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The Temperature Dependence of Intracellular pH in Isolated Frog Skeletal Muscle: Lessons Concerning the Na^+-H^+ Exchanger

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Abstract. We used ^{31}P NMR to investigate the temperature-dependence of intracellular pH (pH_i) in isolated frog skeletal muscles. We found that $ln[H^+_i]$ is a linear function of $1/T_{abs}$ paralleling those of neutral water (i.e., $H^+ = OH^-$) and of a solution containing the fixed pH buffers of frog muscle cytosol. This classical van't Hoff relationship was unaffected by inhibition of glycolysis and was not dependent upon the pH or $[Na^+]$ in the bathing solution. Insulin stimulation of Na^+-H^+ exchange shifted the intercept in the alkaline direction but had no effect on the slope. Acid loading followed by washout resulted in an amiloride-sensitive return to the (temperature dependent) basal pH_i.

These results show that the temperature dependence of activation of Na^+-H^+ exchange is similar to that of the intracellular buffers, and suggest that constancy of $[H^+]/[OH^-]$ with changing temperature is achieved in the short term by intracellular buffering and in the long term by the set-point of the Na^+-H^+ exchanger. Proton activation of the exchanger has an apparent standard enthalpy change ($\Delta H°$) under both control and insulin-stimulated conditions that is similar to the $\Delta H°$ of the intracellular buffers and approximately half of the $\Delta H°$ for the dissociation of water. Thus, the temperature-dependent component of the standard free-energy change ($\Delta F°$) is unaffected by insulin stimulation, suggesting that changes in Arrhenius activation energy (E_a) may not be a part of the mechanism of hormone stimulation.

Key words: ^{31}P NMR spectroscopy — Frog muscle — pH_i regulation — Na^+-H^+ exchange — Temperature — Enthalpy

Introduction

Living cells regulate their intracellular hydrogen ion concentration ($[H^+]_i$) at a value far removed from electrochemical equilibrium. When temperature changes, the pH of most cells of most organisms, including plant (Aducci et al., 1982) and animal (Roos & Boron, 1981) species also changes, such that pH_i becomes 0.15 to 0.2 pH units more alkaline with every 10°C decrease. This behavior parallels the temperature dependence of the dissociation of water and also the pKs of fixed intracellular buffers, primarily protein histidines (Reeves & Malan, 1976). At any given temperature, therefore, pH_i of cells that behave in this manner is regulated near to neutrality (i.e., $[H^+] = [OH^-]$) as temperature is varied. This has been termed the α-stat hypothesis (Reeves, 1972), since a constant α-imidazole (i.e., the ratio of unprotonated to total protein histidine imidazole), is maintained.

This study concerns the mechanism of α-stat regulation of intracellular pH. Because protons are constantly being produced by metabolism and extruded from the cell by energy-requiring ion-exchangers, the temperature dependence of the intracellular buffers cannot by itself explain the temperature dependence of pH_i. It has therefore been argued that the physiological set-point (i.e., the regulated pH value) for the Na^+-H^+ exchanger, as well as for other pH regulating proteins, is at a constant alkaline deviation from neutral pH (Nattie, 1990). This hypothesis is attractive since it implies a functional integration of pH buffers and active proton extrusion across the cell membrane, which tends to maintain constant $[H^+]/[OH^-]$ and thus a constant charge state of proteins as temperature is varied.

The hypothesis that the activity of a membrane pro-

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ton transporter regulates $[H^+]/[OH^-]$ does, however, have thermodynamic consequences. Over the physiological temperature range, a classical van't Hoff plot of either *ln* or log $\{[H^+] \times [OH^-]\}$ of neutral water *vs.* $1/T_{abs}$ is approximately linear, with a slope proportional to the standard enthalpy change ($\Delta H°$) and an intercept proportional to the standard entropy change ($\Delta S°$) for the dissociation of water. If energetic relationships are to be maintained, regulation of pH_i to a constant deviation from neutrality requires that the reactions that determine pH_i have a similar dependence on temperature, both under control and hormone-stimulated conditions.

To test the hypothesis that pH_i is regulated to a constant deviation from neutrality via α-stat activation of an exchanger, we used ^{31}P NMR to measure the effect of temperature on pH_i of sartorius and gastrocnemius of *Rana temporaria* and *Rana pipiens* under control conditions and while undergoing maneuvers that affect proton production and extrusion. We found that acid loading followed by washout resulted in an amiloride-sensitive return to the temperature dependent basal pH_i. In addition, we were able to confirm that the van't Hoff values for $\Delta H°$ for the intracellular buffers and for transmembrane H^+-extrusion are similar to each other and approximately 1/2 the $\Delta H°$ for the dissociation of water. These results are independent of metabolic rate, of the overall driving force for proton extrusion and of hormone-stimulation. Stimulation of Na^+-H^+ exchange with insulin resulted in intracellular alkalization, the magnitude of which was temperature independent. Thus, the temperature-dependent component of the standard free-energy change ($\Delta F° = \Delta H° - T\,\Delta S°$) is unaffected by insulin stimulation, and changes in Arrhenius activation energy ($E_a = \Delta H° + RT$) may not be a part of the mechanism of hormone stimulation.

These results are consistent with α-stat regulation of the exchanger, and with temperature-independent mechanisms of proton-activation and hormone stimulation. Various aspects of this work have been presented previously in preliminary form (Dawson & Elliott, 1985; Marjanovic et al., 1994).

Materials and Methods

Calibration Solutions and pH Measurements

Solutions of composition shown in Table 1 were made up with double glass-distilled water using analytical reagent grade chemicals obtained from Sigma or British Drug Houses (UK). Magnesium chloride (0.1 or 1 M stock solution) was standardized by titrating Mg^{2+} with EDTA (ethylenediamine-N,N,N′,N′-tetracetic acid) using Solochrome Black as an indicator, or by titrating Cl^- using a commercially available assay kit (Sigma). The pH of the solutions was adjusted using 1 M HCl or KOH.

