Characterization of the Effect of pH on the Excitation-Contraction Coupling System of Canine Masseter Muscle*

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Abstract—The effect of pH on the excitation-contraction coupling system of canine masseter muscle was studied by evaluating the functional integrity of the sarcoplasmic reticulum (SR) and myofibrils. Increasing proton concentration (pH 7.0–5.8) significantly reduced oxalate supported SR calcium uptake velocity, while Ca^{2+} -stimulated, Mg^{2+} -dependent ATPase activity was unaffected by pH. The efficiency ratio of calcium transport, or the coupling ratio ($\mu\text{moles Ca}^{2+}$ transported/ $\mu\text{moles ATP}$ hydrolyzed), decreased from 1.094 ± 0.042 at pH 7.0 to 0.946 ± 0.036 at pH 6.0 ($P < 0.05$) and to 0.780 ± 0.024 at pH 5.8 ($P < 0.01$). Myofibrillar pCa ($-\log [\text{free Ca}^{2+}]$)-ATPase activity was unaffected between pH 7.0 and pH 6.5. At pH 6.0, increasing Ca^{2+} concentration inhibited myofibrillar ATPase activity, and this inhibitory phenomenon was accentuated at pH 5.8. Kinetic analysis of the myofibrillar pCa-ATPase data, utilizing double-reciprocal plots, demonstrated an increase in K_m at low pH. It is concluded that acidosis significantly uncouples calcium transport from ATP hydrolysis in the SR of masseter muscle and significantly alters myofibrillar ATPase activity. It is hypothesized that these defects may explain an observed depression in skeletal muscle cell function during ischemia.

In skeletal sarcoplasmic reticulum (SR), Arkhipenko et al. (1) found an uncoupling of calcium uptake from ATP hydrolysis during limb tourniquet ischemia with accompanying accumulation of lipid peroxidation products. It has been previously shown that the generation of free radicals can cause an uncoupling of oxalate-supported calcium uptake from the Ca^{2+} -stimulated, Mg^{2+} -dependent ATPase (Ca^{2+} -ATPase) activity of the SR calcium pump in myocardium (2–4) and in masseter muscle (5). However, the mediators for this uncoupling process in the ischemic muscle have not been conclusively identified.

A significant process that would fuel this cascade and lead to a breakdown of the excitation-contraction coupling system during ischemia is the generation of protons.

There is abundant evidence that during ischemia, there is a switch from aerobic to anaerobic metabolism with increased rates of glycolysis and, therefore, the generation of protons at an increased rate (6). The accumulation of these hydrogen ions leads to a decrease in intracellular pH of the muscle, a finding now documented by direct intracellular pH measurements (7) and nuclear magnetic resonance studies (8). However, to date, the effect of increasing proton concentration on the excitation-contraction coupling system in masseter muscle has not been critically examined. To aid our understanding of the action of acidosis on masseter muscle, we need to characterize the effect of pH on this system. One method of critically analyzing this problem would be to examine those processes at a subcellular level that are responsible for the maintenance of the normal excitation-contraction coupling mechanism within the masseter muscle cell.

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Intracellularly, there are two prime candidates that have been identified as important subcellular organelles involved in the excitation-contraction coupling mechanism: the SR and the myofibril. The SR is the intracellular network of tubules that has been incriminated as the source of coupling calcium within the muscle cell and, via an adenosine triphosphate (ATP) dependent calcium pump, is responsible for actively sequestering cytosolic calcium, resulting in relaxation. There is currently a large body of evidence (1, 9) incriminating sarcoplasmic function as one of the first intracellular processes to breakdown as a result of ischemia. The contractile proteins, which are actively responsible for shortening and tension development, have also been incriminated in the intracellular breakdown of the excitation-contraction coupling system as a result of the ischemic process.

It was, therefore, the purpose of this study to characterize the effects of increasing proton concentration on calcium transport and Ca^{2+} -ATPase activity of SR and myofibrillar pCa-ATPase activity of 'masseter muscle' and to determine whether the results of this in vitro study can correlate with and explain the mechanical dysfunction of ischemic skeletal muscle.

