Biochimica et Biophysica Acta, 430 (1976) 352–365

BBA 47085

Ca^{2+} DEPENDENCE OF TENSION AND ADP PRODUCTION IN SEGMENTS OF CHEMICALLY SKINNED MUSCLE FIBERS

RONALD M. LEVY, YOSHIKI UMAZUME and MARTIN J. KUSHMERICK

Department of Physiology and Biophysical Laboratory, Harvard Medical School, 25 Shattuck Street, Boston, Mass. 02115 (U.S.A.)

(Received October 31st, 1975)

SUMMARY

Both ADP production and tension have been measured in segments of chemically skinned fibers contracting at different Ca^{2+} concentrations. Full mechanical activation occurred between pCa 7.00 and pCa 6.50. The total ATPase was due to both actomyosin and non-actomyosin ATPase. Actomyosin ATPase was observed at pCa 7.09 without accompanying tension. The Ca^{2+} dependence of tension was steeper than actomyosin ATPase. This finding implies some rate constants of the mechanochemical cycle are Ca^{2+} dependent. Non-actomyosin ATPase was measured in fibers stretched beyond overlap of the thick and thin filaments. Sarcoplasmic reticulum was isolated and sarcoplasmic reticulum activity was measured in vitro under the same conditions as the single-fiber experiments. Non-actomyosin ATPase in the single fibers was found to be small compared to maximally activated actomyosin ATPase but larger than the ATPase that could be attributed to sarcoplasmic reticulum activity.

INTRODUCTION

Segments of a single fiber [1, 2] possess two useful properties that facilitate the study of Ca^{2+} regulation of contraction: (A) the three-dimensional structural organization of intact muscle is maintained and (B) it is possible to control the internal environment of the fiber. It is thus possible to measure the ATPase and other relevant enzymatic activities of single fibers whose mechanical activity is also recorded. We studied, in the same fiber, the mechanical and chemical responses as a function of Ca^{2+} concentration. If Ca^{2+} acts as a switch to mediate the number of cross-bridge cycles occurring at any instant but does not effect the kinetic steps within a cycle, then a parallel and graded increase in chemical and mechanical activity should be observed as the Ca^{2+} concentration is increased.

Experiments with troponin [3, 4] coupled with studies of tropomyosin movement [5–7] following activation have formed the basis of our understanding of the way in which Ca^{2+}-protein interactions remove the inhibition of actomyosin ATPase. Within

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

the context of this regulation Ca^{2+} is viewed as a "trigger substance" or "switch", where the number of active actin sites is a function of the amount of Ca^{2+} bound to troponin C. Numerous experiments suggest Ca^{2+} may regulate contraction in more complex ways. The binding curves of Ca^{2+} to myofibrils, troponin or regulated thin filaments [8–10] are much less steep than are mechanical or chemical indicators of contractile activity [4, 10–12]. Futhermore, both rigor complexes and force generating complexes can potentiate actin cofactor activity and Ca^{2+} binding [13, 14]. Finally, experiments on Ca^{2+} binding to myosin [15] coupled with observations of changes in intensity of myosin X-ray reflections during activation of muscle stretched beyond overlap [7, 16] suggest Ca^{2+} may effect cross-bridge mobility directly.

Skinned single fiber preparations potentially contain a variety of enzymes whose activity might be mistaken for actomyosin ATPase. A series of experiments was made to identify the magnitude and kinds of non-actomyosin ATPases in our preparation. These experiments consisted of measurement of (A) ATPase rates in the absence of Ca^{2+}, (B) ATPase in fibers stretched beyond overlap, (C) sarcoplasmic reticulum activity in vitro under conditions similar to the single fiber experiments as a check on (B) and finally (D) myokinase activity in our single fiber preparation.

MATERIALS AND METHODS

Preparation of fibers and mechanical apparatus. A bundle of muscle fibers was dissected from the ventral part of semitendinosus muscle of *Rana pipiens*, placed onto a glass slide after immersion in a Relaxing solution and covered with paraffin oil. A segment of a single muscle fiber, 1 cm long and free of endplate, was dissected under a dissecting microscope. All other experimental procedures were performed at 4 C°. The fiber segment was mounted on a force transducer in a chamber that consisted of a plexiglass block with a series of rectangular wells drilled out [11]. Each well contained a different bathing solution. The sensitivity of the force transducer was 0.1 mV/mg tension and the compliance was 0.2 μm/mg tension. The sarcolemma was made permeable by soaking the fiber in a glycerol-containing solution for 20 min and then in a Lubrol-containing solution for 30 min. The middle section (4–6 mm) was cut out. Both ends of the fiber segment were tied with silk thread and remounted on the apparatus. The sarcomere length was measured by optical diffraction with a helium-neon laser and was set to 3.0 μm. The diffraction pattern remained uniform along the entire segment. During contraction the average sarcomere length did not change but the diffraction lines broadened. The original pattern was restored on relaxation. The length of each fiber was measured at the end of the experiment with a micrometer attached to the force transducer.

Solutions. Each chamber contained 0.1 ml of bathing solution. The following solutions were used: Glycerol solution: (50%, v/v), 4 mM potassium EGTA (ethyleneglycol-bis-(β-aminoethylether)-*N*,*N'*-tetraacetic acid) and 10 mM imidazole, pH 7.0. Relaxing solution: 140 mM KCl, 10 mM imidazole, pH 7.0, 4 mM potassium EGTA, 0.5 mM $MgCl_2$ and 5 mM ATP. Lubrol solution: Relaxing solution containing 0.5 % (w/v) Lubrol (ICI America Inc.). Contracting solutions of various Ca^{2+} concentrations (pCa 6.50, 6.86, 6.97 and 7.09) were made by adding appropriate amounts of $CaCl_2$ to EGTA, keeping the total EGTA concentration fixed at 4 mM and taking the apparent stability constant of Ca^{2+}-EGTA complex to be $10^{+6.68}$ M^{-1} at pH 7.0 at 4 °C [17].

