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COOPERATIVE BINDING OF CALCIUM TO GLYCERINATED SKELETAL MUSCLE FIBERS

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SUMMARY

The binding of $^{45}Ca^{2+}$ to glycerinated rabbit psoas fibers was measured by means of a double isotope technique. With 5 mM Mg^{2+} (no ATP) binding was half-maximal at $1.4 \cdot 10^{-6}$ M Ca^{2+} and the maximal amount bound was 1.6 μmol/g protein. At $< 50\%$ saturation, the Scatchard plot had a positive slope and the Hill coefficient was 2.2. At greater than 50% saturation, the Scatchard plot was linear with a negative slope ($K' = 0.8 \cdot 10^6$ M^{-1}) and the Hill coefficient was 1.0. In the absence of Mg^{2+}, binding was half-maximal at $3 \cdot 10^{-7}$ M Ca^{2+} and the maximal amount bound was 2.9 μmol/g protein. The Scatchard plot indicated two classes of sites with K' values of about $2 \cdot 10^7$ and $2 \cdot 10^6$ M^{-1}. The Hill coefficient in the mid-saturation range was approx. 0.6. The data indicate that in the presence of Mg^{2+} binding to about half of the total Ca^{2+} binding sites is suppressed and there is a strong positive cooperativity involving half of the remaining sites.

INTRODUCTION

In the presence of a low intracellular Ca^{2+} concentration ($< 10^{-7}$ M) relaxation of vertebrate striated muscle is maintained through the action of a regulatory protein complex (troponin-tropomyosin) bound to the actin filament [1, 2]. The contractile process is activated by the binding of Ca^{2+} to troponin [3, 4]. Recent studies have revealed interesting cooperative features of this interaction. It has been established that troponin is a complex of three subunits, namely, a Ca^{2+}-binding subunit (Tn-C), an inhibitory subunit (Tn-I) which suppresses actin-myosin interaction, and a component (Tn-T) which binds the complex to tropomyosin [5–7]. The Tn-C subunit binds four Ca^{2+} with an affinity that is partly dependent on interactions with Tn-I and Mg^{2+} [8]. According to Bremel and Weber [9] the affinity of Ca^{2+} for two of the sites is significantly enhanced when cross-bridges (myosin) form attachments ("rigor complexes") with the actin filaments. Hence, it would seem more appropriate to treat Ca^{2+}-troponin affinity as a property of the complete contractile system rather than of isolated Tn-C subunits. From this standpoint, it seems reasonable to suppose that Ca^{2+}-troponin affinity is a dynamic property which is altered as cross-bridges attach and detach during the contraction-relaxation cycle. As discussed

elsewhere [1, 10], such a hypothesis might account for certain mechanical properties of intact muscle which still have no explanation. The present picture of the molecular control mechanism is further complicated by recent suggestions in the literature [11–15] that myosin may also have Ca^{2+}-binding sites with a regulatory function.

To obtain more information about Ca^{2+} binding in a physiological context, we have undertaken a systematic study of the interaction of Ca^{2+} with glycerol-extracted rabbit psoas muscle fibers. In this preparation, the myofilament lattice is presumably in a state very similar to that of living muscle and such mechanical variables as cross-bridge attachment, filament overlap, and filament motion are, in principle, amenable to experimental manipulation. On the other hand with a more complex system there is a greater degree of uncertainty in the location of binding sites. By combining data from both complex and purified preparations, it should be possible to make some reasonable deductions about the localization, binding affinity, and physiological function of Ca^{2+} binding sites in organized contractile systems. In this communication, we report on the effects of Mg^{2+} on the binding of Ca^{2+} to glycerinated psoas fibers in the absence of ATP. Our experimental approach bears some similarity to that described in a recent paper by Marston and Tregear [16].

METHODS AND MATERIALS

Rabbits were killed by intravenous injection of Nembutal and thin strips of psoas muscle were tied to wood sticks at body length. The muscle bundles were extracted for 24 h at 2–4 °C with a solution containing 50 % glycerol, 1 % Triton X-100, and 10 mM imidazole (pH 7.0). The bundles were then split further, transferred to a fresh solution of 50 % glycerol, 10 mM imidazole (pH 7.0), and stored at −20 °C. All measurements were made on fibers which had been extracted for at least 4 weeks. The average sarcomere length of the fibers used in this study, as determined by phase contrast microscopy, was 2.5–2.7 μm.

