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Response of Chloride Efflux from Skeletal Muscle of *Rana pipiens* to Changes of Temperature and Membrane Potential and Diethylpyrocarbonate Treatment

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Summary. Efflux of $^{36}Cl^-$ from frog sartorius muscles equilibrated in two depolarizing solutions was measured. Cl^- efflux consists of a component present at low pH and a pH-dependent component which increases as external pH increases.

For temperatures between 0 and 20°C, the measured activation energy is 7.5 kcal/mol for Cl^- efflux at pH 5 and 12.6 kcal/mol for the pH-dependent Cl^- efflux. The pH-dependent Cl^- efflux can be described by the relation $u = 1/(1 + 10^{n(pK_a - pH)})$, where u is the Cl^- efflux increment obtained on stepping from pH 5 to the test pH, normalized with respect to the increment obtained on stepping from pH 5 to 8.5 or 9.0. For muscles equilibrated in solutions containing 150 mM KCl plus 120 mM NaCl (internal potential about −15 mV), the apparent pK_a is 6.5 at both 0 and 20°C, and n = 2.5 for 0°C and 1.5 for 20°C. For muscles equilibrated in solutions containing 7.5 mM KCl plus 120 mM NaCl (internal potential about −65 mV), the apparent pK_a at 0°C is 6.9 and n is 1.5. The voltage dependence of the apparent pK_a suggests that the critical pH-sensitive moiety producing the pH-dependent Cl^- efflux is sensitive to the membrane electric field, while the insensitivity to temperature suggests that the apparent heat of ionization of this moiety is zero. The fact that n is greater than 1 suggests that cooperativity between pH-sensitive moieties is involved in determining the Cl^- efflux increment on raising external pH.

The histidine-modifying reagent diethylpyrocarbonate (DEPC) applied at pH 6 reduces the pH-dependent Cl^- efflux according to the relation, efflux = $\exp(-k \cdot [DEPC] \cdot t)$, where t is the exposure time (min) to DEPC at a prepared initial concentration of [DEPC] (mM). At 17°C, k^{-1} = 188 mM · min. For temperatures between 10 and 23°C, k has an apparent Q_{10} of 2.5. The Cl^- efflux inhibitor SCN^- at a concentration of 20 mM substantially retards the reduction of the pH-dependent Cl^- efflux by DEPC. The findings that the apparent pK_a is 6.5 in depolarized muscles, that DEPC eliminates the pH-dependent Cl^- efflux, and that this action is retarded by SCN^- supports the notion that protonation of histidine groups associated with Cl^- channels is the controlling reaction for the pH-dependent Cl^- efflux.

Key Words skeletal muscle · Cl^- efflux · Cl^- channel · pH · muscle membrane · temperature · diethylpyrocarbonate

Introduction

Conclusive demonstration that frog skeletal muscle is permeable to Cl^- as well as to K^+ was provided by Boyle and Conway (1941). For resting muscle in normal physiological salt solutions at an external pH of 7.4 about two-thirds of the membrane conductance is ascribable to Cl^- and one-third to K^+ (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960).

Chloride conductance drops as external pH is decreased with an apparent pK_a near 7 in *Rana temporaria* (Brooks & Hutter, 1962; Hutter & Warner, 1967*c*). Cl^- efflux in skeletal muscle fibers also depends on external pH. On lowering external pH from alkaline values to pH 5 both Cl^- efflux and conductance drop with an indistinguishable pH dependence (Hutter & Warner, 1967*b*,*c*). The magnitude of the Cl^- efflux measured at physiological pHs corresponds to that expected for the measured Cl^- conductance with movements of ions in single file along a chain of sites (Hodgkin & Horowicz, 1959). Recent studies using frog skeletal muscle fibers with patch-clamp methods have demonstrated the presence of Cl^- channels (Woll et al., 1987; Woll & Neumcke, 1987). Hence, it has generally been concluded that the external pH-dependent Cl^- efflux is mainly ascribable to movements through Cl^- channels. Since the pH-dependent Cl^- conductance and tracer efflux both have an apparent pK_a near 7 and are sensitive to Zn^{2+}, Hutter and Warner (1967*a*) suggested that the charge on an imidazole moiety controls Cl^- permeability. Potassium conductance, on the other hand, is insensitive to external pH in the range between 5 and 10 (Hutter & Warner, 1967*c*; Blatz, 1984).

At an external pH of 5, Cl^- may exit muscle fibers in part through a different pathway than at alkaline pHs, since the drop in total Cl^- conductance with decreasing pH is greater than the drop in total Cl^-

efflux (Hutter & Warner, 1967*b*,*c*). The Cl^- efflux at low pH has been interpreted to occur mainly through a Cl^- exchange mechanism, at least in depolarized muscle (Hutter & Warner, 1967*b*; Skydsgaard, 1987). Further, there is evidence that salicylate and other aromatic anions stimulate Cl^- exchange in frog muscle (Venosa, Ruarte & Horowicz, 1972). On the other hand, there is also evidence for a measurable Cl^- conductance in acid solutions which increases as the surface membrane is hyperpolarized (Hutter & Warner, 1972; Warner, 1972; Vaughan & Fong, 1978; Loo, McLarnon & Vaughan, 1981; Kotsias & Horowicz, 1990).

The temperature dependence of ion movements through membranes can provide useful information about the permeation mechanisms involved; in particular, measurements of the effects of temperature are needed to characterize more fully the mechanisms of the Cl^- efflux at pH 5 and the pH-dependent Cl^- efflux in frog skeletal muscle and to permit comparisons to be made between these Cl^- fluxes with Cl^- fluxes in other cells.

For purposes of comparison, one can note that the Cl^- exchange mechanism in erythrocytes at physiological pHs has a very large temperature dependence, corresponding to an activation energy of about 30 kcal/mol in the temperature range 0 to 15°C (Dalmark & Wieth, 1972; Brahm, 1977) and about 20 kcal/mol above 15°C (Brahm, 1977). At pHs near 5, on the other hand, the activation energy for Cl^- exchange in erythrocytes falls to a value below 10 kcal/mol (Q_{10} of about 2). Other characteristics of the Cl^- exchange system such as a high degree of selectivity among monovalent anions and saturation kinetics are also greatly diminished at low pHs (Gunn, Wieth & Tosteson, 1975). If at pH 5 the Cl^- efflux in depolarized skeletal muscle mainly moves through a Cl^- flux mechanism similar to that of erythrocytes at pH 5, one might expect to find a comparable temperature dependence and activation energy.