General procedure for experiments at different Ca^{2+} concentrations. In the series of experiments designed to test the effect of Ca^{2+} on force and ATPase each fiber was subjected to a series of three contractions. The first and third contractions served as an internal control in a reference solution (pCa 6.86), where tensions were about 0.6–0.8 those observed at saturating Ca^{2+} levels. The second contraction of the series was the experimental or test contraction and the Ca^{2+} concentration of its bathing solution was varied. To fulfill best the competing requirements of long incubations when pCa is high (to increase ATP breakdowns so it is measurable) and short incubations when pCa is low (to mimimize deterioration of the fiber and non-uniform sarcomere spacing), the incubation time was varied in different solutions: 3 min at pCa 6.50; 4 min at pCa 6.86; 8 min at pCa 6.97; and 20 min at pCa 7.09 and in relaxing solution.

ATPase measurement. The ADP content of the bathing solutions was analysed chromatographically, providing a measure of the ATPase. High pressure liquid chromatography (Lab Data Control, Inc.) was used as a reproducible assay of the ADP concentration in the micromolar range [18]. 30 μl of the 0.1 ml total sample (in duplicate) were injected through the column; an anion exchanger (Pellionex, purchased from Northgate Laboratories and subsequently from Reeve Angel) was used with an isochratic solvent consisting of 25 % (v/v) methanol in 0.2 M KH_2PO_4. The flow rate was 2.3 ml/min through a 100 cm column, 1.6 mm in diameter. Under these conditions ATP and ADP were completely separable from each other and from AMP which eluted near the void volume (Fig. 1). ATP (Sigma) was purified on a DEAE-Sephadex column using a KCl gradient so that the concentration of the ADP impurity was reduced to less than 0.2 % of the ATP concentration.

The transfer of ADP into a bath initially containing no added ADP (10^{-5} M) from a preceding bath containing 0.1 mM ADP was less than 2% of the concentration of ADP in the first bath. This result indicates carryover from the apparatus is negligible.

In order to be able to express the enzymatic activity of the fibers in standard units of μmol/mg protein, the protein content of single fibers was studied as a function of fiber length by measuring the length of individual segments of fibers and combining them into groups whose aggregate length was between 8 and 28 cm, and the protein content of the groups of fibers was measured [19]. This control experiment showed the relationship between fiber length and protein content is linear and the slope is approx. 1 μg protein/mm fiber at a sarcomere length of 2.4 μm for fibers from the frog studied. Only fiber length was measured in all subsequent experiments and this slope was used as an approximate conversion factor so we could express chemical activities per unit protein.

Isolation and ATPase of sarcoplasmic reticulum. Fragmented sarcoplasmic reticulum was isolated following the method of Martonosi [20]. 30–50 g of leg muscle (7–12 frogs) were minced with fine scissors, diluted 4 : 1 (w/v) in Buffer I (0.1 M KCl, 5 mM histidine, pH 7.3) and homogenized for 2 min in a Waring blender chilled to 2–4 °C. The homogenate was spun at 2200 × g for 20 min at 4 °C to remove myofibrils, remaining blood vessels and connective tissue. The supernatant was spun at 10 000 × g for 30 min. The pellet (mostly mitochondria) was discarded and the supernatant was spun at 38 000 × g for 1 h to sediment the sarcoplasmic reticulum fraction. This pellet was suspended in 90 ml (for 50 g minced muscle) of Buffer II (0.5 M KCl, 5 mM histidine, pH 7.3) to dissolve any remaining actomyosin. The suspension was spun at 38 000 × g for 1 h and the pellet was resuspended in 19 ml of Buffer I (for 50 g of minced muscle).

The protein concentration of this last suspension was usually 2–5 mg/ml. The maximum yield obtained in four preparations was 12.5 mg/g total protein. The standard incubation medium for sarcoplasmic reticulum ATPase assays consisted of 10 mM imidazole, pH 7.0, 5 mM $MgCl_2$, 5 mM ATP and 100 mM KCl and was altered to simulate conditions used to study single fiber segments as described in Fig. 4. 2.8 ml of incubation medium containing sarcoplasmic reticulum (0.04 mg sarcoplasmic reticulum/ml) was incubated for 2, 4, 6 min at 35 °C (some incubations were at 4 °C; see Fig. 4). The reaction was stopped by adding 0.5 ml of 6% trichloroacetic acid; Fiske and Subba-Row [21] assays were performed to determine Pi production.

The contribution of non-actomyosin ATPase in single fibers was measured in fibers stretched beyond overlap of the thick and thin filaments.

RESULTS

Tensions and ATPase at the reference calcium concentration. In Fig. 1 ADP production is shown as a function of time and tension time product for three fibers in pCa 6.86, the reference Ca^{2+} concentration for these experiments. We found a linear relationship between ADP production and tension-time product. That is, there is a time-independent proportionality between chemical and mechanical activity at the reference Ca^{2+} concentration.