In two titration experiments, where free $[Mg^{2+}]$ was maintained constant at 3 or 5 mM, the solutions were initially adjusted to a pH of approximately 4.0 and then titrated with KOH, adding enough 0.1 M $MgCl_2$ to keep the free $[Mg^{2+}]$ constant as the pH was raised. The amount of $MgCl_2$ required was calculated from the total concentrations of the ions present, the pH and the association (stability) constants for the possible complexes of phosphocreatine (PCr) and inorganic phosphate (P_i) with H^+, K^+ and Mg^{2+}. The ligand stability constants were taken from Smith and Alberty (1956*a*,*b*).

A pH-sensitive glass electrode (Corning) was used together with a separate KCl/calomel reference electrode with a ground-glass sleeve liquid junction (Pye-Ingold). This electrode combination was used to avoid errors in pH measurement of the type described by Illingworth (1981) for combination pH electrodes, and was found to be free from errors when tested by the method of Illingworth (1981). The pH of each solution was measured before and after acquiring NMR data. Measurements of pH at 4°C were carried out in a cold room, and measurements at 37°C were carried out with the titration solution maintained in a water bath. The pH electrode was calibrated at the experimental temperature with standard buffers (Corning).

Tissue Preparation and Maintenance

This protocol was approved by the Laboratory Animal Care Advisory Committee of the University of Illinois, Urbana-Champaign. Pairs of gastrocnemius or sartorius muscles were dissected from pithed *R. temporaria* or *R. pipiens* in a cold (4–6°C) moist environment and mounted in an experimental chamber (as described by Dawson, Gadian and Wilkie, 1977) fitted within a 20-mm diameter NMR sample tube. The muscles were bathed in oxygenated Ringer's solution (mM: NaCl, 105; KCl, 2.5; $CaCl_2$, 2; PIPES [piperazine-N,N′-bis-2-ethanesulphonic acid], 10) during dissection and throughout the experiment. The pH was adjusted to pH 7.2 at room temperature with HCl and the solution was equilibrated with 100% O_2. PIPES was chosen as the buffer because it contains no phosphate and because the change of its pK with temperature $(-0.0085$ pH units $(°C)^{-1}$; Good et al., 1966) is similar to that of physiological buffers.

Temperature was changed by replacing the Ringer's solution with solution previously equilibrated at or near the temperature required, and by adjusting the thermostat of the NMR probe. Since the probe thermostat senses the temperature of the air near the sample, but not of the sample itself, a calibration curve was constructed using Ringer's solution and a thermocouple in the NMR sample tube. Data acquisition was begun when the probe temperature had stabilized. The temperature control of the NMR probe is accurate to 0.1°C and the steady-state temperature reading varied by no more than 0.3°C.

NMR Spectroscopy

^{31}P NMR spectra were recorded either on a Bruker WM-200 spectrometer operating at 81 MHz (15-μsec pulses at 2-sec intervals, 8000 data points and 5000 Hz spectral width) or on a GN-300 wide-bore spectrometer (General Electric, now Bruker) operating at 121 MHz (12 μsec pulses at 0.5-sec intervals, 8,000 data points, and 4,000 Hz spectral width). Exponential line broadening of 1–5 Hz was applied.

In the studies of calibration solutions, chemical shifts were referenced to that of PCr at pH 7.0, either in the titration solution or in a separate solution containing 0.14 M KCl, 20 mM PCr, 10 mM PIPES and 0–8 mM total $MgCl_2$, so as to yield a free $[Mg^{2+}]$ of 0, 3 or 5 mM. The chemical shift of PCr changed by only 0.07 ppm when the free $[Mg^{2+}]$ was raised from 0 to 5 mM, and was unaffected by changing the temperature over the range 4°–37°C or by variations in ionic strength between 0.15–0.25 M.

In the muscle studies, two to six hundred scans were collected for

each spectrum, requiring 7 to 20 min. Data collection was continued until the P_i peak, which is very small in resting aerobic muscle, could be clearly distinguished. The pH_i was calculated using the calibration curves determined in the present study for solutions containing 5 mM total magnesium at 4°, 21° or 37°C, with interpolation from these curves when the experimental temperature differed from the calibration temperatures.

Construction of the pH Calibration Curves

Calibration curves were constructed from plots of pH, determined as described above, *vs.* chemical shift of P_i (Fig. 1). Henderson-Hasselbalch titration curves for a single ionization were fitted to the experimental data using an iterative nonlinear least squares procedure which minimized the sum of squared deviations of the observed *vs.* the calculated end points. Using the calibration curves thus obtained, the pH_i of muscle was calculated from

$$pH_i = pK + \log \frac{\delta - \delta_A}{\delta_B - \delta}, \quad (1)$$

where K is the apparent proton association constant; δ, δ_A and δ_B are the observed and limiting chemical shifts of P at acidic and basic pH respectively, representing the apparent chemical shifts of $H_2PO_4^-$ and HPO_4^{2-}.

Tissue Experiments

Pairs of gastrocnemius muscles were used to define the relationship between temperature and pH_i. Initial spectra were always obtained at 5°C, and subsequent spectra were recorded when the muscles were equilibrated with Ringer's solution at a series of higher temperatures (5°–37°C) in random order. Final spectra were again obtained at 5°C. If pH_i differed by more than 0.1 pH units in the initial and final spectra, the experiment was excluded from the analysis.

To equilibrate the solutions within the intracellular spaces by diffusion, small, paired sartorius muscles were used for studies of the effects of drugs or of manipulations of the bathing solution. One muscle from each frog was used at 5°C while the paired muscle was used at 21°C. Four sartorii were used at the same time. In some experiments, glycolysis was inhibited with Ringer's solution containing 1 mM iodoacetic acid (IAA) adjusted to pH = 7.2 (added from a 0.1 M stock solution which was kept frozen) at room temperature for 20 min; IAA was then removed and the temperature was adjusted to either 5°C or 21°C for the pH_i measurement. This procedure blocks glycolysis effectively, as judged by the fact that exposure of muscle to 2 mM sodium cyanide (NaCN) and 10 mM caffeine, which ordinarily leads to a large intracellular acidosis, did not cause acidification of pH_i in muscles which had been treated with IAA, and by the fact that a lactate methyl peak is ordinarily observed in the ^1H spectrum of caffeine-treated muscles (Shen, Gregory & Dawson, 1996), but is not present following this protocol for IAA treatment.

