ATP, uncomplexed by divalent cations, and the LC2 light chain are interdependent modifiers of the skeletal actomyosin MgATPase activity

Suzanne M. PEMRICK* and Patricia A. MARTINEZ

Department of Biochemistry and Biophysics Program, State University of New York, Health Science Center at Brooklyn, Brooklyn, NY 11203, U.S.A.

In the absence of troponin and tropomyosin, skeletal actomyosin MgATPase activity can be altered by 2–3-fold by divalent cations. The ‘sign’ of this effect (i.e. inhibition or activation) varies with ionic strength. To investigate the mechanism, P_i liberation was analysed at both low and high ionic strength with three concentrations of MgATP and over a wide range of Mg^{2+} concentrations. This procedure separated the effects of two dependent variables, Mg^{2+} and $ATP^{4-/-3-}$ (ATP_{free}), to provide the following observations. (1) ATP_{free} , not Mg^{2+} (nor Ca^{2+}), was the modifier. (2) ATP_{free} was an activator at low ionic strength and an inhibitor at high ionic strength, with half-maximal activation/inhibition occurring between 0.75 and 0.8 mM- ATP_{free} . (3) The rate constants controlling V_{max} with respect to actin were increased up to 3-fold by ATP_{free} at low ionic strength, and decreased up to 3-fold by ATP_{free} at high ionic strength. (4) The effect of ATP_{free} required near-native levels of the LC2 light chain bound to myosin (i.e. 2 mol of LC2/mol of myosin). (5) Sensitivity of P_i liberation to a 50% decrease in the LC2 content of myosin required high ATP_{free} concentrations. It is concluded that LC2 and ATP_{free} are interdependent, non-additive, modifiers of MgATPase. These results are consistent with thin filament regulation of skeletal muscle contraction, and begin to explain why both positive and negative effects on MgATPase have been attributed to LC2.

INTRODUCTION

In the absence of the troponin complex (TN) and tropomyosin (TM), skeletal actomyosin MgATPase activity is sensitive to divalent cations. At low ionic strength, micromolar concentrations of either Mg^{2+} or Ca^{2+} have an inhibitory effect [1–3]. At physiological ionic strength, the opposite effect is noted, i.e. positive sensitivity to micromolar concentrations of Ca^{2+} [3]. The LC2 light chain of myosin has been implicated in both effects [2,3]. As a result, both positive and negative effects of divalent cations have been tentatively attributed to the same mechanism. The present study examined this ‘apparent’ divalent cation sensitivity under controlled conditions of ionic strength plus actin, MgATP, ATP^{4-}/ATP^{3-} (ATP_{free}), Mg^{2+} and Ca^{2+} concentrations. ATP_{free} , and not divalent cations, was the effector at both low and high ionic strength.

The early studies which explored a function for the LC2 light chain in vertebrate striated muscle were activity profiles of actomyosin MgATPase *in vitro*. These reports were contradictory ([4–7] versus [8–10]), as for the ATP_{free} effect described here. With few exceptions [5,7], reports of inhibition following LC2 removal came from studies using low ionic strength, whereas positive effects were observed exclusively at around physiological ionic strength. Similarly, the phosphorylated form of LC2 (LC2-P) has been observed to exert opposite effects at the extremes of ionic strength ([11] versus [12]). These observations led to the hypothesis that LC2 is involved in binding certain polyanions, such as ATP_{free} and/or phosphate (as LC2-P), to become an activator or an inhibitor of the actomyosin MgATPase activity, depending upon the experimental conditions [13]. The present study tested this hypothesis.

MATERIALS AND METHODS

Chemicals

ATP was vanadium-free (Sigma no. A-5294). All other reagents were from Sigma, and were of the highest purity available.

Protein preparation

The preparative procedures for the following were as described previously: unphosphorylated myosin (2 mol of LC2/mol) [4, 11]; G-actin and reconstituted thin filaments [4]; LC2-deficient myosin (1 mol of LC2/mol) and LC2-reassociated myosin (1.85 mol of LC2/mol) from 5,5'-dithiobis(2-nitrobenzoic acid) (Nbs₂)-treated myosin [4,14], with no evidence of LC1 or LC3 depletion or proteolytic degradation [14]. Thiol regeneration after Nbs₂ treatment restored ATPase activity (without actin) to native levels (see below). F-actin was formed from G-actin by addition of 3 M-KCl to give final concentrations of 30–100 mM and was used within a few hours.

