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Differential, Direct Effects of H^+ on Ca^{2+}-Activated Force of Skinned Fibers from the Soleus, Cardiac and Adductor Magnus Muscles of Rabbits*

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Abstract. The effect of acidosis on Ca^{2+}-activated force generation was studied in rabbit soleus, left ventricular, and adductor magnus muscles. Fibers were skinned (sarcolemma peeled off or mechanico-chemically disrupted) to facilitate direct manipulation and standardization of their intracellular ionic milieus according to bathing solution composition. Skinned single skeletal and small bundles of cardiac fibers were mounted in a photodiode force transducer and activated by immersion in buffered-Ca^{2+} bathing solutions. The magnitude of steady state isometric force at each $[Ca^{2+}]$ was determined at pH 7.0 and 6.5 (paired data) at both 1 mM and 10 mM Mg^{2+} in order to detect artifacts of errors in calculated $[Ca^{2+}]$. All bathing solutions contained: 7 mM total EGTA [ethyleneglycol-bis-(β-amino-ethylether)-N,N′ tetra-acetic acid], 70 mM $(Na^+ + K^+)$, 2 mM $MgATP^{2-}$ (Mg adenosine triphosphate), 15 mM CP^{2-} (creatine phosphate), 15 units/ml CPK (creatine phosphokinase), imidazole (adjusted ionic strength to 0.15 M), and propionate anion at 23 ± 1° C. Maximum tensions were similar at both $[Mg^{2+}]$s but less at pH 6.5 than at pH 7.0, with the following order of mean magnitude of acidotic depression adductor > cardiac > soleus. The proportionately greater acidotic depression of submaximum (relative to maximum) forces that occurred only at 1 mM Mg^{2+} (cardiac > adductor > soleus) implicates acidotic depression of Ca^{2+}-activated force as a major cause of decreased cardiac contractility.

Key words: Striated muscle – Contraction – Acidosis – Muscle types – Ca^{2+}-activation.

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Introduction

Acidosis has a direct, pronounced negative inotropic effect on cardiac muscle [6, 24, 28–30, 34] and may be responsible for the early, rapid decline in force generation during myocardial ischemia [44]. In this regard, excess H^+ appears to act primarily intracellularly; the depression of force from acidosis due to elevation of extracellular P_{CO_2} is greater and reaches steady state more quickly than that in response to equivalent extracellular acidosis created by decreasing $[HCO_3^-]$ at constant P_{CO_2}, as would be expected from the more rapid equilibration of gases versus ions across cell membranes. Apparently also due to the more rapid equilibration of CO_2, elevation of extracellular P_{CO_2} causes a transient negative inotropic effect even when extracellular pH is maintained at a normal value by simultaneously increasing extracellular $[HCO_3^-]$ [34].

However, the specific intracellular mechanisms responsible for the depressant effect of acidosis on cardiac contractility remain obscure. These mechanisms must be unique to cardiac muscle, or at least different for skeletal and cardiac muscles, since twitch and tetanic forces of rat soleus muscle are enhanced, rather than depressed, by acidosis from increased extracellular P_{CO_2} [31]. Alterations in metabolism or supply of high energy phosphate compounds necessary for contraction appear unrelated to the effect [44]. There are no associated changes in series elasticity [40]; therefore, passive mechanical properties probably do not contribute. Increases in intracellular $[K^+]$ are associated with respiratory acidosis uniquely in cardiac muscle but appear unrelated to changes in contractility [34].

There are several possible mechanisms whereby H^+ might exert its negative inotropic effect. On the basis of current theories of muscle contraction, either the sarcoplasmic Ca^{2+} level or the response of the contractile proteins to Ca^{2+} might be altered. The sarcoplasmic reticulum binds more Ca^{2+} at acid pH and thus

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might produce a decrease in the amount of Ca^{2+} released internally for activation of contractile proteins. The effect appears similar for both mammalian cardiac muscle and skeletal muscle [25,26], however, and so would not account for the selective depressant effect of acidosis on the myocardium. Cardiac muscle contraction appears uniquely dependent on a slow inward Ca^{2+} current across the sarcolemma during the plateau phase of the action potential. But during acidosis the changes in action potential associated with alterations of this current appear dissociated from depression of force generation [34], although the evidence for mammalian myocardium is only indirect [42].

The effect of H^+ might be directly on the contractile-protein force-generating apparatus; as originally proposed by Katz and Hecht [20], H^+ might reduce the effect of Ca^{2+} on troponin and thus lower force generation. A pH-dependence of Ca^{2+}-binding to isolated troponin has not been demonstrated [14] but acidotic depression of Ca^{2+}-sensitivity of the force-generating apparatus has been noted in glycerinated dog papillary muscle [39] and skinned fibers from frog skeletal [5,13,37] and rat cardiac muscles [13]. The effect appears to be very similar, however, for frog skeletal and rat cardiac muscles [13]. Moreover, in all of these studies a chelator was used to buffer $[Ca^{2+}]$, and such chelators have a pH-dependent affinity for Ca^{2+}. Thus, part or all of the observed changes in Ca^{2+}-sensitivity attributed to acidosis could have been artifacts of incorrect binding constants used in calculating $[Ca^{2+}]$ (concentration of free Ca^{2+}) of the activating bathing solutions. However, maximum tension at saturating Ca^{2+}-concentrations is not subject to these errors and was observed to be depressed in cardiac muscle by Schädler [39] and in skeletal muscles by Robertson and Kerrick [37], though not in other studies [5,13].

The purpose of the present study was to evaluate alteration of the Ca^{2+}-activation of contractile proteins as a mechanism in the negative inotropic action of acidosis by comparing isometric tensions generated in rabbit skeletal and left ventricular fibers at 7.0 and 6.5 under identical intracellular ionic conditions.

Methods

Skinned Fiber Preparations. Adductor magnus (fast-twitch skeletal), soleus (slow-twitch skeletal) and left ventricular (cardiac) muscle fibers of female New Zealand white rabbits were studied. The animals were sacrificed by an overdose of sodium pentobarbital, the heart was removed while still beating, and the skeletal muscles were excised immediately afterwards. Fresh tissue was used on each experimental day.

Small bundles of cardiac fibers (40 – 150 μm wide, 1 – 3 mm long) were obtained from a slice of left ventricular tissue (apical) by homogenization in a relaxing solution (see below) as described by Kerrick and Best [21]. The sarcolemmas of the individual cells in each cardiac bundle were either mechanically disrupted during homogenization or chemically skinned by the high concentrations of EGTA (7 mM) present in all bathing solutions. As evidence that these fibers were skinned, further treatment of selected bundles (30 min soak) with detergents (1% Triton X-100 and 1% or 0.5% Brij-58) did not alter absolute force generation at any $[Ca^{2+}]$ or pH. The homogenate was diluted with relaxing solution and kept in ice for the remainder of the day.

