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THE ROLE OF PHOSPHATE COMPOUNDS IN THAW CONTRACTION AND THE MECHANISM OF THAW RIGOR

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SUMMARY

1. Frozen frog sartorius muscles, when thawed rapidly, contracted in 5 sec performing up to 300 g·cm external work per g muscle. The contraction was characterized by a release of Ca^{2+} and rapid utilization of ATP.

2. In muscles pre-treated with 0.38 mM FDNB in a bicarbonate saline solution the efficiency based on the total ATP used was 17.3 ± 1.8 g·cm work per μmole (4% at −10 kcal per mole ATP being split to ADP). In addition to the actomyosin ATPase, the following enzyme systems were found to be active: adenylate kinase, adenylate deaminase and the formation of hexose monophosphates. ATP:creatine phosphotransferase was completely inhibited. The muscles went rapidly into rigor while much active tension was maintained.

3. In untreated aerobic muscles, the thaw contraction used ATP and phosphorylcreatine. This was followed by a phase of lengthening under the load and rigor developed only 20–30 min later at 24°. This pre-rigor phase was accompanied by increased levels of ADP.

4. When rigor developed (both FDNB-treated and untreated muscles) the sarcoplasmic level of ADP was very low. Thus, the development of rigor requires the absence of both ADP and ATP.

INTRODUCTION

There is increasing evidence that all types of contraction in skeletal muscle occur by a similar biochemical mechanism which requires the liberation of free Ca^{2+} from the sarcoplasmic reticulum and the utilization of ATP[1–3]. Most of the liberated calcium ions travel to the region of overlap of the thick and thin filaments but some are liberated externally. This liberation occurs in contractions produced by electrical pulses[4], high external potassium concentration[4], acetylcholine[5], and at the onset of FDNB-induced rigor at 20° (see ref. 6).

When a skeletal muscle is frozen and subsequently thawed, a contraction develops and soon afterwards the muscle becomes stiff and inextensible in 'thaw rigor'.

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Although thawed muscle can shorten to 30% of rest length, the force developed is very small[7] so little or no work is done. Rapid splitting and deamination of ATP occurs in thawed muscle[8], but the actual mechanism of this type of contraction has not been studied.

The experiments reported here were done to determine whether ATP was utilized and Ca^{2+} was liberated from striated muscle during thaw contraction and the subsequent rigor and to study the mechanism of the development of thaw rigor. The techniques used depended on the finding that FDNB can irreversibly and completely inhibit ATP:creatine phosphotransferase (EC 2.7.3.2) *in vivo* at 0° without affecting the ability of the muscle to contract and relax normally[9–11]. A preliminary report of part of these experiments has been given[12].

EXPERIMENTAL PROCEDURE

Paired sartorius muscles from female frogs (*Rana pipiens*) were used. The muscles were dissected and the pelvic bone was split in the midline without damage to either muscle. A loop of cotton thread tied to the distal tendon and a stainless steel wound clip in the pelvic bone connected the muscle to hooks on the apparatus so each muscle maintained its *in vivo* attachments at both ends. The muscles rested 2 to 3 h at 24° in a dish of oxygenated bicarbonate saline solution (87.7 mM NaCl, 25.0 mM $NaHCO_3$, 3.7 mM KCl and 2.0 mM $CaCl_2$, saturated with CO_2–O_2 (5:95, v/v)). The muscles were then used directly or were treated with 0.38 mM FDNB in bicarbonate saline solution for 40 min at 0° (see refs. 9 and 11). Such treatment allows the direct measurement of ATP changes by completely inhibiting ATP:creatine phosphotransferase[11]. The muscles were mounted on an afterloaded isotonic lever at rest length, recooled to 0° and frozen by immersion in a 2:1 (v/v) mixture of chlorotrifluoromethane–dichlorodifluoromethane (Freon 13–Freon 12) cooled to −180°. Contraction was induced by thawing the muscles completely in 1 to 2 sec by flooding with approx. 80 ml bicarbonate saline at 24°. Such a rapid thawing induced a contraction which reached its maximal degree of shortening in 10 sec. Contractions lasting 5 sec were used to study the chemical changes since by this time the muscles had done most of the work but had not yet reached the fully shortened isometric condition. The thawed muscles were refrozen for assay by immersion into the freezing mixture. For longer periods used to study the onset of rigor, untreated muscles were used aerobically, and after thawing were refrozen for assay after various intervals. The assay techniques routinely used in this laboratory were used to measure changes in ATP, ADP, AMP, P_i, free creatine, glucose 6-phosphate, glucose 1-phosphate, and fructose 6-phosphate (see refs. 6, 10 and 13). Hypoxanthine nucleotides were identified by paper chromatography as described previously[14]. Lactate was measured by oxidation with NAD^+ and L-lactate:NAD^+ oxidoreductase (EC 1.1.1.27)[15] using a Turner model 111 fluorimeter and the materials supplied by Boehringer Mannheim Corp., New York, N.Y.