The effect of external pH on pH_i was investigated by placing paired sartorius muscles in the standard Ringer's solution adjusted to the same pH at different temperatures (pH = 7.2 at 5°C and 21°C) or with the pH varying so that $[H^+]/[OH^-]$ remained constant (pH = 7.2 at 5°C and pH = 7.4 at 21°C). A similar protocol was used to study the effects of variation in external sodium. After measuring pH_i in the standard bathing solution at either 5° or 21°C, muscles were placed in solution in which the Na^+ was replaced by an equal concentration of N-methyl-D-glucamine (NMDG), and the pH_i measurement was repeated.

In other experiments Na^+-H^+ exchange was stimulated in the presence of insulin. The pH_i was determined first under control conditions at the experimental temperature and then the muscles were exposed to 2 μM insulin (Moore, 1981). After 30 min of incubation at 21°C, muscles were returned to the experimental temperature and the pH_i was again measured. The temperature dependence of pH_i recovery from an acid load was measured by exposing frog sartorius muscles to 20 mM NH_4Cl for 45 min in the absence and presence of 1 mM amiloride at both 5° and 21°C, as described by Putnam, Roos & Wilding (1986).

Data Analysis

To distinguish between monophasically linear and biphasic curves, multiple regression analysis (Weisberg, 1985) was used to test for the presence of two linear segments:

$$y = y_1 + y_2 = (a_1 + b_1x) + (a_2 + b_2x). \quad (2)$$

To do this, the data were divided between two limbs at each interval along the x-axis, between experimentally obtained points. The case producing the least sum of squares of the deviation was then tested to determine whether a_1, b_1 were significantly different from a_2, b_2. If the two limbs produced significantly different values ($P < 0.05$), the biphasic solution was accepted. If not, the plot was treated as being monophasic, with single values for a and b.

Results

Construction of pH Calibration Curves

We first calibrated the pH measurement at different temperatures, in order to establish the accuracy of the method for use in solution and tissue experiments. Effects of altering magnesium concentration were investigated because free Mg^{2+} is known to have a pronounced effect on the calibration curves, because free $[Mg^{2+}]_i$ is not known with certainty at any temperature, and because this quantity may vary with temperature. Figure 1 shows the resonance frequency of the P_i peak over the pH range 8.4 to 4.25, 21°C with 5 mM $MgCl_2$. The resonance frequency of PCr is unaffected by pH in the physiological range, but it does shift at pH values less than 6. Table 1 gives the parameters describing standard titration curves for a single ionization, fitted to data such as that shown in Fig. 1.

The apparent pK was affected by temperature, being higher at 4°C than at 21°C or 37°C. At all three temperatures, the presence of 5 mM $MgCl_2$ shifted the curve to the left (i.e., to slightly lower pH values) and lowered the apparent upper (basic, δ_B) limit relative to that of the zero-Mg^{2+} curve while the lower (acidic, δ_A) limit was relatively unaffected. These changes led to the apparent pK in the presence of 5 mM $MgCl_2$ being about 0.1 pH unit lower than in the absence of Mg^{2+} at each temperature.

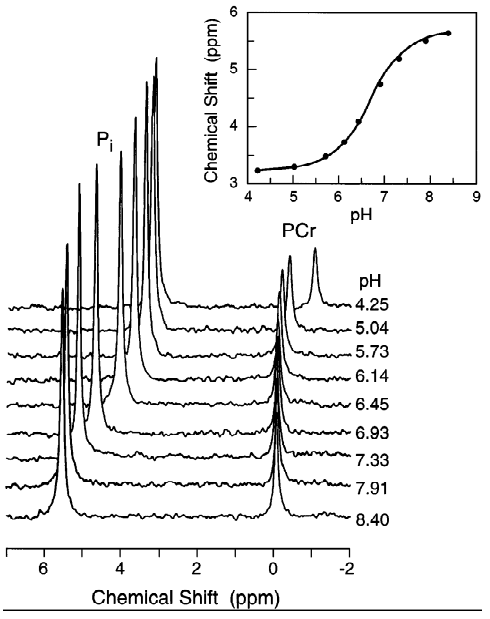

Fig. 1. The variation in the chemical shift of P_i with pH. The stacked ^{31}P NMR spectra were obtained from a solution containing 140 mM KCl, 10 mM KP_i, 5 mM Na_2PCr and 5 mM $MgCl_2$ (ionic strength = 0.18 M) at 21°C.

A complication which arises when titrating a physiological solution with a particular level of total magnesium is that, as the pH is changed, free $[Mg^{2+}]$ varies as a consequence of different amounts of magnesium being bound to P_i, since HPO_4^{2-} binds magnesium more strongly than does $H_2PO_4^-$. For a solution of 10 mM P_i and 5 mM magnesium, the free $[Mg^{2+}]$ decreased from almost 5 mM at the acidic end of the P_i titration curve to around 3.2 mM at the basic end. The variation in free $[Mg^{2+}]$ can be minimized by altering total magnesium and pH simultaneously, such that free $[Mg^{2+}]$ remains constant. When free $[Mg^{2+}]$ was kept constant at 3 or 5 mM, the resulting parameters were similar to those describing the titration curve with 5 mM total magnesium.

The Temperature Dependence of Intracellular pH

Figure 2 shows a typical ^{31}P NMR spectrum obtained from isolated frog gastrocnemius muscle at the end of an experiment lasting several hours, during which the muscle was subjected to repeated temperature changes. The intensity of the P_i peak corresponds to a $[P_i]$ in the muscle of about 2 mmol (kg wet wt.)$^{-1}$. In two experiments, muscles were maintained at either 4° or 20°C for 24 h; the position of the P_i peak at the end of this period did not differ from that measured at the beginning. There was, however, a tendency for the pH to shift in the acid direction over a period of several hours if the temperature was varied. For this reason, initial and final pH at 5°C were always compared as described in Materials and Methods.

