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Changes in Intracellular pH Caused by High K in Normal and Acidified Frog Muscle

Relation to Metabolic Changes

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ABSTRACT We examined the effect of depolarization on intracellular pH (pH_i) of normal ($\text{pH}_i \sim 7.37$) and acidified (pH_i 5.90–6.70) frog semitendinosus muscle using microelectrodes. A small bundle was superfused with a Na^+ -free buffered solution (10 mM HEPES, 100% O_2 , pH 7.35) containing either 2.5 or 25 mM K^+ . An NH_4Cl prepulse was used to lower pH_i . At normal pH_i , depolarization usually produced a slight (0.04) alkalinization, followed by a fall in pH_i of ~0.2. In contrast, in all 25 acidified bundles pH_i rose by 0.1–0.7. The rise was greater the lower the initial pH_i . It could be imitated by caffeine and blocked by tetracaine and thus was, most likely, initiated by release of calcium. We ascribed the alkalinization to hydrolysis of phosphocreatine (PCr); 2,4-dinitrofluorobenzene abolished it. Biochemical analysis on fibers at the peak of alkalinization showed PCr to be reduced by one-half, while PCr in normal fibers that had been depolarized for the same period (4–6 min) showed no change. We postulated that low pH_i slows glycolysis with its associated ATP formation by reducing glycogenolysis and particularly by reducing conversion of fructose-6-phosphate to fructose-1,6-diphosphate through inhibition of phosphofructokinase (PFK), an enzyme which is known to be highly pH sensitive. Thus PCr hydrolysis would be required to replace much of the hydrolyzed ATP. This postulated effect on PFK is in agreement with the finding that glucose-6-phosphate (in near-equilibrium with fructose-6-phosphate) was increased nearly fivefold in the depolarized acid fibers, but not in the depolarized normal fibers. However, fructose-1,6-diphosphate also increased significantly; 3-phosphoglycerate was not affected. This suggests an additional acid-induced bottleneck between the latter two substrates. We measured the intrinsic buffering power, β , of frog semitendinosus muscle with small pulses of NH_4Cl . It was found to vary with pH_i according to $\beta = 144.6 - 17.2 (\text{pH}_i)$.

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INTRODUCTION

When a skeletal muscle is depolarized, the resulting release of calcium from its intracellular stores sets in motion two chains of events. One may lead to mechanical changes in the muscle, the other manifests itself by mobilization of glycogen and accelerated glycolysis. Each of these chains is affected by intracellular pH (pH_i), and each in turn affects pH_i . The experiments reported here are examples of this reciprocity.

In a previous study (Abercrombie and Roos, 1983) we examined the effect of depolarization by 50 mM K⁺ on pH_i of frog semitendinosus muscle. Since pH_i (and V_m) measurements were made with microelectrodes, violent muscle movements had to be avoided. The muscles were, therefore, put in solutions of double-normal tonicity, which greatly reduces the mechanical response while Ca²⁺ release is left intact (Hodgkin and Horowicz, 1957; Gordon and Godt, 1970; Taylor et al., 1975). We observed, simultaneously with the start of depolarization, a striking but transient fall in pH_i by as much as 0.5. When the experiment was carried out in a poorly buffered medium, this pH_i change was accompanied by a significant fall in external pH (pH_o) in the immediate vicinity of the fiber. The fall in pH_i could be imitated by 1–3 mM caffeine, which is known to release Ca²⁺ from intracellular stores (Lüttgau and Oetliker, 1968; Weber and Herz, 1968), and could be prevented by 2 mM tetracaine, which blocks such release (Almers and Best, 1976). We ascribed the internal and external acidification to the increased production of a weak acid, most likely lactic, resulting from Ca²⁺-stimulated glycolysis.

In this paper we describe experiments on muscles that were exposed to 25 mM K⁺ not only at normal pH_i but also after pH_i had been artificially lowered. Fiber movement was only slight so that isotonic media could be used. Depolarization of the normal fibers led to a slow fall in pH_i , a not unexpected finding. In the acid fibers, however, a significant rise in pH_i was observed which, in general, was more striking the more acid the fibers. Biochemical analyses were performed in both normal and acid fibers in order to correlate the pH_i response to metabolic changes.

A preliminary report on this work has been published (Moreno et al., 1989).

METHODS

 pH_i and V_m Measurements

A small bundle of 20–30 fibers was dissected from the dorsal head of the semitendinosus muscle of *Rana pipiens* (obtained from J. M. Hazen and Co., Albion, VT). The bundle was mounted in a 1.1-ml chamber and superfused at 22°C at a rate of 1.5 ml/min. All solutions were gassed with 100% O₂ and buffered to pH 7.35 with 10 mM HEPES. The control solution was Na⁺ free; it contained (in mmol/liter) the Na⁺ substitute *N*-methyl-d-glucammonium (NMDG), 120; K⁺, 2.5; Ca²⁺, 4; Cl⁻, 130; EDTA, 0.1. The absence of Na⁺ obviated Na⁺/H⁺ exchange-mediated pH_i recovery which would otherwise occur at low pH_i (Putnam et al., 1986). V_m was measured with a conventional 3 M KCl-filled electrode (10–20 MΩ resistance), pH_i with a liquid membrane electrode, using a neutral ion carrier (Amman et al., 1981) as described by Reuss and Costantin (1984). The pH electrodes had slopes of 55–60 mV/pH unit and 90% response time of a few seconds when they were switched from a 50-mM phosphate buffer solution at pH 7 to a 50-mM phosphate buffer at pH 8. In several

experiments we used double-barreled microelectrodes (modification of method described by Aickin, 1981); one barrel was filled with 3 M KCl, the other with the neutral ion carrier. The electronics of the experimental arrangement have been described previously (Putnam, 1985).