Outflux of $^{45}Ca^{2+}$

Each muscle was incubated for 12 h at 5° in 12 ml bicarbonate saline, continuously gassed with CO_2–O_2 (5:95, v/v), containing in addition 2.0 μC $^{45}Ca^{2+}$ per ml. $^{45}Ca^{2+}$ in the interstitial fluid space was washed out[16] and the muscles were suspended

individually in small glass chambers containing 2.0 ml non-radioactive bicarbonate saline at 24° continuously gassed with CO_2–O_2 (5:95, v/v). Each chamber was constructed with a Teflon stopcock so drainage was rapid and complete. The bathing solution was replaced at regular intervals (2.5 or 5.0 min) and the rate of outflux of $^{45}Ca^{2+}$ from the muscles was determined by counting each volume of bathing solution in a liquid scintillator[17]. After establishing a base line for 60 min the experimental muscle of the pair was frozen as described above and allowed to thaw during the next minute in the bicarbonate saline in the chamber. Outflux was measured for 60 min after thawing and compared to the outflux from the control muscle of the pair.

RESULTS

Outflux of $^{45}Ca^{2+}$

The outflux of $^{45}Ca^{2+}$ is shown in Fig. 1 for one pair of muscles and was similar in two other pairs studied. The outflux for each collection interval was averaged over the duration of that interval. The circles and dashed line represent the control muscle. The experimental muscle (triangles and solid line) was frozen at the time indicated and thawed as described above. The uppermost curve is the kymogram of the experimental muscle lifting a 6 g load and lengthening 10 min later under the same load.

