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THE ACTIVATION BY Ca^{2+} OF THE ATPase OF EXTRACTED MUSCLE FIBRILS WITH VARIATION OF IONIC STRENGTH, pH AND CONCENTRATION OF MgATP

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SUMMARY

1. The alteration of the Ca^{2+} requirements of the ATPase activity of fibrils from rabbits and crabs at varying ionic strength, pH and concentration of MgATP (*i.e.* $MgATP^{2-} + MgHATP^{-}$) was investigated.

2. Under physiological conditions, it was found that the ATPase activity of rabbit and crab fibrils after an initial increase decreased steeply when the Ca^{2+} concentration is raised above $1 \cdot 10^{-4}$ M. This is a primary effect of the over-optimal Ca^{2+} concentration and not a secondary one caused by the influence of accompanying ions.

3. The Ca^{2+} requirements for ATP splitting by rabbit fibrils remain constant at an ionic strength from 0.1 to 0.2 and for a MgATP concentration in the range from 0.5 to 10 mM. At $I = 0.05$ it is about 5 times smaller than at 0.1. When the pH is decreased from 8 to 7, the Ca^{2+} requirements are increased some 10 times but only 3 times when the pH is varied between 7 and 6.

4. In crab fibrils, there is no alteration of the Ca^{2+} requirements when the ionic strength is varied between 0.05 and 0.2, but a reduction of the pH from 8.0 to 6.0 raises the Ca^{2+} requirements for half activation and for threshold by a factor of 10. Changing the MgATP concentration increases the Ca^{2+} requirements only in the range from 1 to 5 mM, while the concentration required in 0.5 mM is identical with that at 1 mM, and 10 mM corresponds to 5 mM.

5. It can be deduced from the experimental results that at a pH above 6.0 maximal activation is always obtained if the Ca^{2+} concentration is $5 \cdot 10^{-5}$ M. By contrast, relaxation is only achieved when the Ca^{2+} concentration is below $1 \cdot 10^{-7}$ M for pH 7.0 and $I > 0.1$ or below $1 \cdot 10^{-8}$ for pH > 7.0 or $I < 0.1$.

6. To achieve complete relaxation, an ethyleneglycoldiaminotetraacetate (EGTA) concentration of 1 mM is sufficient, even when there is a large degree of contamination by Ca^{2+} as long as the pH stays above 6.5.

INTRODUCTION

The extent of dissociation of the actomyosin[1,2] in relaxed fibrils can be calculated from a comparison of the ATPase activities of contracted and relaxed fibrils and of

Abbrevation: EGTA, ethyleneglycoldiaminotetraacetate.
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purified myosin. For this calculation to be valid, the activities of the fully contracted and fully relaxed fibrils must be used.

The conditions necessary for Ca^{2+} to fully or partially activate living muscle[3,4] and extracted fibrils [5–7] under approximately physiological conditions have been extensively studied. At marked deviations from physiological conditions, however, little is known about activation by Ca^{2+}. This paper reports a study of Ca^{2+} activations under varying conditions of ionic strength, pH and MgATP concentrations. A knowledge of such factors is important, since the dependence of fibril activation upon Ca^{2+} concentration even under physiological conditions has proved to be much more complicated than was previously thought.

The ATPase activity is taken as a measure of the fibril activation. The fibrils used are from either rabbit or crab *Maia squinado*, the latter being chosen since Ca^{2+} activities have been studied in this species in the living muscle.

RESULTS

The dependence of the ATPase activity upon the Ca^{2+} concentration under standard conditions

Under approximately physiological conditions (I = 0.1, pH around 7, concentration of MgATP about 2 mM), the dependence of the activation of the fibrillar ATPase activity was investigated. At a given concentration of added Mg^{2+} and ATP, the addition of Ca^{2+} (Table I and Fig. 1, filled symbols) in form of $CaCl_2$ leads to decreasing amounts of the MgATP complex (*i.e.* $MgATP^{2-}$ + $MgAHTP^{-}$) due to the competition between Ca^{2+} and Mg^{2+} for ATP and to increasing concentrations Mg^{2+} and of the CaATP complex (*i.e.* $CaATP^{2-}$ + $CaHATP^{-}$) with an increase in the concentration of Ca^{2+} (Table I).