When the pH_i effect of depolarization in normal fibers was studied, the bundle was first superfused for 30–40 min with the control solution. The electrodes were then introduced into a fiber, and after several minutes K^+ was raised to 25 mM by equivalent reduction of NMDG, while Cl^- remained unchanged. The high K^+ was maintained for 9–13 min. When the pH_i effect of depolarization was studied in the acidified fibers, the bundle was prepulsed for 1–2.5 h with a solution containing 20 mM NH_4Cl . The control solution was then readmitted. This results in a fall of pH_i below control, the “undershoot” (Boron and De Weer, 1976). 20–30 min later the fiber was impaled. After a few minutes of stable pH_i , a solution (still Na^+ free) containing 25 mM K^+ (Cl^- remaining unchanged) was admitted. The course of pH_i was followed for another 5–20 min.

The composition of the bathing solutions requires some comment. (a) We found that 25 mM was the highest K^+ concentration that allowed us to maintain impalement throughout depolarization, provided the Cl^- concentration was kept constant. At constant $[K^+] [Cl^-]$ product, on the other hand, contractures were the rule. The difference in behavior is probably due to the much slower rate of depolarization under constant Cl^- conditions (compare Figs. 1 and 2 with Fig. 3). (b) We had to keep Na^+ (100 mM) in the NH_4Cl -containing solution, because in its absence plateau acidification was greatly slowed so that exposure time needed to obtain a significant undershoot became so long as to damage the muscle. Raising NH_4Cl concentration above 20 mM also had a detrimental effect. We ascribe the effect of Na^+ removal on plateau acidification to reduced activity of the Na^+ pump. Aickin and Thomas (1977a) observed a similar effect in mouse soleus muscle. Indeed, 10 μM ouabain nearly completely abolished plateau acidification in our frog muscles (unpublished observations), in agreement with the findings of Aickin and Thomas (1977b) in mouse soleus. Apparently a large fraction of the entering NH_4^+ in frog muscle is carried on the pump.

In some experiments the pH_i effect of caffeine (1 or 1.5 mM) rather than high K^+ was observed, both at normal and low pH_i . In other studies acidified fibers were exposed to 25 mM K^+ or to caffeine in the presence of 0.2 mM tetracaine ($pK_a' 8.24$). This drug was added to the superfuse after NH_4Cl exposure and maintained throughout the remainder of the experiment. In a few studies 2,4-dinitrofluorobenzene (0.38 mM) was added in the same way as tetracaine and ~1 h was allowed before exposing the fibers to 25 mM K^+ .

Buffering Power

The intrinsic buffering power at 2.5 mM K^+ was derived from the rise in pH_i resulting from adding small amounts of NH_4Cl to the Na^+ -free bathing solution. Both normal and acid fibers were tested. The initial pH_i of the former group ranged between 7.1 and 7.4, of the latter between 6.1 and 7.0. The acid fibers were tested at least one-half hour after NH_4Cl had been removed. At $pH_i \geq 7.0$, 3 mM NH_4Cl was used; at lower pH_i values, 1 mM was used. These pulses raised pH_i by 0.05–0.3. The $[NH_4^+]_i$ was calculated from $[NH_3]_i$ (assumed to be the same as $[NH_3]_o$) and pK_a' of ammonium (9.2). Buffering power was taken as $\Delta[NH_4^+]_i / \Delta pH_i$ on the assumption that before the pulses the fibers contained no NH_4^+ .

Muscle Sampling for Biochemical Measurements

In a separate series of experiments on both acid and normal bundles, pH_i and V_m were first followed as described above. In five acid bundles, at the peak of the depolarization-induced pH_i response, the electrodes were rapidly withdrawn, and 36–83 s later metabolism of the muscle was stopped by flooding the chamber with liquid nitrogen-cooled freon. The chamber

was placed in dry ice, the tendons of the frozen bundle were cut off, and the bundle was then stored at -70°C for biochemical measurements. For comparison, prepulsed bundles of the other leg of the same five frogs were frozen at normal (2.5 mM) K^{+} . A similar procedure, also on five bundles, was followed in normal muscles. Here the bundles were frozen when 25 mM K^{+} had reduced V_m to 60% of its original value (4–6 min after the start of depolarization), a voltage comparable to that at which the prepulsed bundles were frozen. This matching of the moment of freeze-fixation in acidified and normal fibers was greatly facilitated by the slow rate at which the fibers depolarized. Again, bundles of the other leg of the frogs were frozen in 2.5 mM K^{+} for comparison. In addition, three bundles at normal pH_i were kept in 25 mM K^{+} until the pH_i had fallen to a stable value, 9–13 min after start of depolarization, and then frozen. Unfortunately, substitution of Na^{+} by either NMDG or choline produced a sticky residue on the surface of the fibers after they had been dried, which interfered with the analysis. Therefore, all solutions used in the biochemical studies contained Na^{+} . The values for pH_i and V_m in the normal and acid fibers were in the range of those obtained in the absence of Na^{+} . This makes sense since the rate of $\text{Na}^{+}/\text{H}^{+}$ exchange-mediated pH_i recovery is only of the order of 0.06 units per min (Putnam et al., 1986). The rise in pH_i upon depolarization of the acid fibers conformed also with that observed in the absence of Na^{+} .

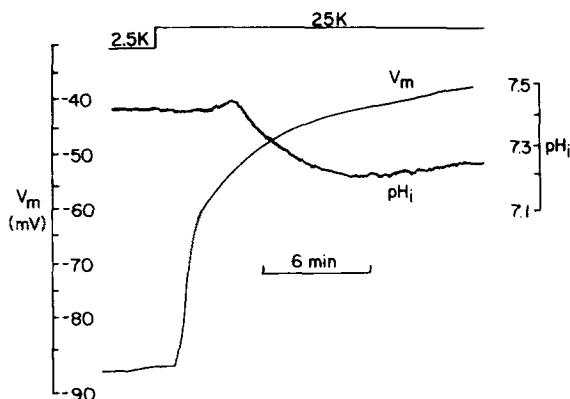


FIGURE 1. Time course of pH_i and V_m of a fiber. When external $[\text{K}^{+}]$ was raised from 2.5 to 25 mM, $[\text{NMDG}^{+}]$ (substituted for Na^{+}) was reduced correspondingly, while $[\text{Cl}^{-}]$ was maintained at 130 mM. External pH 7.35. Initial pH_i 7.42. The biphasic pH_i course in response to depolarization shown here was seen in 11 of 16 fibers.

