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CALCIUM BINDING TO RABBIT SKELETAL MYOSIN UNDER PHYSIOLOGICAL CONDITIONS

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SUMMARY

At a free Mg^{2+} concentration of 1.0 mM, myosin binds one Ca^{2+} per molecule when the Ca^{2+} concentration is 20 μM, a value in the concentration range expected during contraction of skeletal muscle. Mg^{2+} alters Ca^{2+} binding in a complex manner, not by simple competition. In the range from 20 to 100 μM Mg^{2+} it produces positive cooperativity between the high-affinity Ca^{2+} binding sites, in addition to shifting binding to higher Ca^{2+} concentrations. High-affinity Ca^{2+} binding is not significantly affected by the addition of ATP, increase in ionic strength to 0.1 and changes in temperature. Ca^{2+} binding did not increase actin-activated ATPase activity in the absence of regulatory proteins, but rather inhibited it.

INTRODUCTION

It is generally accepted that vertebrate skeletal muscle contracts when the thin filaments of the myofibril make the transition from the off- to the on-state [1, 2]. This transition is caused by binding of Ca^{2+} to troponin [2, 3]. It is not known whether myosin filaments must also be switched on for contraction. Interest in this question was greatly heightened several years ago when Haselgrove [4] and Huxley [5] reported a reduction in the intensity of the 143 meridional reflections (considered indicative of the position of the bridges; cf. ref. 5) after stimulation of fibers that had been stretched beyond overlap. Unfortunately, an unambiguous interpretation of this observation could not be made for several reasons, discussed in detail by Huxley [5]. However, shortly thereafter, on–off regulation of mollusk myosin was demonstrated in vitro by Kendrick-Jones et al. [6], who demonstrated furthermore that the switching mechanism was triggered by Ca^{2+}. Although countless experiments have shown that isolated rabbit myosin in vitro is not switched off in the absence of Ca^{2+} since it

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

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interacts freely with pure actin under these conditions (for a summary see refs 3 and 7), one may wonder whether myosin behaves differently in vivo. As a first step in exploring this possibility we investigated, several years ago, whether myosin in vitro is at least capable of binding Ca^{2+} in the presence of Mg^{2+} at Ca^{2+} concentrations which may occur in the cytoplasm during contraction. A summary of the data was published as an abstract [8] and a more detailed description of the data is found in the Ph.D. thesis of Bremel [9].

Provided that the concentration of free ionized Mg^{2+} in the cytoplasm is between 0.1 to 1.0 mM, we observed that myosin released and bound significant amounts of Ca^{2+} when the concentration of free Ca^{2+} was varied between 0.1 and 20 to 30 μM. These myosin preparations, however, when hydrolyzing ATP with pure actin (no regulatory proteins) as a cofactor were not activated by Ca^{2+}; on the contrary, they were somewhat inhibited.

METHODS AND MATERIALS

Myosin

Myosin was prepared from minced back and leg muscles, extracted briefly with KCl–phosphate buffer (0.3 M KCl, 0.2 M phosphate buffer, pH 6.5 [10]). After precipitation of myosin by 15-fold dilution with water, the muscle residue was separated by filtration through cheese cloth, and the myosin was allowed to settle over several hours [11]. After decantation of the supernatant, the precipitate was collected in a continuous flow attachment. It was redissolved in 0.6 M KCl, and actomyosin removed by precipitation at 0.28 ionic strength [12]. If necessary this step was repeated once. Myosin was further purified by one or two more precipitations at 0.05 ionic strength.

Actin

Actin was extracted from acetone powder with 0.5 mM ATP, 0.2 mM $CaCl_2$, 5.0 mM Tris buffer, pH 8.5 (0 °C) [13], polymerized, and treated with 0.8 M KCl, pH 8.5, to remove the regulatory proteins from the filaments [14]. It was depolymerized by dialysis against the extraction solution and stored in the refrigerator for use over 2–3 weeks [13].

Enzymatic assays

Actomyosin was prepared from actin and myosin by mixing 2.3 mg myosin with 1 mg actin in 0.6 M KCl. ATPase activity was measured in a medium of 0.052 ionic strength, 20.0 mM imidazole, pH 7.0, 4.0 mM total ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA); 0.15 mg/ml actomyosin, 5 min incubation with 1.0 mM MgATP at 25 °C. Inorganic phosphate was determined according to Taussky and Schorr [15].