Small bundles of fibers were obtained from the skeletal muscles by blunt dissection in mammalian Ringer's solution. Single fibers were isolated in silicone oil and stripped of their sarcolemmas in an infused bubble of relaxing solution as described by Donaldson and Kerrick [8]. Thus, in contrast to the homogenized cardiac tissue, the sarcolemma was totally removed by peeling from each individual skeletal fiber.

The skinned fibers retain longitudinal integrity of the contractile proteins, as evidenced by their ability to generate and support tension. Contraction and relaxation are induced by appropriate $[Ca^{2+}]$ in the bathing solution (buffered with EGTA). Despite the presence of mitochondria and sarcoplasmic reticulum, the skinned fibers are not dependent upon or influenced by normal excitation-contraction coupling mechanisms, intracellular H^+ buffering, or metabolism since their internal $[Ca^{2+}]$, pH, and $[MgATP^{2-}]$ are determined by bathing solution composition. Thus, the force-generating apparatus is functionally isolated, and its behavior can be studied under known ionic conditions.

A disadvantage is that all soluble enzymes and mediators, such as cyclic adenosine monophosphate, should diffuse out of the preparation becoming greatly diluted in the bathing solution and thus losing their influence. Furthermore, the application of the findings to in vivo mechanisms is dependent upon similarity of the bathing solutions to intracellular fluid.

Bathing Solutions. The bathing solutions approximated the intracellular environment in important constituents and concentrations and provided adequate buffering of $[Ca^{2+}]$, $[H^+]$, and $[MgATP^{2-}]$. With glass-distilled water as solvent to limit Ca^{2+} contamination, the stock solutions consisted of K_2H_2EGTA [potassium ethyleneglycol-bis-(β-amino-ethylether)-N,N′ tetra-acetic acid], calcium propionate, magnesium propionate, Na_2H_2ATP, Na_2CP, CPK, potassium propionate, propionic acid and imidazole. A computer program employing binding constants from the literature was designed to solve the complex equilibrium for each bathing solution, calculate the buffer capacity, and calculate and adjust the ionic strength to 0.15 M [8].

The independent variables were pH (7.0, 6.5) and $[Mg^{2+}]$ (1 mM or 10 mM). Normal intracellular pH (37° C) is 7.0 [1,34,43]; pH 6.5 simulates myocardial intracellular acidosis in vivo during ischemia [19,34]. Normal $[Mg^{2+}]$ is thought to be in a mM range [32]. Although Mg^{2+} significantly affects the Ca^{2+}-activated force generation of skinned fibers [3,8,9,12] the affinity of EGTA for Mg^{2+} is approximately 10^5 less than for Ca^{2+} [8]. Thus, changing $[Mg^{2+}]$ has no significant effect on pH-dependent errors in calculated $[Ca^{2+}]$, and any effects of H^+ that are modified by Mg^{2+} must be from direct effects on the contractile apparatus.

For each combination of pH and $[Mg^{2+}]$, a set of bathing solutions varying in $[Ca^{2+}]$ from pCa $(-\log_{10}[Ca^{2+}]M) = 8$ (relaxing solution) to 3.0 were mixed. In all bathing solutions $[K^+ + Na^+] = 70$ mM, $[MgATP^{2-}] = 2$ mM, $[CP^{2-}] = 15$ mM, [CPK] = 15 units/ml, total [EGTA] = 7 mM. Imidazole was used as a H^+ buffer; imidazole concentration was varied to maintain ionic strength at 0.15 M [11] (varying imidazole concentrations does not affect skinned fiber force generation) [8]. Na^+, added as Na_2H_2 ATP and Na_2CP, was approximately 36 to 46 mM and thus varied only slightly as a function of pH and $[Mg^{2+}]$. $[K^+ + Na^+]$ was less than normal in vivo K^+ level to maintain ionic strength at 0.15 M despite the addition of EGTA (to buffer Ca^{2+}); large variations in $[K^+]$ do not

affect skinned fiber force generation [8]. The relaxing solution (pCa = 8) for pH 7.0 and 1 mM Mg^{2+} was used for skinning and storage of fibers.

$MgATP^{2-}$ is the normal substrate for the contractile proteins, and CP and CPK constitute the primary, rapid-acting in vivo regenerating system for $MgATP^{2-}$. Concentrations similar to those normally found in muscle [8,22] were used. The ATP-regenerating system is particularly important in experiments with this preparation because gradients in [$MgATP^{2-}$] within the fibers could occur due to diffusion limitations during contraction. The CP and CPK concentrations were such that increasing them did not alter Ca^{2+}-activated skinned fiber tension generation [8].

Bathing solutions were mixed in bulk (except for the addition of CPK) and stored frozen in amounts required for one experiment. Multiple checks of accuracy of solution mixing are described elsewhere [8]. At the time of an experiment, bathing solutions were brought to room temperature, pH was checked, and CPK was added. Solutions were placed in 1-ml slots of a springform tray and topped with silicone oil to prevent evaporation. Room temperature was maintained at 23 ± 1° C. The fibers were transferred quickly from one solution to another by lowering, repositioning, and raising of the solution tray under the mounted fibers.

Ionic conditions characteristic of the sarcoplasm under normal and ischemic conditions at 37° C were used, even though these experiments were conducted at 23 ± 1° C, because we were primarily interested in simulating in vivo conditions. The inherent assumption that the behavior of the mammalian contractile apparatus at lower temperatures is representative of in vivo performance appears to be valid based upon preliminary data from this laboratory as discussed later. We elected to use room temperature to minimize diffusion limitations for $MgATP^{2-}$ in the skinned fibers.

Isometric Tension Measurement. Following skinning, cardiac fiber bundles or single skeletal muscle fibers (approximately 1 – 3 mm long) were mounted by their ends in the forceps of photodiode relatively noncompliant force transducers [8,21] and they were slowly stretched until they supported 1 – 2 mg of force. The mounted fibers were immersed sequentially in solutions of differing ionic compositions and the isometric tension generated in each solution was continuously recorded. Base-line tension generation was established as the steady-state voltage output recorded with a fiber bundle or single fiber immersed in relaxing solution. Only steady-state (unchanged for 1 min or longer) deviations in voltage from the base-line were used as measures of tension generation in each bathing solution. Using steady-state tensions eliminated the possible effects of sarcoplasmic reticulum or mitochondria on the ionic environment of the contractile proteins, as these effects would be manifested as tension transients in the buffered bathing solutions.