Chemical Analysis of Individual Fibers

The bundles were dried under vacuum at -35°C . Single fibers were teased apart at room temperature. Portions of each fiber (usually 2–4 mm long) were then stored separately (Lowry and Passonneau, 1972). After drying, the bundles and the dissected fibers were stored under vacuum at -70°C , where the metabolites are stable indefinitely.

Each of four fibers in a bundle was assayed in duplicate for ATP, phosphocreatine (PCr), glucose-6-phosphate (G6P), fructose-1,6-diphosphate (FDP), and lactate. In two bundles (eight fibers), 3-phosphoglycerate (3PGA) was also measured, again in duplicate. The methods are based on highly specific enzymatic reactions in which a pyridine nucleotide is either oxidized or reduced. To achieve the sensitivity needed, the nucleotide product (NAD^{+} , NADH , or NADPH) was amplified as much as 20,000-fold by "enzymatic cycling" (Lowry, 1980).

To keep the reagent blank values to a manageable size, the analytical reactions were carried out under oil in small volumes (1–5 μl). Samples $\sim 1 \mu\text{g}$ in weight (0.5–1.5 mm in length) were weighed ($\pm 2\%$) on a quartz fiber fish-pole balance (Lowry, 1953) and then heated in 1 μl of either weak NaOH or weak HCl. Aliquots of the alkaline extract were used for ATP, PCr, and

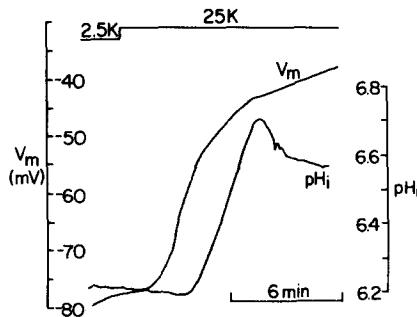


FIGURE 2. Time course of pH_i and V_m of a fiber when external $[K^+]$ was raised from 2.5 to 25 mM. The fiber had been acidified by an NH_4Cl prepulse to pH_i 6.21. Composition of the solutions was as in Fig. 1. Depolarization is associated with a rise in pH_i of 0.52. The pH_i is still elevated 7.5 min after the beginning of the rise.

3PGA assays; aliquots of the acid extracts were used for the rest. The methods were essentially as described by Hintz et al. (1982) except in the case of 3PGA, which is adapted from the macro procedure of Lowry et al. (1964). The pooled coefficients of variation for replicates were 0.9% for G6P and 3PGA, 1.2% for ATP, 1.5% for PCr, 7.2% for FDP, and 9.8% for lactate. The high coefficient for lactate is due to the extreme danger of contamination, while that for FDP is due to the extremely low concentrations in most of the fibers.

RESULTS

The resting V_m of the normal fibers was -86 ± 1.5 mV (mean \pm SEM, $n = 16$), and the resting V_m of the acidified fibers was -81 ± 1.1 mV ($n = 25$). Exposure to 25 mM K^+ at constant Cl^- concentration resulted in a very slow depolarization (Figs. 1 and 2). This slowness is probably the result of K and Cl^- ions entering the muscle from the unstirred layer faster than they are replenished from the bulk. Because of the relatively slow rate of superfusion (the ratio superfusion rate/chamber volume was only 0.023 s^{-1}), it would take many minutes for the layer's composition to reach equality with that of the superfusate. (At constant $[K^+] [Cl^-]$ no net transmembrane fluxes occur; accordingly, depolarization was much more rapid [Fig. 3]). We found a striking difference in the pH_i response to depolarization between normal and acid muscle. At normal pH_i (7.37 ± 0.04 , $n = 16$) 11 fibers responded with a slight rise (0.041 ± 0.009 , range 0.01–0.14), which was maximal 0.8–6.5 min after the start of depolarization. Eventually the pH_i of all fibers fell. After 3–11 min a stable pH_i value

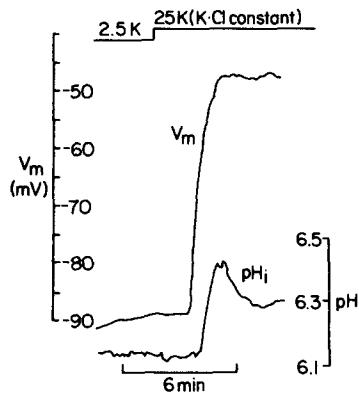


FIGURE 3. Time course of pH_i and V_m of an acidified fiber (pH_i 6.13) upon depolarization in 25 mM K^+ . In contrast to the experiments shown in Figs. 1 and 2, $[Cl^-]$ in the depolarizing solution had been reduced 10-fold (to 13 mM) so that $[K^+] [Cl^-]$ remained unchanged. Note that V_m reaches its steady value much faster than in the first two figures. Depolarization produces a steep and marked rise in pH_i .

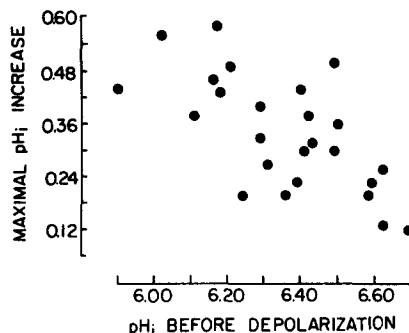


FIGURE 4. Relationship between maximal rise in pH_i produced by depolarization in 25 mM K⁺ and pH_i before depolarization in 25 acidified fibers. In general, the rise is greater the lower the initial pH_i.

was reached, 0.22 ± 0.07 below control. Fig. 1 illustrates the biphasic pH_i course seen in most fibers. A similar course was usually observed with 1–1.5 mM caffeine, concentrations low enough that they did not produce significant fiber movement. In contrast, in the 25 acid fibers (pH_i 5.90–6.69) depolarization produced without exception a significant rise in pH_i that was 3–15 times that in the normal fibers. The pH_i reached its maximal value a few minutes after the start of depolarization. In most cases it then declined and leveled off to a value higher than before depolarization. An example of the pH_i response at starting pH_i of 6.21 is given in Fig. 2. In general, the rise in pH_i was greater the lower the initial value, as shown in Fig. 4. The effect of high K⁺ on pH_i in acid muscle could be imitated by 1–1.5 mM caffeine (Fig. 5): in six fibers (mean pH_i 6.63) caffeine raised pH_i by an average of 0.33. The rise in pH_i produced by either K⁺ or caffeine could be blocked by tetracaine (nine experiments). A low dose (0.2 mM), resulting in an intracellular concentration of ~2 mM, was effective.