With regard to the pH-dependent Cl^- efflux, since the pK_a of the imidazole moiety in histidine is temperature dependent ($dpK_a/dT = -0.02\ °C^{-1}$, Greenstein & Winitz, 1961), if the drop in Cl^- conductance when pH is lowered is due to protonation of a histidine residue associated with the Cl^- channel, one might expect a shift of 0.4 in the apparent pK_a of the Cl^- efflux-pH relation when the temperature is changed by 20°C. Shifts in pK_a may also result from changes in membrane potential, if the access or binding of protons to the critical site is voltage dependent.

The experiments to be presented reveal a relatively low temperature dependence for the efflux at pH 5, and no measurable pK_a shift with change in temperature. On the other hand, a shift in apparent pK_a was observed in response to a change in membrane potential.

Another productive approach for testing if a proposed chemical group or residue is involved in a permeation mechanism is to examine the effects of specific covalent alterations of that group. For this reason we have examined the effects of DEPC at pH 6 which specifically carbethoxylates histidyl residues in proteins (Mühlrád, Hegyi & Tóth, 1967; Ovádi, Libor & Elödi, 1967; Mühlrád, Hegyi & Horányi, 1969).

Materials and Methods

The efflux of $^{36}Cl^-$ from sartorius muscles isolated from the frog *Rana pipiens* was measured in these experiments. Both muscles from the same frog were used; in most experiments, one muscle served as a control for the other. Muscles were attached to stainless steel frames and were placed in an isotonic K_2SO_4 solution for 30 min, during which time contractures were completed. Muscles were then loaded with K^+ and Cl^- by a 1- to 2-hr soak in one of the KCl plus NaCl solutions described below at a pH of 7.4. After transient volume changes were over, muscles were placed in a similar KCl plus NaCl solution prepared from neutralized $H^{36}Cl$ (New England Nuclear) for at least another hour. The specific activity of the solutions containing $^{36}Cl^-$ was in the range of 4 to 20 μCi/ml.

$^{36}Cl^-$ efflux was measured by suspending the muscles in a series of tubes containing 4 ml of various (inactive) solutions. Muscles were transferred to new tubes after timed intervals (generally 5 min). The fluid of each tube was mixed in a counting vial with a scintillation cocktail mixture, and the radioactivity was measured in a scintillation counter. The radioactivity remaining in the muscle at the end of the experiment was measured by placing the muscle in distilled water, for 1 hr in one tube and then in a second tube overnight (at least 12 hr), followed by scintillation counting as with the other tubes. Most but not all of the $^{36}Cl^-$ remaining in the muscle appears in the first tube, and the rest appears in the second tube (Venosa et al., 1972).

In earlier experiments the scintillation cocktail mixture contained 50% Scintiverse (Fischer Scientific) and 50% Triton-X (Emulsion Engineering). Sixteen ml of this cocktail was added to each of the 4-ml fluid samples from the muscles. In later experiments the scintillation cocktail was 100% Ecoscint A (National Diagnostics). Twelve ml of this cocktail was added to each of the 4-ml fluid samples from the muscles.

After correction for background, $^{36}Cl^-$ efflux was calculated as the fraction of counts lost from the muscle and expressed as an apparent efflux rate coefficient, k. The average amount of $^{36}Cl^-$ in the muscle during any collection interval was estimated by the sum of the activity found in all the subsequent collection samples and the distilled water samples plus one-half of the activity leaving the muscle during the given collection period. Throughout, the efflux rate coefficient is referred to simply as "Cl^- efflux" and has the units of min^{-1}. In general, efflux rate coefficients in a given solution were taken as the average of the values for two or three collection intervals (10–15 min).

The solutions used in this study are identified by the concentration (in mM) of K^+ and Na^+ (as chloride salts) used in their preparation, for example "150 K^+/120 Na^+ solution" or "7.5

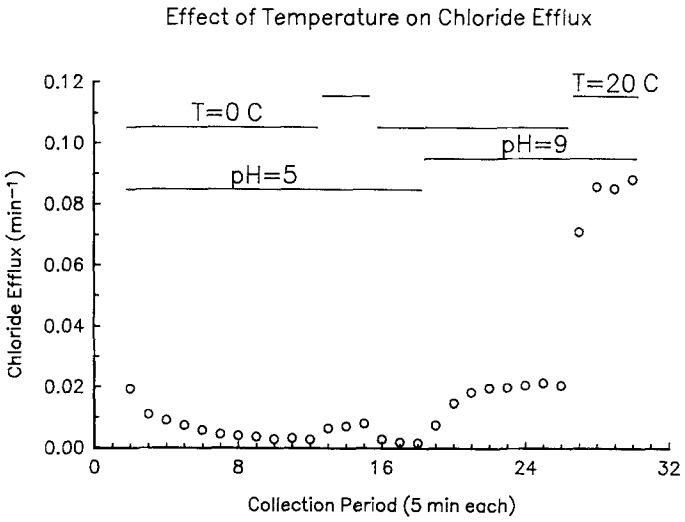

Fig. 1. Increase in Cl efflux in response to increase in temperature from 0 to 20°C for external pH 5 and 9. Description given in text. Exp. ref. CF5H.

K^+/120 Na^+ solution." In addition, solutions contained 1 mM $CaCl_2$ and 5, 10 or 20 mM MES, PIPES, HEPES, $H_2PO_4^-/HPO_4^{2-}$, TAPS, or Tris buffer, depending on the pH of the solution. The pH of each solution was adjusted at the temperature at which it was applied to the muscle. In solutions where NaCl and KCl provide nearly all the osmotically active particles Boyle and Conway (1941) showed that as KCl concentration is varied at constant NaCl concentration the steady-state volumes of frog muscle are constant. For these conditions, the final internal K^+ and Cl^- concentrations increase as external KCl concentration is increased (Boyle & Conway, 1941; Spalding, Swift & Horowicz, 1986). Thus, the fiber volumes equilibrated in 150 K^+/120 Na^+ solution are the same as those equilibrated in 7.5 K^+/120 Na^+ solution and are nearly the same as in normal Ringer's fluid.

All solutions in the collection tubes were equilibrated to the temperature desired using temperature baths prior to being applied to the muscles.

Treatment with DEPC was performed as follows: solutions containing DEPC with $H_2PO_4^-/HPO_4^{2-}$ buffer which had been adjusted to pH 6.0 at the desired temperature were used to fill a series of collection tubes. Just before application to the muscle the tubes were allowed to equilibrate for 25 to 30 min to the preset temperature of the water bath for each muscle to be treated. To minimize loss of reagent to hydrolysis the DEPC was added to the solution immediately before this equilibration period. Since DEPC hydrolyzes spontaneously to ethanol and CO_2 with a half life of about 69 min at 20°C (Fedorcsák & Ehrenberg, 1966), the concentration of DEPC to which muscles were exposed was somewhat less than the initial prepared concentration. In the presentation of the experiments and in the analysis, the initial prepared concentration is used.