Even though overall length changes of the mounted fiber bundles did not occur, this did not assure that sarcomere behavior was isometric or similar under the various ionic conditions. Also of concern is the potential artifact introduced by the damaged ends of the mounted skinned fibers. Based upon measurements obtained by T. Iwazumi (unpublished observations) from analysis of first order light (laser) diffraction patterns of our preparation, clamping the fibers in the transducer damages approximately 0.05 to 0.1 mm of the length of the fiber. The characteristic sarcomere length for our mounted soleus fibers in pH 7.0 relaxing solution is approximately 2.2 – 2.3 μm; and prior to contraction the longitudinal variation in sarcomere lengths is less than 0.1 μm. During gradual activation, longitudinal non-uniformity of sarcomere lengths (variations > 0.1 μm) is associated with initial force generation of less than 1.5 kg/cm² cross-sectional area. Soleus fibers demonstrating longitudinal uniformity of sarcomere lengths during maximum contracture generated forces of approximately 2 – 3 kg/cm². Some skinned fibers that generated high initial forces had no discernable striation pattern during maximum contracture. Since the loss of striations occurred

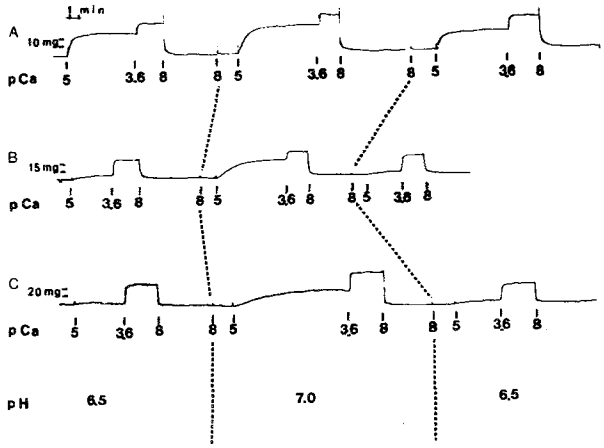

Fig. 1. Protocol for paired data collection. Tension records are for fibers from muscles from the same rabbit: *A.* soleus (diameter = 50 μm), *B.* cardiac (diameter = 100 μm) and *C.* adductor magnus (diameter = 70 μm). For each record, abscissa shows time and ordinate tension. [Mg^{2+}] = 1 mM in all bathing solutions and pCa was changed as indicated below each time marker. For every record, the first and third contractures were at pH 6.5 and the second one at pH 7.0

over their entire length, perhaps myofibrils were misaligned rather than that some sarcomeres were shortening with others in series lengthening. In contrast to initial magnitude of force generation, decline of force with each contracture may be unrelated to sarcomere nonuniformities.

Protocol for Data Collection. Because the bundles and fibers varied in diameter and thus absolute maximum force generation, normalization of forces was required for comparison between them. Scaling of absolute forces according to cross-sectional fiber area at pH 7.0 could not be used because the bundles of cardiac cells varied in longitudinal integrity between the forcep tips. Each absolute force was normalized to selected maximum force for the same fiber or bundle and expressed as a percentage or proportion of the selected maximum [8,22] for purposes of comparison.

For each fiber, force generated at each subsaturating [Ca^{2+}] was converted to a percentage of maximum force at saturating [Ca^{2+}] at the same pH and [Mg^{2+}]. A protocol producing "steps" of force was used for determining force at subsaturating [Ca^{2+}]s. After equilibration in a relaxing solution at a given pH and [Mg^{2+}] for at least 1 min, the fiber was contracted at a subsaturating [Ca^{2+}] and then at saturating [Ca^{2+}] at the same given pH and [Mg^{2+}] as illustrated in Figure 1. Each steady state submaximum force was divided by its associated maximum force and multiplied by 100 to convert it to a percentage of maximum force at the same pH and [Mg^{2+}]. For data analysis a single mean value of percentage of maximum tension for each subsaturating [Ca^{2+}] was used for each fiber; this value was treated as a datum rather than a mean.

For comparison of maximum force generation as a function of pH or [Mg^{2+}] a second "bracketing" protocol was used (illustrated in Donaldson and Kerrick) [8]. At each [Mg^{2+}] the fibers were relaxed, contracted, and relaxed at saturating [Ca^{2+}]s alternately at pH 7.0 and 6.5. The mean absolute maximum tension for pH 6.5 was expressed as a proportion of that at 7.0 for the same fiber at the given [Mg^{2+}]. The range of saturating [Ca^{2+}]s at each pH was determined for each fiber and bundle and the same [Ca^{2+}] was used at pH 7.0 and 6.5 for determining the pH dependent maximum tension ratios. Mg^{2+} dependent maximum tension ratios at pH 7.0 were determined in a similar manner.

As is evident from comparison of the first and third contractures in Figure 1, each bundle and fiber could be contracted and relaxed many times with little decline in maximum force per contraction at a given pH. Thus, at each [Mg^{2+}] the effects of the change in pH were studied at any [Ca^{2+}], independent of time- and contraction-dependent variables, by alternately contracting each fiber at the two pH's. Self-paired data for fibers and bundles were collected at a variety of [Ca]s for (a) pH 7.0 and 6.5 for 1 mM Mg^{2+} and (b) pH 7.0 and 6.5 for 10 mM Mg^{2+}. Thus for a given fiber or bundle, at each Ca^{2+} level, usually four contractures of alternating pH were executed; the mean percentage of tension of the two "stepped" contractures at each pH or the mean maximum tension pH 6.5 to 7.0 ratio was used for analysis. Similarly, mean maximum tension 1 mM to 10 mM Mg^{2+} ratios at pH 7.0 were determined from self-paired contractures.

Although self-paired data were obtained at multiple [Ca^{2+}]s, it was not possible to study the entire range of subsaturating [Ca^{2+}]s for any single fiber or bundle; therefore data were collected from multiple fibers and hearts. When steady-state forces were inadvertently not achieved or a bathing solution was contaminated (verified by assay) during an experiment, the data were eliminated; this accounts for differences in sample sizes at subsaturating [Ca^{2+}]s.

The cross-sectional area of a skeletal fiber was calculated from a diameter of the mounted fiber in relaxing solution (pH 7.0, 2 mM $MgATP^{2-}$) on the assumption that the fibers were cylindrical. Skeletal fibers initially generated forces of approximately 2.0 kg/cm^2 cross sectional area. Skeletal and cardiac fibers were discarded when maximum tension generation declined to less than 50% of original value at pH 7.0, 2 mM $MgATP^{2-}$ or less than 1 kg/cm^2 for skeletal fibers.