Ca^{2+} determination

All solutions used for Ca^{2+} binding were stored in acid-washed labware. All solutions were analyzed by atomic absorption in a Perkin–Elmer spectrophotometer, model 403, using internal standards. Ca^{2+} present in myosin was extracted with a solution of 2.0 mM EGTA, 1–5 mM $MgCl_2$ and measured by atomic absorption as above, in the presence of 1 % $CaCl_2$ "ultrapure".

Ca^{2+} binding

Before use myosin was washed four times with ten pellet volumes of a solution containing 40 mM KCl, 10 mM imidazole, pH 7.0, and the appropriate Mg^{2+} concentration to be used in the experiment. For measuring Ca^{2+} binding myosin was equilibrated with ^{45}Ca in a medium containing 40 mM KCl, 10 mM imidazole, pH 7.0, 10.0 mM [^{3}H]glucose [16], Ca^{2+}–EGTA/EGTA in varying concentrations (total EGTA decreased from 90 μM at 0.1 μM free Ca^{2+} to 30 μM at maximal free Ca^{2+}), Mg^{2+}, ATP and temperature as indicated in the legends. pH was measured in each mixture since a shift of one-half of a pH unit alters 10-fold the value for free Ca^{2+} at a given ratio of Ca^{2+}–EGTA/EGTA. Glucose was not bound and did not affect ATPase activity. Myosin was separated from the incubation medium by 15 min centrifugation at 12 000 $\times g$, forming a pellet containing about 50–60 mg/ml protein. Control experiments showed that 98 % of the protein was collected in the pellet in the absence of ATP, and about 85 % in its presence. These values were used for calculation of bound Ca^{2+}. Ca^{2+} was extracted from the weighed pellets with 0.1 M cold Ca^{2+}. To that purpose pellets were thoroughly dispersed in the presence of the cold carrier Ca^{2+}, first by hand and second by vigorous use of the "vortex" shaker. Afterwards myosin was removed from the extract by precipitation with trichloroacetic acid. Only the extracts of the pellets were counted since the Ca^{2+} content of the supernatants was similar to the initial value with a maximal reduction of 10 % at the appropriate Ca^{2+} concentrations. At concentrations of 100 μM Mg^{2+} and free Ca^{2+} above 1.0 μM unbound Ca^{2+} in the pellet constituted 33 to 25 % of the total radioactivity in the pellet. This relatively high fraction of unbound Ca^{2+} is due to the presence of Ca^{2+} buffer. For the calculation of specific activity we took the sum of the contaminating (in solutions and myosin) and the added Ca^{2+}. We calculated free Ca^{2+} using two different values for

$$K = \frac{(\text{total free EGTA}) \times (Ca^{2+})}{(\text{CaEGTA})}$$

at pH 7.0, $1.9 \cdot 10^{-7}$ M (calculated from Schwarzenbach's constants [17]) and $1 \cdot 10^{-6}$ M (calculated for pH 7.0 from Ogawa's measurements at pH 6.8 [18]). In the absence of ATP we calculated free Ca^{2+} according to the expression

$$Ca^{2+}_{free} = \pm \sqrt{K \cdot Ca_u^{2+} + \frac{(EGTA_T + K - Ca_u^{2+})^2}{4}} - \left(\frac{EGTA_T + K - Ca_u^{2+}}{2}\right)$$

(Ca_u^{2+} = total unbound Ca^{2+}, i.e. free Ca^{2+} + CaEGTA; $EGTA_T$ = total EGTA; K = one of the two K just mentioned). We compared binding curves calculated with the two different constants because, at the lower buffer concentrations used, the shape of the two curves is significantly different. However, all important features, such as positive cooperativity and a second class of low-affinity binding sites, appear with both calculations. We used the same constants for 20 and 0 °C since we had measured previously [19] that temperature did not cause a significant change in the apparent dissociation constant at pH 7.0.