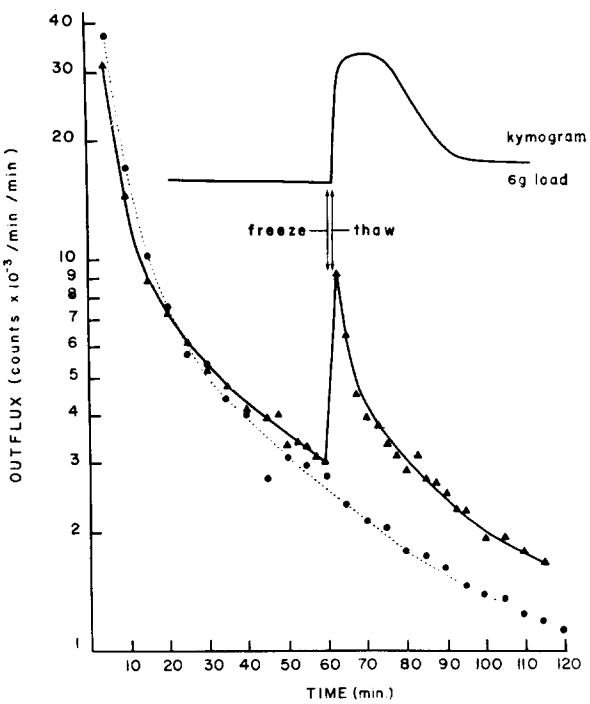

Fig. 1. Outflux of $^{45}Ca^{2+}$ from normal frog sartorius muscle at 25° during a thaw contraction. After pretreatment for 12 h at 5° in oxygenated bicarbonate saline containing 2.0 μC $^{45}Ca^{2+}$ per ml, the outflux of $^{45}Ca^{2+}$ (counts/min per min) into 2.0 ml vol. of non-radioactive bicarbonate saline was measured. The outflux was averaged over the duration of each collection interval, 2.5 or 5.0 min. The experimental muscle was rapidly frozen and thawed after 60 min. ●, control; ▲, experimental muscle. The top curve is the kymogram of the contraction lifting a 6 g weight isotonically and reextending 10 min later under the same load.

There was a temporary burst of $^{45}Ca^{2+}$ release after thawing, coinciding with the onset of shortening. This increased outflux of $^{45}Ca^{2+}$ from the experimental muscle diminished gradually during the contraction and remained greater than the outflux from the control muscle even 60 min after the onset of contraction.

Utilization of ATP

First, it had to be tested that the presence of ATP was required for thaw contraction. A muscle was pretreated with FDNB as described above and stimulated electrically to do work at 0° until the ATP content was depleted[11]. It did not contract when rapidly frozen and thawed even when loaded with only 0.5 g. The other member of the pair was treated similarly with FDNB but not electrically stimulated. It had a normal content of ATP (2.0–2.5 μmoles/g), and gave a typical thaw contraction.

A series of FDNB-treated sartorius muscles were mounted on an isotonic lever and afterloaded with various weights (5 to 35 g) at rest length. One member of each pair serving as the control was frozen as described above and taken for assay. The other member of the pair was frozen, thawed rapidly by flooding with bicarbonate saline as described above, refrozen after a 5-sec contraction and taken for assay. Such rapidly thawed muscles were able to lift loads up to 35 g and performed up to 300 g·cm external work per g muscle. As in electrically stimulated contractions, heavier loads were lifted at slower rates for shorter distances. At 24°, the initial velocity of shortening against a 15 g load was 0.7–0.8 cm/sec, compared to 8 cm/sec in normal muscle[18]. The ability to lift considerable loads and perform external work is an important difference found between these rapidly thawed and slowly thawed muscles[7,19,20].

The chemical changes observed in this series of experiments of FDNB-treated muscles are given in Table I. The results are expressed as the difference of the experimental muscle compared to its paired control. There was a very large ATP utilization

TABLE I

SUMMARY OF AVERAGED CHEMICAL CHANGES IN FDNB-TREATED SARTORIUS MUSCLES DURING A 5-SEC THAW CONTRACTION AT 0°

Muscles, treated with 0.38 mM FDNB in bicarbonate saline at 0° gassed with N_2–CO_2 (95:5, v/v) were rapidly frozen in a Freon mixture (chlorotrifluoromethane–dichlorodifluoromethane (2:1, v/v)) at −180°, thawed rapidly as described in the text, and refrozen for assay after 5 sec during the rising phase of a thaw contraction. Contractions were isotonic against loads varying from 5 to 35 g. Values given are the means ± S.E. of the differences between a control and a contracted muscle pair. The probability of the mean values being different from zero was calculated by Fisher's distribution of *t* for paired samples.