The large dose of sodium pentobarbital used in sacrificing the rabbits did not appear to alter maximum absolute force generation since similar forces were generated by equal diameter fibers and bundles from animals killed by a blow to the head. Also, there was no statistically significant difference ($P = 0.05$) in cardiac fiber Ca^{2+} sensitivity (pH 7.0, 1 mM Mg, pCa's 5.0, 5.2, 5.4, 5.6, 3.6) between bundles from rabbits sacrificed with sodium pentobarbital versus a blow to the head.

The observed force per cross sectional area for the skinned skeletal fibers appears low relative to that measured for intact mammalian skeletal muscle [7] but is underestimated due to swelling of the skinned fibers in our relaxing solution. Correcting for this swelling, the lower limit of force per skinned skeletal fiber cross-sectional area in this study was 1.6–2.3 kg/cm^2 [16].

Results

Maximum Force Generation

Table 1 shows the means of the self-paired ratios of maximum force generations for pH 6.5 and 7.0 for the fiber types according to [Mg^{2+}]. All of the ratios are significantly less than 1.0 ($Ps < 0.05$, one-tailed t-tests), and the order of mean magnitude of depression at both [Mg^{2+}]'s is: adductor > cardiac > soleus. There is no statistically singificant ($Ps < 0.1$, two-tailed t-tests) difference between the pH dependent mean ratio for the soleus fibers and the cardiac fibers at either Mg^{2+} concentration. Although the pH dependent mean ratio for the adductor fibers is always significantly different from that for the soleus ($Ps < 0.05$, two-tailed t-tests), there does appear to be less of a depression of the adductor fibers' maximum tension at 10 mM Mg^{2+} as compared with that at 1 mM Mg^{2+} ($P < 0.005$, two-tailed t-test of adductor 1 mM and 10 mM Mg^{2+} mean ratios). Thus the acidotic depression of maximum tension does not appear to be a function of [Mg^{2+}] except for the adductor fibers, and the adductor fibers are the most sensitive to acidosis at physiological [Mg^{2+}] and saturating [Ca^{2+}].

Table 1. Mean maximum tension ratio pH[a] according to [Mg^{2+}] and fiber type

[Mg^{2+}]	Soleus	Cardiac	Adductor
1 mM	0.88 ± 0.02 (8)[b]	0.82 ± 0.04 (5)	0.70 ± 0.02 (6)
10 mM	0.92 ± 0.04 (7)	0.83 ± 0.04 (5)	0.80 ± 0.02 (12)

[a] Maximum tension pH 6.5/Maximum tension pH 7.0
[b] ± Standard error of mean (fiber population)

Table 2. Mean maximum tension ratio [Mg^{2+}][a] at pH 7.0 according to fiber type

Soleus	0.97 ± 0.03 (7)[b]
Cardiac	0.94 ± 0.02 (7)
Adductor	1.07 ± 0.04 (9)

[a] Maximum tension 1 mM Mg^{2+}/Maximum tension 10 mM Mg^{2+}
[b] ± Standard error of mean (fiber population)

Furthermore, increasing [Mg^{2+}] did not appear to greatly affect maximum force generation at pH 7.0. Table 2 shows means of self-paired ratios of maximum force generation for 1 mM and 10 mM Mg^{2+} (pH = 7.0). Only the cardiac Mg^{2+} dependent mean ratio is significantly less than 1.0 ($P < 0.02$, one-tailed t-test) indicating that the maximum force generation at saturating Ca^{2+} concentration increased slightly as [Mg^{2+}] was increased. The Mg^{2+} dependent ratios for cardiac bundles were all less than 1.0 whereas those for both types of skeletal fibers were ± 1.0.

Ca^{2+}-Sensitivity at 1 mM Mg^{2+}

The Ca^{2+}-tension curves for soleus, cardiac, and adductor fibers for 1 mM Mg^{2+} at pH 7.0 and 6.5 are displayed in Figures 2, 3 and 4. Since the submaximum forces generated at each subsaturating [Ca^{2+}] were converted to percentages of the maximum tension generated at the same pH, the differences in absolute tension are not evident in these curves. The curves show the relative forces generated at given [Ca^{2+}]s and thus reflect changes in Ca^{2+}-sensitivity of the skinned fiber force generating apparatus per se.

At pH 7.0, the soleus and cardiac fibers were activated at similar [Ca^{2+}]s and had a lower range of subsaturating [Ca^{2+}]s than the adductor fibers. A comparison of the position of the Ca^{2+}-tension curve at pH 6.5 relative to that at pH 7.0 for each fiber type (Figs. 2, 3 and 4) shows a differential depressant effect

Table 3
Rabbit Soleus
Means of percentage maximum tensions, standard errors of the means (± S.E.M.) and sample size (n) according to pCa, [Mg^{2+}] and pH

pCa	Mg^{2+} = 1 mM		Mg^{2+} = 10 mM	
	pH = 7.0	pH = 6.5	pH = 7.0	pH = 6.5
5.6	29.2 ± 3.40 (6)	12.4 ± 1.36 (5)		
5.5	47.3 ± 3.15 (6)	27.4 ± 3.85 (7)		
5.4	55.1 ± 3.97 (9)	36.4 ± 3.94 (9)		
5.3	56.3 ± 4.71 (7)	48.8 ± 6.95 (4)		
5.2	75.4 ± 3.28 (7)	66.6 ± 5.19 (7)		
5.1	77.1 (1)	78.1 (1)	8.1 ± 2.41 (5)	
5.0	84.9 ± 2.61 (7)	74.1 ± 3.33 (7)	11.3 ± 2.39 (6)	4.5 ± 0.60 (7)
4.8	95.8 ± 2.34 (6)	93.7 ± 3.95 (5)	33.7 ± 3.34 (6)	23.9 ± 5.66 (5)
4.7			58.5 ± 3.62 (9)	35.7 ± 6.09 (5)
4.6	95.4 ± 1.67 (4)	86.8 ± 2.33 (3)	60.4 ± 4.19 (8)	42.3 ± 4.76 (8)
4.5				56.2 ± 5.81 (6)
4.4	97.6 ± 2.46 (4)	96.0 ± 6.21 (4)	64.6 ± 1.90 (6)	71.3 ± 2.61 (4)
4.2	100	100		
4.0	100	100	29.4 ± 2.03 (2)	91.3 ± 0.04 (2)
3.8			89.6 ± 3.94 (3)	95.8 (1)
3.6	100	100	94.5 ± 1.62 (4)	96.2 ± 3.02 (3)
3.4			100	100
3.2			96.3 ± 0.69 (2)	
3.0			95.4 ± 1.66 (4)	100