Because we interpreted the rise in pH_i to be due to PCr hydrolysis (see Discussion), we examined the effect of the metabolic inhibitor, 2,4-dinitrofluorobenzene (0.38 mM), which is known to specifically block this process by inactivating creatine phosphokinase (Infante and Davies, 1965). The drug blocked 25 mM K⁺-induced alkalinization in acid fibers (Fig. 6), and had a slight acidifying effect in normal fibers.

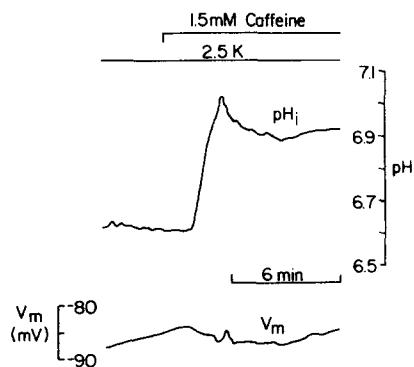


FIGURE 5. Effect of 1.5 mM caffeine on pH_i in an acidified fiber (pH_i 6.62) maintained in 2.5 mM K⁺. The pH_i rapidly rises by 0.4 and then levels off to a slightly lower value.

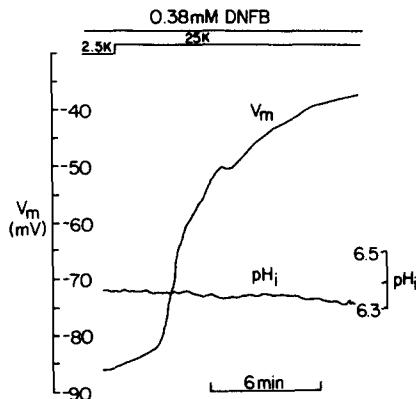


FIGURE 6. Time course of pH_i and V_m of an acidified fiber (pH_i 6.43) in the presence of 0.38 mM 2,4-dinitrofluorobenzene. Otherwise, composition of the superfusates was the same as in Figs. 1 and 2. The drug blocks the pH_i response to depolarization.

Buffering Power

We found that intrinsic buffering power, β , increased with decreasing pH_i . A similar trend has been found in homogenates of cat skeletal, cardiac, and smooth muscle (Furusawa and Kerridge, 1927), in barnacle muscle (Boron, 1977; Boron et al., 1979), in rat renal mesangial cells (Boyarsky et al., 1988), and in sheep cardiac Purkinje fibers (Vaughan-Jones and Wu, 1990). The relationship between β and pH_i obtained by the least-squares method is given by $\beta = A + B(\text{pH}_i)$, where $A = 144.6 (\pm 20.2)$ and $B = -17.2 (\pm 3.1)$ with a correlation coefficient of 0.64 ($n = 48$, Fig. 7) (higher degree terms were unnecessary since their standard errors are so high as to include zero).

The intrinsic buffering power of sartorius muscle of *Rana temporaria* as reported by Curtin (1986) seems to be unrelated to pH_i (6.4–7.4). On the other hand, the theoretical curve that she computed from the concentrations and pK_a 's of carnosine, histidine, and phosphate, the principal cytosolic buffers, agrees rather well with our own regression line. Thus, at pH_i 6.1 the β from regression is 40, from computation 41; at pH_i 6.7, 29 and 33; and at pH_i 7.4, 17 and 13, respectively. Most of Curtin's measurements were made with CO_2 pulses. Even though she used incorrect values for pK'_1 of H_2CO_3 at ionic strength 0.15 M and 20°C (6.12 rather than 6.22 [see Putnam, 1988, who drew attention to this common misreading of Harned and Bonner, 1945]) and for the solubility of CO_2 under the same conditions (0.0377

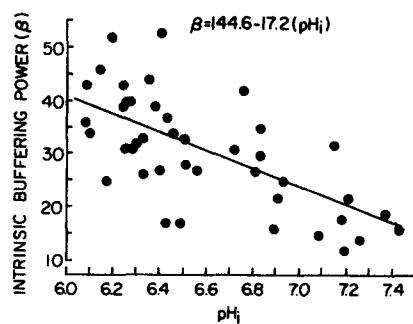


FIGURE 7. Relationship between intrinsic buffering power, β , and pH_i . A least-squares fitting led to the equation: $\beta = 144.6 - 17.2(\text{pH}_i)$. The data were derived from both normal and acidified fibers.

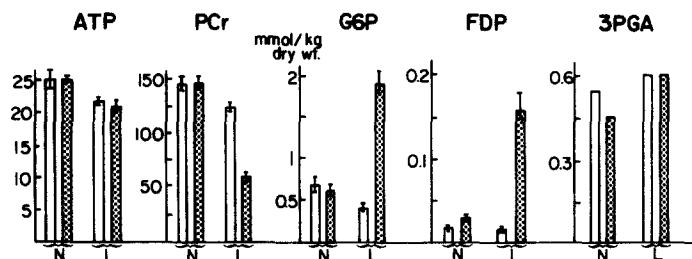


FIGURE 8. Summary of biochemical measurements on individual fibers. Each column shows the average \pm SEM of measurements on 20 fibers, 4 from each of 5 bundles. Each fiber was analyzed for all five compounds. Only two of the five bundles (eight fibers) were analyzed for 3PGA. For this reason SEM marks have been omitted, but agreement between the two bundles was excellent. Open bars, fibers in 2.5 mM K⁺; stippled bars, fibers in 25 mM K⁺. All measurements in 25 mM K⁺ were done 4–6 min after start of depolarization. N indicates normal fibers (average pH_i 7.48); L indicates acidified fibers (average pH_i 6.43). To convert mmol/kg dry weight in this and the next table into mmol/kg cell water, the data should be multiplied by 0.322 (Baylor et al., 1983) (the slight and uncertain amount of fiber swelling in the depolarized fibers is neglected).