Results

Temperature Activation of Acid-Resistant and pH-Dependent Chloride Efflux

The observations to be presented first deal with the effect of temperature on Cl^- efflux from sartorius muscles at pH 5 and at either pH 8.5 or 9. Various protocols, some of which are illustrated in this report, were employed. In most experiments paired muscles from the same frog were used with one muscle acting as a control. In other experiments protocols were devised so that both experimental and control values of Cl^- efflux could be obtained from the same muscle.

An experiment employing a single muscle is illustrated in Fig. 1. In this experiment a muscle was equilibrated in solutions containing 150 mM K^+ and 120 mM Na^+ (150 K^+/120 Na^+). After loading with $^{36}Cl^-$, the muscle was exposed to solutions free of radioactive isotope for the remainder of the experiment. At the start, the muscle was placed in solution at pH 5 and 0°C. During the first seven to eight collection periods of 5 min each, the radioactivity collected gradually decreased[1].

After the efflux became relatively stable, the muscle was placed for 15 min in a pH 5 solution at 20°C. In response, Cl^- efflux increased. When the muscle was returned to 0°C, Cl^- efflux returned promptly to a low value. In this muscle at an exter-

[1] To understand this decline requires some comment. Using sartorius muscles from *R. pipiens* comparable in size to those used in this report and under similar experimental arrangements, diffusion of tracer from the extracellular space has been shown to be essentially complete in 15 min (Venosa, 1974; Venosa & Horowicz, 1981). The further decline in Cl^- efflux over the next 20 to 25 min is likely due to the fact that some individual fibers have efflux rate constants greater than the average of most fibers and the internal $^{36}Cl^-$ is lost more rapidly from these fibers than from the others during the first portion of the efflux at pH 5, 0°C. This effect is discussed in greater detail in connection with the experiment illustrated by Fig. 4.

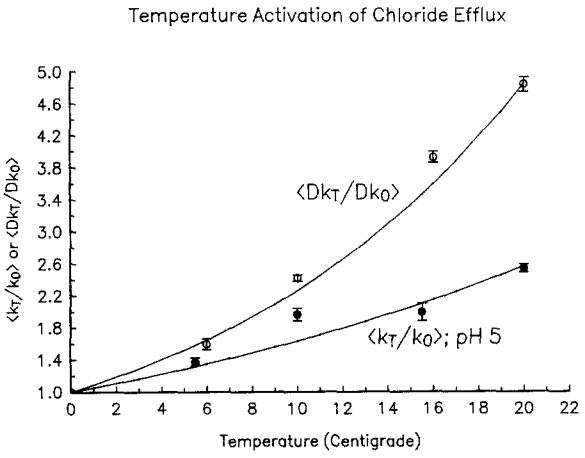

Fig. 2. Summary of temperature dependence of Cl^- efflux at external pH 5 and of Cl^- efflux increment on stepping from pH 5 to 9. Each efflux is normalized to 0°C. Filled circles are k_T(pH = 5)/k_0(pH = 5), where k_T(pH = 5) is the rate coefficient for Cl^- efflux at temperature T and k_0(pH = 5) is the rate coefficient at 0°C, both coefficients measured at an external pH of 5. Open circles are Dk_T/Dk_0, where $Dk_T = k_T$(pH = 9) − k_T(pH = 5) and $Dk_0 = k_0$(pH = 9) − k_0(pH = 5). Curves shown are plots of Eq. (1) with E = 7.52 or 12.6 kcal/mol. Symbols give averages of several experiments. Bars through each symbol give one standard error of the mean (SEM) above and below the mean. The number of determinations based on muscle pairs was 4 for all means below 20°C. For 20°C, 86 and 76 determinations were made for k_T(pH = 5)/k_0(pH = 5) and Dk_T/Dk_0, respectively. Hence, these means heavily weight the average activation energies.

nal pH of 5, the ratio of efflux at 20°C to the average efflux at 0°C was 3.2.

Next, keeping the temperature constant at 0°C, external pH was increased to 9 for 40 min; during this time the Cl^- efflux rose and, after 15 min, became relatively stable. The temperature was raised again to 20°C during the last 20 min of the experiment, producing a large further rise in Cl^- efflux. The ratio of the increment in efflux on going from pH 5 to 9 at 20°C to the increment for the same pH step at 0°C was 4.2 in this muscle. In general, as in this experiment, the pH-dependent Cl^- efflux (measured as the increment on going from pH of 5 to 9) is more sensitive to temperature than is the Cl^- efflux component present at pH 5. Other experiments showed that the effects of raising pH and of raising temperature at high pH are reversible.

Figure 2 gives a summary of the observations on the temperature sensitivity of the two components of Cl^- efflux for several temperatures in the range between 0 and 20°C. In this figure are plotted the Cl^- efflux at pH 5 normalized to 0°C (k_T/k_0) and the normalized increment in Cl^- efflux on going from pH 5 to 9 (Dk_T/Dk_0). The curves are fits of the normalized efflux, y, given by the following form of the Arrhenius equation:

$$y = \exp[ET/(542.8(T + 273.2))] \quad (1)$$

where T is the temperature on the centigrade scale, and E is the activation energy in calories/mole. For Cl^- efflux at pH 5, E is 7.52 kcal/mol and the equivalent Q_{10} is 1.6; for the pH-dependent Cl^- efflux increment, E is 12.6 kcal/mol and the equivalent Q_{10} is 2.3.

These results show that the activation energy of the Cl^- efflux at pH 5 in depolarized frog skeletal muscle is similar to the activation energy of the Cl^- flux in erythrocytes at pH 5.

Effect of Temperature on the Functional Relation of Cl^- Efflux to External pH

We turn now to the effects of temperature on the pH dependence of Cl^- efflux, examining both the apparent pK_a and the steepness of the relation. Since the pK_a of the imidazole group in histidine is temperature dependent ($dpK_a/dT = -0.02\ °C^{-1}$), if the decline in Cl^- efflux at low pH is due to protonation of a histidine moiety associated with the Cl^- channel, one might expect a 20°C increase in temperature to lower the apparent pK_a of the pH-dependent Cl^- efflux by about 0.4 pH units. The steepness of the pH relation is an indicator of the number of titratable groups involved and the cooperativity of the channels mediating pH-dependent Cl^- efflux.