Table 4
Rabbit Cardiac
Means of percentage maximum tensions, standard errors of the means (± S.E.M.) and sample size (n) according to pCa, [Mg^{2+}] and pH

pCa	Mg^{2+} = 1 mM		Mg^{2+} = 10 mM	
	pH = 7.0	pH = 6.5	pH = 7.0	pH = 6.5
5.6	16.7 ± 4.17 (4)	4.7 ± 2.72 (4)		
5.5	22.1 ± 2.88 (6)	6.9 ± 2.52 (4)		
5.4	45.3 ± 2.99 (10)	19.3 ± 3.43 (9)		
5.3	46.8 ± 8.75 (6)	18.1 ± 3.31 (10)		
5.2	71.7 ± 3.09 (10)	27.9 ± 4.71 (11)		
5.1	74.7 ± 3.82 (4)	31.0 ± 6.50 (3)		
5.0	81.0 ± 1.69 (10)	36.0 ± 5.25 (11)		
4.8	88.0 ± 3.23 (12)	78.9 ± 3.53 (10)	13.3 ± 3.79 (5)	5.4 ± 1.66 (5)
4.6	95.9 ± 2.93 (7)	88.4 ± 2.85 (7)	47.1 ± 3.88 (7)	15.0 ± 4.76 (7)
4.5				21.6 ± 4.03 (5)
4.4		102 ± 2.00 (2)	60.5 ± 3.16 (6)	37.4 ± 2.24 (6)
4.3			62.4 ± 2.74 (3)	42.5 ± 8.90 (4)
4.2	100	100	67.8 ± 3.89 (5)	65.7 ± 9.59 (7)
4.0	100	100	79.3 ± 3.45 (2)	79.0 (1)
3.8			90.4 ± 4.09 (2)	90.3 (1)
3.6	100	100	94.2 ± 0.99 (3)	94.9 ± 1.34 (3)
3.4			99.6 ± 1.52 (2)	100
3.2			100	
3.0			96.1 ± 0.54 (2)	98.0 ± 2.87 (4)

of acidosis on Ca^{2+}-sensitivity of the fibers, as follows: cardiac > adductor > soleus.

The solid line for each curve represents the non-linear least-squares regression fitting of the Hill equation [18]:

$$\frac{\%T}{100} = \frac{[Ca^{2+}]^n}{Q + [Ca^{2+}]^n},$$

(where %T is percentage of maximum tension at the given subsaturating [Ca^{2+}] and n and Q are derived constants) to all of the data, rather than just the fiber means that are represented by the symbols. Hill n, a constant determined in the regression analysis, gives information related to the curve steepness and thus the degree of cooperativity in the tension-generating system during Ca^{2+}-activation; a value greater than 1.0 is evidence of positive cooperativity in the system [45]. The pCa of 50% tension, or midpoint for each solid curve is $-(1/n)\log_{10}Q$; these values are given for each curve in the figure legends. For all Ca^{2+}-tension curves

Table 5
Rabbit Adductor Magnus
Means of percentage maximum tensions, standard errors of the means (± S.E.M.) and sample size (*n*) according to pCa, [Mg^{2+}] and pH

pCa	Mg^{2+} = 1 mM		Mg^{2+} = 10 mM	
	pH = 7.0	pH = 6.5	pH = 7.0	pH = 6.5
5.5	4.1 ± 0.68 (2)	0 (3)		
5.4	18.3 ± 3.00 (4)	14.6 ± 5.51 (3)		
5.3	24.0 ± 8.61 (6)	13.4 ± 4.60 (6)		
5.2	39.2 ± 4.63 (8)	17.6 ± 3.74 (8)		
5.0	61.9 ± 4.72 (11)	27.0 ± 3.81 (11)		
4.8	75.3 ± 1.74 (9)	62.1 ± 3.65 (9)	19.9 ± 4.54 (5)	6.6 ± 2.74 (4)
4.7			38.3 ± 7.75 (6)	33.6 ± 7.76 (7)
4.6	84.7 ± 3.91 (5)	74.9 ± 4.04 (5)	61.4 ± 5.25 (6)	46.7 ± 5.24 (8)
4.4	88.2 ± 5.30 (2)	88.8 ± 5.45 (2)	73.4 ± 4.18 (4)	79.4 ± 5.23 (4)
4.2	100	100	82.5 ± 3.07 (4)	75.8 ± 6.65 (5)
4.0	100	100	90.6 ± 3.63 (2)	
3.8			94.7 (1)	92.3 ± 4.11 (2)
3.6	100	100	96.5 ± 3.52 (6)	97.9 ± 3.17 (4)
3.4			100	100
3.2			97.1 ± 0.85 (3)	
3.0			99.4 ± 0.63 (3)	97.4 ± 1.54 (4)

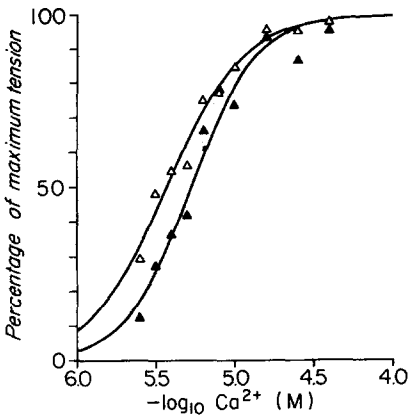

Fig. 2. Means of percentages of maximum tension for soleus fibers at 1 mM Mg^{2+} and pH 7.0 and 6.5. Standard errors of the means and fiber populations are in Table 3. Hill *n* and pCa at midpoint of the curves are 1.79 and 5.43 for pH 7.0 and 2.09 and 5.28 for pH 6.5. (△ pH 7.0; ▲ pH 6.5)

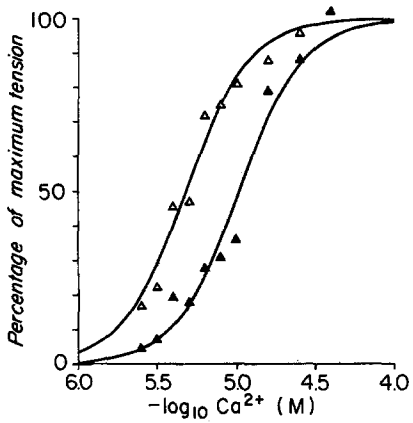

Fig. 3. Means of percentages of maximum tension for cardiac fibers at 1 mM Mg^{2+} and pH 7.0 and 6.5. Standard errors of the means and fiber populations are in Table 4. Hill *n* and pCa at midpoint of the curves are 2.14 and 5.32 for pH 7.0 and 2.14 and 4.98 for pH 6.5. (△ pH 7.0; ▲ pH 6.5)

at 1 mM Mg^{2+}, Hill *n* was approximately 2; therefore, the steepness of the curves does not appear to be a function of pH.