Compound	*Changes observed ± S.E. (μmoles/g wet wt.)*	*Number of pairs studied*	*Probability*
P_i	+3.92 ± 0.16	38	$P<0.001$
ATP	−2.23 ± 0.10	24	$P<0.001$
ADP	−0.18 ± 0.04	7	$0.01>P>0.001$
AMP	+0.78 ± 0.12	7	$P<0.001$
Total adenine nucleotide	−1.04 ± 0.24	7	$0.01>P>0.001$
Free creatine	−0.22 ± 0.13	22	$0.2>P>0.1$
Glucose 6-phosphate	+0.54 ± 0.10	4	$0.02>P>0.01$
Glucose 1-phosphate	+0.04 ± 0.004	4	$0.01>P>0.001$
Fructose 1-phosphate	+0.06 ± 0.02	4	$0.1>P>0.05$
Total hexose monophosphate	+0.65 ± 0.11	4	$0.01>P>0.001$

for the work done, -2.23 ± 0.10 μmoles/g, representing virtually all the ATP present. The apparent efficiency (external work per μmole ATP used) was 34.5 ± 3.7 g·cm per μmole which is much lower than in electrically stimulated contractions, *i.e.* approx. 150 to 200 g·cm/μmole[9,10,13]. At —10 kcal/mole ATP for the free energy, the apparent overall efficiency for these thaw contractions was 8 %; however, the efficiency based on the total amount of ATP used was really about 4 % (17.3 g·cm/μmole) since, as is discussed below, the activity of adenylate kinase (EC 2.7.4.3) made available twice as much energy as would have been delivered from just the terminal phosphate of ATP (see ref. 9). The observed change in P_i (3.92 ± 0.16 μmoles/g) was much greater than the net change in ATP and this was not due to breakdown of phosphorylcreatine, for the change in free creatine was -0.22 ± 0.13 μmole/g, that is, in the reverse direction. However, this change was only 2 % of the free creatine content and is not significantly different from zero ($0.2 > P > 0.1$) though there may have been a small loss of creatine from the muscle due to the technique of thawing.

The difference of the observed change of ATP compared with the observed change of P_i represents complete conversion of ADP formed by the actomyosin ATPase (EC 3.6.1.3) to ATP and AMP by adenylate kinase, and further cycling of the newly produced ATP to ADP by actomyosin ATPase. The reasons for this conclusion follow. There was no equivalent increase in ADP at the end of the 5-sec contraction; in fact, the ADP content decreased. There was incorporation of P_i into hexose monophosphates (see Table I), and therefore, the total inorganic phosphate change equals the observed change of P_i *plus* the change of hexose phosphate. Incorporation of P_i into hexose monophosphates has also been found in sartorii going into FDNB-induced rigor[6] and in isometric contractions of FDNB-treated sartorius muscles[26] when ATP breakdown significantly increased the P_i content. The total P_i produced (4.56 μmoles/g) equals twice the observed change in ATP (*i.e.* 2×2.23 μmoles/g) within experimental error as expected with adenylate kinase activity. Hence, the continued activity of actomyosin ATPase (and probably the sarcoplasmic reticulum and other membrane ATPases) and adenylate kinase resulted in splitting of ATP to the monophosphate level by use of both terminal phosphates in ATP.

However, a large increase of AMP did not occur. Rather, there was a large decrease in total adenine nucleotides. The difficulties in measuring quantitative changes of hypoxanthine nucleotides in $HClO_4$ extracts of muscle have been previously discussed[14]. The molar absorbance coefficient of FDNB in the bicarbonate saline used was maximal at 240 mμ (1.3×10^4) and was 0.96×10^4 at 260 mμ, and therefore spectrophotometric measurements of the quantities of hypoxanthine nucleotides are unreliable in the presence of FDNB. Nevertheless IMP was identified in the extracts of two pairs of thawed muscles tested by paper chromatography and was absent in the control muscles. This provided direct evidence for the activity of adenylate deaminase (EC 3.5.4.7). These results, the activity of adenylate kinase, and the finding of WEBSTER[21] that IMP was the main product of dephosphorylation and deamination of ADP by myofibrillar preparations at pH 7 indicate that the observed decrease in total adenine nucleotides is an accurate measure of IMP production (see Table I).