The results are presented in Fig. 3*A* in the form of a classical van't Hoff plot, treating pH_i as an equilibrium constant for the reaction(s) which determine its value:

$$ln\,[H^+]_i = -7.51 - 2648\,(1/T_{abs}) \qquad (3)$$

$$r = 0.89 \quad n = 32$$

The data are highly linear and least-squares fitting did not yield an improved fit to two lines, rather than a single line. The van't Hoff plot shown in Fig. 3*A* serves to divide the standard free energy change ($\Delta F° = \Delta H° - T\,\Delta S°$) for the mechanism which determines pH_i into its temperature-dependent and independent components. If $\Delta H°$ is independent of temperature, then the slope = $-\Delta H°/R$ and the *y*-intercept = $\Delta S°/R$, where *R* is the gas constant. On this basis, the apparent $\Delta H°$ is constant over the temperature range studied at 22 ± 2.3 (SD) kJ · mol^{-1} and the apparent $\Delta S°$ = −62.4 ± 6.1 (SD) J · mol^{-1}. In agreement with earlier literature (and with the near linearity over short ranges of the reciprocal of a linear relationship), a linear correlation was also observed between pH and *T* (°C):

$$pH_i = 7.46 - 0.013\,T\,(°C) \qquad (4)$$

$$r = 0.86 \quad n = 32$$

Temperature Dependence of Intracellular Buffers

The major pH buffers in frog skeletal muscle are (*i*) HCO_3^-; (ii) P_i; (iii) imidazole groups of carnosine and protein histidine residues. Under the conditions of the present study only the last one contributes significantly to pH buffering in the muscle, since intracellular $[HCO_3^-]$ is negligible in the bicarbonate-free Ringer's solution, and the ^{31}P NMR spectra show that $[P_i]$ is low (<3 mM). Frog skeletal muscle contains approximately 10 mM carnosine (β-alanylhistidine) and approximately 36 mM protein histidine (Godt & Maughan, 1988). Figure 3*B* shows the temperature dependence of pH in a model solution which mimics the intracellular ions and buffers of frog skeletal muscle. The regression of the model solution data is described by the equation:

$$ln\,[H^+]_i = -5.88 - 3146\,(1/T_{abs}) \qquad (5)$$

$$r = 0.95$$

The apparent $\Delta H°$ for pH buffering in this solution is 26 ± 2.7 (SD) kJ · mol^{-1}, and is not significantly different from that for pH_i.

Table 1. The effect of temperature and $[Mg^{2+}]$ on the P_i titration curve

Solution	Ionic strength (m)	t(°C)	Apparent pK ± SE	δ_A(ppm)± SE	δ_B(ppm) ± SE
140 mM KCl 10 mM KP_i*	0.21	4	6.83 ± 0.02	3.29 ± 0.01	5.66 ± 0.02
20 mM Na_2PCr		21	6.75 ± 0.01	3.19 ± 0.01	5.70 ± 0.01
140 mM KCl	0.16	37	6.77 ± 0.02	3.12 ± 0.02	5.75 ± 0.02
10 mM KP_i					
140 mM KCl		4	6.71 ± 0.02	3.19 ± 0.02	5.49 ± 0.02
10 mM KP_i	0.17	21	6.64 ± 0.02	3.20 ± 0.01	5.58 ± 0.02
5 mM $MgCl_2$		37	6.65 ± 0.02	3.14 ± 0.02	5.61 ± 0.02
90 mM KCl					
20 mM KP_i	0.175	21	6.55 ± 0.04	3.30 ± 0.03	5.55 ± 0.05
5 mM Na_2PCr					
13 mM $MgCl_2$					
140 mM KCl					
10 mM KP_i	0.17	21	6.70 ± 0.02	3.18 ± 0.02	5.65 ± 0.03
3 mM *free* $[Mg^{2+}]$					
140 mM KCl					
10 mM KP_i	0.175	21	6.65 ± 0.02	3.21 ± 0.02	5.64 ± 0.03
5 mM *free* $[Mg^{2+}]$					

Values of the apparent pK and limiting resonance frequencies at acidic (δ_A) and basic (δ_B) pHs for inorganic phosphate (P_i) were obtained from calibration curves (Fig. 1). Numbers given are fitted parameters and the standard errors (±SE) of residual deviations from the best fit curve (*see* Methods for details). The ionic strength of the titration solution used for the 4°C experiments was 0.21 M, compared with 0.17 M for the 21°C and 37°C experiments. The higher ionic strength at 4°C would be expected to lower the apparent pK relative to the experiments at 0.17 M ionic strength. The size of this effect can be estimated from the published data as about 0.03–0.04 pH units (Seo et al., 1983). The true effect of changing temperature between 21° and 4°C on the pK was thus probably slightly underestimated in the present study.
* KP_i = mixture of K_2HPO_4 and KH_2PO_4 in ratio necessary to give certain pH.

Temperature Dependence of Acid Production and Extrusion

We performed a number of experiments to assess whether changes in metabolism, transmembrane proton or sodium gradients, proton activation or hormone stimulation of transmembrane sodium-proton exchange could be responsible for the changes in pH_i with temperature. These studies, performed at 5°C and 21°C on paired sartorius muscles, are summarized in Table 2. The control data indicate the same dependence of pH_i upon temperature in sartorius as in gastrocnemius muscles. Taken together, the results show $pH_i = 7.41 \pm 0.07$ ($n = 16$) at 5°C and $pH_i = 7.14 \pm 0.06$ ($n = 16$) at 21°C, yielding a dependence of pH_i upon temperature of 0.14 pH units/10°C.