ATPase assays

Activity is expressed as nmol of $P_i \cdot s^{-1} \cdot (nmol \text{ of myosin site})^{-1}$. The Ca/EDTA- and K/EDTA-ATPase assays were as described previously [4]. Values were 1–1.3 s^{-1} for the CaATPase and 12–17 s^{-1} for the K/EDTA-ATPase, irrespective of LC2 content. Actomyosin was premixed immediately before the ATPase assay, by mixing F-actin at 0.6 M-KCl and then adding soluble myosin (to 6 μM final concentration), and stirring by hand. A positive displacement micropipette, designed for viscous solutions (Rainin), was used to transfer samples of the actomyosin premix to the ATPase reaction mixtures of lower ionic strength. Each reaction mixture was stirred by hand for 5–10 s immediately after

Abbreviations used: ATP_{free} , ATP^{4-}/ATP^{3-} ; LC2, one of three low- M_r subunits [light chains (LC)] of skeletal myosin, located at the head/rod junction, homologous to the regulatory LCs of myosin-linked regulatory systems; LC2-P, phosphorylated LC2; HMM, heavy meromyosin (the soluble, two-headed fragment of myosin); S-1, subfragment 1 (the soluble head region of myosin); TN, the troponin complex, consisting of TN-C (Ca^{2+} -binding)-TN-I (inhibitory)-TN-T, (tropomyosin-binding); troponin A and troponin B, the original names for TN-C and (TN-I plus TN-T) respectively; TM, tropomyosin; regulated actin (or thin filaments), F-actin plus TN and TM; Nbs₂, 5,5'-dithiobis(2-nitrobenzoic acid).

* To whom correspondence should be addressed, at: Box 41, SUNY, Brooklyn, 450 Clarkson Avenue, Brooklyn, NY 11203, U.S.A.

the addition of actomyosin, and again, for an additional 5–10 s, just before the start of the reaction. The MgATPase activity was measured in 2 ml of 20 mM-imidazole-Cl (pH 7, 25 °C), 2 mM- K_2 EGTA, 0.02–1 mM- Mg^{2+} (at constant MgATP), 0.6–2.0 mM-MgATP, 7.5–77.5 mM-KCl (ionic strength curve), and 50 or 100 μ l of actomyosin premix or myosin. Ionic strength was kept constant by the addition of KCl to adjust for changes in both $[Mg^{2+}]$ and $[ATP_{free}]$. These calculations were checked using the acto-heavy meromyosin (HMM) MgATPase [3] and found to be precise, i.e. ionic strength varied by less than 0.0002 mM. The reaction was started by the addition of ATP (as an $MgATP/Mg^{2+}/ATP_{free}$ equilibrium solution), run for 3–12 min (plus mechanical stirring), and terminated with 1 ml of 10% trichloroacetic acid. Liberation of P_i was measured [15] and corrected against appropriate blanks (ATP, actin) and MgATPase activity in the absence of actin.

Filament stability versus ionic strength

Turbidity measurements of insoluble myosin were carried out at 340 nm (Gilford Model 260 Spectrophotometer) under the conditions employed for the ionic strength profile of the actomyosin MgATPase (2 mM-MgATP and 0.067 or 2 mM- ATP_{free}).

Calculation of Ca^{2+} , Mg^{2+} , and ATP_{free} concentrations

A series of Ca^{2+} stock solutions were prepared by varying the ratio of K_2 EGTA/ Ca EGTA at constant ionic strength and pH, assuming a K_d of 1.9×10^{-7} M for Ca^{2+} and EGTA, correcting for a 10-fold dilution and $CaATP$ formation in the ATPase medium [1]. A K_d of 4×10^{-5} M for Mg^{2+} and ATP_{free} (pH 7) was estimated [1] from the stability constants given by O'Sullivan & Perrin [16], adjusting for K^+ and pH. This K_d value falls within the range 0.035–0.045 mM determined by ^{31}P -n.m.r. measurements under physiological conditions [17,18].

