio (A/V) of the muscle fiber. Since the units conventionally used for M_{OH} are (mol/cm^2-surface)/sec and for A/V (=2/radius) are cm^2-surface/cm^3-volume, both have to be multiplied by 10^3 to convert them to units of (millimol/cm^2-surface)/sec and cm^2-surface/liter, respectively. Keeping the conventional units for M_{OH} and A/V and making the conversion factors explicit, $D[OH^-]$ is given by the relation

$$D[OH^-] = 2 \cdot 10^6 \cdot M_{OH}/r, \tag{C2}$$

where r is the radius of the fiber in cm. Substituting Eq. (C2) into Eq. (C1) and rearranging terms, one obtains the following expression for M_{OH},

$$M_{OH} = \frac{DpH_i \cdot r \cdot \beta}{2 \cdot 10^6} \tag{C3}$$

From constant field theory, P_{OH} is given by the expression,

$$P_{OH} = \frac{M_{OH}RT\{1 - \exp(-VF/RT)\}}{VF\{[OH^-]_o - [OH^-]_i \exp(-VF/RT)\}10^{-6}} \quad (C4)$$

where V is the membrane potential; F, R, and T have their usual significance; and the factor 10^{-6} in the denominator is needed to convert concentrations from units of millimol/liter to mol/cm^3. Substituting Eq. (C3) into Eq. (C4) one has

$$P_{OH} = \frac{D\mathrm{pH}_i \cdot r \cdot \beta \cdot RT\{1 - \exp(-VF/RT)\}}{2VF\{[OH^-]_o - [OH^-]_i \exp(-VF/RT)\}} \quad (C5)$$

Consequently, measurement of $D\mathrm{pH}_i$, r, β, and V at an experimentally set pH_o permits one to calculate P_{OH} *once DpH_i is corrected for pH_i changes associated with buffer entry* into the fiber. We turn, therefore, to a consideration of the effects of buffer anion entry, which in these experiments is mainly $CAPS^-$.

Experimentally, entry of buffer anions is estimated by measuring the increment in the rate of change of pH_i when external buffer concentration is increased at constant pH_o. It is assumed that the rate of change of pH_i due to OH^- entry is unaltered since the pH_o is kept constant, and that the increment in the rate of change of pH_i, $\delta D\mathrm{pH}_i$, is ascribable solely to the increment in external buffer anion concentration, $\delta[B^-]_o$, consequent to the increment in total external buffer concentration, δT_B. The increment in the influx of buffer anion, δM_B, is assumed to be proportional to $\delta[B^-]_o$. Since the membrane potential does not change significantly for δT_B in the high K^+ solutions used for the experiments where pH_i are measured, the equation for buffer anion permeability, P_B, can be written as

$$P_B = \frac{\delta M_B RT\{1 - \exp(-VF/RT)\}}{VF\delta[B^-]_o} \quad (C6)$$

in which the term $[B^-]_i \exp(-VF/RT)$ usually present in the denominator is assumed negligible. Since the pK_a of CAPS is 10.4 and pH_i is in the range of 7.4 to 7.6 for a typical experiment, all but a negligible portion of the entering B^- is rapidly protonated. The equation relating $\delta[B^-]_o$ to δT_B is

$$\delta[B^-]_o = \frac{\delta T_B \cdot 10^{-6}}{1 + 10^{(pK_a - pH_o)}}. \quad (C7)$$

where the factor 10^{-6} in the numerator is needed to convert δT_B, which is conventionally expressed as millimol/liter, to mol/cm^3 which is required for the dimensions of $\delta[B^-]_o$ in Eq. (C6). Finally, the equation relating the increment of influx of B^-, δM_B, to the increment in rate of change of pH_i, $\delta D\mathrm{pH}_i$, is

$$\delta M_B = \frac{\delta D\mathrm{pH}_i \cdot r \cdot \beta}{2 \cdot 10^6} \quad (C8)$$

which is analogous to Eq. (C3) and again assumes that all but a negligible fraction of B^- becomes protonated on entering the myoplasm. Substituting Eq. (C7) and (C8) into (C6), one gets

$$P_B = \frac{\delta D\mathrm{pH}_i \cdot r \cdot \beta \cdot RT\{1 - \exp(-VF/RT)\}\{1 + 10^{(pK_a - pH_o)}\}}{2VF\delta T_B} \quad (C9)$$

Using this equation, P_B can be calculated from the measurements of $\delta D\mathrm{pH}_i$, r, β, V, the experimentally set values of δT_B, pH_o, and the other known values. Once P_B is known, one can correct the total $D\mathrm{pH}_i$ for B^- entry and use the corrected value in Eq. (C5) to obtain P_{OH}.

Appendix D

Relative Permeabilities of Anions Based on Membrane Potential Measurements

Constant field equations have been used to estimate the relative permeabilities of the anions tested. We first consider the case where only OH^- and B^- were changed when pH_o was raised from its initial value of 7.2 using phosphate buffer. Phosphate buffer anions, at the concentrations used, do not make a measurable contribution to the resting membrane potential (*unpublished observations*).