The top row of Table 2 shows that blocking glycolysis with iodoacetic acid has no effect on pH_i at either temperature. The rest of Table 2 concerns transmembrane proton extrusion. Replacement of sodium with NMDG has no effect on pH_i at either temperature studied. In addition, pH_i is not affected by whether the Ringer's solution is maintained at constant pH or constant $[H^+]/[OH^-]$ as the temperature is changed. There is, however, a significant effect of stimulation of Na^+-H^+ exchange with insulin, and this is similar at the two temperatures. In four paired experiments, insulin caused an alkaline shift of 0.15 ± 0.02 pH at 5°C and 0.13 ± 0.01 pH at 21°C. These data yield an apparent $\Delta H°$ for the process regulating pH_i in the presence of insulin which is not significantly different from the control, and an apparent $\Delta S°$ which is more negative by 4.7 ± 0.08 $J \cdot mol^{-1}$ ($P < 0.01$).

To observe the temperature dependence of proton activation of Na^+-H^+ exchange, we investigated the temperature-dependence of pH_i following an acid load, with and without blockage of the exchanger. Figure 4 shows one such experiment on frog sartorius at 21°C, confirming an acid shift immediately following NH_4^+ washout, as well as the expected amiloride sensitive return to the initial pH_i. In 6 experiments at 21°C, exposure to NH_4^+ resulted in an acid shift of 0.28 ± 0.05 (SE), $P < 0.01$, and pH_i returned to its initial value following NH_4^+ washout ($\Delta pH_i = 0.04 \pm 0.02$ SE, $P > 0.1$). The corresponding

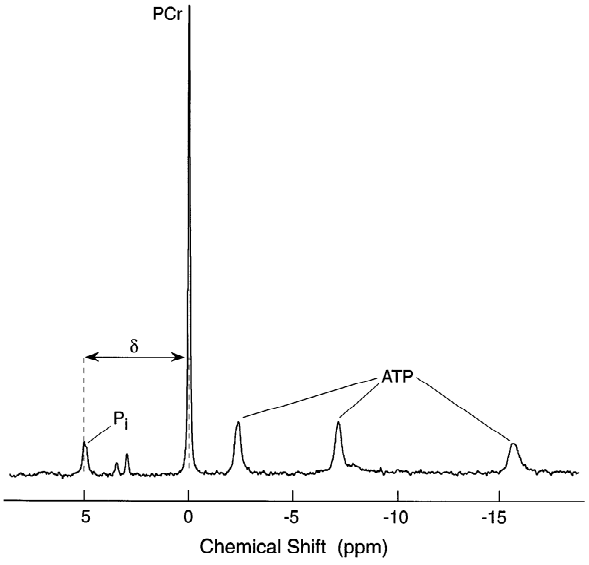

Fig. 2. ^{31}P NMR spectrum of isolated gastrocneumius muscle from *R. pipiens* at 21°C in Ringer's solution (pH = 7.2). The spectrum was obtained at 120 mHz and is the average of 300 scans at 2-sec intervals. Peaks from P_i, PCr and ATP are visible. The vertical line through the P_i peak has a chemical shift (δ) of 5.05 ppm, corresponding to a pH_i of 7.2.

numbers at 5°C are: control − NH_4^+ = 0.19 ± 0.04 (SE), n = 4, $P < 0.01$; control − washout = 0.03 ± 0.02 SE, n = 4, $P > 0.1$. In two experiments, amiloride had no effect on the acid shift in pH_i following exposure to NH_4^+, but it completely inhibited the alkaline return.

Discussion

In these experiments, we have shown that both the intracellular buffers and the set point for the Na^+–H^+ exchanger have the same temperature dependence as pH_i of isolated frog skeletal muscle and that of neutral water. In addition, the apparent standard free energy (ΔF°) for activation of the exchange during insulin stimulation has the same temperature sensitivity, but a more negative temperature independent component, compared to the processes that determine pH_i under control conditions. Thus, the temperature-dependent component of the standard free-energy change is unaffected by insulin stimulation, and changes in Arrhenius activation energy (Ea) may not be a part of the mechanism of hormone stimulation.

Accuracy of pH_i Measured as a Function of Temperature

To understand the control of pH_i in intact cells, it is necessary to measure pH_i with the maximum possible accuracy and with minimal disturbance to the cell. Measurement of the resonance position of intracellular P_i using ^{31}P NMR meets the second criterion, and its insensitivity to physiological changes in a number of vari-

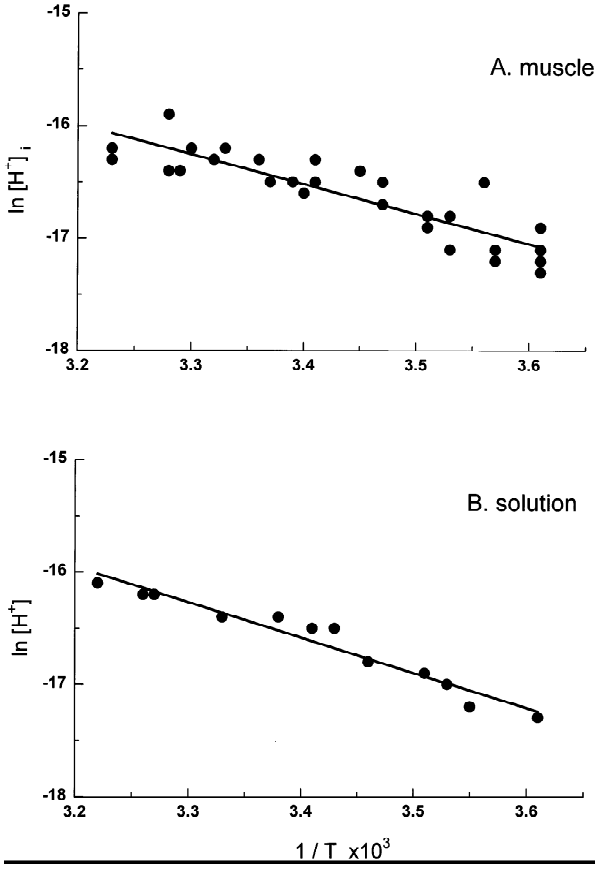

Fig. 3. (*A*) The temperature-dependence of *ln* [H^+] in isolated frog skeletal muscle. The data from six experiments were fitted by linear regression: $ln\ H = -\Delta H°/RT_{abs} + \Delta S°/R$ to determine apparent standard entropy change (Δ*S*°) and apparent standard enthalpy change (ΔH°). T_{abs} is the absolute temperature (Kelvin); *R* is the gas constant (8.31 J · mol^{-1} · T^{-1}). (*B*) The temperature-dependence of *ln* [H^+] in a model solution containing pH buffers present in frog muscle: 10 mM carnosine, 36 mM histidine and 2 mM P_i (pH 7.1 at 21°C).