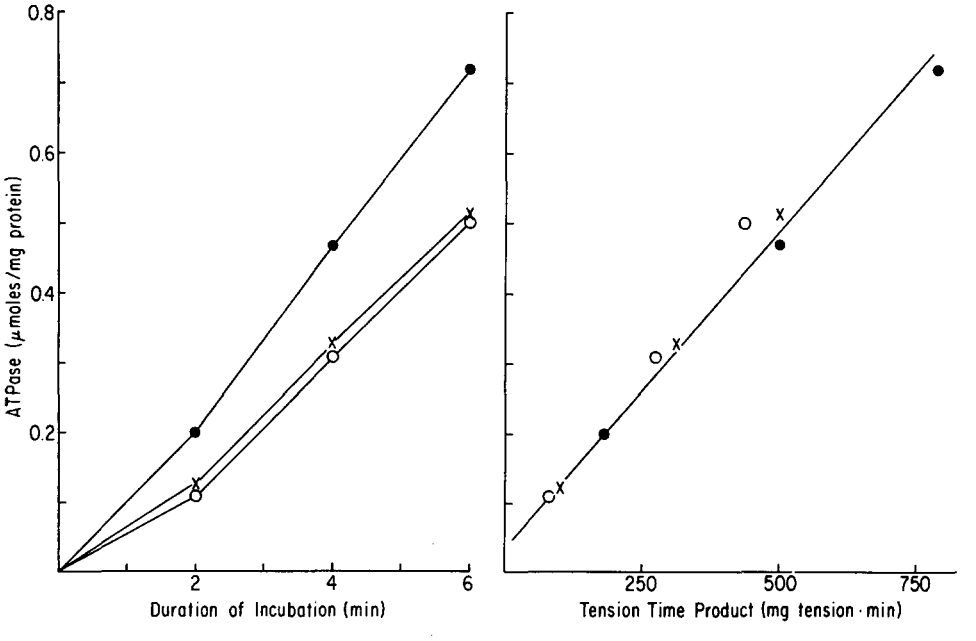


Fig. 1. Left: The ADP production is plotted as a function of time for three fibers contracting in pCa 6.86. Both ● and ○ are from the same frog. The initial ATPase rate is smaller than when the fibers reach maximum tension. Between 2 and 6 min the ATPase rates are linear and tension is constant. Right: ADP production for the three fibers shown on the left is plotted against tension-time product (mg tension-min). This graph indicates there is a good correlation between amount of ATPase and tension at pCa 6.86.

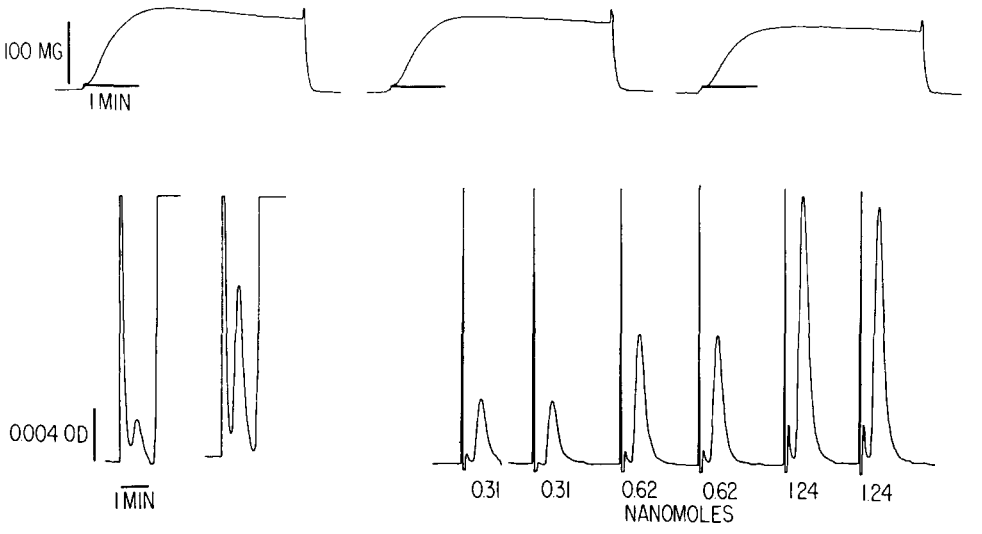


Fig. 2. Top: Photograph of tension traces of three repeated contractions of the same fiber at pCa 6.86 at 4 °C. The horizontal bar also indicates immersion into contracting solution and the true zero of tension. The difference between the zero of tension before and during a contraction is a reproducible artifact due to the sensitivity of the tension transducer to the heights of solutions in the plexiglass bath. The upward blip on the tension record indicates immersion of the fiber into Relaxing solution. Bottom: On the left are chromatographs of the ADP peak of the solution in which the first contraction shown above occurred along with a blank showing the ADP content of the solution before the fiber was immersed. On the right we show a standard curve of absorbance vs. nmol of ADP injected. The large spike before the ADP peak is due to the void volume. ATP elutes last (reading from left to right) and can be seen as a signal off scale due to large amounts of ATP in the experimental solutions shown on the left. There is no ATP peak in the standard ADP chromatographs. The baseline is very stable even after large scale deflections due to passage of large amounts of ATP through the monitor.

Ca^{2+}-dependent relationship between tension and ATPase. We present in Fig. 2 a typical force trace of three contractions at pCa 6.86 elicited in the same fiber together with a chromatograph of the bathing solution used for the first contraction. The delay before the onset of contraction was less than 5 s and complete activation occurred in about 60 s. The deterioration in tension after three contractions was about 20 % in this case. The chromatographs have been included to show the reproducibility of the assay as well as peak height differences we obtained in assaying a blank as compared to a solution in which the fiber had been immersed. It can be seen in Table I that for every fiber studied both mechanical and chemical activity were increased at the higher calcium concentration. A comparison of the columns listing mechanical and chemical activities for the first and third contractions indicate some deterioration occurred in most fibers, the relative decrease in tension being somewhat greater than the decrease in chemical activity. There are clear seasonal or batch differences. The mean mechanical and chemical activities at the reference Ca^{2+} concentration decreased progressively from the batch where the experimental contractions occurred in pCa 6.50 to the batch where the experimental contractions were at pCa 7.09. The experimental results shown in Table I were obtained from March through July, and were performed sequentially. Possibly some of these differences were due to decreasing fiber diameter since

TABLE I

TENSION AND ATPase IN SINGLE FIBER SEGMENTS AT 3.0 μm

All measurements were internally controlled. Individual values and means $\pm$S.D. are given.