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rather than 0.0496 mmol/kg per mmHg [Harned and Davis, 1943]), this does not affect the general distribution of her data.

Biochemical Measurements

The biochemical data are summarized in Figs. 8 and 9. As shown in Fig. 8, acidification by itself (pH_i 6.27–6.61, five bundles) only slightly reduced ATP, PCr, G6P, and FDP concentrations. However, 4–6 min after the start of high K⁺-induced depolarization the composition of the acid fibers had strikingly changed (pH_i just before depolarization, 6.16–6.59; at time of withdrawal of the electrodes from the depolarized fibers, 6.62–6.78; five bundles). Their PCr concentration had been reduced by half, while G6P had increased nearly fivefold and FDP tenfold. 3PGA remained unaffected, as did ATP. In contrast, depolarization of the normal fibers had no statistically significant effect on PCr, G6P, and FDP. An analysis was also made on three bundles at normal pH_i after a longer period of depolarization, 9–13 min, at which time pH_i had fallen by ~0.2 to a stable value. Here we observed a fall

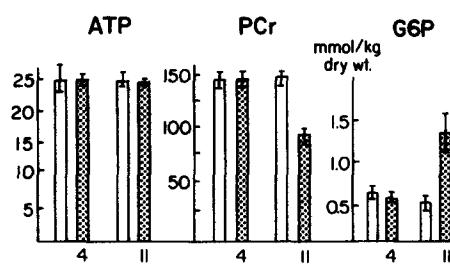


FIGURE 9. Summary of biochemical measurements on individual normal fibers (average pH_i 7.47). The fibers in 25 mM K⁺ were analyzed either 4 or 11 min after start of depolarization. The 20 fibers (from 5 bundles) analyzed after 4 min are the same as those shown in Fig. 8. The 12 fibers after an average of 11 min were derived from 3 bundles. Open bars, fibers in 2.5 mM K⁺; stippled bars, fibers in 25 mM K⁺.

in PCr and a rise in G6P (Fig. 9), but the changes were less than those observed after brief depolarization of the acid fibers. Lactate was the only other substance measured. Since unknown amounts of lactate must have left the superfused muscle, no quantitative importance can be attached to these measurements; they have not been included in Figs. 8 and 9. It is, however, significant that lactate was present in all categories of fibers, though at widely varying concentrations. Part of this variability may have a technical basis (see Methods).

DISCUSSION

At normal pH_i , depolarization of frog semitendinosus muscle produced a very slow intracellular acidification of ~ 0.2 , which in most cases was preceded by a brief alkalinization of ~ 0.04 . Dubuisson (1939, 1950) inferred a similar biphasic behavior of pH_i from the observed external pH changes in the bicarbonate-containing film covering the gastrocnemius muscle when it was tetanically stimulated. Abercrombie and Roos (1983), recording intracellularly, found that hypertonic semitendinosus fibers became acid when exposed to 50 mM K^+ , but both rate and degree of pH_i fall were greater than those found in the present studies, and no initial alkalinization was seen. As in this previous study, we ascribe the acidification observed in our normal fibers (average resting pH_i 7.37) to the enhanced production of acid, most likely lactic, by Ca^{2+} -stimulated glycolysis induced by depolarization. The initial alkalinization of ~ 0.04 is probably due to PCr hydrolysis. It corresponds to removal of 0.7 mmol of H ions from each liter of cell water ($\beta = 17.6$, see Fig. 7), due to partial conversion of hydrolysis-derived HPO_4^{2-} to $H_2PO_4^{-1}$. If no other processes involving H ions took place, a total of $(0.7 \times 10^{7.37-6.8}) + 0.7 = 3.3$ mmol of HPO_4^{2-} was generated from an equal amount of PCr (6.8 is the apparent pK_a of $H_2PO_4^{-1}$ at 25°C and $\mu = 0.16$ M [Edsall and Wyman, 1958]). Our failure to demonstrate biochemically such a small fall in PCr is not surprising since it approximately equals the SEM of the measurements (Fig. 8).

The main feature of this work is the striking rise in pH_i of the acid fibers when they are exposed to 25 mM K^+ . In general, the lower the pre-exposure pH_i , the greater was the increase. At pH_i 5.9 the alkalinization amounted to 0.44 pH units; at pH_i 6.7 it amounted to 0.12 pH units (Fig. 4). Note that even the smallest degree of alkalinization was three times that observed at normal pH_i .

Using the DMO method for measuring pH_i , Connell (1978) observed high K^+ -induced alkalinization in frog sartorius muscle even without NH_4Cl prepulsing. Possibly the very low starting pH_i of the muscles, 6.6, may explain his findings.

It is unlikely that the high K^+ -induced rise in pH_i is due to transmembrane events. (a) Both of the known membrane-bound mechanisms in frog muscle that could remove H^+ equivalents, namely, Na^+/H^+ exchange and $(Na^+ + HCO_3^-)/Cl^-$ exchange (Abercrombie et al., 1983; Putnam et al., 1986), were inoperative, since no Na^+ and hardly any HCO_3^- were present. Even though the prepulsing solution contained Na^+ , the subsequent 20–30 min of exposure to Na^+ -free superfusate before raising K^+ should have been sufficient to get rid of nearly all Na^+ (Putnam et al., 1986). (b) A leakage pathway for H^+ (or HCO_3^-) also would not explain the pH_i response. The electrochemical gradient for H^+ (and for HCO_3^-) favored inward rather than outward movement of H^+ equivalents throughout the period of

alkalinization in all but a few of the most acid fibers. Moreover, when the pH of the external solution after the NH₄Cl pulse was reduced to 6.2 (10 mM PIPES, pH_i 6.19–6.50, *n* = 4), 25 mM K⁺ again achieved a striking alkalinization despite an inward gradient for H⁺.