Table 2. Effects of maneuvers which alter H^+ production, Na^+-H^+ gradients or exchanger activity

t(°C)	5	21	
Control	7.31 ± 0.04	7.06 ± 0.02	
1 mM Iodoacetic acid	7.29 ± 0.02	7.04 ± 0.02	(4)
Control	7.45 ± 0.02	7.17 ± 0.04	
$-[Na^+]_e$	7.44 ± 0.04	7.17 ± 0.04	(4)
Constant pH_e	7.46 ± 0.03	7.12 ± 0.04	
Constant $(H+/OH-)_e$	7.45 ± 0.04	7.09 ± 0.03	(4)
Control	7.43 ± 0.03	7.21 ± 0.01	
5 μM Insulin	7.58 ± 0.02	7.34 ± 0.02	(4)

Means ± SE are given for number of paired experiments in parenthesis.

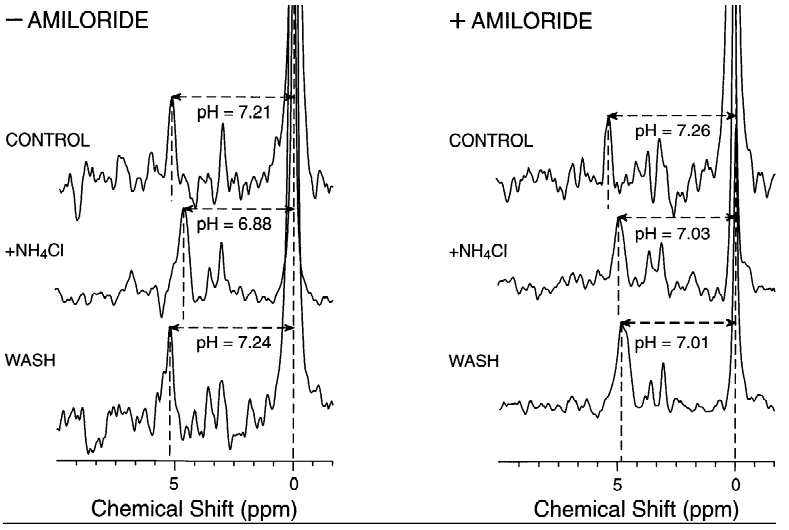

Fig. 4. Effect of an acid load on pH_i in the presence and absence of 1 mM amiloride at 21°C. After a control period (upper spectra) muscles were incubated for 45 min in Ringer's solution containing 20 mM NH_4Cl. The middle spectra were obtained immediately after NH_4Cl removal and the final data (lower spectra) were obtained 1 hr later.

ables, including ionic strength, protein concentration, substitution of K^+ for Na^+, etc. is well established. Assessment of possible sources of error indicates that ^{31}P NMR has a similar accuracy to that of pH electrodes (Madden et al., 1991). While it is not possible to rule out all interfering effects in the measurement of pH_i by ^{31}P NMR or any other method, we were particularly concerned about the effects of changes in temperature and free Mg^{2+}, both of which have quantitatively significant effects on the resonance position of P_i (Roberts, Wade-Jardetzky & Jarketzky, 1981; Jacobson & Cohen, 1982; Seo et al., 1983). The concentration of Mg^{2+} may well change with temperature in frog skeletal muscle, as it does in red blood cells (Marjanovic et al., 1993). While theoretically it is possible to measure $[Mg^{2+}]_i$ using ^{31}P NMR, in practice this method has low sensitivity and reproducibility, especially in the face of temperature-dependent changes in peak shape (Roman, 1992). We therefore calibrated our pH measurement in the presence of 0 and 5 mM Mg^{2+}, which brackets the outermost limits of the values for $[Mg^{2+}]_i$ (Godt & Maughan, 1988).

The temperature-dependence observed for the pK of P_i agrees with previous work (Bates & Acree, 1943; Seo et al., 1983), and establishes that there is no effect of Mg^{2+} over the physiological range. At any given temperature, however, there is a pronounced effect of Mg^{2+} on the basic region of the titration curves and on the basic endpoint (δ_B), in agreement with previous studies carried out at room temperature (Jacobson & Cohen, 1982; Seo et al., 1983). The most obvious interpretation is that, in the presence of Mg^{2+}, phosphate exists in two forms (HPO_4^{2-} and $MgHPO_4^0$) which are in fast exchange on the NMR time-scale and thus give an ''averaged'' chemical shift. By analogy with other phosphate groups that show changes in chemical shift on Mg^{2+} binding (for instance ATP), it is reasonable to expect the chemical shifts of HPO_4^{2-} and $MgHPO_4^0$ to be different, and that the basic endpoint of the titration (δ_B) should be progressively more affected by increasing levels of Mg^{2+}.