pCa of second (test) contraction	Fiber reference number	Tension (mg)			ATPase (ΔADP μmol/min per mg protein)		
		Control		Test	Control		Test
		First contraction pCa 6.86	Third contraction pCa 6.86	Second contraction test pCa	First contraction pCa 6.86	Third contraction pCa 6.86	Second contraction test pCa
6.5	18	78	72	99	0.050	0.050	0.069
	24	130	123	162	0.089	0.079	0.112
	25	142	130	172	0.134	0.119	0.128
	26	113	110	158	0.108	0.099	0.158
	30	131	117	155	0.093	0.090	0.115
	31	130	107	159	0.092	0.098	0.110
	$n = 6$	121$\pm$23	110$\pm$20	151$\pm$26	0.094$\pm$0.027	0.089$\pm$0.023	0.115$\pm$0.029
6.86	38	62	53	55	0.056	0.059	0.058
	39	100	81	100	0.076	0.070	0.072
	41	119	94	109	0.111	0.103	0.103
	42	127	112	127	0.105	0.104	0.108
	43	105	85	91	0.087	0.078	0.076
	44	86	86	93	0.057	0.058	0.057
	$n = 6$	100$\pm$24	85$\pm$19	96$\pm$24	0.082$\pm$0.023	0.080$\pm$0.021	0.079$\pm$0.022
6.97	47	74	64	34	0.055	0.051	0.033
	48	60	57	29	0.056	0.057	0.044
	49	94	87	56	0.072	0.069	0.055
	50	105	94	50	0.068	0.068	0.050
	55	73	62	50	0.064	0.067	0.057
	56	50	55	34	0.052	0.060	0.045
	64	86	89	25	0.075	0.070	0.036
	65	81	75	30	0.069	0.070	0.036
	66	85	75	40	0.075	0.065	0.038
	67	93	91	40	0.077	0.076	0.037
	68	60	56	32	0.051	0.054	0.037
	$n = 11$	78$\pm$17	73$\pm$15	38$\pm$10	0.065$\pm$0.010	0.064$\pm$0.008	0.043$\pm$0.008
7.09	71	87	75	0	0.062	0.059	0.009
	72	72	72	0	0.060	0.058	0.011

we noticed that the body weight of the frogs purchased during the experiment progressively decreased and there appeared to be a correlation between the body weight of the frog and average fiber diameter. These differences emphasize the value of our protocol wherein each fiber served as its own control.

Mechanical or chemical activity in the experimental contraction are expressed as a percentage of the activity averaged over the first and third reference (pCa 6.86) contractions in Fig. 3. The data in Fig. 3 represent total measured ATPase and thus include both actomyosin and non-actomyosin ATPase. The sigmoidal dependence of chemical activity on Ca^{2+} is flatter than the dependence of mechanical activity on Ca^{2+}. There is a significant ATPase at pCa 7.09 where no tension was observed. This

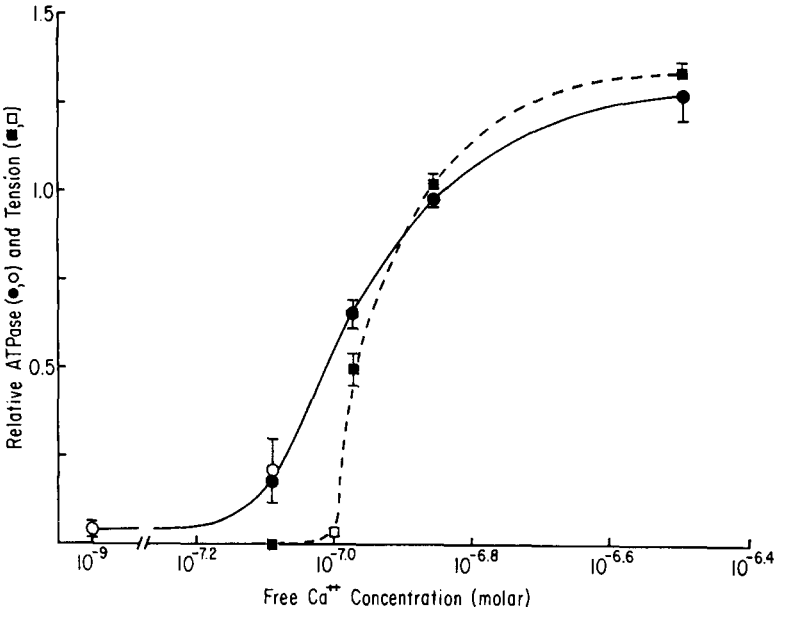


Fig. 3. Mean values of tension and ATPase expressed as a fraction of the value found at pCa 6.86. ATPase (●) and tension (■) are internally controlled. ATPase and tension of the second, experimental contraction, are expressed as a fraction of average values of the first and third control (pCa 6.86) contractions. ATPase (○) and tension (□) are independent experiments without internal controls. The value for ATPase and tension in these experiments are normalized to the overall average of ATPase and tension observed in pCa 6.86. The number of fibers at each pCa are given in Tables I and II. Error bars indicate one S.E. of the mean.

observation is explored in more detail in experiments presented below at a sarcomere length of about 5 μm.

A clearer demonstration of the different functional dependence of the force and ATPase on Ca^{2+} concentration is possible when we (A) evaluate the data in a way that compares the chemical and mechanical activity in the same fiber and (B) subtract a maximal value for the non-actomyosin ATPase from total ATPase. The main result is the ratio of chemical to mechanical activity is dependent on Ca^{2+} concentration. As Ca^{2+} concentration is lowered there is more ADP produced per unit tension. The ADP breakdown per unit tension at pCa 6.97 is about 35 % greater than at pCa 6.5, the saturating Ca^{2+} concentration.