Figure 3 illustrates one protocol used to determine the effect of temperature on the increase of Cl^- efflux produced by raising external pH from pH 5. In each experiment a pair of muscles from the same frog was employed. One muscle was used to determine the maximum increment in Cl^- efflux (providing the basis for normalization) by raising external pH from 5 to 8.5, and the other was used to determine the increment in efflux produced by an intermediate pH (6.7 in Fig. 3). At pH 5, after an initial hour of washout at 0°C (the first 50 min not shown), the temperature of both muscles was raised to 20°C for 15 min and then lowered back to 0°C. This allowed a Q_{20} for the efflux at pH 5 of each muscle to be calculated. Next, keeping the temperature constant at 0°C, external pH was raised for 40 min, one muscle being exposed to pH 8.5, the other to pH 6.7. During this time the efflux from each muscle increased and became relatively constant. For the final 20 min of the experiment the temperature of both muscles was raised to 20°C. It is apparent that the efflux at pH 6.7 did not increase as

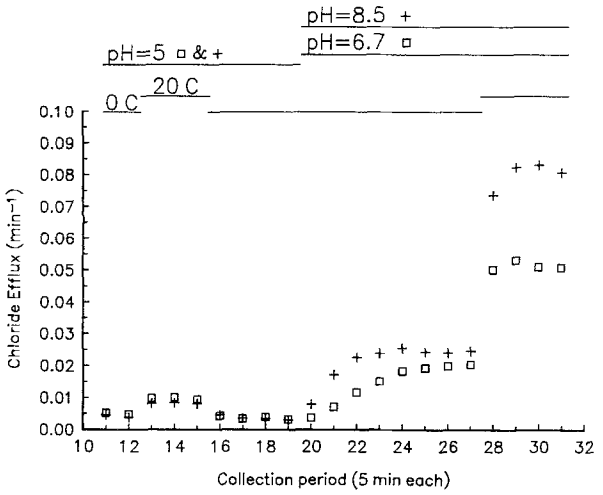

Fig. 3. Effect of temperature on the pH dependence of Cl efflux employing one type of protocol. Symbols give the measured Cl efflux from a pair of muscles for the conditions indicated. Description given in text. Exp. ref. CN9E/F.

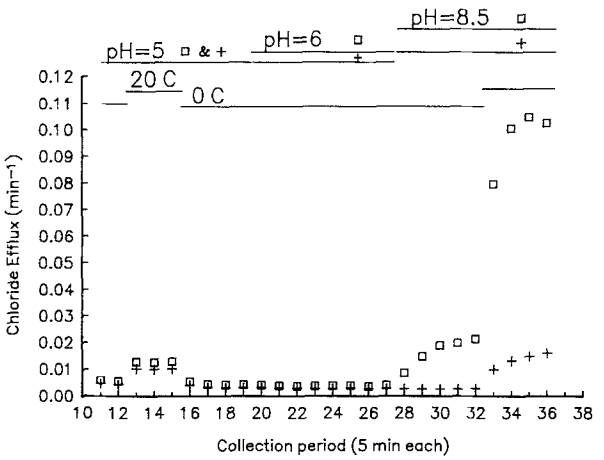

Fig. 4. Effect of temperature on the pH dependence of Cl efflux employing a protocol different from that of Fig. 3. Symbols give the measured Cl efflux from a pair of muscles for the conditions indicated. Description given in text. Exp. ref. CP2A/B.

much as the efflux at 8.5. There is a change in the ratio (increment in Cl^- efflux produced by raising pH from 5 to 6.7)/(increment produced by raising pH from 5 to 8.5) from an average value of 0.76 at 0°C (periods 26 and 27) to an average value of 0.62 at 20°C (periods 29 and 30). The protocol of Fig. 3 was used for test pHs of 6.3 and above.

In order to measure more accurately any small increases in Cl^- efflux at pHs of 5.7 and 6.0 above the baseline efflux at pH 5, a different protocol was used. The rationale for this protocol is that, even in a steady state, Cl^- efflux at constant pH declines slowly throughout long experiments using whole muscle. Sartorius muscles are composed of several hundred fibers each having a slightly different rate constant for efflux. Fibers with more rapid rate constants clear their internal $^{36}Cl^-$ sooner than those with slower rate constants; consequently, the average efflux rate constant from the whole muscle slowly declines with time. If external pH is changed from 5 to a test pH that has little or no effect on the individual fiber efflux rate constants, and at least 30 min is allowed for the test pH to equilibrate the extracellular space and to obtain efflux in the new steady state, this slow decline of efflux from whole muscle may obscure the effect of the test pH. Thus, to make clear the effects of such test pHs it is necessary to utilize a control muscle which remains at pH 5 throughout the period.

Figure 4 shows an experiment using such a protocol. As before, a pair of muscles was used and after an initial washout at pH 5, the effect of an increase from 0 to 20°C was determined. Then, at 0°C, the external pH of one muscle (open symbols) was raised to 6.0 for 40 min (collection periods 20 through 27), while the other muscle was kept at pH 5 (crosses) for use in estimating the decline in baseline Cl^- efflux. At 0°C there was no significant increase of efflux at pH 6. Next, the external pH of the first muscle was increased to 8.5 and that of the second muscle to 6 for the next 25 min (periods 28 through 32). The increment of Cl^- efflux in the first muscle at pH 8.5, 0°C at the end of 25 min was used to normalize efflux measurements in that muscle during the earlier periods (periods 20 through 27). For the last 20 min the temperature was raised to 20°C, keeping the external pH 8.5 in the first muscle, and 6 in the second. The rise in efflux for a comparable temperature increase at pH 6 was greater than at pH 5 (periods 13 through 15). The average normalized increment produced by pH 6 at 20°C was 0.09 and is significantly different from zero. Other experiments (e.g., *see* Figs. 7 and 11*B*) generally reveal an increase in Cl^- efflux for temperatures in the range 17 to 23°C when external pH is raised from 5 to 6.

A summary of the effects of temperature on the relative rates of Cl^- efflux at various external pHs is given in Fig. 5. The midpoint of the relation between Cl^- efflux and external pH (pH = 6.5) is not measurably different at 0 and 20°C for muscles equilibrated in 150 mM K^+/120 mM Na^+. The relation is steeper at 0 than at 20°C: below pH 6.5 the relative Cl^- efflux is greater at 20 than at 0°C and above pH 6.5 it is less. A least-squares fit of the equation

$$\text{relative efflux} = 1/(1 + 10^{n(pK_a - pH)}) \quad (2)$$

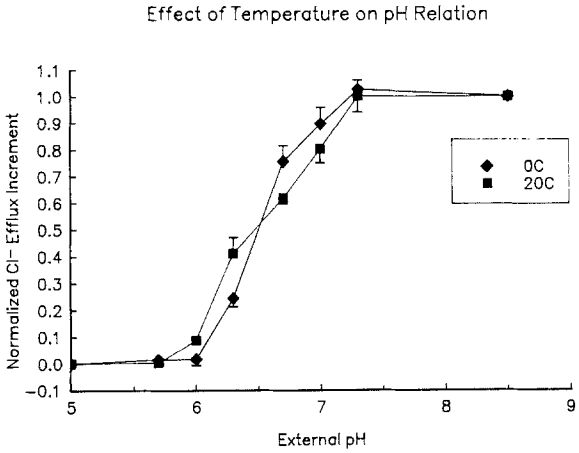

Fig. 5. Relation between normalized Cl efflux increment, N, and external pH for 0 and 20°C. Normalization was done with respect to the Cl^- efflux increment for the step from pH 5 to 8.5 at the appropriate temperature. In symbols, at a fixed temperature $N = \{k(\text{pH} = x) - k(\text{pH} = 5)\}/\{k(\text{pH} = 8.5) - k(\text{pH} = 5)\}$, where $k(\text{pH} = 5)$, $k(\text{pH} = 8.5)$, and $k(\text{pH} = x)$ are the rate coefficients for Cl efflux at external pHs of 5, 8.5 and x, respectively. Filled diamonds give average increments for 0°C; filled squares give average increments for 20°C. For clarity, half-bars give one SEM either above or below the mean depending on symbol. Where half-bars are not apparent, they fall within the size of symbols. Either six or eight determinations using muscle pairs were used for each point.