Ca^{2+}-Sensitivity at 10 mM Mg^{2+}

The Ca^{2+}-tension curves at pH 7.0 were displaced in the direction of higher [Ca^{2+}]s as [Mg^{2+}] was increased (Figs. 5–7), indicating depression of Ca^{2+}-sensitivity as a function of [Mg^{2+}] for all fiber types. The order of the fiber types according to relative Ca^{2+}-sensitivity at pH 7.0 changed to soleus > adductor > cardiac and differences between the three types were less than at 1 mM Mg^{2+}. Slightly less steepness is apparent in the cardiac and soleus curves at pH 7.0, 10 mM Mg^{2+} (see legends for Figures 5, 6 and 7 for Hill *n* and $-(1/n)\log_{10}Q$ values for each Ca^{2+}-tension curve).

Unlike at 1 mM Mg^{2+}, decreasing pH from 7.0 to 6.5 had very little effect on the position of the curves along the pCa axis. However, the curves for the cardiac and soleus fibers become steeper again, with a Hill *n* of approximately 2.0, when pH is decreased to 6.5 at 10 mM Mg^{2+}.

Reversibility

On the basis of the paired data, the effects observed from alterations in pH and [Mg^{2+}] appear completely

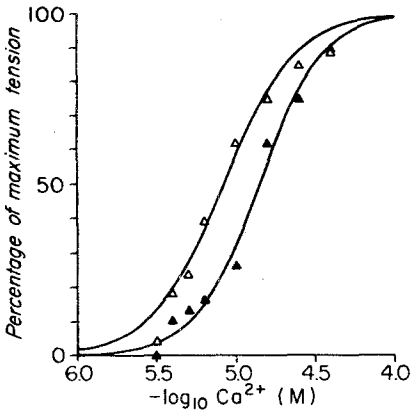

Fig. 4. Means of percentages of maximum tension for adductor magnus fibers at 1 mM Mg^{2+} and pH 7.0 and 6.5. Standard errors of the means and fiber populations are in Table 5. Hill *n* and pCa at midpoint of the curves are 1.90 and 5.08 for pH 7.0 and 2.15 and 4.85 for pH 6.5. (△ pH 7.0; ▲ pH 6.5)

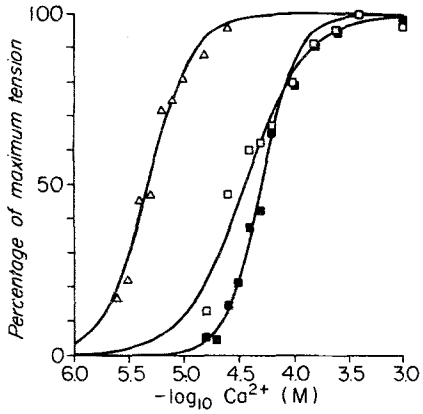

Fig. 6. Means of percentages of maximum tension for cardiac fibers at 10 mM Mg^{2+} and pH 7.0 and 6.5. Cardiac curve for pH 7.0 at 1 mM Mg^{2+} from Figure 3 repeated here for reference. Standard errors of the means and fiber populations are in Table 4. Hill *n* and pCa at midpoint of the curves are 1.55 and 4.45 at pH 7.0 and 2.61 and 4.29 at pH 6.5. Symbol key same as in Figure 5

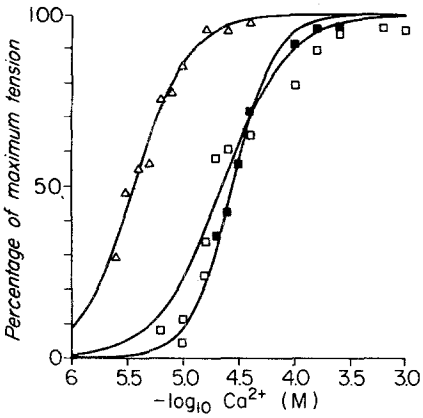

Fig. 5. Means of percentages of maximum tension for soleus fibers at 10 mM Mg^{2+} and pH 7.0 and 6.5. Soleus curve for pH 7.0 at 1 mM Mg^{2+} from Figure 2 repeated here for reference. Standard errors of the means and fiber populations are in Table 3. Hill *n* and pCa at midpoint of the curves are 1.47 and 4.65 at pH 7.0 and 2.29 and 4.56 at pH 6.5. (△ pH 7.0, 1 mM Mg^{2+}; □ pH 7.0, 10 mM Mg^{2+}; ■ pH 6.5, 10 mM Mg^{2+})

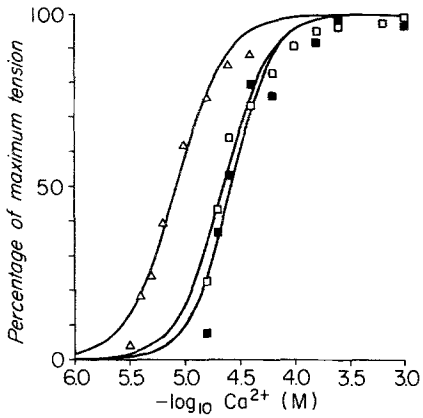

Fig. 7. Means of percentages of maximum tension for adductor magnus fibers at 10 mM Mg^{2+} and pH 7.0 and 6.5. Adductor curve for pH 7.0 at 1 mM Mg^{2+} from Figure 4 repeated here for reference. Standard errors of the means and fiber populations are in Table 5. Hill *n* and pCa at midpoint of the curves are 2.25 and 4.63 for pH 7.0 and 2.33 and 4.55 at pH 6.5. Symbol key same as in Figure 5

reversible (Fig. 1), reproducible for a given fiber, and independent of time, number, and order of fiber contractures.

Discussion

Direct Effects of H^+ on Maximum Force Generation

The acidotic reduction of maximum force for all muscle types at both [Mg^{2+}]s cannot be explained by errors in the apparent binding constant for $CaEGTA^{2-}$ since [Ca^{2+}] was increased to saturating level for every fiber at both pH 7.0 and 6.5. At each [Mg^{2+}], the ranges of saturating [Ca^{2+}]s at pH 7.0 and 6.5 for fibers of each type overlapped; thus maximum tensions achieved at these [Ca^{2+}]s could be compared.

Our results are in agreement with the findings of Schädler [39] in glycerinated dog papillary muscle and Robertson and Kerrick [37] in skinned frog skeletal fibers. The negative results of Chen-Lui and Endo [5] may be due to the normalization of forces according to cross-sectional fiber area; the transverse myofilament spacing decreases in glycerinated rabbit psoas fibers with acidosis [38], although changes in lattice volume were found to have no effect on force generation in skinned crayfish fibers [2]. Fabiato and Fabiato [13] did

not indicate the method used for normalization of tensions, and the reason for their negative result is not known.