Experiments at approx. 5 μm. The ATPase at pCa 7.09 described in the preceding section may be due to actomyosin without force generation or represent a non-actomyosin ATPase. To distinguish between these possibilities, experiments were made at 3.0 and approx. 5 μm where overlap of the thick and thin filaments does not occur (Table II).

In the absence of Ca^{2+}, ADP production was about 0.003 μmol/mg protein per min at both 3 and $\simeq$ 5 μm. Eisenberg and Moos [22] reported the activity of purified rabbit heavy meromyosin was 0.020 μmol/mg per min at 20 °C. This value corresponds to about 0.002 μmol/mg total protein per min at 4 °C and may be the source of the observed ATPase in Relaxing solution. At approx. 5 μm there was Ca^{2+}-activated ATPase. The average ATPase activity at approx. 5 μm was approximately the same at

TABLE II

EFFECT OF OVERLAP OF MYOSIN AND ACTIN FILAMENTS ON SINGLE FIBER ATPase

Mean values of all experiments are given. The experiments listed in the second row were internally controlled: each fiber was incubated in pCa 7.09 at 3.0 μm and at approx. 5 μm the ATPase of these fibers was also measured in Relaxing solution, (pCa 9) at approx. 5 μm.

$[Ca^{2+}]$ (M)	Tension (relative to maximal isometric) at 3.0 μm	ATPase rate (μmol/mg protein per min $\pm$S.E.)	
		3.0 μm sarcomere length	5 μm sarcomere length
$10^{-9.0}$	0	0.003 ± 0.0004 $n = 8$	0.003 ± 0.0003 $n = 15$
$10^{-7.09}$	0	0.015 ± 0.002 $n = 7$	0.008 ± 0.0007 $n = 7$
$10^{-6.86}$	0.7	0.080 ± 0.005 $n = 18$	0.011 ± 0.002 $n = 5$

pCa 7.09 and pCa 6.86 and was three times higher than in the absence of Ca^{2+} (Relaxing solution). This result provides an upper limit for non-actomyosin Ca^{2+}-activated ATPase and could reflect a Ca^{2+}-dependent activation of myosin ATPase. The ATPases in pCa 6.86 and pCa 7.09 at approx. 5 μm provide an estimate of the total non-actomyosin ATPase, which is about 0.010 μmol/mg protein per min.

The ATPase activities at pCa 7.09 for seven fibers were first studied at 3.0 μm and then at approx. 5 μm. All ATPase activities at 3.0 μm were greater than at approx. 5 μm and in five of the seven fibers the activity at 3.0 μm was greater than twice that obtained at approx. 5 μm in the same fiber. No tension was observed in any of these fibers. Thus the difference between the rates at 3.0 and approx. 5 μm, 0.007 μmol/mg protein per min, is due to Ca^{2+}-activated actomyosin ATPase in the absence of tension.

Sarcoplasmic reticulum ATPase. We performed a series of experiments in vitro to determine to what extent we could account for the non-actomyosin ATPase on the basis of sarcoplasmic reticulum activity, because the treatment of the single fiber segments with glycerol and Lubrol does not necessarily abolish sarcoplasmic reticulum ATPase in the fibers. The results are given in Fig. 4. Maximal ATPase activity, 1.65 μmol/mg sarcoplasmic reticulum protein per min, was observed in a standard incubation medium consisting of trace amounts of Ca^{2+} (no EGTA buffer), 100 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole, pH 7.0, at 35 °C. When the incubation medium was buffered at pCa 6.86 (4 mM total EGTA) and all other conditions were unchanged, the ATPase rate was very similar. We expected that both Lubrol and deoxycholate would abolish sarcoplasmic reticulum activity in vitro. While activity was totally abolished after sarcoplasmic reticulum was incubated for 5 min in 1 % (w/v) deoxycholate, substantial activity remained after 30 min incubation in 2 % (w/v) Lubrol. Even though Lubrol and not deoxycholate was the detergent used in the single fiber experiments, the combined effect of low temperature (4 °C) and low $MgCl_2$ (0.5 mM) also abolished in vitro sarcoplasmic reticulum activity completely (see + and ○ in Fig. 4) and this was the temperature and $MgCl_2$ concentration which was chosen for all our single fiber experiments.

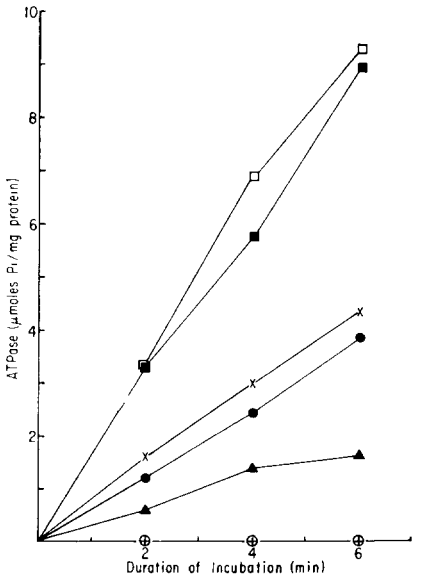


Fig. 4. Plot of ATPase vs. time for sacroplasmic reticulum incubated as follows: □, 10 mM imidizole, pH 7.0, 5 mM $MgCl_2$, 5 mM ATP and 100 mM KCl, 35 °C (standard incubation medium); ■, standard incubation medium except Ca^{2+} buffered at pCa 6.86 with EGTA (total [EGTA] = 4 mM); X, sarcoplasmic reticulum pre-incubated for 30 min in relaxing solution containing 2 % Lubrol at 4 °C and then incubated in standard incubation medium; ●, standard incubation medium except 0.5 mM $MgCl_2$, and Ca^{2+} buffered at pCa 6.86; ▲, standard incubation medium except at 4 °C; ○, sarcoplasmic reticulum pre-incubated for 5 min with 1 % deoxycholate in relaxing solution at 4 °C and then incubated in standard incubation medium; +, pCa 6.86 with EGTA, 10 mM imidizole, pH 7.0, 0.5 mM $MgCl_2$, 5 mM ATP, 140 mM KCl, 4 °C, that is the incubation conditions of the single fiber experiments when the pCa was 6.86.