For the binding measurements thin bundles 2.0–2.5 cm in length, containing no more than six fibers, were separated under a dissecting microscope and transferred to beakers containing 2 ml of buffer solution of the following composition: 100 mM KCl, 20 mM imidazole (pH 7.0), 0.1 mM $^{45}CaCl_2$, 5 mM [^{3}H]glucose, and varying concentrations of ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) to adjust the free Ca^{2+} concentration. The concentration of $MgCl_2$ was either 0 or 5 mM, as indicated in Results. The free Ca^{2+} concentration was calculated according to the equations given by Ogawa et al. [17]. In these calculations we used an apparent Ca-EGTA binding constant (at pH 7.0) of $10^{6.14}$. This value was obtained experimentally by Godt [18] under solvent conditions similar to those used in this study. The total Ca^{2+} concentration included contaminant Ca^{2+}, as determined by atomic absorption spectroscopy.

Fiber bundles were incubated in the buffered Ca^{2+} solutions for approx. 20 min at room temperature. An aliquot of the buffer solution was removed and analyzed for both ^{45}Ca and ^3H with a Beckman LS-100 liquid scintillation counter and external standards. At the end of the incubation period the fiber bundles were transferred to 2 ml of a solution containing 0.19 M Na_2CO_3, 0.1 M NaOH, 4 mM $CuSO_4$, 0.95 mM sodium tartrate and digested by heating at 100 °C [19]. After cooling, an aliquot of the alkaline digest was analyzed for protein [20] and another

aliquot was analyzed for ^{45}Ca and ^{3}H. The [^{3}H]glucose served as a marker for solvent space within the fiber bundles. Bound Ca^{2+} was readily calculated from the $^{45}Ca/^{3}H$ ratio of the fibers relative to that of the buffer solution.

In later studies, the fiber bundle was transferred after incubation to 1 ml of fresh buffer solution containing excess non-radioactive carriers (10 mM glucose, 5 mM $CaCl_2$) to elute ^{3}H and ^{45}Ca. The elution buffer was then analyzed for radioactivity as above and the fiber bundle was digested and analyzed for protein. Both methods gave identical results.

For comparative purposes binding studies were also carried out with washed myofibril preparations. These were prepared from rabbit back muscle essentially as described by Fuchs and Briggs [4], with minor modifications, and stored in 50 % glycerol at −20 °C. Known concentrations of myofibrillar protein (3–4 mg/ml) were incubated in the Mg^{2+}-containing buffer (final volume 2 ml) exactly as described for the glycerinated fiber bundles. The myofibrils were separated by centrifugation and the isotopes were extracted from the pellets with fresh buffer solution containing excess non-radioactive glucose and $CaCl_2$. Bound Ca^{2+} was then determined as described above.

RESULTS

Binding curves obtained for glycerinated fibers in the presence and absence of Mg^{2+} are shown in Fig. 1. In the presence of 5 mM $MgCl_2$, the Ca^{2+} concentration at half saturation ($S_{0.5}$) was $1.4 \cdot 10^{-6}$ M and saturation was reached at a Ca^{2+} concentration of approx. $6 \cdot 10^{-6}$ M. The value for maximum bound Ca^{2+} (N) was arbitrarily taken as the mean of all measurements made at $[Ca^{2+}] > 6 \cdot 10^{-6}$ M. This value was 1.61 μmol Ca^{2+}/g protein (Table I). In the absence of Mg^{2+} the

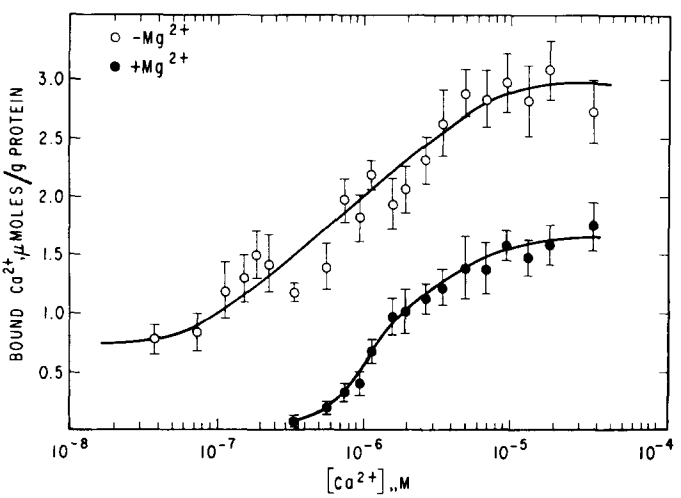

Fig. 1. The binding of Ca^{2+} to glycerinated rabbit psoas muscle fibers in the presence (●–●) and absence (○–○) of 5 mM $MgCl_2$. Each point is mean of 4–16 determinations. Vertical bars indicate standard errors.