This difficulty of the variation of concentrations can be overcome if instead of adding $CaCl_2$, the Ca^{2+} is added as CaATP. When this is done, as is seen in Table I, the concentrations of ATP ions, MgATP and Mg^{2+} remain constant (for calculation see MATERIALS, METHODS AND CALCULATIONS). The concentration of the CaATP complex is equal to or greater than that of Ca^{2+}.

TABLE I

THE CONCENTRATION OF Ca^{2+}, CaATP, MgATP AND OF FREE ATP OF THE EXPERIMENTS SHOWN IN FIG. 1

Molar concn. of Ca^{2+} adjusted by		*Resulting conc. (mM) of*				
Addition of	*For experiment*	*Ca^{2+}*	*CaATP*	*MgATP*	*Mg^{2+}*	*Free ATP*
$CaCl_2$	▲	1.40–8.7	0.01–1.35	1.52–0.62	0.46–1.4	0.45–0.05
	●, ■	0.58–8.7	0.05–1.35	1.52–0.62	0.45–1.4	0.4 –0.06
Calcium-CaEGTA	△	approx. 0–0.03	approx. 0–0.01	approx. 1.5	approx. 0.42	approx. 0.42
	○	approx. 0–0.2	approx. 0–0.2			
CaATP	□	0.1–4.0	0.13–5.0			

Fig. 1 shows the plot of the effective concentration of Ca^{2+} against the ATP splitting activity in crab and rabbit fibrils (filled symbols, Ca^{2+} increased by addition of $CaCl_2$; unfilled symbols, Ca^{2+} increased by adding CaATP). It is seen that on the addition of both $CaCl_2$ and CaATP the activation of the ATPase activity falls off

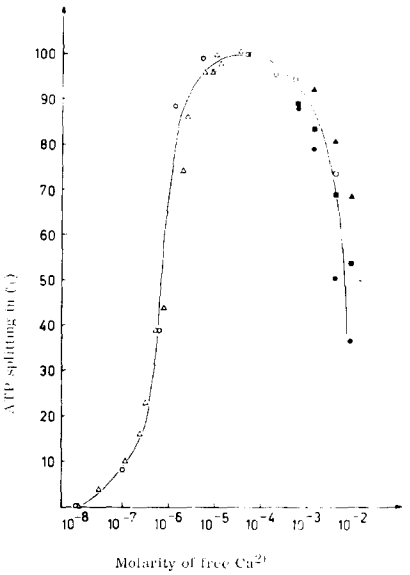


Fig. 1. The dependence of fibril ATPase activities on the Ca^{2+} concentration. Ordinate splitting rate relative to the activity in the presence of $5 \cdot 10^{-5}$ M Ca^{2+}. For calculation of the percentage splitting of ATP, the value at $1 \cdot 10^{-9}$ M Ca^{2+} was taken to be zero. ○, □, ● and, ■ rabbit; △ and ▲, crab; for further meaning of the symbols see Table I. Adjustment of Ca^{2+} concentration: $< 5 \cdot 10^{-5}$ M with 2 or 2.5 mM calcium-EGTA + potassium-EGTA (○, △); $> 5 \cdot 10^{-5}$ M with $CaCl_2$ (▲, ●, ■) or with a Ca^{2+}–ATP mixture (□). All assay solutions contain 10 or 15 mM Tris–maleinate or 20–40 mM imidazole and KCl. *I* approx. 0.1, pH approx. 7.0; concentration of fibrils, 0.1–0.3 mg protein per ml; temp. 20°.

steeply as soon as the concentration of Ca^{2+} surpasses $1 \cdot 10^{-4}$ M. Since the change in the accompanying ions is completely different for $CaCl_2$ and CaATP (Table I), the effect of over-optimal Ca^{2+} concentrations is due to a primary effect upon the activation and not secondary to an induced change of the concentration of accompanying ions. The effect of over-optimal Ca^{2+} concentrations differs somewhat in extent in different preparations (*cf.* Fig. 1 ○ ● *vs.* □ ■) but is present in both rabbit and crab fibrils (Fig. 1 ● ■ and ▲).