Kinetic analyses

The apparent K_d (K_{app}) for actin and V_{max} were estimated from the mean velocities of three duplicate experiments, by a weighted linear regression analysis [19] of plots of $1/v$ versus $1/[actin]$ (0.1–20 μ M). Modifier potency (i.e. the $[ATP_{free}]$ at half-maximal effect) was evaluated from plots of v versus $[ATP_{free}]$. Half-maximal activation by ATP_{free} was defined as the $[ATP_{free}]$ at which activator efficacy was equal to one-half of the sum of P_i liberation values at the lowest and highest $[ATP_{free}]$. Half-maximal inhibition by ATP_{free} was defined as the $[ATP_{free}]$ at which inhibitor efficacy was equal to one-half of the difference between P_i liberation values at the lowest and highest $[ATP_{free}]$.

RESULTS

Studies at low ionic strength

At an ionic strength of 55 mM, the MgATPase activity of actomyosin (no TN or TM) was inhibited by 30–40% (e.g. from $3.75 s^{-1}$ to $2.4 s^{-1}$) by intermediate concentrations (0.02–0.2 mM) of either Mg^{2+} or Ca^{2+} . The two divalent cations together were not more effective than either one alone. This inhibitory effect required actin. Similar effects have been reported previously [1,3]. Subsequent experiments focused on the inhibitory effect of Mg^{2+} in the absence of Ca^{2+} .

The effect of Mg^{2+} was proportional to the actin concentration (Fig. 1). In fact, increasing the $[Mg^{2+}]$ from 0.02 to 1 mM decreased both the K_{app} (for actin) and the V_{max} by the same degree (2–3-fold). As a result, Lineweaver–Burk plots of $1/v$ versus $1/[actin]$ were nearly parallel (see inset to Fig. 1). Concentrations of Mg^{2+} above 1 mM were not analysed in this study.

In the above experiments, the $[MgATP]$ was held constant at 1 mM. Two variables were changing in reciprocal manner: as $[Mg^{2+}]$ increased from 0.02 to 1 mM, $[ATP_{free}]$ decreased from 2.0 to 0.04 mM. Thus the effect could be due to inhibition by Mg^{2+} or activation by ATP_{free} . To distinguish between these two possibilities, the experiment was repeated at three different concentrations of MgATP. In the inset to Fig. 2, each of the data points for a particular $[Mg^{2+}]$ represented both different ATP_{free} and different MgATP concentrations. This procedure separated the three variables. Because the three curves were not superimposed with respect to $[Mg^{2+}]$, it was unlikely that Mg^{2+} was the modifier. To test the alternative hypothesis, the data were replotted versus $[ATP_{free}]$ to give a single curve (Fig. 2). This was especially true at high ATP_{free} concentrations. Below 0.5 mM- ATP_{free} , there was a 10–15% variability in P_i liberation among the three MgATP concentrations when plotted versus ATP_{free} (Fig. 2). Two additional experiments provided a plausible explanation for this variability.

In the first experiment, $[ATP_{free}]$ was held constant (at 0.1 mM), and the Mg^{2+} concentration was increased from 0.2 to 0.8 mM, along with the usual increase in $[MgATP]$. P_i liberation varied randomly by less than 12%, between 2.45 and $2.70 s^{-1}$ (five concentrations tested), suggesting that neither Mg^{2+} nor MgATP was the modifier. This experiment also verified a 12% scatter in the data. In the reciprocal experiment, $[Mg^{2+}]$ was held constant (at 0.2 mM) while increasing the ATP_{free} (from 0.1 to 0.27 mM) and MgATP (from 0.6 to 2.0 mM) concentrations. P_i liberation increased by 30%, from 2.0 to $2.6 s^{-1}$. This was the expected efficacy of ATP_{free} over this range of ATP_{free} concentrations (see Fig. 2). Therefore, at low ionic strength, ATP_{free} was the principal modifier.

Fig. 1. Inhibitory effect of Mg^{2+} (in the absence of Ca^{2+}) upon MgATPase activity at low ionic strength (55 mM)

Actin concentrations (μ M) were: 0 ($\times$), 0.6 ($\circ$), 1.2 ($\bullet$) and 3.7 ($\blacktriangle$), in the presence of 0.6 μ M-myosin heads and 1.0 mM-MgATP. Data are expressed as means $\pm$ S.E.M. ($n = 3$ experiments). Inset: relationship between K_{app} (for actin) and V_{max} at low (0.02 mM; $\circ$) and high (1 mM; $\bullet$) Mg^{2+} concentrations.