Chemical mechanism of rigor mortis

It is commonly supposed that the onset of rigor mortis is characterized by the disappearance of ATP[6,22–24]. The results of FDNB-treated muscles might have been

interpreted as support of this view, for complete breakdown of ATP was found and the muscles rapidly became stiff and inextensible on the completion of shortening. However, in the untreated muscles, as is shown in Fig. 1, the loaded muscles lengthened spontaneously and completely after the thaw contraction. In fact, once extended under a load, the untreated muscle would contract again, if it were unloaded within 10–15 min of contraction at 0–4°. It was only about 20–30 min after the onset of thawing that the muscles became stiff and inextensible. They did not shorten or develop force during this development of rigor. We, therefore, studied the ATP, ADP and AMP contents of untreated muscles at various times after the onset of a thaw contraction (Fig. 2). The muscles were frozen, rapidly thawed as described above,

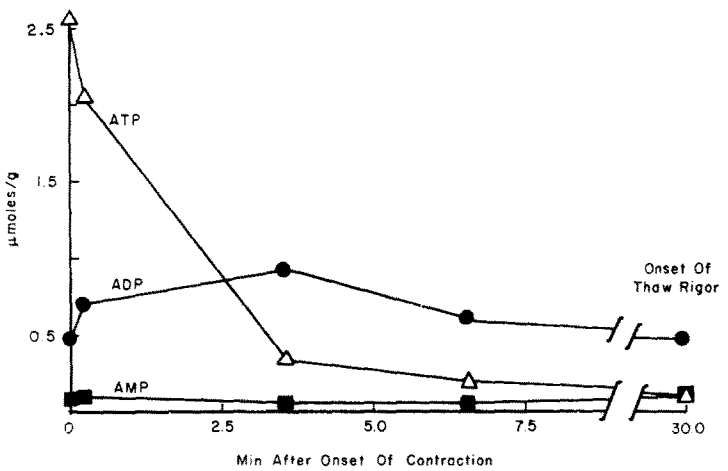

Fig. 2. Adenine nucleotide changes during thaw contraction of untreated frog sartorius at 24° and subsequent reextension under a 10-g load. Symbols used: △, ATP; ●, ADP; ■, AMP. Resting levels (µmoles/g wet wt. of muscle) are given at initial time (0). Each muscle was rapidly frozen and thawed, then refrozen for chemical assay after various intervals. Stiffness characteristic of rigor occurred 20 to 30 min after the onset of contraction. Measurement of nucleotide levels in the muscles was performed by reactions coupled with NADH oxidation.

contracted isotonically with a 10-g load and each was refrozen after various intervals for assay. The shapes of the kymograms of contractions were similar to that shown in Fig. 1, but reextension under the 10-g load occurred at about 5 min. The ATP content rapidly fell to levels that were unable to maintain the contraction against the load of 10 g, and the muscle lengthened. The ADP content increased at this time and remained slightly elevated during the lengthening phase. The AMP content remained relatively constant throughout. Only when the ATP content was less than 0.2 µmole/g, and the ADP content was equal to the resting levels, 0.5 µmole/g (the amount bound to actin), did rigor develop. Therefore, the absence of sarcoplasmic ATP and ADP is necessary for the development of rigor; in the absence of ATP, if ADP is present, lengthening, not rigor, occurs in a loaded muscle.

DISCUSSION

The results show that contractions induced by freezing and thawing proceed by a mechanism similar to other types of muscle contraction. It seems likely that

the formation of ice crystals during the freezing and thawing mechanically damages the lateral cisternae of the sarcotubular system which were the sites from which the Ca^{2+} was released[25]. Once released into the sarcoplasm, the Ca^{2+} activated the contractile mechanism. This rapidly utilized ATP as did the transport mechanism for calcium in the sarcotubular system and possibly other mechanisms.