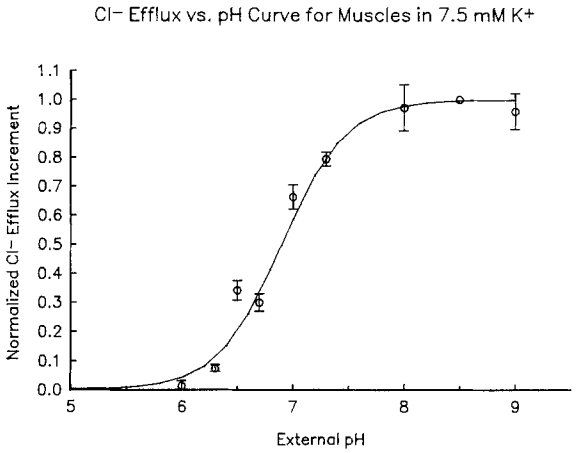

Fig. 6. Relation between normalized Cl efflux increment and external pH at 0°C for muscles equilibrated in solutions containing 7.5 mM KCl and 120 mM NaCl. For normalization procedures and symbol definition, *see* legend of Fig. 5. Curve is drawn according to Eq. (2) with $n = 1.5$ and $pK_a = 6.9$. Each symbol gives the mean of several experiments. Bars through each symbol give one SEM above and below the mean. The number of determinations was 7 for pH 9, 16 for pH 8, and 8 for pHs below 8. All determinations at pH 8.5 were normalized to 1.

to the points in Fig. 5 yields $pK_a = 6.5$ for both temperatures and $n = 2.5$ for 0°C and 1.5 for 20°C.

Relation between Chloride Efflux and External pH for Muscles Equilibrated in Solutions Containing 7.5 mM External Potassium

In addition to temperature, the apparent pK_a of the pH-dependent Cl^- efflux may be influenced by transmembrane potential. If the moiety sensitive to external pH is located within the membrane electric field, then a change in transmembrane potential should shift the midpoint of the relation between Cl^- efflux and external pH. If muscle fibers are equilibrated in solutions with low external K^+ (i.e., less than the 150 mM employed in the experiments above), the internal potential will be more negative and, for any given external pH, the local pH (in the vicinity of the pH-sensitive group) should be lower. Consequently, Cl^- efflux should be lower and the midpoint of the relation between Cl^- efflux and external pH should shift to higher pH.

Experiments were performed on muscle pairs equilibrated in 7.5 mM K^+ and 120 mM Na^+ (membrane potential −65 mV, Hodgkin & Horowicz, 1959) for comparison with muscles equilibrated in 150 mM K^+ and 120 mM Na^+ (membrane potential −15 mV, Spalding et al., 1986) using a protocol similar to that of Fig. 3, but at a constant temperature of 0°C (i.e., the periods at 20°C were omitted). In the initial collection periods, the external pH was 5, and in the final periods, external pH was raised to 8.5 for the control muscle and to a test pH for the other muscle.

The average increment in Cl^- efflux on raising the pH from 5 to various test pHs, normalized to the increment on raising pH to 8.5 is shown in Fig. 6. The curve is calculated from a least-squares fit of Eq. (2) which yields $pK_a = 6.9$ and $n = 1.5$. Comparing Figs. 5 and 6 shows a midpoint shift from pH 6.5 to 6.9 when external K^+ was lowered from 150 to 7.5 mM. This supports the notion that the critical pH-sensitive moiety producing the pH-dependent Cl^- efflux is located within the membrane field.

It is important to recall that the NaCl concentration was the same (120 mM) for the equilibating solutions containing 7.5 mM KCl and 150 mM KCl. For this condition, Boyle and Conway (1941) demonstrated that the steady-state volume of frog muscles is the same in the two solutions because the NaCl concentration is the same.

Properties of Cl^- Efflux after Exposure to Diethylpyrocarbonate

Since the apparent pK_a of the external pH-dependent Cl^- efflux is in the range of 6.5 to 7.0, it has

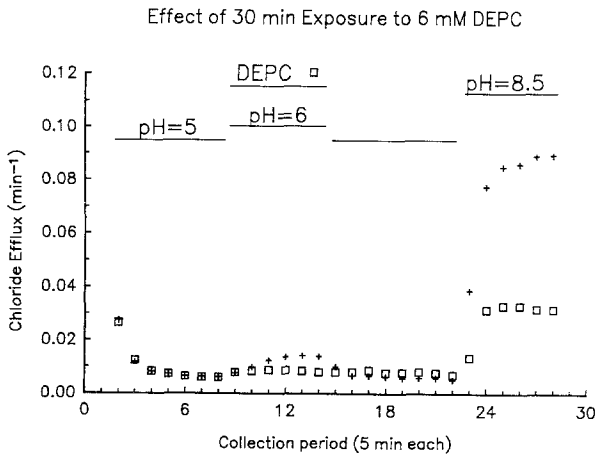

Fig. 7. Effect on Cl efflux of 30-min exposure to 6 mM DEPC at 17°C. Symbols give the measured Cl efflux from a pair of sartorius muscles. Exp. ref. CT3N/O.

been suggested that protonation of histidine residues may be the key reaction underlying this dependence (Hutter & Warner, 1967*c*). To further test this notion, we examined the effects of treating muscles with diethylpyrocarbonate (DEPC) at pH 6, which specifically carbethoxylates histidyl residues in proteins (Mühlrád et al., 1967; Ovádi et al., 1967; Mühlrád et al., 1969).

Information about the kinetic features of the effects produced by DEPC was obtained by varying exposure times at fixed initial DEPC concentrations as well as by varying initial DEPC concentration at a fixed exposure time. Figure 7 gives a representative experiment showing the inhibition of Cl^- efflux produced by exposure to DEPC at a nominal concentration of 6 mM for 30 min, at 17°C.