TABLE I

CALCIUM-BINDING CHARACTERISTICS OF GLYCERINATED RABBIT PSOAS MUSCLE FIBERS

	$+Mg^{2+}$	$-Mg^{2+}$
N (μmol/g)*	1.61 ± 0.08 (25)	2.89 ± 0.13 (28)
K' (M^{-1})	$0.8 \cdot 10^6$	$2 \cdot 10^7$ $2 \cdot 10^6$
$S_{0.5}$ (M)	$1.4 \cdot 10^{-6}$	$2.9 \cdot 10^{-7}$
R_s	12	> 200
Hill coefficient	2.2 (< 50 % saturation) 1.0 (> 50 % saturation)	≈ 0.6

* Values are given as mean ± S.E. Number of measurements is indicated in parentheses. ($P < 0.001$, Student t-test).

binding curve spanned a much larger range of free Ca^{2+} concentrations. Half-saturation was observed at $[Ca^{2+}]$ of $3 \cdot 10^{-7}$ M and the maximum bound Ca^{2+} was 2.89 μmol/g protein. It will be noted that at 10^{-7} M Ca^{2+} there was no detectable binding in the presence of Mg^{2+} whereas in the absence of Mg^{2+} there was clearly a significant binding at less than 10^{-7} M Ca^{2+}.

The steep slope of the binding curve in the presence of Mg^{2+} is suggestive of a positive interaction between binding sites. For a binding system which consists of a single class of non-interactive sites the ratio of $[Ca^{2+}]$ at 90 % saturation to $[Ca^{2+}]$ at 10 % saturation (R_s factor, ref. 21) should be 81. R_s values of less than 81 are strongly indicative of positive cooperativity whereas R_s values greater than 81 could be consistent with either negative cooperativity or heterogeneous, independent binding

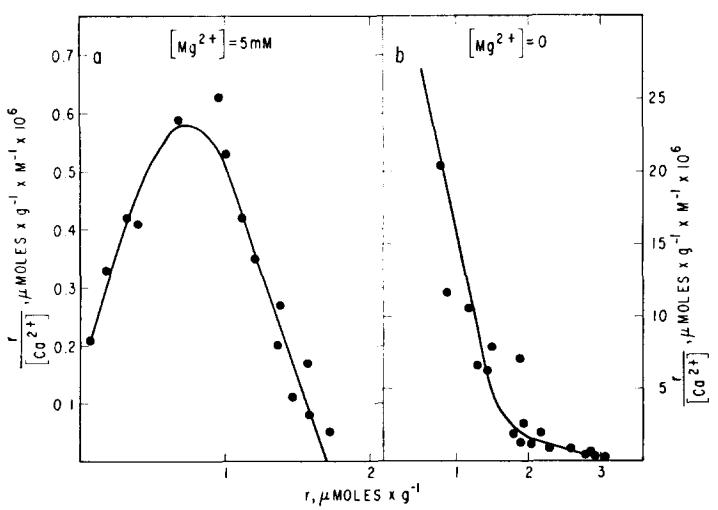

Fig. 2. Scatchard plots of the data of Fig. 1 for the presence (A) and absence (B) of Mg^{2+} (r, bound Ca^{2+}).

sites. As shown in Table I, the R_s was 12 in the presence of Mg^{2+} and over 200 in the absence of Mg^{2+}.

Further information was obtained from Scatchard plots (Figs. 2A and 2B) of the data in Fig. 1. In the presence of Mg^{2+}, the slope was positive until about half of the sites were filled. A positive slope is considered to be a diagnostic test of positive cooperativity [22]. At greater than half-saturation, the plot was negative and linear and intercepted the abcissa at 1.7 μmol Ca^{2+}/g protein, close to the value expected from the binding curve in Fig. 1. The apparent affinity constant (K') estimated from the linear portion of the plot was $0.8 \cdot 10^6$ M^{-1}. For the Mg^{2+}-free condition the Scatchard plot was consistent with the assumption of two classes of binding sites. Apparent affinity constants estimated from the linear portions of the plot were roughly $2 \cdot 10^7$ and $2 \cdot 10^6$ M^{-1} (Table I).