The maximum error in determining pH_i that could result from the effects of Mg^{2+} is simply the difference in the pH_i derived from the zero and 5 mM $MgCl_2$ curves for the same value of the chemical shift. We determined $pH_i = 7.43$ in frog gastrocnemius muscle at 4°C using the ''4°C, 5 mM total $MgCl_2$'' calibration curve (Table 1). If the ''4°C, zero $MgCl_2$'' curve had been used, the pH would have been estimated as 7.34. For 20–25°C, intracellular pH was found to be 7.14. Use of the zero-Mg curve would have led instead to a value of 7.17. The problems are clearly worse at alkaline values of pH_i, and this means that the errors introduced by incorrect estimates of $[Mg^{2+}]_i$ are worse, for example, in frog skeletal muscle at low temperatures (where $pH_i \sim 7.4$) than in most mammalian tissues at 37°C, in which pH_i is thought to be near 7.0. We conclude that the size of the error due to Mg^{2+} in the measurement of absolute values of pH_i depends on the range in which pH_i lies, and is less than 0.1 pH units. The slope of pH_i as a function of temperature has an accuracy of better than 0.03 pH/10°C, which is the effect of changing free $[Mg^{2+}]$ by 5 mM over the temperature range studied.

Temperature-dependence of pH_i

The values of pH_i reported here compare well with earlier results obtained by NMR or pH electrodes in isolated frog muscle in bicarbonate-free solutions (e.g., pH_i 7.5 at 4°C, Dawson et al., 1977; 7.18 at 22°C, Abercrombie, Putnam, & Roos, 1983; 7.09 at 20°C, Curtin, 1986; 7.05 at 25°C, Bolton & Vaughan-Jones, 1977). They also agree with the in vivo results of Malan, Wilson and Reeves (1976), who used equilibration of a weak acid to find a change with temperature of −0.0147 pH units/°C in skeletal muscle of temperature-acclimatized frogs.

These results are not unique to frog muscle; a similar temperature dependence of pH_i has been reported in all studies of isolated tissue of which we are aware. These include a difference in pH_i at two temperatures in mouse soleus muscle (pH_i 7.23 at 28°C compared with 7.07 at 37°C; Aickin & Thomas, 1977), in cardiac muscle (pH_i became 0.21 pH units more alkaline when the temperature was lowered from 35 to 21°C; Ellis & MacLeod, 1985) and in rat uterine smooth muscle (pH_i 7.48 at 4°C and 7.09 at 37°C; Dawson & Wray, 1985). pH_i becomes more alkaline (~0.15 pH units/10°C) as temperature is lowered in red cells from humans and from a hibernating species of ground squirrel (Marjanovic et al., 1993), as well as in nucleated chicken red blood cells (Kozma et al., 1995), and in an osteoblast-like rat cell line (0.13 pH/10°C between 17° and 37°C; Dascalu, Nevo & Korenstein, 1992). The same principle holds for maize root tips, in which pH changes by 0.2 pH/10°C between 4° and 28°C (Aducci et al., 1982). The similarity of the results in a variety of isolated tissues, including those from plants, from both ectothermic and endothermic animals and from hibernators, suggests fundamental similarities in the mechanisms responsible for the temperature dependence of pH regulation across tissues and species.

While pH_i of all isolated tissues appears to respond to changes in temperature in a similar manner, the same is not true of pH_i within tissues of intact organisms. In some cases, such as frog skeletal muscle (data of Malan et al., 1976 compared to that reported here) or dog brain and heart (in vivo data of Swain et al., 1991 compared to that of Ellis & MacLeod, 1985), the change in pH_i with temperature is the same in vivo as in vitro. In other cases, temperature-dependent alterations in blood pCO_2 are superimposed. For example, in white muscle of intact newts, pH changes by 0.18 pH/10°C, while in lungless red-backed salamanders it changes by 0.41 pH/10°C (Johnson et al., 1993). In hibernators, blood pH is maintained constant (termed pH-stat regulation) due to increased CO_2 as the temperature is lowered. This has variable consequences for pH_i, presumably depending upon tissue mechanisms for handling CO_2 (Malan, 1982). Such studies indicate that the temperature dependence of gas exchange can affect pH_i in vivo, shifting it away from that observed in isolated tissues.

What Causes the Temperature Dependence of pH_i?

Reeves & Malan (1976) suggested that the change in pH_i with temperature could be explained by the temperature dependence of the pKs of the intracellular buffers. Their calculations predicted variation in pH_i with temperature which agreed well with the in vivo measurements of Malan et al. (1976). We found experimentally that the pH of the model solution that mimics the buffers present in isolated frog muscle has the same temperature dependence as that of the isolated muscle. We conclude that whatever mechanism is responsible for the temperature dependence of pH_i is closely linked to the temperature dependence of the pH of intracellular buffers. In particular, the temperature dependence of the pKs of intracellular buffers ensures that when temperature is changed, a new pH_i, which is similar to the steady-state pH_i of the isolated tissue, is attained instantaneously. Neither we nor anyone else have observed a time dependence in the shift of pH_i in isolated tissues with change in temperature.

Intracellular pH is maintained at its set-point, near to the pKs of the intracellular buffers, but far removed from transcellular electrochemical equilibrium, by the mechanisms which remove H^+ from the cytosol. The constancy of pH_i when a tissue is in a physiological steady-state represents the balance between the rate of H^+ removal and the rate at which H^+ (i) enters the cell down its electrochemical gradient and (ii) is produced intracellularly by metabolism. As temperature increases, the rates of these processes can be expected to increase, perhaps to variable extents. However, since metabolism is the largest source of protons in skeletal muscle, and since in resting muscle, glycolysis is the starting point for both aerobic and anaerobic metabolism, the possibility that temperature dependence of proton production contributes to the temperature dependence of pH_i is virtually eliminated by the lack of effect of blocking glycolysis with iodoacetate.

We are left with the fact that the temperature dependence of the steady-state pH_i must result from the temperature dependence of proton extrusion as well as that of the intracellular buffers. The parameters that affect proton extrusion are (i) the gradient against which protons are extruded, (ii) the driving force for extrusion and (iii) the rate or the set point of the proton transporters.

An obvious consideration is the fact that, if the temperature dependence of the extracellular pH is not the same as that of pH_i, the gradient against which the protons are extruded is altered as the temperature changes. We have found that pH_i of isolated frog muscle is the same function of temperature between 5°C and 21°C, irrespective of whether extracellular pH or $[H^+]/[OH^-]$ is maintained constant. This finding is similar to that of Swain et al. (1991) who found that, in anesthetized dogs, cardiac and brain pH_i were the same function of temperature between 37°C and 26°C, irrespective of whether blood pH or $[H^+]/[OH^-]$ was held constant. We conclude that the temperature-dependence of proton extrusion does not depend upon that of extracellular pH.