Myokinase activity in single fiber segments. Since the equilibrium constant for myokinase is about 0.3–1.0, it is possible that myokinase activity could be manifest either as an increase or decrease of ADP. Activity might lead to a synthesis of ADP, because the initial concentration of ADP is 0.2 % ATP. However, the initial AMP concentration is even less than the ADP concentration and furthermore the ADP production in relaxing solution was not affected when the AMP concentration was increased to 50 μM. Only when single fiber segments were incubated in relaxing solution containing 0.5 mM AMP was an additional ADP production observed. If there is very substantial diffusional lag of ADP out of the single fiber, it is possible that as the contraction progresses the internal ADP concentration increases to such high levels that myokinase converts ADP to ATP plus AMP. Diffusion experiments of nucleotide out of mechanically skinned fibers [41] indicate the diffusion constant for ADP out of a similar preparation is too large to create a substantial diffusional lag of ADP out of a single fiber; diffusion experiments have not been performed on fibers skinned chemically with glycerol and Lubrol and it would be desirable to measure AMP and IMP directly to assure myokinase activity is completely absent.

DISCUSSION

We have studied simultaneously both chemical and mechanical activities in single fibers made to contract in solutions containing different Ca^{2+} concentrations. We first discuss the magnitude of the ATPase rates observed. Secondly, our main finding that an increase in the ratio of chemical to mechanical activity as the Ca^{2+} concentration is lowered is considered with reference to biochemical models of contraction. Finally we explore the relationship between our sarcoplasmic reticulum ATPase in vitro and estimates of sarcoplasmic reticulum ATPase based on measurements of Ca^{2+} pumped.

Chemical activity of the single fiber preparation. The maximal activation of ATPase activity by Ca^{2+} in the single fiber preparation was 34-fold: 0.003 μmol/mg protein per min in relaxing solution (pCa about 9) to 0.115 μmol/mg protein per min at pCa 6.50. There was a 10-fold increase in activity between pCa 7.09 and pCa 6.50. Over this range of Ca^{2+} concentration, the Ca^{2+} sensitivity of single fibers is similar to myofibrillar suspensions [4, 23]. Taking differences of temperature into account, the maximal ATPase observed at pCa 6.50 is comparable (within a factor of two) to the maximal ATPase of myofibrillar suspensions [12], the V of actin-activated myosin ATPase [22] and the steady-state rates of ATP splitting in whole muscle [24].

The difference between activities at approx. 5 and 3.0 μm in pCa 7.09 (0.007 μmol/mg protein per min) is due to actomyosin ATPase without measurable tension because this ATPase depends on overlap of myosin and actin containing filaments. Perhaps a branch in the biochemical cycle that involves actomyosin in which no force is generated predominates at this Ca^{2+} concentration. There are, however, alternative Ca^{2+}-dependent mechanisms that might explain the observed chemical activity without mechanical activity (e.g. force generated per bridge is Ca^{2+} dependent) which cannot be distinguished by our experiments.

Ca^{2+}-dependent relationship between chemical and mechanical activity. The obvious effect of Ca^{2+} in removing inhibition of actomyosin activity by binding to troponin is that mechanical and chemical activation curves have a similar appearance. However, we found a relative increase in chemical activity over mechanical activity as Ca^{2+} concentration is decreased. The ratio of chemical to mechanical activity at pCa 6.97 is about 1.35 times that at pCa 6.86 (1.5 times if no subtraction for non-actomyosin ATPase is made).

We have attributed at pCa 7.09 the difference in ATPase between 3.0 and approx. 5 μm to Ca^{2+} activation of actomyosin without tension. It is possible that a tension of up to 2 mg was generated at pCa 7.09 (since this was the noise level of the force transducer). A 2 mg force would require at least a 9-fold increase in the ratio of chemical to mechanical activity at pCa 7.09 as compared to average control activities at pCa 6.86. Schädler's data [25] indicated relatively more chemical than mechanical activity at low Ca^{2+} concentrations but different bundles of fibers were used for force and ATPase measurements. We reported, as a preliminary result [26] that the change in the ratio of chemical to mechanical activity in fibers contracting at 21 °C might be Ca^{2+} dependent. However, no internal controls were possible then because of deterioration of the preparation at that high temperature. We did not observe any granulation or residual force on relaxation in the present series of experiments.

There has been a series of mechanical experiments which studied whether Ca^{2+} effects the kinetic properties of cross-bridge interactions with thin filaments. Julian

[2] using briefly glycerinated fibers and Endo [27] and Podolsky and Teichholz [28] using mechanically skinned single fibers obtained force velocity relations at different Ca^{2+} concentrations. The assumption behind the experiments was mechanical V reflected cross-bridge turnover rate. Both Endo [27] and Julian [2] observed a decrease (approx. 2-fold) in the V when Ca^{2+} concentration was lowered below saturation. However, Podolsky and Teichholz [28] found V to be independent of Ca^{2+} levels and the different results were attributed to the presence of an internal load [29]. Our consistent finding of increased chemical with respect to mechanical activity as the Ca^{2+} concentration is progressively decreased from $10^{-6.50}$ to $10^{-7.09}$ M indicates Ca^{2+} not only determines the number of cross-bridges activated but in some way affects cross-bridge kinetics. Two related questions naturally arise. Is our finding consistent with a 2-fold decrease in mechanical V when Ca^{2+} concentration is decreased? Can our results be interpreted within the context of present models of contraction?