The dependence of the ATPase activity upon the Ca^{2+} concentration during variation of ionic strength, pH and MgATP concentration

Fig. 2a and more markedly Fig. 2b demonstrate an increase in the Ca^{2+} requirements of about 5 times for threshold, half-activation and full activation in rabbit fibrils when the ionic strength is increased from 0.05 to 0.10. A further increase to 0.20 does not further influence the Ca^{2+} requirements of the ATP splitting (*cf.* Curves 2 to 4 in Fig. 2a and the corresponding symbols of curve 2 in Fig. 2b)*. By contrast, the Ca^{2+}

*This finding agrees well with that of WEBER *et al.*[8] who were unable to detect a significant difference in Ca^{2+} requirements (pH 6.6) after changing the ionic strength from 0.09 to 0.15.

requirements of crab fibrils are not at all influenced by the ionic strength, even between 0.05 and 0.1 (see Fig. 1 in ref. 6). However, the absolute magnitude of the ATPase activation decreases with increasing ionic strength (see Fig. 2a for rabbit and Fig. 1 for crab in ref. 6).

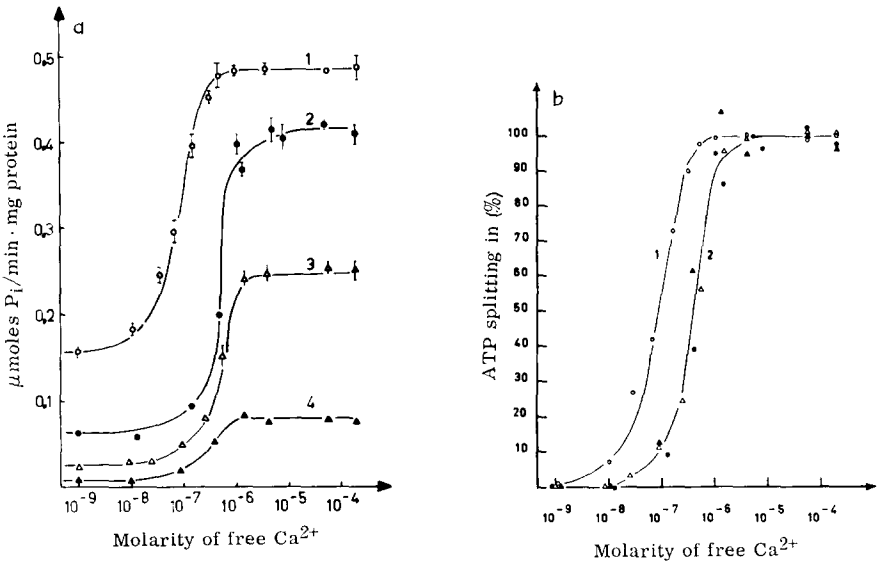


Fig. 2. Influence of ionic strength on the Ca^{2+} requirements of the ATPase activity of rabbit fibrils. Ordinate of b: as in Fig. 1. The symbols apply to both figures: ○—○, I = 0.05; ●—●, I = 0.10; △—△, I = 0.15; ▲—▲, I = 0.20. All media contained 2 mM ATP, 2 mM $MgCl_2$, 2 mM calcium-EGTA + potassium-EGTA or 0.1–0.4 mM $CaCl_2$, 10 mM Tris–maleinate and KCl; temp., 20°; pH approx. 7.0. The vertical bars in a represent S.E.; where they are not drawn their size equals that of the symbol.