Two membrane pH regulating systems are known to exist in frog muscle: an amiloride-sensitive Na^+-H^+ exchanger which accounts for 90% of the proton efflux, and a SITS-sensitive H^+-Na^+-Cl^--HCO_3^- exchanger

which accounts for the remainder of the flux (Putnam & Roos, 1986). The driving force for Na^+-H^+ exchange is the electrochemical gradient for sodium entry into the cytoplasm. We found that there is no effect on pH_i at either 5°C or 21°C when the sodium in the bathing solution is completely replaced with NMDG. This is consistent with the observations of Abercrombie et al. (1983) that, at 22°C, sodium removal does not affect steady state pH_i in frog muscle, although it does affect the return of pH_i following an acid load (Putnam et al., 1986). We conclude that the temperature dependence of pH_i is not due to any temperature dependence of the driving force for sodium entry.

We are left with only one possibility: that the temperature dependence of steady state pH_i must be due to the temperature dependence of the set point of the Na^+-H^+ exchanger, and to a lesser extent that of the H^+-Na^+-Cl^--HCO_3^- exchanger. The amiloride sensitive return of pH_i to its initial value following exposure to NH_4^+, at both high and low temperatures, clearly shows that the Na^+-H^+ exchanger is primarily responsible for maintenance or return of pH_i in frog skeletal muscle under conditions of proton loading.

Steady-state Thermodynamics of Proton Extrusion

Several forms of the Na^+-H^+ exchanger of eukaryotic cells have recently been cloned, and all show a great deal of homology (Noël and Pouysségur, 1995). For the ubiquitous amiloride-sensitive and growth-factor activatable NHE_1, a group which includes the exchanger of skeletal muscle, phosphorylation and Ca/calmodulin interaction are thought to be likely mechanisms for hormone stimulation, acting through protonation of a regulatory site that is distinct from the transporter site (Wakabayashi, Shigekawa & Pouysségur, 1997). Although steady-state thermodynamics cannot provide detailed molecular mechanisms, and although the classical van't Hoff approach used here is undoubtedly an oversimplification, these studies do yield evidence in favor of some proton activation and hormone stimulation mechanisms and are inconsistent with others.

For example, binding of activators and substrates induces conformational changes in many proteins which cause alterations in heat capacity ($C_P = \Delta H/\Delta T$), and thus a nonlinear slope of the van't Hoff plot (Alber et al., 1982). The fact that the van't Hoff plot for the mechanisms regulating pH_i is linear suggests that these induced-fit mechanisms are not a part of H^+-activation of the exchanger. Similarly, any mechanisms which involve changes in Arrhenius activation energy ($E_a = \Delta H° + RT$) upon hormone stimulation, are inconsistent with the equality of $\Delta H°$ under control and insulin-stimulated conditions.

It has been suggested that maintenance of a constant ratio of intracellular $[H^+]/[OH^-]$ when temperature is changed results from dependence of activity of the Na^+-H^+ exchanger on degree of ionization of the imidazole groups on the exchanger protein (Nattie, 1990). Ionization of imidazole groups has been shown to explain temperature dependent properties of a number of cellular enzymes and thus to underlie a number of biological adaptation processes (Somero & White, 1985). If constancy of imidazole ionization determines the set point of the exchanger, it follows from the Henderson-Hasselbalch equation that the pH_i thus determined will have an apparent $\Delta H°$ similar to that for the relevant imidazole groups on the exchanger protein. Our finding that apparent $\Delta H°$ for pH_i is constant over the physiological temperature range, as is that for imidazole, and is quantitatively similar to that of a buffer solution dominated by imidazoles, is consistent with this suggestion. Since *changes* in ionization of imidazoles have large enthalpic effects, the mechanism which determines the set point of the exchanger must either preserve the imidazole ionization state or offset the enthalpic consequences of any such changes.

Dependence of exchanger activity on ionization of imidazole groups, either specific or to a regulatory site or for the balance between stability and lability of the protein as a whole, could preserve constant heat capacity, and thus provide effective euthermic regulation of pH_i. Hormone stimulation must decrease the actual free energy change ($\Delta F = \Delta H - T\Delta S$) for activation of the exchanger, in order that the set point occur at a higher value of pH_i. A change in the temperature-insensitive apparent ΔS, rather than a change in ΔH or E_a, would allow effective euthermic regulation of pH_i by maintaining a constant energetic relationship among the various relevant ionic equilibria.

Entropy-driven modifications of enzyme activity have been known for many decades, and typically they involve changes in the hydration-state of the protein (Lauffer, 1975; Silva & Webber, 1993). Formation of hydrophobic bonds, together with release of protein-bound water, provides the driving force for such reactions. By analogy with known entropy-driven processes, the effect of insulin could involve the relative exclusion of water from the regulatory site. Such a mechanism would be consistent with the small observed change in apparent $\Delta S°$.

Overall, our results provide thermodynamic evidence that is consistent with, and extends, the α-stat hypothesis. We have found that the apparent $\Delta H°$ for the fixed intracellular buffers (26 kJ/mol) and for proton activation of the Na^+-H^+ exchanger (22 kJ/mol) are constant over the physiological temperature range and similar to that for protein production by ionization of neutral water (1/2 of the 56 kJ/mol $\Delta H°$ for production of both

H^+ and OH^-). Insulin stimulation alters the temperature-independent component, and not the temperature-dependent component of the free-energy change for proton activation of the exchanger. Thus, in addition to its role in preservation of constant charge state of proteins and Donnan distribution ratios under control conditions, as argued by the α-stat hypothesis, pH_i regulation also maintains temperature-independent energetic relationships among ionization of water, intracellular buffers and the regulatory site(s) of the exchanger under both control and hormone-stimulated conditions.

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