In order to measure Ca^{2+} binding in the presence of MgATP and free Mg^{2+} we added 140 μM ATP and 200 μM $MgCl_2$ which gives a final concentration of 100 μM MgATP and 40 μM free ATP, assuming a binding constant of 40 μM for

Mg^{2+} and ATP [20]. Free ionized Ca^{2+} was calculated, assuming a dissociation constant for Ca ATP of 90 μM [20] according to the formula:

$$Ca^{2+}_{free} = \left(\sqrt{K \cdot Ca_u^{2+} + \frac{(EGTA_T + 1.44K - Ca_u^{2+})^2}{4}} - \frac{EGTA_T + 1.44K - Ca_u^{2+}}{2}\right) \times \frac{1}{1.2}$$

(Ca_u^{2+} = total unbound Ca^{2+}, i.e. Ca EGTA+CaATP+free Ca^{2+}; $EGTA_T$ = total EGTA; K = dissociation constant for Ca EGTA, as described above.

$$1.444 = 1 + \frac{\text{free ATP}}{K_{D,\,CaATP}}).$$

This simplified expression is valid only if the concentration of unchelated ATP is not altered significantly with increasing Ca^{2+} concentration. At the highest free Ca^{2+} concentration of about 30 μM the value for unchelated ATP falls from 40 to 33 μM and consequently 1.444 K should become 1.36 K. This is not a large change, which furthermore creates a very small error since 1.44 K is only one term in a larger sum. Therefore, we used this expression over the whole range of Ca^{2+} concentrations. Taking account of the CaATP resulted in a correction for the calculated values for ionized free Ca^{2+} of maximally 15–20 %. One experiment was performed slightly differently (Fig. 3; 1000 μM Mg^{2+}). Before the experiment, myosin was washed twice with a 10-fold volume of a solution containing 1.0 mM $MgCl_2$, 10 mM imidazole, pH 7.0, 40 mM KCl, 20 μM ^{45}Ca and 10 mM [^{3}H]glucose in order to adjust the contaminating Ca^{2+} in the myosin to the final desired specific activity. After the second wash the pellets were diluted to a protein concentration of 12 mg/ml with a solution containing 1.0 mM $MgCl_2$ and 40 mM KCl. To measure binding, Ca^{2+}–glucose mixtures of the same specific activity were added to give the desired concentrations of free Ca^{2+}, glucose (10 mM) and a final protein concentration of 2 mg/ml. No EGTA buffer system was present. The stock salt solutions had been checked by atomic absorption and were found to contain less than 1 μM total Ca^{2+}.

RESULTS

Fig. 1, a Scatchard plot of Ca^{2+} binding in the presence of 100 μM Mg^{2+}, shows considerable heterogeneity: the upward swing and the flat trail suggest positive cooperativity in addition to low-affinity binding. Although the latter may result from negative cooperativity it is just as likely that it represents another independent class of low-affinity binding sites. The existence of low-affinity binding is quite apparent even when minimized by using a dissociation constant for CaEGTA, $K_{pH\ 7.0} = 1 \cdot 10^{-6}$ M [18] that is five times higher than the Schwarzenbach constant [17] we usually employ. (Whereas at very high buffer concentrations an increase in the dissociation constant only shifts the curve (binding versus free Ca^{2+}) along the abscissa, at low buffer concentrations the shape of the curve is altered. Increasing the value for K_D compresses the curve over the abscissa, thereby reducing the apparent low-affinity binding. This difference in shape is illustrated by Fig. 2, the raw data to Fig. 1.)

With increasing concentrations of ionized Mg^{2+} Ca^{2+} binding decreased in a complex manner. It seems that the shape of the binding curve was altered by raising the Mg^{2+} concentration from 3 to 100 μM, the slope becoming steeper (Fig. 3). At