When pH was changed from 5 to 6 in the presence of DEPC, Cl^- efflux increased less than in the control muscle which was not exposed to DEPC. After exposure to DEPC the muscles were returned to the initial pH solution for 40 min in order to obtain a baseline Cl^- efflux. The Cl^- efflux for the DEPC-treated muscles during this washout at pH 5 was slightly higher than in the control muscle. (Note that the points for the DEPC-treated muscle were consistently higher than the points for the control, untreated muscle after exposure to DEPC.) In the final 30 min the external pH was raised to 8.5; it is clear that the increment in Cl^- efflux for the DEPC-treated muscle was less than in the untreated muscle. In other experiments, a comparison of these final efflux increments on switching external pH from 5 to 8.5 showed that for a 30-min exposure the pH-dependent efflux was more inhibited after exposure to 6 mM DEPC than to 3 mM DEPC, and that for 3 mM DEPC the pH-dependent Cl^- efflux was more inhibited after a 60-min exposure interval than after 30 min.

The progressive decline of the uninhibited fraction of the pH-dependent Cl^- efflux with increasing exposure time to 3 mM DEPC is shown in Fig. 8*A*. The calculated curve shown, which approximates the averaged data from several experiments, is given by the exponential function, $u = \exp[-t/(63 \text{ min})]$, where t is the exposure time to 3 mM DEPC.

For a constant exposure time of 30 min, the uninhibited pH-dependent efflux declined monotonically as the initial DEPC concentration applied was increased. The averaged values obtained for several DEPC concentrations in the range from 0.75 to 12 mM is shown in Fig. 8*B* (squares). The calculated curve is given by the function, $u = \exp\{-[\text{DEPC}]/(6 \text{ mM})\}$, where [DEPC] is the initial DEPC concentration. (The data with 20-mM SCN^- will be described later.)

Since the inhibition of the pH-dependent Cl^- efflux with either variable exposure time or with variable DEPC concentration can be approximated by simple exponential functions, all the results were pooled and expressed in terms of the product [DEPC] · (exposure time). For example, all the inhibitions with [DEPC] · (exposure time) = 45 mM · min were averaged together. The outcome of this analysis is displayed in Fig. 9. It is clear that the average pooled inhibitions are well approximated by the exponential function

$$u = \exp\{-k \cdot [\text{DEPC}] \cdot (\text{exposure time})\} \quad (3)$$

where $k^{-1} = 188$ mM · min. Beyond 360 mM · min, Cl^- efflux cannot be accurately measured because at long exposure times or high DEPC concentrations the efflux at pH 5 becomes large and, more importantly, unstable.

The small increments in Cl^- efflux at pH 5 after DEPC treatment (Fig. 7, periods 15–22) were somewhat variable. The correlation between the (DEPC treated/untreated) ratio of Cl^- efflux at pH 5 and the uninhibited fraction of pH-dependent efflux is given in Fig. 10 for all experiments employing DEPC. In most cases, when the uninhibited fraction of the pH-dependent efflux is greater than 0.5, Cl^- efflux at pH 5 is relatively little affected. However, when the pH-dependent efflux is more than half-inhibited, there are sizeable increases in Cl^- efflux at pH 5 after DEPC in most cases. The explanation for the increase in Cl^- efflux at pH 5 after exposure to DEPC at high concentrations and long intervals is unclear, but one possibility is the development of a nonspecific leak.

Experiments with DEPC were performed at 10 and 23°C for comparison with the effects obtained

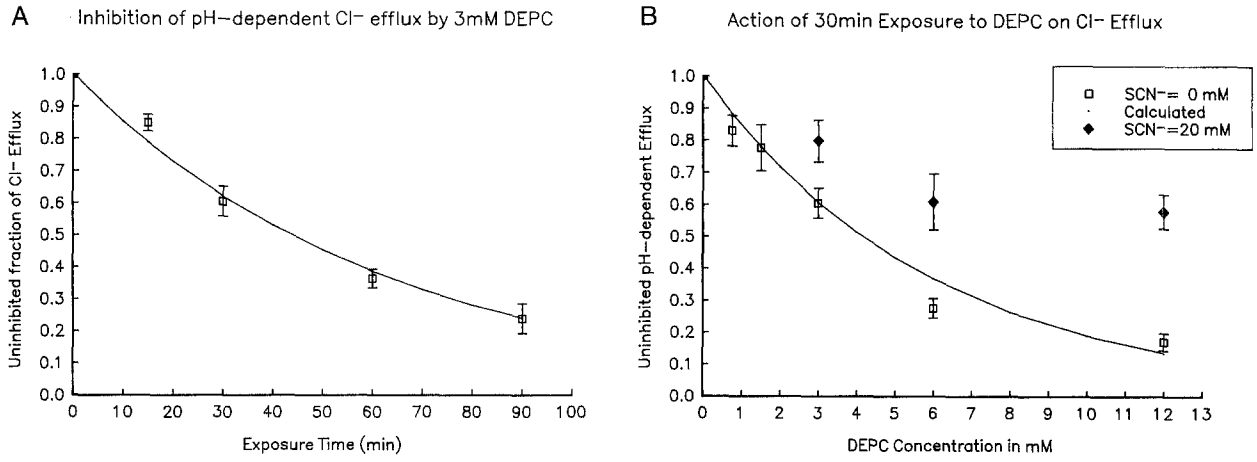

Fig. 8. Effect on the uninhibited fraction of the pH-dependent Cl efflux of exposure time at 3 mM DEPC (*A*) and of nominal DEPC concentration at an exposure time of 30 min (*B*). Experiments performed at 17°C. A typical protocol employed is given in Fig. 7. The uninhibited fraction is taken as the increment in Cl^- efflux produced by raising external pH from 5 to 8.5 in a muscle previously exposed to 3 mM DEPC, normalized to the Cl^- efflux increment produced in the control muscle not exposed to DEPC (*see* last 30 min of efflux measurements on paired muscles in Fig. 7). Symbols give means of several experiments, ranging from 4 to 10. For *A*, curve drawn according to equation $u = \exp[-t/63 \text{ min}]$, where t is exposure time to 3 mM DEPC. For *B*, curve drawn according to equation $u = \exp\{-[\text{DEPC}]/6 \text{ mM}\}$, where [DEPC] is the DEPC concentration. Filled diamonds in *B* denote means for DEPC-treated muscles in the presence of 20 mM SCN^- (*see* Fig. 11*A* for typical experiment). Bars through symbols give one SEM above and below the mean.