In FDNB-treated muscles, resynthesis of ATP from phosphorylcreatine, by glycolysis and by oxidative phosphorylation was prevented[11]. Nevertheless, several reactions involving phosphate compounds did occur. Measurement of the net change of ATP *per se* does not represent the total ATP utilized whenever adenylate kinase is operative since this enzyme allows a *de facto* usage of both terminal phosphates of ATP. Adenylate kinase and adenylate deaminase activity resulted in the rapid accumulation of IMP. Measurement of the incorporation of P_i into hexose monophosphates was necessary to get an accurate measure of the total increase in P_i which was equal to the total ATP utilized (ATP originally present and that produced from ADP by adenylate kinase). The small increases of the hexose monophosphates observed after rapid freezing and thawing mean that the early steps of the glycolytic pathway can function to take up P_i in 5-sec contractions of thawed FDNB-treated muscles. This was also shown to occur at 0° when rigor developed slowly[6] and in electrically stimulated isometric contractions lasting longer than 3 sec (see ref. 26). These findings raise the question whether the increase in hexose monophosphates indicates an accompanying increased rate of lactate production with contractions lasting 5 sec or less, or an enhancement of the glycolytic path only up to the isomerase or phosphofructokinase level. Preliminary results[27] indicate that the rate of lactate production remains very low and constant during brief contractions of FDNB-treated muscles. It is known that there is delay in the increase of lactate production following continued stimulation of normal sartorii[28].

A major finding was that rigor developed only in the absence of both ATP and ADP in the sarcoplasm. This happened rapidly in the FDNB-treated muscles (Table I) so that rigor mortis was developed at the end of the 5-sec contraction phase. In the untreated muscles, the rapid induction of thaw contraction allowed a separation of a contraction phase, a pre-rigor phase and a rigor phase. In these untreated muscles the ATP level was sustained by the available phosphorylcreatine stores for ATP: creatine phosphotransferase was not inhibited, and by glycolysis which is known to occur in frozen-thawed muscle[29]. As the ATP level decreased, the force of contraction was not maintained and the muscle was extended by the load. The thawed and lengthened muscle at 24° did not go into rigor until approx. 20 to 30 min after thawing. The period of lengthening and pre-rigor was accompanied by increased levels of ADP. When rigor developed, levels of ATP and AMP were less than 0.2 μmole/g (Fig. 2). Moreover, at the time, there was no free ADP, for the ADP content was equal to the resting, control level; resting aerobic mitochondria are in a steady state with very low levels of free ADP[30].

These results, therefore, provide an extension to the conclusions of ERDÖS[22], BENDALL[23] and NAUSS AND DAVIES[6] that rigor occurs in the absence of ATP. The new finding is that rigor requires the absence of ADP in addition to the absence of ATP. The design of the previous experiments could not distinguish a pre-rigor state in which ATP levels were low, for in these experiments rigor developed slowly from a resting state and the ADP content never exceeded the ATP content. Furthermore,

the onset of rigor was correlated with diminishing ATP contents not with ADP content. The results of the present experiments also mean that the conditions for rigor are similar in whole muscle and in glycerinated fibers and actomyosin threads. In these muscle models any polyphosphate (*e.g.* pyrophosphate, ADP or ATP) 'plasticizes' the model system under appropriate conditions[24]. The polyphosphate available during the lengthening and pre-rigor phases of untreated thawed muscles was found to be ADP. No increased levels of ADP were found in FDNB-treated muscles (*cf.* Table I and Fig. 2) and these muscles went into rigor at the completion of shortening. Hence, the conclusion was drawn that lengthening with the load required the presence of ADP. Only when both the ATP and ADP levels in the sarcoplasm were very low did rigor occur.

Therefore, muscle contraction (*i.e.* shortening and tension development) requires the presence and utilization of ATP and Ca^{2+}. Relaxation requires the absence of free Ca^{2+} in the presence of ATP. ADP in living muscle acts in a manner similar to the plasticizers of muscle model systems and allows the lengthening of loaded muscles when ATP is depleted. The stiffness characteristic of the developed rigor state occurs only in the absence of any polyphosphate.

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