Materials and Methods

Healthy, adult dogs were anesthetized with sodium pentobarbital (25 mg/kg, i.v.), and their masseter muscle was rapidly removed and placed in ice-cold 0.9% NaCl. All subsequent procedures were carried out in a 4°C cold room. The muscle was cleaned of fatty and connective tissue, diced with scissors and then homogenized (1 g muscle/4 vol. 10 mM imidazole buffer, pH 7.0) in a Sorvall Omnimixer for 1 min at top speed, two times, with a rest interval of 1 min.

1) Sarcoplasmic reticulum (SR): An aliquot of this homogenate was saved and designated as unfractionated homogenate; calcium transport by the homogenate was studied under conditions that optimize SR calcium uptake as previously described by Solaro and Briggs (10), which allows for the characterization of SR function in unfractionated homogenates to determine if the

calcium uptake and Ca^{2+} -ATPase activities of unfractionated homogenates of muscle correspond to these activities in SR fractions isolated from the same homogenates. The homogenate was centrifuged at 10,000 g for 20 min. The pellet was rehomogenized in 4 vol. of 10 mM imidazole and spun again at 10,000 g for 20 min. The supernatant from this and the previous centrifugation were combined, poured through four layers of cheesecloth, and then centrifuged at 12,000 g for 15 min. The supernatant was filtered through eight layers of cheesecloth and centrifuged at 31,000 g for 90 min. The pellets from this spin were rehomogenized by use of a Potter-Elvehjem homogenizer with a Teflon pestle in 1 M KCl, 10 mM imidazole buffer and then centrifuged at 145,000 g for 60 min. The SR pellet was rehomogenized in 30% sucrose, 20 mM Tris-HCl, pH 7.0. Protein concentration was determined by the method of Lowry et al. (11). The isolated SR were kept at -20°C for 1-4 days.

The velocity of calcium uptake by the SR was determined in the presence of oxalate by use of the Millipore filtration technique. All incubations were carried out at 27°C. The composition of the incubation solution was 100 mM KCl, 20 mM imidazole buffer, pH 7.0-5.8, 10 mM NaN_3 , 10 mM potassium oxalate, 5 mM Na_2ATP , 5 mM MgCl_2 , and 200 μM CaCl_2 with 0.05 μCi $^{45}\text{Ca}/\text{ml}$. The reaction was started by the addition of 0.09-0.13 mg SR protein/ml reaction bath, and aliquots were taken at various time intervals over 2 min and filtered through Millipore filters of 0.45 μm pore diameter; the filtrate was then counted in a Beckman liquid-scintillation spectrometer. The velocity of calcium uptake is presented as μmol Ca^{2+} per mg SR protein per min. The reaction is linear over the 2 min period.

Ca^{2+} -stimulated, Mg^{2+} -dependent adenosine triphosphatase (ATPase) activity of the SR was determined utilizing the same medium as SR calcium uptake velocity except that $^{45}\text{CaCl}_2$ was omitted. ATPase activity was determined as the rate at which inorganic phosphate (Pi) was liberated during the incubation. Samples were analyzed for inorganic phosphate by the method of Penney (12). The ATP hydrolysis associated

with the uptake of calcium was calculated as the difference between the ATPase activity in the presence and in the absence of 0.02 M EGTA [ethyleneglycol bis (oxyethylene nitrilo) tetraacetic acid] and was expressed as $\mu\text{mol Pi}$ per mg SR protein per min.

2) Myofibrils: Myofibrils of masseter muscle were isolated by a method similar to that of Solaro (13), except the first two washes were performed in 3 pellet vol. of 10 mM imidazole buffer, pH 7.0. This preparation has been independently reviewed by Sheurer and Bahn (14) and found to yield the most physiologic representation of contractile protein function and to be free of phospholipid (SR, sarcolemma and mitochondria) contamination. Following the 1% Triton X-100 washes, the myofibrils were then washed in 10 pellet vol. of 2 mM EGTA, 2 mM MgCl_2 , 60 mM KCl and 30 mM imidazole buffer, pH 7.0. Myofibrillar ATPase activity was determined by measuring inorganic phosphate liberated in the incubation medium with varying free Ca^{2+} concentrations. The reaction was started by addition of protein and was stopped at predetermined times by adding samples of the incubation solution to equal amounts of iced 10% trichloroacetic acid. Inorganic phosphate was determined by the method of Penney (12). ATPase reactions were run at 27°C for up to 8 min with about 0.5 mg/ml myofibrillar protein. Less than 15% of the ATP was hydrolyzed during these reaction times. Myofibrils were incubated in 2.14 to 2.34 mM Na_2ATP , 4.0 mM MgCl_2 , 83–89 mM KCl, 30 mM imidazole to achieve a constant 2 mM Mg^{2+} -ATP, 2 mM free Mg^{2+} , and ionic strength of 0.12. Various pCa's were achieved by varying the total EGTA at a constant 50 μM CaCl_2 .