Hill [30–32] has published a comprehensive series of papers that describes in detail the necessary connection between the rate constants, steady-state concentrations of intermediates in a cyclic model of contraction, and the free energies and force functions. Under the simplest assumption that the number of ATP molecules split per cycle is independent of Ca^{2+} concentration (this might well be unreasonable since ATP may be split by actomyosin via alternate pathways that are also regulated by Ca^{2+}) a decrease in mechanical V due to a change in Ca^{2+} concentrations could reflect a decrease in the chemical activity of the muscle. It does not necessarily follow that the ratio of chemical activity to mechanical activity would also decrease. As an example we reproduce in a brief appendix a kinetic scheme with plausible rate constants published by Taylor [33]. A decrease in the rate of cross-bridge attachment produces a decrease in both the turnover rate and the concentration of attached bridges, but the decrease in turnover rate is smaller than the decrease in the concentration of attached bridges. This is one hypothetical mechanism whereby a decrease in the turnover rate is accompanied by an even greater decrease in the observed force.

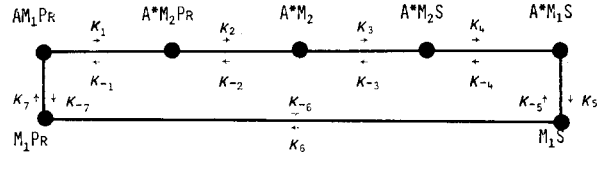
Sarcoplasmic reticulum activity

The largest potential source of non-actomyosin ATPase was thought to be the sarcoplasmic reticulum and therefore we undertook an extensive series of control experiments with sarcoplasmic reticulum in vitro to test whether our method of estimating non-actomyosin ATPases by stretching a muscle beyond overlap seriously effected that estimate of ATPase. There are essentially two methods of evaluating the sarcoplasmic reticulum ATPase: one can measure the Ca^{2+} bound per fiber and then calculate the ATPase per Ca^{2+} bound or one can subject isolated sarcoplasmic reticulum to the same incubation conditions as our single fibers and measure the ATPase directly. We chose the second method. Our finding of a large decrease in isolated sarcoplasmic reticulum activity when total Mg^{2+} in the medium is decreased from 5 to 0.5 mM or temperature is lowered from 35 to 4 °C agrees with previous reports [34, 35]. We found that 5-min treatment with deoxycholate abolished sarcoplasmic reticulum activity as previously reported by Martonosi [20] and Selinger et al. [36]. While incubation with 2 % Lubrol decreased the activity by 60 %, when the temperature was lowered to 4 °C and the total Mg^{2+} concentration lowered to 0.5 mM, no sarcoplasmic reticulum ATPase was observed; these are the incubation

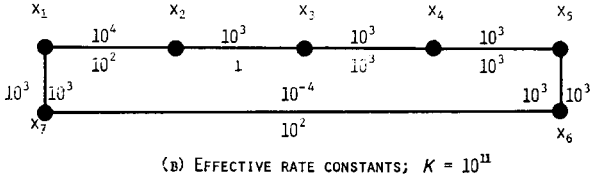
conditions of our single fibers. Although our yield of 1.5 % sarcoplasmic reticulum protein/total protein is in agreement with published values [37] there remains the possibility we (and others as well) lost some important sarcoplasmic reticulum fraction during isolation. Therefore it is of interest to arrive at an estimate of the sarcoplasmic reticulum ATPase from measurements of Ca^{2+} transport made by others. At room temperature, 1 mM $MgCl_2$, in the absence of oxalate, uptake equivalent to 1.5 μmol/mg sarcoplasmic reticulum has been reported [38] (3 mmol Ca^{2+}/l fiber/volume)$\times$(10^{-8} l/mm fiber)$\times$(1 mm/μg total protein)$\times$(μg total protein/0.02 μg sarcoplasmic reticulum protein). This amount is substantially higher than values reported for isolated sarcoplasmic reticulum both with and without oxalate [35, 39] and the discrepancy may arise because extraction of sarcoplasmic reticulum is incomplete or damages the preparation. Using a Ca^{2+} to ATP ratio of 2 [40], sarcoplasmic reticulum ATPase during the first few hundred milliseconds in a single fiber at room temperature and 1 mM $MgCl_2$ would be about 0.014 μmol ATP/mg total protein. Since Ca^{2+} accumulation is very dependent on $MgCl_2$ concentration [34] and temperature [35], accumulation should be substantially less than 0.014 μmol ATP/mg total protein per contraction in our single fiber preparation. The non-actomyosin ATPase actually measured was 0.010 μmol ATP/mg total protein per min. On the basis of our measurements of sarcoplasmic reticulum ATPase in vitro and the sarcoplasmic reticulum calculated from Ca^{2+} uptake data, we conclude the non-actomyosin ATPase observed in our single fiber preparation cannot be attributed to the sarcoplasmic reticulum.

APPENDIX

Taylor [33] has discussed a model of a cyclic mechanochemical reaction. We have used it for illustrative purposes to explore the changes that can occur in the concentrations of chosen intermediates and the overall cycle rate when rate constants for one or more transitions are altered. Reactions involving substrate, product or



(A) MECHANO-CHEMICAL CYCLE



(B) EFFECTIVE RATE CONSTANTS; $K = 10^{11}$

Fig. 5. Mechanochemical cycle redrawn from Taylor [33]. Effective rate constants listed are reprinted from Taylor [33]. K is the effective equilibrium constant.

actin are second order but are treated as first order by including the concentration of the second reactant in the rate constant.