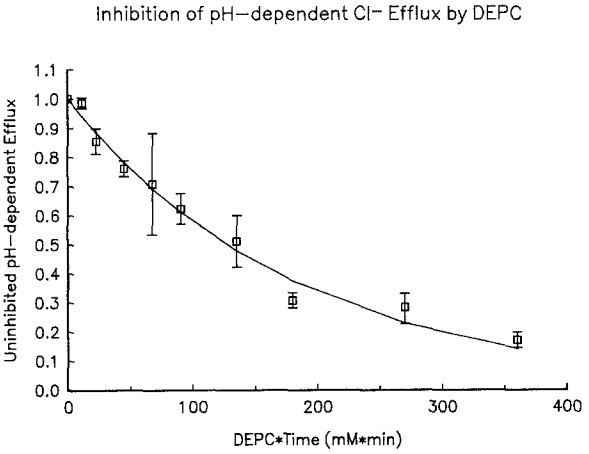

Fig. 9. Inhibition of pH-dependent Cl^- efflux expressed in terms of the product [DEPC] · (exposure time), where [DEPC] is given in units of mM and exposure time in min. Temperature was 17°C. *See* text for details. Curve is drawn according to equation $u = \exp\{-k \cdot [\text{DEPC}] \cdot (\text{exposure time})\}$, where $k^{-1} = 188$ mM · min. Symbols give means; bars give one SEM above and below the means. The number of measurements included in each mean varied between 4 and 13.

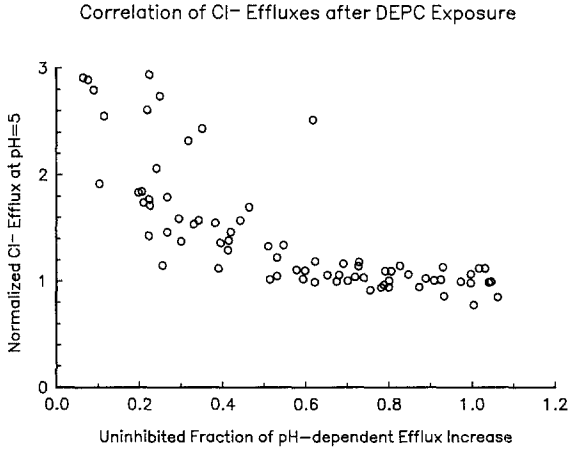

Fig. 10. Correlation between the Cl^- efflux at pH 5 in DEPC-treated muscle (normalized to that in paired untreated muscle) and the uninhibited fraction of pH-dependent Cl^- efflux for each muscle treated with DEPC. Temperature of experiments was 17°C.

at 17°C. Lowering the temperature decreases the rate at which DEPC inhibits the pH-dependent Cl^- efflux, whereas raising the temperature increases it. The average DEPC 'rate constants'—that is, k of Eq. (3)—were determined and could be fit by the Arrhenius equation with an activation energy of 13.94 kcal/mol. The equivalent Q_{10} is 2.5.

Reduction of Diethylpyrocarbonate Effects in Presence of SCN^-

In order to define further the location of the groups with which DEPC interacts to inhibit the pH-dependent Cl^- efflux, the effect of the Cl^- channel blocker SCN^- on the inhibition produced by DEPC was examined. Figure 11*A* gives an experiment which compares the efflux increment when external pH is

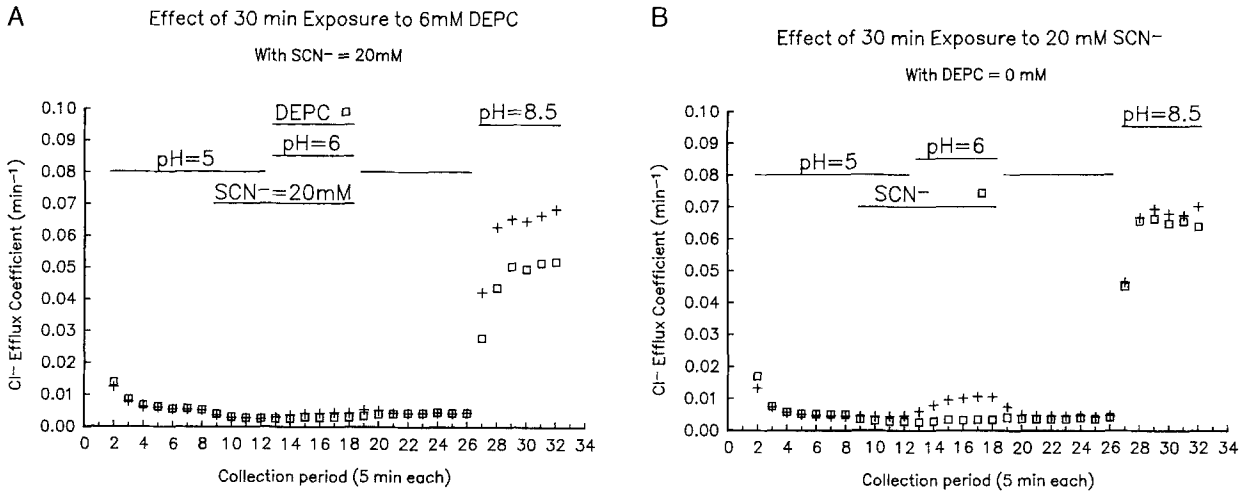

Fig. 11. Effect of 20 mM SCN^- on Cl^- efflux with and without DEPC treatment at a temperature of 17°C. (*A*) The measured Cl efflux from a pair of sartorius muscles exposed to 20 mM SCN as indicated. During the last 30 min of exposure to SCN (collection periods 13 through 18), one muscle was exposed to 6 mM [DEPC] at pH 6 while the other was exposed to pH 6 with no added DEPC. Exp. ref. CV3G/H. (*B*) The Cl efflux from a muscle pair only one of which was exposed to 20 mM SCN . For collection periods 13 through 18 both muscles of each pair were exposed to pH 6. Exp. ref. CV5N/O. *See* text for description of aims and conclusions.

changed from 5 to 8.5 in a muscle treated with DEPC (6 mM for 30 min) in the presence of 20 mM SCN^- with that in a muscle exposed to SCN^- alone. After the initial washout in solutions at pH 5, both muscles were exposed to 20 mM SCN^- at pH 5 for 20 min during which time the Cl^- efflux declined and stabilized at lower values. Maintaining [SCN^-] constant, external pH was then raised to 6 for 30 min, while DEPC was applied at 6 mM to one muscle; a small increase in Cl^- efflux occurred during this interval. Then both muscles were returned to pH 5 solution free of SCN^- and DEPC for 40 min. During the final 30 min of the experiment the external pH was raised to 8.5. In this experiment and others like it, the DEPC treatment inhibited the increment in Cl^- efflux at pH 8.5 less when SCN^- was present during application of DEPC; for example, compare Fig. 11*A* with Fig. 7. The protection given by 20 mM SCN^- for three concentrations of DEPC is illustrated in Fig. 8*B*.