We conclude that the rise in pH_i must be due to intracellular events, most likely breakdown of PCr. In confirmation, 2,4-dinitrofluorobenzene, which specifically blocks PCr hydrolysis (Infante and Davies, 1965), abolished the alkalinization (Fig. 6). The alkalinization can in part be explained by a larger fraction of the released HPO₄²⁻ being protonated in the acid fibers than in the normal ones. For instance, at pH_i 6.3 three times as many H ions are removed as at 7.3. Since, however, the buffering power at 6.3 is about twice that at 7.3 (Fig. 7), the same degree of PCr hydrolysis in the acid fibers would raise pH_i by only 50% more than in the normal fibers. This is not nearly enough to explain the alkalinization that was observed. It follows that much more PCr must have been hydrolyzed at the lower pH_i. In agreement, 4–6 min of depolarization reduced PCr in five acid bundles (average pH_i 6.39) to half the concentration observed in five polarized acid bundles (average pH_i 6.41), whereas there was no measurable difference between the two groups in the five normal bundles whose average pH_i was 7.50 (Fig. 8).

The amount of HPO₄²⁻ released in the acid fibers, and thus the amount of PCr broken down, between the initial pH_i just before depolarization and the highest pH_i during depolarization can be calculated from these pH_i values (Keifer and Roos, 1981) if no other H⁺-consuming or -producing processes take place. The calculation takes into account the change in intrinsic buffering power between the two pH_i's as well as the increase in buffering due to the progressive release of HPO₄²⁻:

$$\text{HPO}_4^{2-} \text{ released} = \left(\frac{K + H_b}{H_b} \right) \left(\log \frac{H_a}{H_b} \right) \left(A - \frac{1}{2} B \log [H_a \cdot H_b] \right),$$

where H_a is the initial H ion concentration just before depolarization, H_b is the H ion concentration at the peak of the depolarization-induced alkalinization, *A* and *B* are the constants in the linear regression of intrinsic buffering power on pH_i, 144.6 and –17.2, respectively (see above), and *K* is the apparent dissociation constant of H₂PO₄⁻, 10^{-6.8}. (Fiber swelling during depolarization would reduce buffering power by 14% at most [Abercrombie et al., 1983]. The precise degree of swelling at the time of H_b is uncertain and has not been taken into account). The calculated values for HPO₄²⁻ released varied over a wide range (7–37 mmol/liter cell water) in the 25 fibers. This is to be expected, given the wide range of the pH_i changes (see Fig. 4). In four of the experiments biochemical analyses were made on bundles that were freeze-fixed at close to the peak of alkalinization, that is, when H_b represented the intracellular H ion concentration. The calculated reductions in PCr were 18.8, 11.5, 15.3, and 26.3 mmol/liter cell water, while the directly measured reductions were 32.6, 15.9, 10.8, and 20.2, respectively. It is not surprising that the agreement between theoretical and experimental values is only fair. The calculated values were derived from only one fiber per bundle, whereas the measured values represent the average of four fibers per bundle (not necessarily including the impaled one). Furthermore, the intrinsic buffering power was derived from the regression line, and may have differed significantly from that pertaining to the particular impaled fiber. Finally, other acid-producing or -consuming processes may have taken place. Even

though the ATP hydrolyzed was restored (ATP levels remained unchanged, Fig. 8) and thus did not affect cytosolic H ion concentration, some lactic acid was produced (see below).

The breakdown of PCr is interpreted as serving to restore the ATP, hydrolyzed in response to Ca^{2+} release from intracellular stores, into ADP and inorganic phosphate. Although not enough Ca^{2+} was released to evoke a full-fledged contracture, some movement on application of 25 mM K^+ probably took place (and often could be observed), which must have been associated with ATP breakdown. This role of Ca^{2+} in initiating the events is in agreement with the effects of caffeine and tetracaine. The former raised pH_i in the acid fibers, while the latter prevented the alkalinization. It must be admitted that the intracellular activity may have reduced the amount of Ca^{2+} released (Nakamaru and Schwartz, 1972) and also the mechanical response to the release of Ca^{2+} (Fabiato and Fabiato, 1978). This would explain why fiber movement in 25 mM K^+ was less in the acid fibers than in the normal ones.

The question arises why such a striking early breakdown of PCr was seen in the acid depolarized fibers but not in the normal depolarized ones. The answer probably is that in the acidified muscle, but not in the normal one, the rate of glycolysis, and thus the rate of ATP generated, was insufficient to replenish the ATP lost. Acidification may have acted at several sites of the glycogenolysis-glycolysis chain to achieve this effect. An early acid-sensitive site involves the conversion (by phosphorylase kinase) of glycogen phosphorylase b to the more active a form; the conversion accelerates glycogenolysis. Danforth (1965) found in electrically stimulated anoxic frog muscle that acidification by CO_2 delayed the appearance of phosphorylase a and even reduced its amount. It is possible that this had also taken place in our muscles (even though they were only mildly "stimulated" by 25 mM K^+ and not deprived of oxygen). This would reduce the amount of glucose available for glycolysis, since the superfusate contained no glucose. A second and perhaps more important reason for depressed glycolysis involves the pH sensitivity of the conversion of fructose-6-phosphate (F6P) into FDP by phosphofructokinase (PFK), an important controlling enzyme in the glycolytic pathway. Trivedi and Danforth (1966) showed that a reduction in the pH of cell-free extracts of frog and mouse muscle by less than 0.1 unit can lead to a more than 50-fold reduction in the activity of PFK. If these findings can be applied to a system of cells such as muscle, one might expect that in acid muscle depolarization would facilitate the PFK step less than in normal muscle. This could lead to an elevation of F6P (substrate of PFK) and G6P (which is in near equilibrium with F6P), and would reduce the amount of glycolytically generated ATP. PCr hydrolysis would thus be required for ATP restitution. As Fig. 8 indicates, G6P was, indeed, greatly increased in the acid fibers after 4–6 min of depolarization, but remained essentially unchanged in the normal depolarized fibers. ATP did not change in either condition. The situation is, however, more complicated than indicated thus far. The release of relatively large amounts of phosphate derived from PCr hydrolysis in the acid fibers, as well as the inferred increase of F6P and also of FDP (see below), all three known activators of PFK (Passonneau and Lowry, 1962; Lowry and Passonneau, 1966) may have opposed the PFK inhibition. Yet the rise in G6P suggests that the overall effect of acidification must have been inhibitory; the reduction in available glucose (see above) by itself would certainly not have raised G6P and might even have lowered it. Some glycolysis was still taking place in the