The increase in pH from 6.0 to 8.0 raises not only the absolute magnitude of the ATPase activation in rabbit fibrils (*cf.* Figs. 3a and 3 of the preceeding paper[2]) but also lowers the Ca^{2+} concentration necessary for this activation (Fig. 3b). This lowering of the Ca^{2+} concentration appears to be least in the pH range from 6.5 to 7.0*. On the other hand, changes of pH between 6.0 and 8.0 have little influence upon the ATPase activity of crab fibrils. Fig. 4a shows only a slight diminution of the absolute ATPase activity between 6.0 and 8.0 (*cf.* Fig. 3 of the preceeding paper[2]). More strongly effected is the Ca^{2+} concentration necessary for half-activation for which the Ca^{2+} requirements decrease by one tenth when the pH shifted from 6.0 to 8.0 (Fig. 4b). This decrease in the Ca^{2+} requirements appears to be smaller for the pH range of 6.5 – 7.0 than for the range 6.0 – 6.5 or 7.0 – 8.0 .This is similar to rabbit fibrils.

A variation of the MgATP concentration does not always alter the absolute activity. In rabit fibrils it is at a maximum and practically constant for MgATP concentrations between 1 and 5 mM. When the MgATP concentration is reduced to

* Likewise, WEBER *et al.*[8] do not find a clear-cut shift of the Ca^{2+}requirements when the pH is increased from 6.0 to 7.0.

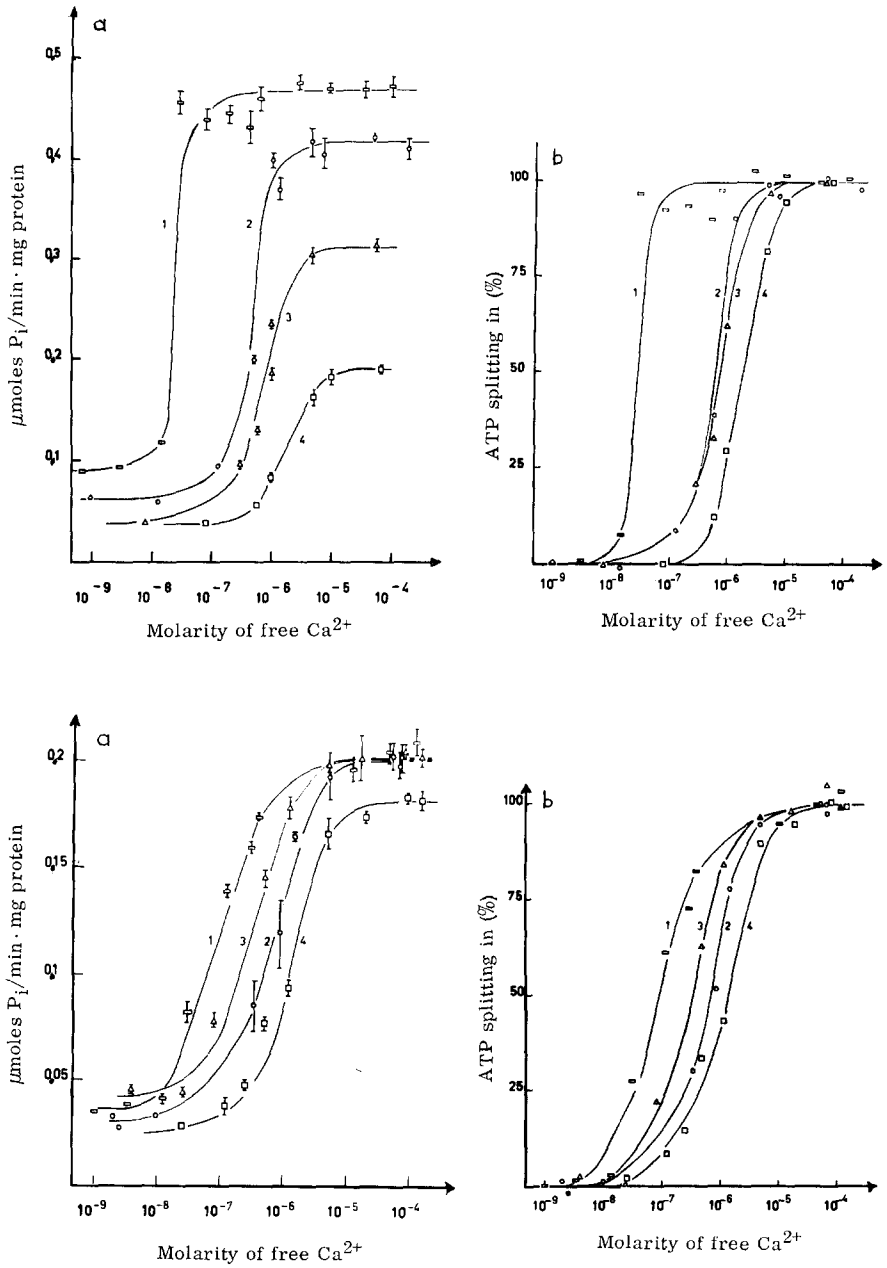
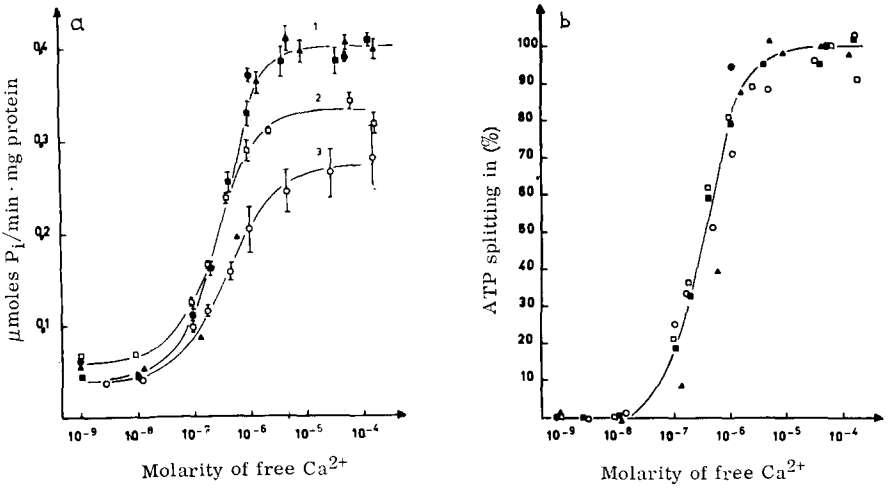