For the initial high K^+ sulfate equilibrating solution, the membrane potential, V_0, is equal to the K^+ equilibrium potential. This is given by the relation

$$V_0 = V_K = \frac{RT}{F} \ln \frac{f_{K_o}[K^+]_o}{f_{K_i}[K^+]_i} \quad (D1)$$

where R, T, F, $[K^+]_o$, and $[K^+]_i$ have the usual significance and f denotes the activity coefficient with subscript identifying the ion and space. If we let r equal the ratio of the K^+ activity coefficient in the external space to that in the internal space, then Eq. (D1) can be transformed to give

$$r = \frac{f_{K_o}}{f_{K_i}} = \frac{[K^+]_i}{[K^+]_o} \exp(V_0 F/RT) \quad (D2)$$

When external K^+ is replaced by Cs^+ at pH_o 7.2, the membrane potential takes on a new value, V_1, which is approximated by the relation

$$V_1 = \frac{RT}{F} \ln \frac{P_{Cs} f_{Cs_o}[Cs^+]_o}{P'_K f_{K_i}[K^+]_i} \quad (D3)$$

where P_{Cs} and P'_K represent the membrane permeability to Cs^+ and K^+, respectively, in the cesium sulfate solution at pH_o 7.2. Although we allow for OH^- permeability in this analysis, the terms involving OH^- are not included in Eq. (D3) since they make negligible contributions when pH_o and pH_i are both about 7.2 as was discussed in connection with Eq. (A1) for the case of H^+. Indeed, the membrane permeability for OH^-, P_{OH}, is smaller by about a factor of five than the estimated value of P_H given by Izutsu (1972). Assuming that the activity coefficients for Cs^+ and K^+ are equal in the external solutions, then Eq. (D3) can be transformed to give the ratio of the Cs^+ permeability to K^+ permeability

$$\frac{P_{Cs}}{P'_K} = \frac{[K^+]_i}{r[Cs^+]_o} \exp(V_1 F/RT) \quad (D4)$$

Equation (D4) permits the calculation of the relative permeability of Cs^+ to K^+ 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. (D2). It is assumed throughout that $[K^+]_i$ = 140 mM (*see* Venosa, 1979).

When the pH_o of the cesium sulfate solution was made alkaline in the experiment illustrated in Fig. 3, the resulting hyperpolarization was clearly the combined effect of OH^- and buffer anion movements

from the external space into the myoplasm. For this situation, the membrane potential can be described by the relation

$$V_2 = \frac{RT}{F} \ln \frac{P_{Cs} f_{Cs_o} [Cs^+]_o}{P'_K f_{K_i} [K^+]_i + P_{OH} f_{OH_o} [OH^-]_o + P_B f_{B_o} [B^-]_o} \quad \text{(D5)}$$

where P_{OH} and P_B are the hydroxide and buffer anion permeabilities, $[OH^-]_o$ and $[B^-]_o$ are the external hydroxide and buffer anion concentrations, and the f's are the activity coefficients of the designated ion in the space denoted by the subscript. The terms associated with the internal concentrations of OH^- and B^- in the numerator of the logarithmic expression are omitted owing to their smallness for the short exposure periods to the alkaline solutions. Dividing both numerator and denominator of the logarithmic expression of Eq. (D5) by $P'_K f_{K_i}$ yields

$$V_2 = \frac{RT}{F} \ln \frac{(P_{Cs}/P'_K) r [Cs^+]_o}{[K^+]_i + R_{OH} [OH^-]_o + R_B [B^-]_o} \quad \text{(D6)}$$

where the following definitions for relative anion permeabilities with respect to P'_K are made

$$\frac{P_{OH} f_{OH_o}}{P'_K f_{K_i}} = R_{OH}, \quad \frac{P_B f_{B_o}}{P'_K f_{K_i}} = R_B. \quad \text{(D7)}$$

When pH_o is kept constant and the buffer concentration is raised from $[B^-]_o$ to $[B^-]_{o+}$, as in the experiment of Fig. 3, the membrane potential takes on a new value V_3 which can be approximated by a relation similar to Eq. (D6)

$$V_3 = \frac{RT}{F} \ln \frac{(P_{Cs}/P'_K) r [Cs^+]_o}{[K^+]_i + R_{OH} [OH^-]_o + R_B [B^-]_{o+}}. \quad \text{(D8)}$$

Equations (D6) and (D8) represent two independent measurements for two separate experimental circumstances and have only two unknowns: R_{OH} and R_B. All concentrations are known or can be calculated, r is obtained from V_0 and (P_{Cs}/P'_K) from V_1 by using Eq. (D2) and Eq. (D4), respectively. By appropriate transformations of Eqs. (D6) and (D8) the following expressions can be obtained for R_β and R_{OH}:

$$R_B = \frac{\{(P_{Cs}/P'_K) r [Cs^+]_o\} \{\exp(-V_3 F/RT) - \exp(-V_2 F/RT)\}}{[B^-]_{o+} - [B^-]_o} \quad \text{(D9)}$$

and

$$R_{OH} = \frac{(P_{Cs}/P'_K) r [Cs^+]_o \exp(-V_2 F/RT) - [K^+]_i - R_B [B^-]_o}{[OH^-]_o}. \quad \text{(D10)}$$