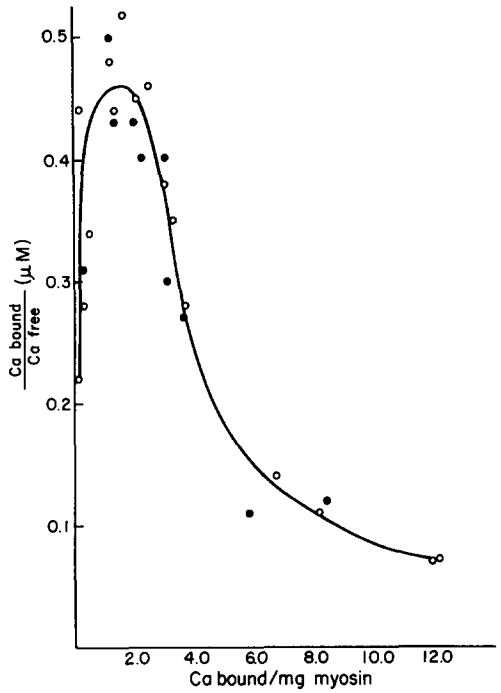

Fig. 1. Scatchard plot of Ca^{2+} binding at 18 °C (open) and 0 °C (closed symbols). Ionized Ca^{2+} was calculated with $K_{D,\,pH7.0} = 1.0 \cdot 10^{-6}$ M for CaEGTA. 100 μM $MgCl_2$, no ATP, pH adjusted to 7.0 for each temperature; myosin No. 2100.

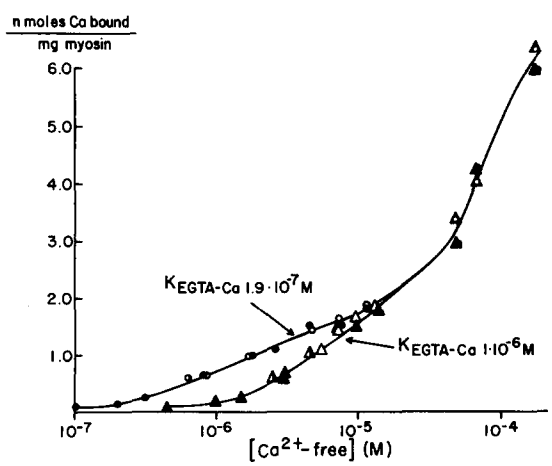

Fig. 2. Comparison of the binding curves calculated with 2 different $K_{D,\,pH7.0}$ for CaEGTA, $1.9 \cdot 10^{-7}$ and $1 \cdot 10^{-6}$ M, respectively. Same data, same symbols as in Fig. 1.

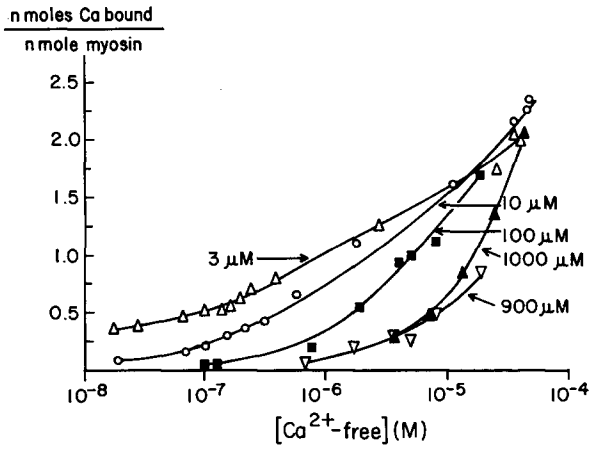

Fig. 3. Change of Ca^{2+} binding with increasing concentrations of free Mg^{2+} (concentrations indicated on figure). No ATP, 18 °C, except for curve with 1000 μM Mg^{2+} at 2 °C. Myosin No. 1,900 (900 μM Mg^{2+}), 2,000 (3–100 μM Mg^{2+}) and 3,500 (1000 μM Mg^{2+}; this measurement was made 3 years later in the absence of a Ca^{2+} buffer system). $K_{D,pH7.0}$ for CaEGTA = $1.9 \cdot 10^{-7}$ M.

20 μM ionized Ca^{2+} there was very little change in binding when the concentration of free Mg^{2+} was raised from 10 to 100 μM; on increasing the Mg^{2+} concentration further, from 100 to 1000 μM, Ca^{2+} binding fell from 2 to 0.7–1.3 moles/mole myosin (Table I, Fig. 3). At 1000 μM Mg^{2+} the Scatchard plot of the data no longer showed positive cooperativity.