Fig. 3 and 4. The Ca^{2+} requirement for ATP splitting by extracted rabbit (Fig. 3) and crab (Fig. 4) fibrils at different pH. Ordinate of 3b and 4b: as in Fig. 1. The symbols represent identical pH values in all figures: Curves 1, pH 8.0; O—O, pH 7.0; △—△ pH 6.5; □—□, pH 6.0. Adjustment of Ca^{2+} concentrations: at pH 8.0 with 3 mM calcium-EGTA + potassium-EGTA or 0.0025–0.26 mM $CaCl_2$, at pH 7.0 with 2 mM calcium-EGTA + potassium-EGTA or 0.1–0.4 mM $CaCl_2$, at pH 6.5 for rabbit fibrils as at pH 7.0, for crab fibrils with 10 mM calcium-EGTA + potassium-EGTA or 0.1 mM $CaCl_2$, at pH 6.0 for rabbit fibrils as at pH 7.0 for crab fibrils with 15 or 10 mM potassium-EGTA or 0.1 mM $CaCl_2$. All media contained 2 mM ATP, 2 mM $MgCl_2$, 15 mM Tris-maleinate and KCl. *I* approx. 0.1; temp., 20°. Vertical bars as in fig. 2a.



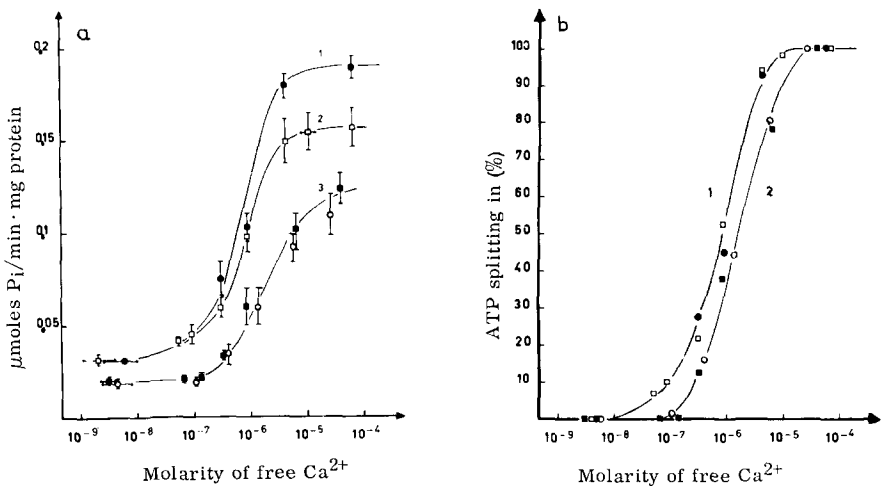


Fig. 5 and 6. Ca^{2+} requirements for ATP splitting by extracted rabbit (fig. 5) and crab (fig. 6) fibrils at different concentrations of MgATP. Ordinate of 5b and 6b: as in Fig. 1. Meaning of symbols:

□—□, 0.31 mM MgATP, 0.19 mM Mg^{2+}, 0.19 mM ATP;
●—●, 0.71 mM MgATP, 0.29 mM Mg^{2+}, 0.29 mM ATP;
△—△, 1.5 mM MgATP, 0.5 mM Mg^{2+}, 0.5 mM ATP;
■—■, 4.2 mM MgATP, 0.8 mM Mg^{2+}, 0.8 mM ATP;
○—○, 9.0 mM MgATP, 1.0 mM Mg^{2+}, 1.0 mM ATP; All media contained 10 mM Tris–maleinate (rabbit) or 20 mM imidazole (crab), 2 mM calcium-EGTA + potassium-EGTA or 0.1–0.5 mM $CaCl_2$, and KCl at I = 0.1, pH approx. 7.0; temp., 20°. Vertical bars as in Fig. 2a.

0.31 mM* the activation of the ATPase activity is significantly lower and at 8.9 mM it falls to about two thirds of the maximal activation (Fig. 5a). In spite of this, the Ca^{2+} requirements for activation are not observably affected by MgATP concentration over the whole range from 0.3 to 8.9 mM (Fig. 5b).

Crab fibrils show a somewhat different behavior regarding changes in MgATP concentration from that of rabbit fibrils. They show at 5 mM MgATP a decrease in the absolute magnitude of the ATPase activity (Fig. 6a, and in ref. 2, Fig. 4)**, and the Ca^{2+} requirements at 4.3 mM and at 9 mM MgATP are higher than at 0.3 and 0.7 mM (*cf.* Fig. 6b with Fig. 5b).

Conditions for relaxation and contraction

In any investigation of muscular contraction, it is often necessary to compare contractile systems under conditions of relaxation and contraction. It is often assumed that the contractile system is fully activated when the concentration of Ca^{2+} is about $1 \cdot 10^{-5}$ M. However, this value applies only at pH 7 and above; in the range of pH 7–6, the maximally activating concentration of Ca^{2+} increases to $3 \cdot 10^{-5}$ M (*cf.* Figs. 3 and 4). Again, Ca^{2+} concentrations above $1 \cdot 10^{-4}$ M actually reduce the activation (*cf.* Fig. 1); so in order to produce maximum activation, it is necessary to have a Ca^{2+} concentration of about $5 \cdot 10^{-5}$ M and a pH greater than 6.

The threshold concentration for Ca^{2+} is generally assumed to lie at $1 \cdot 10^{-7}$ M or below. However, this is only true in rabbit fibrils when the pH is 6 and in crab fibrils when the concentration of MgATP is greater than 5 mM. In all other conditions, the threshold concentration for Ca^{2+} is between $1 \cdot 10^{-8}$ and $1 \cdot 10^{-9}$ M for both rabbit and crab fibrils.