The total concentrations of metals and ligands in incubation solutions were calculated by specifying the desired pH, free Ca^{2+} , free Mg^{2+} , Mg^{2+} -ATP and ionic strength by means of the calculator program described by Fabiato and Fabiato (15). Absolute stability constants used in the program are those compiled by Fabiato (Table 1 in ref. 16) in which the $\log_{10}$ of the stability constants of hydrogen-ligand complexes have been increased 0.12, as discussed by Tsien and Rink (17). Added CaCl_2 and EGTA

concentrations were varied to achieve various pCa's.

The pH of each individual solution was adjusted at 27°C following the method of Fabiato and Fabiato (18) and re-checked prior to the initiation of the reaction sequence. All solutions were prepared with deionized, distilled water, using analytical grade reagents. Statistical significance tests were performed between all groups using Student's *t*-test or an analysis of variance when comparing two populations to a control. A value of $P < 0.05$ was accepted as indicating a significant difference.

Results

The effect of pH in the range 7.0 to 5.8 on calcium transport in the unfractionated homogenate with conditions established such that the sarcoplasmic reticulum (SR) is the principal active, calcium transport organelle

Fig. 1. Oxalate supported calcium uptake by the unfractionated homogenate (A: calcium uptake velocity is expressed as $1 \times 10^{-3} \mu\text{moles Ca}^{2+}$ accumulated per min per mg protein.) and isolated sarcoplasmic reticulum (B: calcium uptake velocity is expressed as $\mu\text{moles Ca}^{2+}$ accumulated per min per mg SR protein.), and Ca^{2+} -stimulated, Mg^{2+} -dependent ATPase activity of isolated sarcoplasmic reticulum (C: Ca^{2+} -ATPase activity is expressed as $\mu\text{moles Pi}$ produced per min per mg SR protein.) at varying pH. Each point represents the mean ($n=6-8$) and the bar represents ± 1 S.E.M. Significance of difference: * $P < 0.05$ and ** $P < 0.01$, compared to pH 7.0.

involved is shown in Fig. 1A. There was no difference in calcium uptake rates between pH 6.0 and 7.0, but at pH 5.8, there was a significant decrease ($P<0.01$) in calcium uptake velocity.

Having established that calcium transport by the SR in the unfractionated homogenate can be significantly depressed at pH 5.8, the fragmented SR was isolated in an attempt to further characterize this defect. The depression of SR calcium transport in the unfractionated homogenate by decreasing pH was also demonstrated in the isolated SR. Figures 1B and 1C present calcium uptake velocity and Ca^{2+} -stimulated, Mg^{2+} -dependent ATPase (Ca^{2+} -ATPase) activity of the isolated SR. In this series of studies, Ca^{2+} -ATPase activity was found to be unaffected over the pH range of 7.0–5.8. However, pH 6.0 produced a significant decrease ($P<0.05$) in calcium uptake velocity. At pH 5.8, there was a 26% depression of SR calcium uptake compared with calcium uptake at pH 7.0.

Table 1 presents the efficiency of calcium transport over the pH range of 7.0–5.8 as evaluated by the coupling ratio ($\mu\text{moles Ca}^{2+}$ transported/ $\mu\text{moles ATP}$ hydrolyzed). In this series of studies, a coupling ratio of 1.094 ± 0.042 at pH 7.0 was found which decreased to 1.081 ± 0.051 at pH 6.5 ($P=\text{NS}$). However, at pH 6.0, the coupling ratio had decreased to 0.946 ± 0.036 ($P<0.05$) which further decreased to 0.780 ± 0.024 at pH 5.8. This decrease in coupling ratio represents a significant degree of uncoupling of calcium transport from ATP hydrolysis in the pH range of 6.0–5.8; i.e., the pump system is much less efficient.