Fig. 2. The observed inhibitory effect of Mg^{2+} (2 mM-EGTA) at low ionic strength is due to an activating effect of ATP_{free}

Actomyosin MgATPase activity was measured in the presence of $3.7 \mu\text{M}$ -actin, $0.6 \mu\text{M}$ -myosin heads and the following concentrations of MgATP (mM): 0.6 ($\times$; inset: $\bullet$), 1.0 ($\bullet$; inset: $\circ$) or 2.0 ($\blacktriangle$) to generate three curves when plotted versus $[\text{Mg}^{2+}]$ and one curve when plotted versus $[\text{ATP}_{\text{free}}]$. Data are expressed as means ($n = 3$) or as means $\pm$ S.E.M. ($n = 3$) in the inset. Range of ATP_{free} : $\times$ (inset: $\bullet$), 0.024–1.2 mM; $\bullet$ (inset: $\circ$), 0.04–2.0 mM; $\blacktriangle$, 0.08–4.0 mM.

Fig. 3. Stimulatory effects of Mg^{2+} (in the absence of Ca^{2+}) upon the MgATPase activity at high ionic strength (95 mM)

Actin concentrations (μM) were: $\circ$, 0; $\times$, 0.3; $\blacksquare$, 0.5; $\blacktriangle$, 2.25, in the presence of $0.6 \mu\text{M}$ -myosin heads and 1.0 mM-MgATP. Data are expressed as means $\pm$ S.E.M. ($n = 2$). Inset: relationship between K_{app} (for actin) and V_{max} at low (0.02 mM; $\circ$) and high (0.4 mM; $\bullet$) Mg^{2+} concentrations.

From the data in Fig. 2, it was possible to estimate modifier potency. Half-maximal activation by ATP_{free} was observed at a concentration of around 0.8 mM.

Studies at high ionic strength

Repeating these experiments at an ionic strength of 95 mM produced opposite effects to those obtained at low salt concentrations: (1) P_i liberation was activated by intermediate concentrations of either Mg^{2+} or Ca^{2+} ; (2) the effect required actin; and (3) both divalent cations together were not more effective than either divalent cation alone. For example, increasing the Ca^{2+} concentration from 0.01 μM to 0.2 mM increased the liberation of

Fig. 4. The stimulatory effect of Mg^{2+} (2 mM-EGTA) at high ionic strength is due to an inhibitory effect of ATP_{free}

Actomyosin MgATPase activity was measured at $2.25 \mu\text{M}$ -actin, $0.6 \mu\text{M}$ -myosin heads, and MgATP at the following concentrations (mM): $\bullet$, 0.6; $\circ$, 1.0; $\blacktriangle$, 2.0, to generate three curves when plotted versus $[\text{Mg}^{2+}]$ (inset) and one curve when plotted versus $[\text{ATP}_{\text{free}}]$. Data are expressed as means, or as means $\pm$ S.E.M. ($n = 2$) in the inset.

P_i by 2.5-fold (from 0.3 to 0.8 s^{-1}) at low $[\text{Mg}^{2+}]$ (0.02 mM), but was without effect at high $[\text{Mg}^{2+}]$ (above 0.2 mM), maintaining an average rate of 0.8 s^{-1} over a 1000-fold increase in $[\text{Ca}^{2+}]$. Somewhat similar results have been reported previously [3]. The following experiments focused upon the effect of Mg^{2+} in the absence of Ca^{2+} .

At high ionic strength, the stimulatory effect of Mg^{2+} was proportional to the actin concentration (Fig. 3). The magnitude of the effect (approx. 2 s^{-1}) was similar at low and high salt concentrations. The relative change (i.e. 7–8-fold) was greater at high salt concentrations because the basal activity was very low (0.24 s^{-1}). With excess actin there was a slight inhibitory response above 0.3 mM- Mg^{2+} (17% in Fig. 3), which was within the scatter of the data. This is demonstrated in the inset to Fig. 3, where Mg^{2+} increased both the V_{max} and the K_{app} (for actin) at 95 mM ionic strength, the opposite of the effect at an ionic strength of 55 mM.