depolarized acid fibers, as shown by the production of lactic acid, but the experimental arrangement did not permit measurement of its rate of production. We would expect it to be less than in the depolarized normal fibers.

While the pH sensitivity of PFK has been confirmed in a number of cell-free preparations (see review by Hoffman, 1976, and also Wu and Davis, 1981), we could find no previous studies on intact cells or tissues that paralleled these findings.

If the only effect of depolarization in the acid fibers had been to provide a bottleneck at the PFK level, the product of the enzyme, FDP, should have been reduced or at least remained unchanged. However, in each of the five acid bundles, but not in normal ones, depolarization raised FDP significantly. The concentration of 3PGA was not affected. This suggests a third acid-induced inhibition located between FDP and 3PGA. The intermediates glyceraldehyde-3-phosphate and 1,3-diphosphoglycerate, interposed between these two compounds are very difficult to measure because of their low concentrations. The important point here is that the overall conversion of glyceraldehyde-3-phosphate to 3-phosphoglycerate involves the release of one proton, and that it has a standard free energy change at pH = 7 of only -3 kcal/mol. It follows that this conversion is quite sensitive to pH, which is in agreement with the biochemical findings.

Fig. 9 shows that when normal fibers were examined after a longer period of depolarization (11 min), their PCr had decreased by one-third, indicating that other pathways for ATP restitution were no longer sufficient. Whether a reduction of PFK activity brought on by the moderate acidification of the muscle and suggested by the increase in G6P plays a significant role here remains uncertain.

The depolarization-induced partial restoration of pH_i that we observed in our acid muscles would serve to reduce the functional impairment resulting from too great an acidity, such as reduced release of Ca²⁺ (Nakamaru and Schwartz, 1972) and reduced mechanical efficacy (Fabiato and Fabiato, 1978). This sustaining effect may also be of significance in the intact muscle under conditions of prolonged activity in which the accumulation of lactic acid might otherwise greatly depress pH_i.

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REFERENCES

- Abercrombie, R. F., R. W. Putnam, and A. Roos. 1983. The intracellular pH of frog skeletal muscle: its regulation in isotonic solutions. *Journal of Physiology*. 345:175–187.
- Abercrombie, R. F., and A. Roos. 1983. The intracellular pH of frog skeletal muscle: its regulation in hypertonic solutions. *Journal of Physiology*. 345:189–204.
- Aickin, C. C. 1981. A double-barrelled micro-electrode suitable for measurement of intracellular chloride activity in guinea-pig vas deferens. *Journal of Physiology*. 320:4P–5P.
- Aickin, C. C., and R. C. Thomas. 1977a. The effect of external Na and amiloride on pH recovery in mouse soleus muscle. *Journal of Physiology*. 269:80P–81P.
- Aickin, C. C., and R. C. Thomas. 1977b. An investigation of the ionic mechanism of intracellular pH regulation in mouse soleus muscle fibers. *Journal of Physiology*. 273:295–316.
- Almers, W., and P. M. Best. 1976. Effects of tetracaine on displacement currents and contraction of frog skeletal muscle. *Journal of Physiology*. 262:583–611.