Since there is a known tight coupling

between contractile protein function and muscle contractility (19, 20) and given the demonstration of impaired mechanical activity, myofibrillar ATPase activities were studied. Furthermore, since myofibrillar ATPase activity is dependent on the amount of free calcium available for contraction (21), ATPase activities were studied over the range of free calcium known to exist intracellularly. The effect of varying pH on contractile protein function as assessed by myofibrillar pCa-ATPase curves is presented in Fig. 2. Between pH 7.0 and pH 6.5, there

Fig. 2. The Ca^{2+} -stimulated, Mg^{2+} -dependent myofibrillar ATPase activity at pH 7.0 (●), 6.5 (△), 6.0 (○) and 5.8 (■). The ordinate is expressed as free calcium concentration between $10^{-5.4}$ and 10^{-7} M. Each point represents the mean ($n=4-6$) and the bar represents ± 1 S.E.M. Significance of difference: * $P<0.01$, compared to pH 7.0.

Table 1. Characterization of the efficiency of isolated sarcoplasmic reticulum calcium transport as a function of pH

pH	n	Coupling ratio ¹	P value ²
7.0	6	1.094 ± 0.042	
6.5	6	1.081 ± 0.051	NS
6.0	6	0.946 ± 0.036	0.05
5.8	4	0.780 ± 0.024	0.01

Values presented are means ± 1 S.E.M. ¹Coupling ratio expressed as $\mu\text{moles Ca}^{2+}$ accumulated per minute per mg SR protein/ $\mu\text{moles Pi}$ produced per minute per mg SR protein. ²The probability, as determined by the analysis of variance that the two populations came from the same control population.

Fig. 3. Double-reciprocal plots of the myofibrillar ATPase activity at pH 7.0 (●), 6.5 (△), 6.0 (○) and 5.8 (■). Each point was derived from the mean value presented in Fig. 2. Calculations: pH 7.0: $V_{\max}=0.1974 \mu\text{moles Pi}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$, $K_m=1.530\times 10^{-7} \text{ mol}^{-1}$, $r=0.999$. pH 6.5: $V_{\max}=0.1986 \mu\text{moles Pi}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$, $K_m=1.556\times 10^{-7} \text{ mol}^{-1}$, $r=0.997$. pH 6.0: $V_{\max}=0.1793 \mu\text{moles Pi}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$, $K_m=2.475\times 10^{-7} \text{ mol}^{-1}$, $r=0.996$. pH 5.8: $V_{\max}=0.1591 \mu\text{moles Pi}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$, $K_m=4.229\times 10^{-7} \text{ mol}^{-1}$, $r=0.991$.

was no difference in the pCa-myofibrillar ATPase curve. With the exception of the first point at pCa=7.0, each point of the pCa-myofibrillar ATPase curve at pH 6.0 was significantly depressed when compared with that at pH 7.0. There was a further shift of the curve down and to the right at pH 5.8.

Since the cleaving of the terminal high-energy phosphate from ATP by the myosin head is an enzymatic reaction, enzyme kinetics were applied utilizing double reciprocal plots, and a calculated Hill coefficient of 2 (22, 23) was obtained. Figure 3 presents the results of such an analysis. The increasing proton concentration between pH 7.0 and 5.8 resulted in increase in K_m values and decrease in $V_{\max}$ for the myofibrillar ATPase activity. This suggests a change in affinity in addition to the significant depression in maximal ATPase activity.

Discussion

It is uncertain whether any rapid intracellular pH changes arise during excitation-contraction coupling of ischemic masseter muscle. Conway (24) predicted that in resting skeletal muscle, intracellular pH should

be lower than extracellular pH. Considering the relationship between the extracellular and intracellular ionic compositions and the Donnan equilibrium, intracellular pH should be approximately 6.0. Contrary to this suggestion, most intracellular pH measurements (dimethyl oxide [DMO], CO_2 , and glass microelectrode procedures) have yielded values from 6.8 to 7.0 (25).