Hill plots of the data are shown in Figs. 3A and 3B and estimated Hill coefficients are listed in Table I. In the presence of Mg^{2+} there is a clear discontinuity at slightly over 50 % saturation. For binding at less than 50 % saturation the Hill coefficient of 2.2 provides clear evidence of a positive interaction involving at least two binding sites. Once these sites are filled further binding occurs by a non-interactive process, as shown by the Hill coefficient of unity. For the Mg^{2+}-free condition the Hill coefficient is approx. 0.6 over the middle range of the binding curve.

A meaningful interpretation of the results rests on the assumption that the Ca^{2+} bound by the fibers is bound only to myofibrils. As shown by Hanson and Huxley [23], 25–30 % of the total protein of glycerinated psoas muscle is extrafibrillar protein. This value is independent of the duration of extraction. We carried out a parallel series of measurements of the binding of Ca^{2+} to well-washed myofibrils under conditions identical to those used for the glycerinated fibers (5 mM $MgCl_2$ present). The results are shown in Fig. 4. At saturating levels of free Ca^{2+}, we obtained a mean bound Ca^{2+} of 2.08 μmol/g myofibril protein (S.E. $\pm$0.15, $n = 25$).

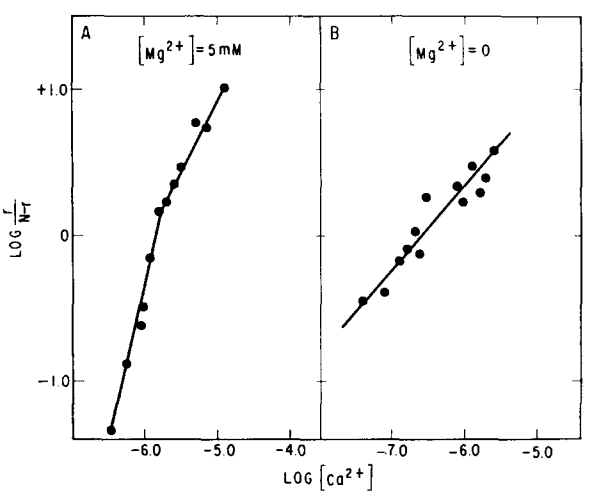

Fig. 3. Hill plots of data of Fig. 1 for the presence (A) and absence (B) of Mg^{2+}. Symbols: r, bound Ca^{2+} in μmol/g; N, maximum bound Ca^{2+}, 1.61 μmol/g in the presence of Mg^{2+}, 2.89 μmol/g in the absence of Mg^{2+}.

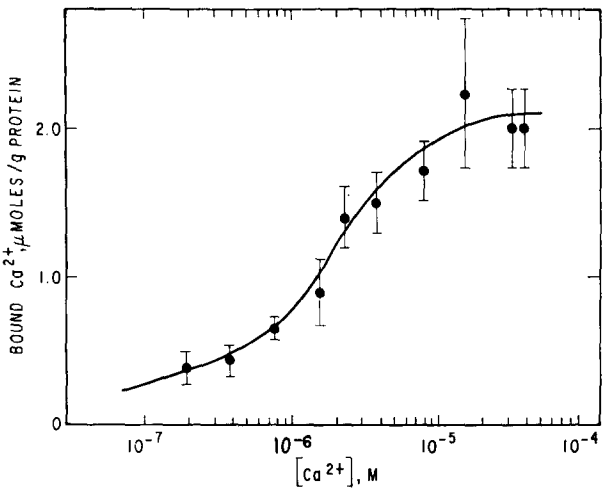

Fig. 4. The binding of Ca^{2+} to myofibrils in the presence of 5 mM $MgCl_2$. Each point is mean of 4–9 determinations. Vertical bars indicate standard errors.

On the basis of the assumption stated above and the data of Hanson and Huxley [23] this value would be equivalent to 1.5–1.6 μmol Ca^{2+}/g fiber protein. The close correspondence between this value and the experimentally determined figure (1.61 ± 0.08 μmol/g fiber protein) leaves us reasonably confident that binding of Ca^{2+} to non-myofibrillar protein can be neglected. The binding curves of the myofibrils and glycerinated fibers are otherwise similar in appearance although the myofibrils appear to bind Ca^{2+} more strongly at lower free Ca^{2+} concentrations. Further studies will be needed to determine whether this difference is real.

DISCUSSION

The main experimental findings of this study are as follows: (1) Mg^{2+} suppresses the binding of Ca^{2+} to about half of the total number of binding sites of glycerinated psoas fibers, and (2) in the presence of Mg^{2+} there is a strong positive cooperativity involving half of the remaining sites.