- AMORENA ET AL. *Changes in Intracellular pH Caused by High K in Frog Muscle*
- Ammann, D., F. Lanter, R. A. Steiner, P. Schulthess, Y. Shijo, and W. Simon. 1981. Neutral carrier-based hydrogen ion selective microelectrode for extra- and intracellular studies. *Analytical Chemistry*. 53:2267–2269.
- Amorena, C., J. K. Manchester, T. J. Wilding, and A. Roos. 1989. Depolarization raises intracellular pH (pH_i) in acid-loaded frog muscle even though it lowers it in normal muscle. *Biophysical Journal*. 55:411a. (Abstr.)
- Baylor, S. M., W. K. Chandler, and M. W. Marshall. 1983. Sarcoplasmic reticulum calcium release in frog skeletal muscle fibres estimated from Arsenazo III calcium transients. *Journal of Physiology*. 344:625–666.
- Boron, W. F. 1977. Intracellular pH transients in giant barnacle muscle fibers. *American Journal of Physiology*. 233:C61–C73.
- Boron, W. F., and P. De Weer. 1976. Intracellular pH transients in squid giant axons caused by CO_2 , NH_3 , and metabolic inhibitors. *Journal of General Physiology*. 67:91–112.
- Boron, W. F., W. C. McCormick, and A. Roos. 1979. pH regulation in barnacle muscle fibers: dependence on intracellular and extracellular pH. *American Journal of Physiology*. 237:C185–C193.
- Boyarsky, G., M. B. Ganz, R. B. Sterzel, and W. F. Boron. 1988. pH regulation in single glomerular mesangial cells. I. Acid extrusion in absence and presence of HCO_3^- . *American Journal of Physiology*. 255:C844–C856.
- Connett, R. J. 1978. Association of increased pH_i with calcium ion release in skeletal muscle. *American Journal of Physiology*. 234:C110–C113.
- Curtin, N. A. 1986. Buffer power and intracellular pH of frog sartorius muscle. *Biophysical Journal*. 50:837–841.
- Danforth, W. H. 1965. Activation of glycolytic pathway in muscle. In *Control of Energy Metabolism*. B. Chance, R. W. Estabrook, and J. R. Williamson, editors. Academic Press, New York. 287–297.
- Dubuisson, M. 1939. Les processus physico-chimiques de la contraction musculaire. *Annales de Physiologie et de Physicochimie Biologique*. 15:461–470.
- Dubuisson, M. 1950. Some chemical and physical aspects of muscle contraction and relaxation. *Proceedings of the Royal Society of London, Series B*. 137:63–70.
- Edsall, J. T., and J. Wyman. 1958. *Biophysical Chemistry*. Academic Press, New York. 442, 452.
- Fabiato, A., and F. Fabiato. 1978. Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. *Journal of Physiology*. 276:233–255.
- Furusawa, K., and P. M. T. Kerridge. 1927. The hydrogen ion concentration of the muscles of the cat. *Journal of Physiology*. 63:33–41.
- Gordon, A. M., and R. E. Godt. 1970. Some effects of hypertonic solutions on contraction and excitation-contraction coupling in frog skeletal muscles. *Journal of General Physiology*. 55:254–275.
- Harned, H. S., and F. T. Bonner. 1945. The first ionization of carbonic acid in aqueous solutions of sodium chloride. *Journal of the American Chemical Society*. 67:1026–1031.
- Harned, H. S., and R. Davis, Jr. 1943. The ionization constant of carbonic acid in water and the solubility of carbon dioxide in water and aqueous salt solutions from 0–50°. *Journal of the American Chemical Society*. 65:2030–2037.
- Hintz, C. S., M. M.-Y. Chi, R. D. Fell, J. L. Ivy, K. K. Kaiser, C. V. Lowry, and O. H. Lowry. 1982. Metabolite changes in individual rat muscle fibers during stimulation. *American Journal of Physiology*. 242:C218–C228.
- Hodgkin, A. L., and P. Horowicz. 1957. The differential action of hypertonic solutions on the twitch and action potential of a muscle fibre. *Journal of Physiology*. 136:17P–18P.

- Hoffman, E. 1976. The significance of phosphofructokinase to the regulation of carbohydrate metabolism. *Reviews of Physiology, Biochemistry and Pharmacology*. 75:1–68.
- Infante, A. A., and R. E. Davies. 1965. The effect of 2,4-dinitrofluorobenzene on the activity of striated muscle. *Journal of Biological Chemistry*. 240:3996–4001.
- Keifer, D. W., and A. Roos. 1981. Membrane permeability to the molecular and ionic forms of DMO in barnacle muscle. *American Journal of Physiology*. 240:C73–C79.
- Lowry, O. H. 1953. The quantitative histochemistry of the brain: histological sampling. *Journal of Histochemical Cytochemistry*. 1:420–428.
- Lowry, O. H. 1980. Amplification by enzymatic cycling. *Molecular Cellular Biochemistry*. 32:135–146.
- Lowry, O. H., and J. V. Passonneau. 1966. Kinetic evidence for multiple binding sites on phosphofructokinase. *Journal of Biological Chemistry*. 241:2268–2279.
- Lowry, O. H., J. V. Passonneau. 1972. A Flexible System of Enzymatic Analysis. Academic Press, New York. 291 pp.
- Lowry, O. H., J. V. Passonneau, F. X. Hasselberger, and D. W. Schulz. 1964. Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain. *Journal of Biological Chemistry*. 239:18–30.
- Lüttgau, H. C., and H. Oetiker. 1968. The action of caffeine on the activation of the contractile mechanism in striated muscle fibers. *Journal of Physiology*. 194:51–74.
- Nakamaru, Y., and A. Schwartz. 1972. The influence of hydrogen ion concentration on calcium binding and release by skeletal muscle sarcoplasmic reticulum. *Journal of General Physiology*. 59:22–32.
- Passonneau, J. V., and O. H. Lowry. 1962. Phosphofructokinase and the Pasteur effect. *Biochemical and Biophysical Research Communications*. 7:10–15.
- Putnam, R. W. 1985. Effect of insulin on intracellular pH in frog skeletal muscle fibers. *American Journal of Physiology*. 248:C330–C336.
- Putnam, R. W. 1988. Na/H exchange. In *Basic Principles of pH Regulation*. S. Grinstein, editor. CRC Press, Boca Raton, FL. 139–153.
- Putnam, R. W., A. Roos, and T. J. Wilding. 1986. Properties of the intracellular pH-regulating systems of frog skeletal muscle. *Journal of Physiology*. 381:205–219.
- Reuss, L., and J. L. Costantin. 1984. Cl/HCO₃ exchange at the apical membrane of Necturus gall bladder. *Journal of General Physiology*. 83:801–818.
- Taylor, S. R., R. Rudel, and J. R. Blinks. 1975. Calcium transients in amphibian muscle. *Federation Proceedings*. 34:1379–1381.
- Trivedi, B., and W. H. Danforth. 1966. Effect of pH on the kinetics of frog muscle phosphofructokinase. *Journal of Biological Chemistry*. 241:4110–4112.
- Vaughan-Jones, R. D., and M.-L. Wu. 1990. pH dependence of intrinsic H⁺ buffering power in the sheep cardiac Purkinje fibre. *Journal of Physiology*. 425:429–448.
- Weber, A., and R. Herz. 1968. The relationship between caffeine contracture of intact muscle and the effect of caffeine on reticulum. *Journal of General Physiology*. 52:750–759.
- Wu, T.-F. L., and E. J. Davis. 1981. Regulation of glycolytic flux in an energetically controlled cell-free system: the effects of adenine nucleotide ratios, inorganic phosphate, pH, and citrate. *Archives of Biochemistry and Biophysics*. 209:85–99.