At high, as at low, ionic strength the Mg^{2+} curves at three MgATP concentrations were not superimposed (see inset to Fig. 4). The maximal apparent activation by Mg^{2+} was obtained at or below 0.1 mM- Mg^{2+} at 0.6 mM-MgATP, and at around 0.5 mM- Mg^{2+} at 2.0 mM-MgATP. Therefore the data were replotted as a function of $[\text{ATP}_{\text{free}}]$ (Fig. 4). Above 0.08 mM- ATP_{free} the data could be fitted to a single curve. Below 0.08 mM- ATP_{free} , however, there was a downward curvature to the data for the two lower MgATP concentrations. To test for a possible effect of Mg^{2+} under these conditions, the $[\text{ATP}_{\text{free}}]$ was held constant at 0.067 mM, while varying the concentration of Mg^{2+} (0.2–0.8 mM) and MgATP (0.6–2.0 mM) (five separate concentrations). The liberation of P_i was nearly constant, at $1.49 \pm 0.12 \text{ s}^{-1}$ at $2.3 \mu\text{M}$ -actin (mean $\pm$ S.E.M.). Therefore Mg^{2+} was not the modifier. This left the possibility of a slight activating effect of MgATP observable only at low ATP_{free} concentrations. As an illustration, at 0.08 mM- ATP_{free} , P_i liberation increased by 26% between 1.0 and 2.0 mM-MgATP, which was 14% above data scatter. At 2.0 mM-MgATP, however, the net inhibitory effect of ATP_{free} was 20-fold.

The data in Fig. 4 were used to estimate the relative potency of ATP_{free} as a modifier at high ionic strength. Half-maximal

Fig. 5. Kinetic analysis of ATP_{free} effect

(a) K_{app} . (for actin); (b) V_{max} . ●, 0.067 mM-; ○, 2 mM-ATP_{free}. Kinetic constants were derived from weighted linear regression analyses [19] in which the mean velocities of three duplicate experiments were plotted with 8–11 actin concentrations (0.1–20 μM) at 0.6 μM-myosin heads.

Fig. 6. Comparison of effect of ATP_{free} as a function of ionic strength and LC2 content

○, △, □, 2 mM-ATP_{free}; ●, ▲, ■, 0.067 mM-ATP_{free}; ○, ●, native LC2 (2 mol of LC2/mol); △, ▲, reassociated LC2 (1.85 mol of LC2/mol); □, ■, LC2-deficient (1.0 mol of LC2/mol) myosin. The shaded area on the left side of the Figure indicates sensitivity to ATP_{free} versus LC2 content; that on the right side of the Figure indicates the activating effect of LC2 removal at high [ATP_{free}] in the presence of 0.3 μM-myosin heads and 1.25 μM-actin. Data are expressed as means of triplicate experiments and were not included in Fig. 5.

inhibition occurred around 0.75 mM-ATP_{free}; therefore potency was similar at low and at high ionic strength.

Quantitative analysis of the effect of ionic strength

At between 35 and 95 mM ionic strength K_{app} (actin) and V_{max} were affected similarly by ionic strength, and ATP_{free} altered both constants to a similar extent (Fig. 5). At 32.5 mM ionic strength, ATP_{free} increased K_{app} (for actin) 3-fold and increased V_{max} 2.5-fold. A 20 mM increase in ionic strength (to 55 mM) increased K_{app} (actin) at low and at high concentrations of ATP_{free} by less than 2-fold, with only a marginal effect upon V_{max} . As a result, ATP_{free} maintained a 2.5-fold stimulatory

effect upon both K_{app} (actin) and V_{max} . At 71 mM ionic strength ATP_{free} had no effect upon the kinetics of P_i liberation, whereas at 95 mM ionic strength ATP_{free} had a pronounced inhibitory effect, expressed as a 2–3-fold decrease in both the K_{app} (for actin) and the V_{max} .

Turbidity measurements

There was no evidence that ATP_{free} solubilized myosin filaments in the presence of millimolar concentrations of MgATP. Turbidity measurements were made of myosin filaments formed under the conditions of the ATPase assays (i.e. at 40–100 mM ionic strength). At 40 mM ionic strength, addition of ATP (as a $Mg^{2+}/MgATP/ATP_{free}$ equilibrium solution) decreased the turbidity from 0.207 to 0.166 absorbance units at 2 mM-ATP_{free}, and from 0.213 to 0.170 units at 0.067 mM-ATP_{free}. At 100 mM ionic strength, the same ATP solution lowered the turbidity reading from 0.198 to 0.167 units at 2 mM-ATP, and from 0.211 to 0.181 units at 0.067 mM-ATP_{free}. This experiment demonstrated: (1) a 5–15 % variability in turbidity, attributable to myosin; and (2) a 15–20 % solubilizing effect of MgATP, which was insensitive to [ATP_{free}].