In order to produce such low Ca^{2+} concentrations for conditions of relaxation, it is usual to add pure ethyleneglycoldiaminotetraacetate (EGTA) (without Ca^{2+}) to the experimental solutions. The resulting Ca^{2+} concentration will depend on the amount of Ca^{2+} contaminating the ATP and the muscle preparation and on the concentration of EGTA. Under the usual experimental conditions, the accidentally introduced Ca^{2+} may reach a value of up to $2 \cdot 10^{-5}$ M (*cf.* MATERIALS, METHODS AND CALCULATIONS). Thus the concentration of EGTA should always be greater than 1 mM to ensure a final Ca^{2+} concentration of less than $1 \cdot 10^{-8}$ M***.

* This suboptimal splitting rate with 0.31 mM MgATP exists even in the presence of a creatine phosphate–phosphocreatinekinase system having at least twice the activity of that of the fibril ATPase. This means that the reduced ATPase rate is not due to a lower MgATP concentration within the 2–3-μ-thick fibrils compared with outside. The suboptimal activity is probably caused by the ratio of free ATP and Mg^{2+} to the MgATP complex. In these experiments, the 0.31 mM MgATP solution contained 0.19 mM free ATP and 0.19 mM Mg^{2+}. When the MgATP concentration was increased by 2.3 times to 0.7 mM, the concentrations of free ions only increased by 1.6 times to 0.3 mM. It is known that free ATP ions and Mg^{2+} in a concentration greater than MgATP inhibit the ATPase activity[9,10,2]. The present experiments apparently demonstrate such an inhibition although the concentration of the ions relative to the concentration of the complex ion is not excessive but is simply high.

** In the experiments of fig. 6a, the elevation of the MgATP concentration above 5 mM does not decrease the ATPase activity further; it did so, however, in earlier experiments[2]. Since this decrease is due to the dissociation of the actin and myosin filaments, it is possible that the different behavior between the two suspensions of fibrils is related to the structure of the fibrils which might hinder dissociation.

*** This is only true for pH 7 and for a MgATP concentration of 1–2 mM. When the pH is less than 6.8, the concentration of EGTA must be increased, for at this pH less Ca^{2+} is bound. It must also be increased when the MgATP concentration is much greater than 2 mM since more Ca^{2+} is introduced by the MgATP as a contaminant.

MATERIALS, METHODS AND CALCULATIONS

The fibril suspension from rabbit and crab (*Maia squinado*) leg muscles were prepared according to PORTZEHL *et al.*[2]. Because of the variable ATPase activities and Ca^{2+} requirements* of different fibril preparations, it was found necessary to determine the Ca^{2+} requirements of one variable using one suspension. Usually the optimal ATPase activity of the fibril preparations changed with time; hence, the dependence of the ATP splitting upon the Ca^{2+} concentration with variable ionic strength, pH or MgATP concentration was measured on the same day.

The determination of the ATPase rate was carried out in a medium containing imidazole or Tris–maleinate, as buffers, MgATP, ATP, KCl, and $CaCl_2$, calcium-EGTA, or potassium-EGTA as required. The exact composition of the medium is given in the legends attached to the figures. The ATPase splitting was measured as liberated P_i (*cf.* ref. 2). In the experiments with creatine phosphate and creatine phosphokinase, the splitting activity was measured as liberated creatine[12]. This is possible since the creatine phosphokinase activity is much greater than the optimal ATPase activity of the fibrils.

Adjustment and calculation of the Ca^{2+} concentration

In the experiments with rabbit fibrils, Ca^{2+} concentrations below $1 \cdot 10^{-5}$ M were obtained by using 2 mM calcium-EGTA and at pH 8 by using 3 mM calcium-EGTA. All greater concentrations of Ca^{2+} were obtained by the appropriate addition of $CaCl_2$. In the crab experiments, a similar method was employed except at pH 6.5 and 6.0 where 10–15 mM EGTA was used. This higher buffer capacity was necessary to compensate for the high (approx. $2 \cdot 10^{-5}$ M) Ca^{2+} content of the crab fibril preparations (see below).