Even though a decreasing reaction velocity of the CP-CPK regenerating system has been found with increasing pH (from 5.0 to 8.0) [27], progressively impaired functioning of the CPK-CP regenerating system did not appear to be responsible for the observed changes in maximum tension. Without an adequate ATP-regenerating system, [$MgATP^{2-}$] gradients, which are a function of fiber (radius)^2, are likely to occur in skinned fibers [15]. Within skinned fibers the regions of significantly lowered [$MgATP^{2-}$] may have increased Ca^{2+}-sensitivity and maximum force generation [3,15]. However, there was no correlation between the magnitude of acidotic changes in maximum tension and fiber or bundle radius, despite two to three fold variations in diameter. Moreover, all effects of H^+ were reversible, and increases in base-line tension with increasing pH were not seen. In more recent studies in this laboratory lowering [$MgATP^{2-}$] to 0.1 mM at both pH 6.5 and 7.5 did not result in enhanced Ca^{2+}-sensitivity or increased maximum tension generation of skinned rabbit cardiac fibers (Donaldson et al., in preparation).

Cooling of the contractile apparatus to 23° C does not preclude interpretation of the results of this study as being related to in vivo mechanisms, since our preliminary data indicate that increasing temperature from 21° C to 30° C causes no appreciable alteration of maximum force generation at pH 7.0 and a slight augmentation of acidotic depression of maximum force. There was no evidence of an increase in baseline tension in the relaxing solutions at 30° C. These effects of temperature were determined by comparing the maximum forces of a given fiber (soleus or cardiac bundle) at 21° C and 30° C (self-paired data); only saturating [Ca^{2+}]s were employed at each pH and temperature to avoid errors from calculated [Ca^{2+}]s (Donaldson, in preparation). Thus, it appears from these preliminary data that acidotic depression of force occurs at higher temperatures and that [H^+] rather than degree of deviation from electrically neutral pH is the more significant variable determining force.

Effect of Mg^{2+} on Ca^{2+}-Sensitivity

The Ca^{2+}-tension curves are too steep to be explained by assuming independent binding sites for Ca^{2+}; thus cooperativity is probably present in the system. The source of interaction cannot be determined from the data, but the degree did not appear to be a function of pH or [Mg^{2+}]. The decrease in steepness of the soleus and cardiac curves (noted previously for frog skeletal but not for rat cardiac muscle [8]) is too slight to be considered significant. If it is real then it should be augmented by increases in pH, since at 6.5 steepness was identical at both [Mg^{2+}]s.

Increasing [Mg^{2+}] from 1 mM to 10 mM dramatically displaced Ca^{2+}-tension curves at pH 7.0 in the direction of higher [Ca^{2+}]s. These results are consistent with prior findings in related investigations [8,9,12,22].

Of interest was the finding that at pH 7.0, the relative Ca^{2+}-sensitivity of the muscle types varied with [Mg^{2+}]. For 1 mM Mg^{2+} the order of Ca^{2+} sensitivity was: soleus > cardiac > adductor, which is in agreement with other data for skeletal muscle at the same [Mg^{2+}] of Kerrick et al. [23], but not with the data on Ca^{2+} binding of Ebashi et al. [10] (in which the order of Ca^{2+} affinities of myosin B for the muscles was white > red > cardiac at 4 mM Mg^{2+}). At 10 mM Mg^{2+}, the order of Ca^{2+} sensitivities for the skinned fibers changed to soleus > adductor > cardiac, which corresponds better, but not completely, to the relative affinity of Ca^{2+} for myofibrillar proteins [10].

Adequacy of Cardiac Skinning

As discussed in methods, the cardiac cells in the bundles appear to have been either mechanically (by homogenization) or chemically (by 7 mM EGTA) skinned since detergents did not alter their submaximum or maximum Ca^{2+}-activated force generation. Furthermore, a similar Mg^{2+} dependence of the acidotic depression of Ca^{2+}-sensitivity was observed with the skeletal fibers which were individually skinned by peeling off their sarcolemmas and thus were not dependent at all upon chemical skinning. Also, homogenized bundles and peeled single cardiac cells have been shown to have very similar Mg^{2+} and H^+ dependence of Ca^{2+}-sensitivity [9,12,13,22], indicating that the homogenization procedure or high EGTA effectively skins the cardiac cells within the bundles.

Direct Effect of H^+ on Ca^{2+}-Sensitivity

The H^+ alterations of Ca^{2+}-sensitivity at 1 mM Mg^{2+} appear to be a direct effect of H^+ on the contractile apparatus. An error in the apparent $CaEGTA^{2-}$ binding constant for pH 6.5, $K'_{6.5} = [CaEGTA^{2-}] \cdot [Ca^{2+}]^{-1} \cdot (7\,mM\text{-}[CaEGTA^{2-}])^{-1}$, might conceivably have caused a pH-dependent artifactual displacement of Ca^{2+}-tension curves in either direction from the "real" curves at pH 6.5, since [Ca^{2+}] was calculated on the bais of uncertain binding of H^+ to $EGTA^{4-}$.

If the assumed H^+ affinity of $EGTA^{4-}$ was too low, then the calculated [Ca^{2+}]s are all too low and the real pH 6.5 Ca^{2+}-tension curves would be displaced farther

to the right. The effect of this type of error is fairly constant over the range of activating $[Ca^{2+}]$s; therefore, it cannot account for the larger displacement of the pH 6.5 and 7.0 Ca^{2+}-tension curves for cardiac fibers compared with those for adductor magnus and soleus fibers.

If, instead, the assumed H^+ affinity of EGTA was too high, then the calculated $[Ca^{2+}]$s are too high and the pH 6.5 curves would be shifted to the left and the effects of acidosis on Ca^{2+} sensitivity minimized. Since the effect of this type of error increases as $[Ca^{2+}]$ decreases, it could account for the larger shift of the pH 7.0 to 6.5 curve for cardiac fibers than for the adductor, but it would mean that the "corrected" soleus pH 6.5 curve would be to the left of the soleus 7.0 curve (enhanced Ca^{2+}-sensitivity of soleus at pH 6.5). This error could lead to an incorrect conclusion that Ca^{2+} sensitivity is depressed at pH 6.5.