ATP_{free} and LC2 are interdependent modifiers

In the presence of TN and TM, LC2-deficient actomyosin retained the Ca^{2+} -sensitive MgATPase activity characteristic of thin filament regulation (i.e. from 0.13 to 1.2 s⁻¹ from 0.01 to 10 μM- Ca^{2+}). This verified the thiol integrity of LC2-deficient myosin preparations [4,20].

In the absence of TN and TM, LC2-deficient actomyosin demonstrated, at best, a 35 % sensitivity of the V_{max} and K_{app} (actin) values to divalent cations, which included the 10–12 % experimental variability described in the previous section.

Since the experiments in Figs. 1–4 demonstrated that sensitivity of MgATPase to divalent cations was due to ATP_{free}, LC2-deficient actomyosin, which was relatively insensitive to divalent cations, should also be insensitive to ATP_{free}. This prediction was verified by an ionic strength profile of P_i liberation as a function of LC2 content and ATP_{free} concentration (Fig. 6). LC2-deficient actomyosin was insensitive to ATP_{free} above 70 mM ionic strength, and was only marginally sensitive to ATP_{free} (35 % change) below 70 mM ionic strength. Therefore sensitivity to ATP_{free} appeared to require near-native levels of LC2 bound to myosin. The reverse was also true: sensitivity of P_i liberation to the LC2 content of myosin was enhanced considerably by high ATP_{free} concentrations. Interestingly, sensitivity to ionic strength was enhanced only by maximal levels of both ATP_{free} and LC2, but was decreased to a similar extent by a decrease in the concentration of either modifier. The inter-relationship between loss of the efficacy of ATP_{free} as modifier, diminution of sensitivity to ionic strength and the effect of LC2 deficiency was completely reversible upon reassociation of LC2 with LC2-deficient myosin (Fig. 6).

The interdependence of LC2 and ATP_{free} as modifiers of P_i liberation demonstrated in Fig. 6 was confirmed in a comparison of K_{app} (actin) and V_{max} for native, LC2-deficient and LC2-reassociated myosin, at different ionic strengths. At low ionic strength (55 mM), a convincing effect of LC2 removal was observed only at high concentrations of ATP_{free}, and this effect was inhibitory [i.e. V_{max} decreased from 8.24 ± 0.32 to 5.00 ± 0.33 s⁻¹ (means $\pm$ S.E.M.; $n = 3$)]. At high ionic strength, the efficacy of the two modifiers was equivalent and non-additive. The MgATPase activity of LC2-deficient actomyosin lost the ability to be inhibited by ATP_{free}. As a result, LC2 deficiency appeared to activate P_i liberation at high ATP_{free} concentrations: the V_{max} was 0.73 and 0.88 s⁻¹ for native and LC2-reassociated actomyosin respectively, and 2.25 s⁻¹ for LC2-deficient acto-

myosin. Furthermore, the magnitude of this apparent activation was similar to the magnitude of the inhibitory effect of ATP_{free} observed with native or LC2-reassociated actomyosin; i.e. from 2.14 ± 0.12 to 0.73 ± 0.075 s⁻¹ or from 1.84 ± 0.14 to 0.88 ± 0.09 s⁻¹ respectively. A similar relationship was observed for the K_{app} (for actin). Therefore it is hypothesized that LC2 and ATP_{free} can be interdependent non-additive modifiers of actomyosin MgATPase activity.

DISCUSSION

The present results demonstrated that the sensitivity to divalent cations of the F-actin MgATPase activity (in the absence of TN and TM) was due to ATP_{free}.

The effect of ATP_{free} was caused by its binding to a site(s) distinct from the hydrolytic site of MgATPase. First, ATP_{free} cannot compete with MgATP for the hydrolytic site [21]. Secondly, ATP_{free} is not considered to be a substrate for either the myosin [21] or the actomyosin ATPase, the latter having almost an obligatory requirement for the divalent cation chelate of nucleotide triphosphates [22]. Thirdly, the efficacy of ATP_{free} was lost upon a decrease in LC2 content (Fig. 6), whereas the hydrolytic site is insensitive to LC2 removal [4,14].