Alterations of intracellular proton concentration may also be involved in some pathological conditions. Katz and Hecht (26) suggest, e.g., that intracellular acidosis may be a cause of the decrease of contractility observed during myocardial ischemia. During the course of both hypoxia and ischemia, utilizing the isolated rabbit intraventricular septum, Coobe and Poole-Wilson (7) have correlated the decrease in developed tension with a decrease in intracellular pH (7.0 to 6.4). According to this concept, protons produced during increased anaerobic glycolysis in ischemia would displace troponin-bound calcium, decreasing the number of actin-myosin interactions.

Even given the difficulties and complex assumptions of extrapolating from various different models of ischemia (variations in species, temperature, skeletal and cardiac muscles, etc.), it would appear that the present study can explain in part the decrease in contractility and fall in tissue pH in masseter muscle. In our system, as the pH falls below 6.5, there is a significant uncoupling of ATP hydrolysis from calcium transport in the isolated sarcoplasmic reticulum (SR). If the SR is capable of serving as both a source and a sink for activator calcium in the excitation-contraction coupling process (27), this uncoupling process would result in a decrease in coupling calcium available for release to troponin, decreased calcium troponin interaction, and a decrease in crossbridge formation. In the physiologic system, this would be expressed as a decrease in tension development. Our data would suggest that at pH 6.5, contractile protein function would not be significantly affected. This conclusion does not agree with the data of Fabiato and Fabiato (18) in the skinned muscle preparation and the data of Kentish and Nayler (28) in a similar preparation of

rabbit white skeletal myofibrils prepared by the Triton-X 100 method. These differences in the pH-sensitivities of masseter muscle and other myofibrillar ATPases cannot account for more than a small part of the contrasting mechanical responses of masseter muscle and other muscle fibers to an acidic pH. Clearly, other explanations are required.

The most dramatic effect of the increase in proton concentration was to shift down the pCa-ATPase relationships for myofibrils to lower pCa value; i.e., to higher Ca^{2+} concentrations. The defect in the contractile proteins induced by acidosis does not appear to be simple. At the pH range of 6.0–5.8, not only is the maximal myofibrillar ATPase activity depressed, but according to the kinetic analysis, there is also an increase in K_m . A possible explanation for this increase in K_m is a decrease in affinity of calcium binding to troponin that would result in less hydrolysis of ATP and a resultant decrease in tension development. This is supported by Fig. 2, which demonstrates that 50% activation occurs at a pCa of 6.65 at pH 7.0. However, at the same pCa, the resultant ATPase activity is 36% and 16% at pH 6.0 and 5.8, respectively. Accompanying this increase in K_m , there is also a decrease in maximal ATPase activity. This observation rules out a simple competitive inhibition mechanism, because even at maximal intracellular free calcium levels, ATPase activity does not approach the values obtained at pH 7.0. The inhibition of the enzyme activity may be the result of an ordered single displacement reaction involving both calcium and ATP, because both of these substrates must bind to different regulatory proteins before hydrolysis of ATP can occur. This may result in our observed depression in maximal ATPase activity. This argument is supported by Reddy et al. (29) who demonstrated that in the ischemic muscle, there was a defect in the regulatory proteins. They found that myosin B isolated from ischemic muscle was not sensitive to EGTA. This loss of sensitivity was thought to reflect a defect in one of the components of troponin. If this is correct, a defect in troponin either in affinity for calcium or in a change in cooperativity would result in a decreased number of

crossbridges formed, a decrease in myofibrillar ATPase activity, and ultimately, a decrease in tension development in masseter muscle.

Thus it would appear that intracellular acidosis, by interrupting the intracellular components of the excitation-contraction coupling system, is able to explain numerous and sometimes divergent observations on the ischemic muscle. Whether these effects are directly or indirectly (by production of free radicals) mediated by protons, has not been conclusively shown. In summary, we have demonstrated that the effect of acidosis on the excitation-contraction coupling system of masseter muscle is characterized by a defect in the calcium transport system of the SR with an uncoupling of calcium transport from ATP hydrolysis, a depression of maximal myofibrillar ATPase activity and an increase in K_m . This state of excitation-contraction uncoupling is capable of explaining some of the major mechanical and biochemical abnormalities in the ischemic masseter muscle.

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