Movement is presumed to accompany the transition from myosin binding at an actin site to myosin binding at a second. We have made the simplest assumption that force is proportional to the concentration of bridges attached to the first actin site in the sequence. Pictures of the cycle including rate constants are listed in Fig. 5. If the total concentration of myosin is set equal to 1 M, the concentrations of the different myosin intermediates are expressed as a fraction of the total directly. Using the rate constants in the diagram as defined by Taylor [33], the number of ATP molecules split per bridge (assuming 1 molecule of ATP split in each completed cycle) is $20\ s^{-1}$ and the concentration of AM_1Pr is 0.002 M. If Ca^{2+} does effect the rate constants, k_7, the rate at which bridges attach to actin, might be modified. For illustrative purposes, we calculated the new cycle rate and concentration of AM_1Pr when the rate at which bridges are formed is decreased by one order of magnitude. In order to satisfy the requirement that $K_{equilibrium}$ for the overall cycle be unchanged k_1 was increased by one order of magnitude. No other rate constants were altered. Both the rate of ATP splitting and the concentration of AM_1Pr decreased but not proportionally. The new chemical rate is $17\ s^{-1}$ and the new concentration of AM_1Pr is 0.0002 M. Assuming the concentration of AM_1Pr is proportional to force, the modification of rate constants described above would lead to a much smaller decrease in chemical activity than mechanical activity.

A change in some Ca^{2+}-dependent rate constant that produces a decrease in the cycle rate can alter both the concentration of attached bridges as shown in the example and the mean force exerted per bridge since detailed balance connects the ratio of rate constants involving attached states to the force produced by those states. This appendix has been included to illustrate that a decrease in the overall cycle rate does not necessarily lead to a more efficient cycle.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Science Foundation. This work was done during the tenure of a Research Fellowship of Muscular Dystrophy Associations of America awarded to Y.U.

REFERENCES

1 Natori, R. (1954) Jikei Med. J. 1, 18–28
2 Julian, F. J. (1971) J. Physiol. Lond. 218, 117–145
3 Ebashi, S., Ohtsuki, I. and Mihashi, K. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 215–223
4 Ebashi, S. and Endo, M. (1968) Prog. Biophys. Biophys. Chem. 18, 123–183
5 Spudich, J. A., Huxley, H. E. and Finch, J. T. (1972) J. Mol. Biol. 72, 619–632
6 Haselgrove, J. C. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 341–352
7 Huxley, H. E. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 361–376
8 Hartshorne, D. J. and Pyun, H. Y. (1971) Biochim. Biophys. Acta 229, 698–711
9 Fuchs, F. and Briggs, F. N. (1968) J. Gen. Physiol. 51, 655–676
10 Weber, A. and Bremel, R. D. (1971) in Contractility of Muscle Cells and Related Processes (Podolsky, R. J., ed.), pp. 37–53, Prentice-Hall, Inc., Englewood Cliffs, N. J.
11 Hellam, D. C. and Podolsky, R. J. (1969) J. Physiol. Lond. 200, 807–819
12 Weber, A. and Herz, R., (1963) J. Biol. Chem. 238, 599–605

13 Bremel, R. D. and Weber, A. (1972) Nat. New Biol. 238, 97–101
14 Bremel, R. D., Murray, J. M. and Weber, A. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 267–275
15 Bremel, R. D. and Weber, A. (1975) Biochim. Biophys. Acta 376, 366–374
16 Haselgrove, J. C. (1975) J. Mol. Biol. 92, 113–143
17 Sillen, L. G. and Martell, A. E. (1964) in Stability Constants of Metal-ion Complexes 2nd edn., Special Publication No. 17, London: The Chemical Society, Burlington House
18 Brown, P. R. (1970) J. Chromatogr. 52, 257–272
19 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
20 Martonosi, A. (1968) J. Biol. Chem. 243, 71–81
21 Fiske, C. H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400
22 Eisenberg, E. and Moos, C. (1968) Biochemistry 7, 1486–1489
23 Weber, A., Herz, R. and Reis, I. (1964) Fed. Proc. 23, 896–900
24 Mommaerts, W. F. H. M. (1969) Physiol. Rev. 49, 427–508
25 Schädler, M. (1967) Pflügers Arch. 296, 70–90
26 Levy, R. M., Umazume, Y. and Kushmerick, M. J. (1975) Biophys. J. 15, 149a
27 Endo, M. (1967) Proc. XVII Gen. Assoc. Jap. Med. Congr. 1, 193–197
28 Podolsky, R. J. and Teichholz, L. E. (1970) J. Physiol. Lond. 211, 19–35
29 Thames, M. D., Teichholz, L. E. and Podolsky, R. J. (1974) J. Gen. Physiol. 63, 509–530
30 Hill, T. L. (1974) Prog. Biophys. Mol. Biol. Part I, 28, 267–340
31 Hill, T. L. (1975) Prog. Biophys. Mol. Biol. Part II, 29, 105–159
32 Hill, T. L., Eisenberg, E., Chen, Y. and Podolsky, R. J. (1975) Biophys. J. 15, 335–372
33 Taylor, E. W. (1973) Curr. Top. Bioenerg. 5, 201–231
34 Panet, R., Pick, U. and Selinger, Z. (1971) J. Biol. Chem. 246, 7349–7356
35 Martonosi, A. and Feretos, R. (1964) J. Biol. Chem. 239, 648–658
36 Selinger, Z., Klein, M. and Amsterdam, A. (1969) Biochim. Biophys. Acta 183, 19–26
37 Weber, A., Herz, R. and Reiss, I. (1963) J. Gen. Physiol. 46, 679–702
38 Ford, L. E. and Podolsky, R. J. (1972) J. Physiol. Lond. 223, 1–19
39 Weber, A., Herz, R. and Reiss, I. (1961) Proc. R. Soc. Lond., Ser. B, 160, 489–501
40 Haselbach, W. and Makinose, M. (1963) Biochem. Z. 339, 94–111
41 Kushmerick, M. J. and Podolsky, R. J. (1969) Science 166, 1297–1298