It was unlikely that the effect of ATP_{free} was caused by another ATP chelate. CaATP was excluded by the presence of EGTA. The concentration of K₂ATP was also negligible, even at high ionic strength [23]. Both Mg²⁺ and MgATP were systematically discounted as modifiers by the present study. By these criteria, therefore, our findings were specific to ATP_{free}.

It was not part of the experimental design to prove that ATP_{free} was the only modifier capable of exerting the same range of effects upon actomyosin MgATPase activity. In fact, the study was initiated because of the qualitative similarity between LC2-*P* and ATP_{free} as modifiers of *P*_i liberation [3]. It is reasonable to question whether other polyanions can substitute for ATP_{free} to cause similar effects. Polyelectrolytes have been known to inhibit the actomyosin ATPase by inhibiting actin-myosin association, thus converting the actomyosin ATPase to a myosin ATPase [24,25]. Heparin, polyglutamic acid and even troponin A and troponin B [26] became bound to actin [25] or to actin-associated proteins [26], and inhibition was reversed upon the addition of excess actin [25,27]. The mechanism of inhibition was distinct from that of the effect of ATP_{free} because: (1) at comparable ionic strength, ATP_{free} was an activator (Figs. 2, 5 and 6); and (2) excess actin did not diminish the efficacy of ATP_{free} as a modifier (Figs 1, 3 and 5).

The mechanism of the effect of ATP_{free} involved an alteration in the rate of *P*_i liberation from actomyosin complexes. Both K_{app} (for actin) and V_{max} were affected by ATP_{free} to a similar extent and exhibited a similar ionic strength profile (Figs. 5a and 5b). In Michaelis-Menten kinetics, the V_{max} term is contained within the rate expression for K_m . Therefore, according to one kinetic interpretation, the ability of ATP_{free} to have a parallel effect upon both the K_{app} and the V_{max} suggested that ATP_{free} altered the rate constants controlling V_{max} . This kinetic relationship was responsible for the parallel Lineweaver-Burk plots (versus [actin]) at low and high ATP_{free} concentrations (see insets to Figs. 1 and 3). As expected, K_{app} (for actin) and V_{max} values were low compared with values obtained with HMM [28,29], but similar to values reported by others working with actin and myosin [6,28,30]. For myosin, but not for HMM, estimates of V_{max} and K_{app} varied depending upon the range of actin concentrations in the Lineweaver-Burk plots, which exhibited a change in slope at around 30 μM-actin [28,30,31]. Structural studies suggested that the primary causative agent was the presence of actin sheaths at 30 μM-actin [28,32,33], since the

biphasic actin plot persisted at high ionic strength, at which myosin is soluble [30]. Fortunately, similar LC effects were observed below and above 30 μM-actin [6,30]. In the present study, the absolute values of K_{app} and V_{max} are not critical, but rather the critical factor was the 2-3-fold change in both constants mediated by the concentrations of LC2 and ATP_{free}.

Some might argue that the actomyosin MgATPase is unsuited for kinetic analysis because of several rate and structural characteristics observable not only with myosin filaments, but also with myosin minifilaments [28-32]: (1) insoluble actomyosin complexes form in the absence of MgATP [28]; (2) a reaction rate which is polyphasic with respect to time [28,32] and biphasic with respect to the actin concentration [28-32]; and (3) superprecipitation at low MgATP concentrations [32,33]. Fortunately, conditions have been chosen which made kinetic assays less troublesome. Above 50 mM ionic strength, and below 30 μM-actin, the reaction rate approached linearity [28,32]. Superprecipitation was prevented by maintaining saturating levels of MgATP. Electron micrographs of the actomyosin assay mixture, before the addition of MgATP, indicated that the predominant structure was the traditional arrowhead-like complexes between the myosin head and actin [28]. Separate myosin filaments were scarce [28]. Upon the addition of MgATP, there was a long-lasting clear phase [28,32,33], due to dissociation of the cross-bridge from actin and a substantial amount of non-filamentous myosin [28,33]. ATP_{free} was reported not to affect the clear phase [33]. In planning an analysis of LC2 function, it must be decided whether it is preferable to work with a full complement of LC2 (as with actomyosin) and select conditions where the reaction rate approaches linearity, or to work with soluble proteins (HMM) which have partially degraded heavy chains [34] and which are deficient in LC2 content [11,34]. In the latter case, an effect of ATP_{free} would be unlikely.