The presence of ATP in saturating concentrations (Fig. 4), or a fall in the temperature from 18 to 0 °C (Figs 1 and 2), or raising the ionic strength to 0.09 (data not shown) did not affect binding significantly under conditions of 100 μM Mg^{2+} and free Ca^{2+} concentrations of 0.01 to 20 μM. Sugden and Nihei [21] found that in the absence of added Mg^{2+} total Ca^{2+} binding (Ca^{2+} ion+CaATP) also was not affected by ATP. Variability of Ca^{2+} binding with different myosin preparations can be evaluated by comparing binding at 100 μM Mg^{2+} in Figs 1 and 3 and at 1000 μM Mg^{2+} in Table II and the two sets of data in Fig. 3.

TABLE I

Ca^{2+} BINDING TO MYOSIN IN THE PRESENCE OF INCREASING Mg^{2+} CONCENTRATIONS

Mg^{2+} (μM)	Moles bound Ca^{2+} per mole myosin
10.0	2.4
20.0	2.1
40.0	1.9
100.0	1.8
200.0	1.6
600.0	1.1
1000.0	0.8
5000.0	0.4

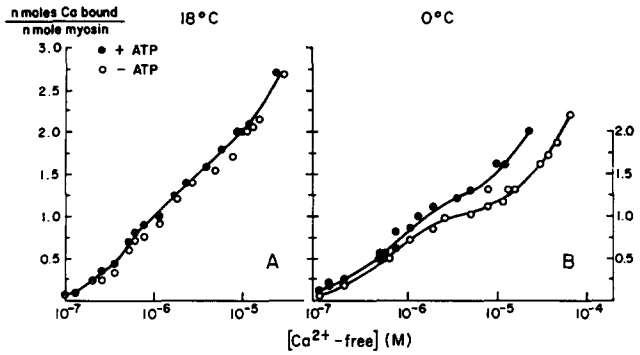

Fig. 4. Effect of ATP on Ca^{2+} binding at 18 and 0 °C. pH adjusted to 7.0 for each temperature. In the absense of ATP 100 μM $MgCl_2$, in its presence 200 μM total $MgCl_2$, 140 μM total ATP. The difference in Fig. 4B is not considered significant because it depends entirely on the assumption that as much myosin remained in the supernatant as had been determined in the control experiments.

In order to learn how Ca^{2+} binding to myosin affects actin–myosin interaction, as measured by actin-activated ATPase activity, we combined myosin with pure actin, free of regulating proteins. Omission of at least troponin is necessary because otherwise any response of myosin would have been obscured by the large effect of Ca^{2+} on troponin. In addition, we kept the Mg^{2+} concentration very low (20 μM) in order to enhance Ca^{2+} effects. Fig. 5 shows that far from promoting interaction with actin, Ca^{2+} caused an inhibition of ATPase activity in the range of concentrations where it is bound.

Inhibition of actin-activated ATPase activity by 0.1 mM Ca^{2+} has always been observed by us and others [22, 23] with pure actin and with tropomyosin–actin [24]. We have even seen it in the presence of troponin, the ATPase activity being about 15 % lower at 0.1 mM than at 8 μM free Ca^{2+} [24]. Whether or not the inhibition by

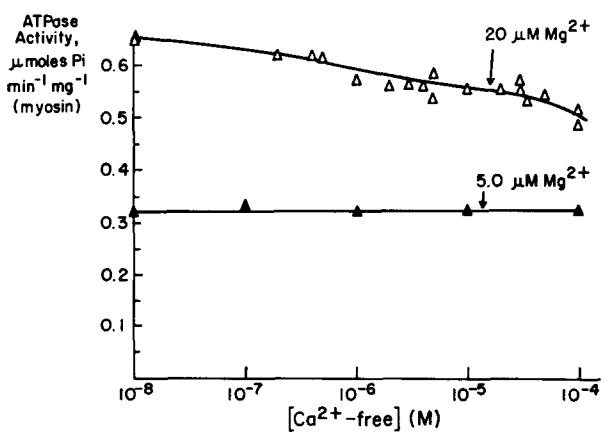

Fig. 5. Effect of Ca^{2+} binding on actin-activated ATPase activity in the absence of regulatory proteins. Upper curve, in the presence of 20 μM, lower curve of 5.0 mM Mg^{2+}. Concentrations of free Ca^{2+} calculated with $K_{D,pH7.0} = 1.9 \cdot 10^{-7}$ M. Temperature, 20 °C.