The Ca^{2+} concentration in the experiments with calcium-EGTA was calculated according to PORTZEHL *et al.*[4]. In the experiment with $CaCl_2$, the necessary amounts of $CaCl_2$, $MgCl_2$ and ATP required to give the desired concentrations of Ca^{2+}, Mg^{2+} and MgATP were calculated according to GRAENICHER-FRICK[13] using the stability constants of MgATP and CaATP given by NANNINGA[14]. In each experiment, the amount of Ca^{2+} contamination was allowed for. This was done by taking a Ca^{2+} content of ATP of 0.2 % (ref. 15), $MgCl_2$ of 0.01 %**, KCl, Tris–maleinate and imidazole of 0.001 %**, rabbit fibrils*** of 4.6 μmoles/g protein[16, 17], and crab fibrils*** of 35 μmoles/g protein.

Under standard conditions (*i.e.* 2 mM ATP, 2 mM $MgCl_2$, 10 mM Tris–maleinate, 58 mM KCl, 0.4 mg protein per ml) the Ca^{2+} contamination of rabbit fibrils amounts to about $8 \cdot 10^{-6}$ M. This value was experimentally verified by measuring the ATPase activity of two fibril suspensions under standard conditions in the presence of 0.01 mM EGTA. From the determined rate values and their dependence on the Ca^{2+} concentration, it can be calculated that the concentration of the contaminating Ca^{2+} must be $8 \cdot 10^{-6}$ M.

* Although the experiments were carried out over a 2-year period, the variation in Ca^{2+} requirements was not nearly as great as those found by WEBER *et al.*[11].

** As indicated by warranty level on the analytical grade preparations of Merck. For Tris–maleinate and imidazole for which no information was available the same Ca^{2+} content as for KCl was assumed. This assumption seems justified by experiments with different concentrations of EGTA.

*** The fibrils were washed in KCl only, never with EDTA.

Under the same conditions, the crab fibrils assay solutions contain about $2 \cdot 10^{-5}$ M Ca^{2+} * corresponding to the high Ca^{2+} content of the washed fibrils. This was obtained from experiments at pH 6 in which the dependence of the ATPase activity upon the Ca^{2+} concentration was determined in the presence of different EGTA concentrations. From the splitting rates in the presence of 2 mM calcium buffer together with the dependence of the splitting upon the Ca^{2+} concentration, in the presence of appropriate concentrations of CaEGTA, it can be deduced that the effective Ca^{2+} concentration must have been about $2 \cdot 10^{-5}$ M. Yet the adjustment of the Ca^{2+} concentration by 2 mM potassium-EGTA will not be affected by this high Ca^{2+} contamination as long as the pH stays above 6.5.

The concentration of MgATP was calculated according the PORTZEHL *et al.*[2], for all experiments except those on which Curves 1, 3 and 4 of Fig. 2a are based.

Protein concentrations were determined by a semimicro-Kjeldahl method, and the pH was measured electrometrically using a compensating meter (Metrohm, Herisau, Switzerland) and a combined glass electrode.

Chemicals

ATP was bought as the disodium salt from P–L. Biochemicals (Milwaukee, Wisc.). EGTA was a gift from Geigy (Basel, Switzerland) but is no longer manufactured, and all other reagents are analytical grade preparations from MERCK (Darmstadt, West-Germany).

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* In an earlier paper[6] a Ca^{2+} contamination of only $5 \cdot 10^{-6}$ M was given. This estimation was necessary in accurate because two of the rate values used (at $I = 0.20$ and $I = 0.15$) cannot be related only to a Ca^{2+} concentration of $4 \cdot 10^{-6}$ M, as was assumed at the time, but must also be related to one of $1 \cdot 10^{-5}$ M, since the rate values were not significantly smaller than the optimal splitting rate.