The ionic strength altered the 'sign' of the ATP_{free} effect, but not the relative potency nor efficacy of ATP_{free} as a modifier (Figs. 2 and 4). At low ATP_{free} levels, ionic strength was only a weak modifier of the actomyosin MgATPase activity (Figs. 5 and 6). Routinely, actomyosin MgATPase exhibited a 2-3-fold variation in activity with a change in ionic strength of 50 to 100 mM-KCl [7,10,35], compared with the 8-fold effect for the acto-S-1 MgATPase [35] and the 20-fold effect observed herein for actomyosin at high ATP_{free} concentrations. It might be argued that chloride is not a physiological anion. According to a recent study on fibres [36], acetate and propionate have disadvantages also. KCl was chosen to vary ionic strength in order to facilitate comparison with previous studies *in vitro* [28-30].

ATP_{free} has been shown to destabilize myosin filament formation in the absence of MgATP [37], but similar effects of ATP_{free} did not appear to occur in the presence of MgATP (see the Results section). This does not exclude the possibility of more subtle structural changes associated with the binding of ATP_{free}.

Sensitivity of *P*_i liberation to ATP_{free} and to LC2 were interdependent effects (Fig. 6), associated with one of the two molecules of LC2 bound to myosin. These results can best be interpreted, at present, in terms of one potent low-affinity ATP_{free} site per myosin molecule, involving 50% of bound LC2. This low-affinity ATP_{free} site may include a heavy chain component, which obviously would persist following LC2 removal. This would explain the residual sensitivity of LC2-deficient actomyosin to ATP_{free} under the optimal binding conditions of low ionic strength (Fig. 6).

This study agrees with reports of no effect on MgATPase activity of LC2 removal at low ATP_{free} concentrations [38,39], and activation at high ionic strength with intermediate concentrations of ATP_{free} [8,10]. However, there were two reports, at low ATP_{free} concentrations, of inhibitory effects following LC2

removal [5,7]. In the fibre study [5] the cofactor was regulated by actin (see below), and in the other report [7] the enzyme was LC2-depleted myosin (0 mol of LC2/mol) produced by protease treatment.

The present study indicates the following: (1) the divalent cation sensitivity of F-actin MgATPase activity is consistent with thin filament regulation of skeletal muscle contraction; (2) ionic strength can change the sign (i.e. stimulation or inhibition) of effects on activity mediated by LC2 and LC2-*P* (see the Introduction section); and (3) LC2 can be an indirect modifier which fine-tunes P_i liberation. This initial study was undertaken with F-actin because it is a non-cooperative system. As such, inactive actomyosin complexes decrease the effective free actin concentration, thereby decreasing total P_i liberation. In contrast, with regulated actin, a co-operative system, inactive actomyosin complexes can potentiate the rate of P_i liberation from the active actomyosin complexes [20]. The latter system would have complicated measurements of ATP_{free} potency.

The physiological significance of the present study is speculative. In the intact muscle, it is unlikely that ATP_{free} levels approach 1 mM [17,18,40] or that they would vary by more than 100 μ M. However, the effect of ATP_{free} may increase in the presence of regulated actin. To cite supportive evidence, below 0.1 mM-ATP_{free}, both the regulated actomyosin MgATPase activity [4] and the myofibrillar MgATPase activity [41] demonstrated an apparent Ca^{2+} -sensitivity which was associated with myosin, and which produced inhibition [4] or activation [41]. The relationship in the two reports between the stimulatory or inhibitory nature of the divalent cation effect and the ionic strength was the same as that described in this study (Figs. 1 and 3) and determined to be due to ATP_{free} (Figs. 2 and 4). This can be explained by a decline in the concentration of LC2-ATP_{free} complexes at high $[Ca^{2+}]$, thereby reversing the effect of ATP_{free} and increasing the efficacy of thin filament regulation upon P_i liberation (termed potentiation). Alternatively, the [LC2-ATP_{free}] may play a role at low-to-intermediate Ca^{2+} levels by affecting the kinetics of LC2 phosphorylation, possibly to favour maintenance of the relaxed state. The latter hypothesis is favoured because it does not require ATP_{free} levels to change with the contractile state. In conclusion, a single modifier, such as LC2, can have different effects on MgATPase activity under different conditions. The present study provided an experimental perspective from which to begin to analyse the effect of thin filament co-operativity upon the potency and exclusivity of LC2-ATP_{free} and LC2-*P* as modifiers of P_i liberation.

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