As a control, Figure 11*B* shows that 20 mM SCN^- alone inhibits the Cl^- efflux increase produced by raising external pH to 6, and that after washing for 40 min in pH 5 solutions free of SCN^-, the Cl^- efflux increment on raising pH to 8.5 was nearly equal to that of muscles not exposed to SCN^-. For comparison, when external pH is raised from 5 to 8.5 while the muscle is still exposed to 20 mM SCN^-, the increment of Cl^- efflux is inhibited, on average, by 80%; i.e., the uninhibited fraction is about 0.2 (*unpublished observations*).

The protection against DEPC afforded by SCN^-, a blocker of the pH-dependent Cl^- channel, is consistent with the notion that the moiety reacting with DEPC to produce the inhibition is associated with the Cl^- channel.

Discussion

It is useful to consider first the evidence for the notion that protonation of an imidazole group, presumably on a histidine residue associated with the Cl^- channel, controls the pH dependence of Cl^- efflux. First, the apparent pK_a of the pH-dependent component of Cl^- efflux (and conductance) is between 6.5 (at internal potential −15 mV) and 6.9 (at internal potential −65 mV) which suggests an imidazole group of histidine. For most free amino acids in solution, the pK_as of alpha and beta carboxyl groups have values of 2.1 ± 0.3 and 4.0 ± 0.2, respectively, and amino groups have values of 9.8 ± 0.7. The pK_as of SH groups have values of 8.5 ± 0.2; the phenolic hydroxyl group of tyrosine has a pK_a of 10 and the guanidine group of arginine has a pK_a of about 12.5. For tabulations of pK_a values *see* Greenstein and Winitz (1961) or Dugas and Penney (1981). Since histidine is the only common amino acid that contains a group with a pK_a near 6.0 it seems the most likely candidate. The studies presented in this report which show that exposure to DEPC at pH 6, conditions specific for carbethoxylation of the *N−1* nitrogen of the imidazole group, can almost completely eliminate the pH-dependent efflux strongly favor the involvement of a histidine residue. Finally, the partial protection

against DEPC action by SCN^-, a Cl^- channel blocker, is consistent with the conjecture that the relevant histidine is associated with the Cl^- channel. All these facts make a case for the notion that protonation of a histidine residue is a controlling factor for the Cl^- channel in frog skeletal muscle.

The only evidence against such a view is that the apparent pK_a is not measurably temperature dependent. The pK_a of the imidazole group of histidine in solution changes by about 0.4 units for a 20°C change (Greenstein & Winitz, 1961). The explanation for this discrepancy is not evident. Presumably the molecular environment of the relevant histidine residue is such that the apparent heat of ionization of the imidazole group is zero (compared to about 7 kcal/mol for free histidine in solution). In this regard, it is of interest to note that the apparent pK_a of the acid inhibition of the Cl^- exchange system in human red cells (about 6.1) is unchanged between 0 and 38°C (Brahm, 1977).

A few kinetic features of the DEPC-modification reaction can be noted. Between pH 5 and 8.5, the time constant for the breakdown of the carbethoxylated product is well over 24 hr (Melchior & Fahrney, 1970); therefore, for the time intervals and pHs employed there was negligible breakdown of product. There is a contrast, however, between the slow rate of reaction with DEPC in muscles as assessed by Cl^- efflux and the rapidity of reaction of DEPC with imidazole or histidine-containing proteins in solution. These reactions are reported to be complete within minutes or even seconds (Ovádi et al., 1967; Melchior & Fahrney, 1970). The long apparent time constants (*see* Figs. 8 and 9) and the apparent Q_{10} (*see* Fig. 11) of the effects of DEPC seem, at least superficially, too large to be ascribable to simple diffusion delays in the extracellular space. Although net diffusion may be slowed by the presence of various extracellular proteins with undetermined histidine content, which may react with DEPC along the diffusion pathways, and by spontaneous hydrolysis of DEPC to ethanol and CO_2 (estimated half life of about 69 min at 20°C and 25 min at 25°C, Mühlrád et al., 1967; Melchior & Fahrney, 1970), the relatively high Q_{10} and the slow kinetics of the DEPC effect suggest that a significant activation barrier limits the access of external DEPC to Cl^- channel reaction sites.

If one accepts that protonation of histidine residues associated with Cl^- channels accounts for the decline in Cl^- efflux when pH is reduced, there is evidence from the steepness of the pH-response curves that more than one histidine group is involved in the transition (*see* Fig. 5). The Hill coefficient (n) for the observed curves is about 1.5 at 20°C and 2.5 at 0°C. The pH response relation is probably steeper than that measured in our experiments since sartorius muscles contain several hundred single fibers, and it is likely that there is dispersion of the apparent pK_a and steepness of the relation between individual fibers.

As noted in the results section, the apparent pK_a of the pH-response relation is 6.5 in muscles equilibrated in 150 mM K^+ (membrane potential about −15 mV), but is 6.9 in muscles equilibrated in 7.5 mM (membrane potential about −65 mV). This shift suggests that the residues controlling the pH-response relation are located within the membrane field. The fraction of the membrane potential sensed by the control residues is uncertain, even if one neglects the possibility that more than one control residue may be involved, since if external H^+ has access to the control residue through the channel pore, which is connected to the myoplasm, then the local $[H^+]$ in the vicinity of the control residue will be a function of both internal and external $[H^+]$, as well as the local potential. Internal $[H^+]$ is generally not in equilibrium with external $[H^+]$ and changes only very slowly in response to changes in external $[H^+]$ when impermeable buffers are used (Bolton & Vaughan-Jones, 1977).

Finally, between 0 and 20°C the measured activation energy is 7.5 kcal/mol for the Cl^- efflux at pH 5 and 12.6 kcal/mol for the pH-dependent Cl^- efflux. The activation energy for Cl^- efflux in depolarized frog muscle at pH 5 is comparable to that of Cl^- exchange in human red cells near pH 5 measured between 0 and 10°C (Gunn et al., 1975). In this regard it is worth noting that Gunn et al. (1975) concluded that the Cl^- flux near pH 5 in human red cells is due to a different transport mechanism than the Cl^- exchange system near pH 7.4 since several properties, in addition to temperature dependence, of the Cl^- fluxes for the two pH regions are very different. The fact that the pH-dependent Cl^- efflux in frog muscle has an activation energy of 12.6 kcal/mol, which is much lower than the activation energy of 30 kcal/mol for Cl^- exchange in red cells at pHs between 7 and 8 (Dalmark & Wieth, 1972; Brahm, 1977), is consistent with the view that a Cl^- exchange system contributes very little to the Cl^- efflux at high pH in frog muscle. This is compatible with the conclusion generally drawn from published findings that the pH-dependent Cl^- efflux in frog muscle is mainly ascribable to the measured pH-dependent Cl^- membrane conductance.

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