Ca^{2+} is an artifact, resulting from protein deterioration, or an attribute of native myosin, and whether, in the latter case, it has physiological significance, is not known. Neither do we know whether Ca^{2+} inhibits by the same mechanism as does elevated free Mg^{2+} (Fig. 5).

DISCUSSION

The purpose of the experiments was to determine whether rabbit myosin binds a reasonable number of Ca^{2+}, at least one for each molecule, in the presence of Mg^{2+} concentrations that may occur in the cell.

Ca^{2+} binding is complex and the total number of binding sites is much larger than one for each molecule (cf. ref. 21), even in the presence of 0.1 mM Mg^{2+}. Since the affinity of most of the sites is too low for accurate measurement we did not determine the total number of binding sites. However, it is possible to state that the number exceeds six (Fig. 1). A myosin molecule contains about 1–2 high-affinity binding sites as suggested by Scatchard plots. We have seen variations between one and two bound ions with different myosin preparations and (a reduction) on aging of the same preparation.

At low concentrations of Mg^{2+} (3 μM) Ca^{2+} binding can be measured at concentrations of free Ca^{2+} below 0.1 μM. This concentration range for Ca^{2+} binding agrees well with earlier observations on the concentration range for Ca^{2+}-activated ATPase activity after removal of Mg^{2+} [25]. Although those measurements were made with myofibrils and not with pure myosin, interaction with actin had been eliminated by the removal of Mg^{2+}, with the result that syneresis no longer took place. Under these conditions, Ca^{2+} stimulation of ATPase activity took place in the concentration range 0.1 to 10 μM Ca^{2+}.

Mg^{2+} affects Ca^{2+} binding in a complex manner which cannot be described as a simple competition. While a general decrease in binding without detailed data has been reported previously [21, 27], we find that increasing the Mg^{2+} concentration from 3 to 100 μM enhances the steepness of the Ca^{2+} binding curve. Scatchard plots suggest positive cooperativity of binding at Mg^{2+} concentrations between 20 and 100 μM. A further increase in Mg^{2+} to 1.0 mM and above shifts Ca^{2+} binding to such high Ca^{2+} concentrations that the shape of the curves could no longer be accurately evaluated. However, even with a free Mg^{2+} concentration of 1.0 mM about one Ca^{2+} is bound for each myosin molecule at a concentration of free Ca^{2+} of 20 μM. That is a Ca^{2+} concentration quite likely to be present in muscle during contraction since about that much is required to saturate troponin. Because of the heterogeneity and complexity of binding we do not know, though, whether at 1.0 mM Mg^{2+} Ca^{2+} binding sites are occupied in the same sequence as at 0.1 mM Mg^{2+}. The slight discrepancy between our values at 1.0 mM Mg^{2+} and those of Kendrick-Jones et al. [6] may reflect differences in the myosin preparations as well as differences in the method of measurement. We avoided any difficulties due to Ca^{2+} contaminations in the stock solutions and in the myosin preparations.

We cannot state whether the high-affinity sites are identical with the binding sites on the 5,5′-dithio-*bis*-(2-nitrobenzoic acid) light chains, studied in the absence of Mg^{2+} on the isolated chains [26].

The binding data suggest that the Ca^{2+} content of myosin undergoes significant

variations during the change from relaxation to contraction in muscle, even if the concentration of free Mg^{2+} should be as high as 1.0 mM. Nevertheless, there is no evidence in vitro that Ca^{2+} binding by myosin enhances actin-activated ATPase activity. To the contrary, Ca^{2+} binding inhibits actin–myosin interaction somewhat. However, recent findings with insect myosin suggest caution in viewing these in vitro ATPase experiments. Insect myosin, which is Ca^{2+} regulated in vivo, easily loses this property during isolation from muscle [23]. Consequently one cannot rule out that rabbit myosin may have lost a regulation that it possessed in vivo. If such deterioration of Ca^{2+} regulation should have taken place, it may have been accompanied by changes in Ca^{2+} binding also. Therefore, one should view binding data as approximations until the question of Ca^{2+} regulation has been unambiguously settled.

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