In the presence of 5 mM Mg^{2+} the fibers bound a maximum of 1.6 μmol/g protein. Correcting for a 25–30 % contamination with non-fibrillar protein, this would be equivalent to about 2.1 μmol Ca^{2+}/g myofibril protein (see above). The ratio of Ca^{2+} to troponin is easily obtainable from data in the literature. The actin content of myofibrils, based on ADP analysis, is 4 μmol/g protein [1] and the molar ratio of actin to troponin is 7 [24]. Thus the troponin content is 0.57 mol/g myofibril protein. A bound Ca^{2+} of 2.1 μmol/g myofibril protein would be equivalent to 3.6 mol Ca^{2+}/mol troponin. This value is in excellent agreement with the known binding ratio (4 mol Ca^{2+}/mol troponin) for the interaction of Ca^{2+} with purified troponin [8] and is also in agreement with the amount of Ca^{2+} bound to reconstituted thin filaments in the presence of myosin and Mg^{2+} [9].

Potter and Gergely [8] have reported that in the presence of millimolar concentrations of Mg^{2+} the four Ca^{2+} binding sites of isolated troponin behave as a

single class with $K' = 5 \cdot 10^6\ M^{-1}$; no evidence was found for cooperativity. It appears that when troponin is incorporated into the native filament lattice binding to two of these sites occurs through a cooperative interaction, at least under rigor conditions. This conclusion follows from the fact that at less than 50 % saturation the slope of the Scatchard plot is positive and the Hill coefficient is 2.2. Similar positive cooperativity has also been reported for the binding of Ca^{2+} to isolated myosin [15], but only with low Mg^{2+} concentrations. The high concentration of Mg^{2+} used in this study (5 mM) was deliberately chosen, on the basis of published data [15], to eliminate the binding of Ca^{2+} to myosin (see below). Unless one assumes that the Ca^{2+} binding properties of myosin are drastically altered when it is linked to actin, it seems reasonable to conclude that the binding sites identified in this study are troponin sites. These results provide further evidence that the physiological behavior of Ca^{2+} receptor sites cannot be accurately predicted from studies with isolated troponin. It is possible to speculate that the observed positive cooperativity is associated with a positive feedback mechanism by which cross-bridge attachment in living muscle facilitates the binding of additional Ca^{2+}. This suggestion rests on the assumption that Ca^{2+}-troponin affinity in the rigor state approximates that found in living muscle when force-generating complexes between myosin and actin are formed. If so, the troponin would, at lower free Ca^{2+} concentrations, bind more Ca^{2+} than would be predicted from the binding curve of isolated troponin. As suggested elsewhere [1, 10], if force is a function of the number of cross-bridge attachments the cooperative effects on Ca^{2+} binding might account for the fact that in both skeletal [25] and cardiac muscle [26] relaxation is delayed as the load is increased.

In the absence of Mg^{2+}, more Ca^{2+} was bound at all levels of free Ca^{2+} concentration. The maximum bound Ca^{2+} (2.9 μmol/g protein) is clearly more than can be accounted for by binding to troponin. The extra Ca^{2+} (1.3 μmol/g fiber protein) is equivalent to roughly 3 mol/mol myosin. This value is fully compatible with the experimentally determined number of high affinity Ca^{2+} binding sites on myosin [13, 14]. The simplest assumption would be that there is a Mg^{2+}-Ca^{2+} competition for binding sites on myosin. There are indications that these sites may play a regulatory role in muscle function [11–15], but at present not enough is known about the true intracellular free Mg^{2+} concentration to enable one to predict whether these sites are ever occupied by Ca^{2+} in living muscle.

The only other double isotope measurements of Ca^{2+} binding to glycerinated psoas fibers are those of Marston and Tregear [16]. Although the binding curves have the same general appearance the results are not directly comparable because of differences in experimental conditions. In particular those workers made their measurements in the presence of MgATP, in which case with the elevation of free Ca^{2+} there would be increased cycling of cross-bridges. Our measurements were made under rigor conditions; cross-bridge attachments were fixed and independent of $[Ca^{2+}]$. Because of the scatter of data points, Marston and Tregear [16] could not reach any conclusions about site heterogeneity or cooperativity in psoas fibers. However, they do show in their Fig. 2B a Scatchard plot for Ca^{2+} binding to glycerinated insect flight muscle which is similar in appearance to the Scatchard plot in our Fig. 2A.

While this paper was in preparation Murray et al. [27] published Ca^{2+} binding data for a reconstituted actomyosin-tropomyosin-troponin system which also in-

dicated the existence of positive cooperativity under conditions of rigor complex formation.

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