Conclusive evidence for the nonartifactual nature of the pH-dependent alterations of Ca^{2+}-sensitivity at 1 mM Mg^{2+} is provided by the data obtained under the same experimental conditions but with $[Mg^{2+}]$ 10 mM. In all fiber types, Ca^{2+}-sensitivity was decreased at 1 mM Mg^{2+} but virtually unaffected by acidosis at 10 mM Mg^{2+}. Yet the total calcium propionate added to achieve a given free $[Ca^{2+}]$ at each pH, and thus the effect of errors in binding constant, was virtually identical at both $[Mg^{2+}]$s.

Each type of error must still be considered, however, because activation occurred in a range of higher $[Ca^{2+}]$s at 10 mM Mg^{2+}. Since the pH 7.0 and 6.5 curves for each fiber type essentially overlap at 10 mM Mg, the assumed H^+ affinity of EGTA could not have been too low. The effect of this type of error should have been very similar despite the higher range of activating $[Ca^{2+}]$'s at 10 mM Mg^{2+}, and thus would not account for the decreased displacement of the pH 6.5 relative to the pH 7.0 Ca^{2+}-tension curves.

The other possible error, an overestimation of H^+ affinity of EGTA should have been minimized in the higher range of $[Ca^{2+}]$s at 10 mM Mg^{2+}. Although the effects of this type of error would be consistent with the finding that the depressant effect of acidosis on adductor and cardiac sensitivity is greatly reduced at 10 mM Mg^{2+}, it cannot explain why acidosis did not enhance soleus sensitivity at 10 mM Mg^{2+}. Similar displacement is seen in the pH 7.0 and 6.5 Ca^{2+}-tension curves of soleus at 1 and 10 mM Mg^{2+}, despite large changes in the displacement of cardiac and adductor curves. Thus the differential depressant effect of H^+ on Ca^{2+}-sensitivity is Mg^{2+}-dependent and cannot be explained by errors in the binding constants used in calculating the $[Ca^{2+}]$s of the bathing solutions. The interaction of Mg^{2+} and H^+ is most likely to be on the contractile machinery itself.

Possible Effect of Sarcomere Length Changes

As mentioned earlier, because of the mounting procedure and the high initial force generation of the skinned skeletal fibers used in this study, longitudinal nonuniformities of sarcomere lengths and significant series elasticity due to damaged ends are unlikely. However, since sarcomere length was not continuously monitored or controlled, the possible effects of these artifacts must be considered. Increased sarcomere shortening could decrease abolute force generation; this effect would be greatest at saturating $[Ca^{2+}]$s yielding less steep pCa-tension curves. Also, if sarcomeres are shortening significantly, ionic effects on the shape of the sarcomere length-tension relationship may be significant.

Based upon the observed pattern of changes in absolute forces and in position and shape of the pCa-tension curves however, the effects of Mg^{2+} and H^+ observed in this study are not likely to be totally accounted for by variable sarcomere shortening during the contractures. Since absolute maximum force generation was essentially unaffected by $[Mg^{2+}]$, the pCa-tension curves would be expected to increase in steepness as Ca^{2+}-sensitivity decreased if the change in position of the pCa-tension curve were due to increasing sarcomere nonuniformities at subsaturating $[Ca^{2+}]$s or plausible changes in shape of the length-tension relationship. Similarly acidotic depression of Ca^{2+}-sensitivity should be associated with increased steepness of the curves if it is due to sarcomere shortening artifacts, despite absolute force depression by acidosis. However, steepness of the curves appeared unaltered by the ionic changes employed in this study.

Site of Mg^{2+}-H^+ Interaction

The exact site or sites of the H^+-Mg^{2+}-Ca^{2+} interaction are not known. Although troponin has a class of sites that binds H^+, Ca^{2+} and Mg^{2+}, binding to these sites does not appear to influence the Ca^{2+}-sensitivity of myofibrillar ATPase [36]. Since Ca^{2+} and Mg^{2+} also bind to myosin, myosin sites are possible loci, [4,35,41]; however, their affinity for H^+ and the contractile significance of Ca^{2+}-binding to them are not known.

Differential Effects of H^+ on Striated Muscle Types

The absolute force generations of cardiac and adductor skinned fibers are significantly depressed at all Ca^{2+} levels in their respective ranges of activating $[Ca^{2+}]$s, whereas that of soleus muscle is generally unaffected. Although these data are consistent with the differential

effect of acidosis on intact rat skeletal and cardiac muscle, they cannot explain the actual enhancement of soleus twitch and tetanic tensions in response to a carbonic acid excess [31]. This effect must be due to other factors besides differences in contractile proteins.

In terms of characteristics of contractile protein and activation, cardiac fibers appear closer to soleus, or slow-twitch fibers than to adductor, or fast-twitch fibers. In both fibers affinity for Sr^{2+} is high relative to that for Ca^{2+} in both activation of force generation [9,23] and binding to troponin [11]. Characteristics of myosin ATPase and light chains are also similar [11]. However, in the observed effects of H^+, cardiac muscle has characteristics similar to both soleus muscle and adductor muscle.

It is interesting that, in this study, maximum tension was always depressed more in the adductor fibers than in the cardiac or soleus, but the greatest acidotic depression of Ca^{2+}-sensitivity at 1 mM Mg^{2+} was seen in cardiac muscle. As a result of the combined effects on maximum tension and Ca^{2+}-sensitivity, the acidotic depression of absolute force generation at subsaturating $[Ca^{2+}]$s was similar for cardiac and adductor fibers (Fig. 1, B and C). On the basis of these results, cardiac and adductor magnus twitch tensions should be similarly depressed by acidosis if the major effect of the excess H^+ is on the contractile proteins.

Significance of Acidotic Depression of Cardiac Force

Since normal intracellular $[Mg^{2+}]$ is estimated to be approximately in a mM range [32] and intracellular pH appears to drop rapidly down to pH 6.5 or below during ischemia [19,33], the pH-dependent alterations of Ca^{2+}-sensitivity and maximum force generation observed in this study are physiologically significant. Cardiac muscle cannot be tetanized, therefore its contractile apparatus is always responding to a subsaturating $[Ca^{2+}]$ even at the peak of twitch tension. For any given subsaturating $[Ca^{2+}]$, even those yielding greater than 50% tension at pH 7.0, tension is greatly diminished when pH is reduced from 7.0 to 6.5 at 1 mM Mg^{2+} (Fig. 3). The added depression of maximum tension, which is not reflected in the percentage of maximum tension data, means that absolute tension at subsaturating $[Ca^{2+}]$ is even lower than that predicted by the curve obtained for cardiac muscle at pH 6.5 and 1 mM Mg^{2+} (Fig. 1 B). Thus, the direct acidotic depression of the contractile protein response to Ca^{2+} in cardiac muscle is probably highly significant in terms of the explanation of the negative inotropic action of excess H^+ and might be responsible for the cessation of contraction during ischemia.

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