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PARALLEL MEASUREMENTS OF BOUND CALCIUM AND FORCE IN GLYCERINATED RABBIT PSOAS MUSCLE FIBERS

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A simple double-isotope procedure has been developed for making simultaneous measurements of bound Ca^{2+} and relative force in glycerinated rabbit psoas bundles containing two fibers. With this preparation it is possible to study Ca^{2+}-troponin interactions coincident with MgATP-induced force development. Over the free $[Ca^{2+}]$ range $6 \cdot 10^{-8}$–$1.2 \cdot 10^{-5}$ M the bound Ca^{2+} varied from 0.25 to 1.65 μmol/g protein. The free $[Ca^{2+}]$ at half-maximal Ca^{2+} saturation was $2 \cdot 10^{-7}$ M while that a half-maximal force was $5 \cdot 10^{-7}$ M. Half-maximal Ca^{2+} saturation was associated with 20% maximal force. The force-$[Ca^{2+}]$ saturation curve showed a steep rise in slope at greater than half saturation. The observed relationship was consistent with a model in which multiple occupancy of troponin Ca^{2+}-binding sites is essential for initiation of cross-bridge cycling.

Introduction

There is general agreement that the contractile mechanism of vertebrate skeletal muscle is activated by the binding of Ca^{2+} to troponin [1]. The mechanism by which Ca^{2+} binding triggers contraction is still not clear but the evidence favors some type of cooperative model. This is most clearly shown in the numerous studies relating force development to free $[Ca^{2+}]$ in a variety of chemically or mechanically 'skinned' fiber preparations [2–5]. In all cases the transition from rest to full activity occurs over an approx. 10-fold range of free $[Ca^{2+}]$; in some studies the range is considerably narrower [6,7]. If the relationship between Ca^{2+} binding and mechanical response followed a simple Michaelis-Menten type scheme the relevant range of free $[Ca^{2+}]$ would span about two orders of magnitude. The steepness of the curve has generally been attributed to a requirement for multiple site occupancy as an essential condition for activation [1] and several authors [7–10] have proposed models in which anywhere from two to six Ca^{2+} must bind to a 'functional unit' in order to activate adjacent cross-bridges. Direct evidence in support of one or another model is still lacking.

More detailed information is available on the Ca^{2+}-binding properties of purified troponin. It is known that troponin (or more specifically, the Ca^{2+}-binding subunit, troponin-C) has four Ca^{2+}-binding sites [11]. Two of these can bind either Ca^{2+} or Mg^{2+} while the other two are specific for Ca^{2+}. On the basis of experiments showing that the relationship between myofibrillar ATPase activity and $[Ca^{2+}]$ is insensitive to variations in $[Mg^{2+}]$, Potter and Gergely [11] concluded that only the Ca^{2+}-specific sites have a regulatory function in muscle activation. On the other hand, every investigator [4,5,12] who has studied the effect of Mg^{2+} on the force-$[Ca^{2+}]$ relationship in skinned fibers has observed a significant effect of Mg^{2+} on Ca^{2+} sensitivity. This is one example of the difficulty in integrating current biochemical and physiological approaches into a comprehensive model of muscle activation. An important dimension which

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

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is missing from all discussions of this subject is precise information on the relationship between bound Ca^{2+} and mechanical output in the intact, working myofilament system. For the past several years we have been using a sensitive double-isotope procedure to characterize the Ca^{2+}-troponin interaction in rabbit psoas muscle fibers extracted with detergent and glycerol [13–15]. These studies were done with fibers in a state of rigor. In this report, we describe a procedure for making simultaneous measurements of both bound Ca^{2+} and force in psoas fibers during isometric contraction in the presence of MgATP.

Methods

Preparation of fibers

Rabbits were killed by intravenous injection of sodium pentobarbital and thin strips of psoas muscle were tied to sticks after the attachments were cut. Such bundles shortened to a sarcomere length (2.0–2.3 μm) less than the in situ sarcomere length (2.6–2.9 μm). The shorter sarcomere length range was preferred, since it provided optimal overlap of the thick and thin filaments. Following the procedure of Taylor [16], the bundles were first extracted for at least 6 h at 0°C with a solution containing 80 mM KCl, 20 mM imidazole (pH 7.0), 2.5 mM dithiothreitol, 5 mM EGTA and 1% Triton X-100. They were then extracted for 24 h at 0°C with a solution of 80 mM KCl, 20 mM imidazole (pH 7.0), 2.5 mM dithiothreitol and 50% glycerol. The bundles were then transferred to fresh glycerol solution (same composition) and stored at −20°C. The bundles were generally used after 5 days of extraction and discarded after about 3 months. During this period there were no changes observed in force generation, Ca^{2+} sensitivity, or troponin-C content. The mean sarcomere length of each bundle was determined by phase contrast microscopy, as described previously [14].

Mechanical measurements

Force development was measured with an RCA 5734 transducer connected to a Gould recorder. The basic apparatus was similar to that of Ashley and Moisescu [4]. Bundles containing two fibers were obtained with the aid of a dissecting microscope and mounted in a lucite chamber. One end of the bundle was glued with a Duco cement/acetone mixture to a glass rod connected to the anode pin of the transducer. The other end was held by jeweller's forceps mounted in a micrometer slide for adjustment of length. The experiments described in this report were done at room temperature (22°C).

Measurement of Ca^{2+} binding

The method for measuring bound Ca^{2+} was based on procedures previously described [13,14], but was modified to allow for measurement of bound Ca^{2+} in association with mechanical response. The fiber bundle was mounted in the muscle chamber and immersed in a solution containing $^{45}CaCl_2$ and [^{3}H]glucose, the latter serving as a marker for solvent space. The free [Ca^{2+}] was initially set at a saturating concentration (10^{-5} M) with EGTA and the length was adjusted to give a just perceptible tension response. Contraction was initiated by the addition of 5 mM MgATP. When a steady force was attained EGTA was injected into the bath with a microliter syringe to reduce the [Ca^{2+}] to some predetermined value. Adequate mixing was obtained with a gentle stream of air. When the new steady force was attained the fiber bundle was carefully lifted out of the bath, still attached to the transducer, and then transferred with jeweller's forceps to 0.5 ml of a neutral buffer solution containing unlabelled carriers (10 mM glucose, 5 mM $CaCl_2$) to elute the isotopes. The eluate was analyzed for ^{45}Ca and ^{3}H by scintillation counting and the fiber bundle was analyzed for protein by a procedure modified from that of Lowry et al. [26], as described previously [13,14]. Bound Ca^{2+} was calculated on the basis of the $^{45}Ca/^3H$ ratio of the eluate relative to that of the incubation solution. Relative force for each Ca^{2+}-binding measurement was expressed in terms of the force after relaxation relative to that at 10^{-5} M Ca^{2+}. To determine the bound Ca^{2+} at saturation a large number of fiber bundles were removed from the chamber while they were still in 10^{-5} M Ca^{2+} and analyzed as described.

Solutions

The solutions used for the Ca^{2+}-binding measurements contained 80 mM KCl, 20 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 5 mM [^{3}H]glucose, 100 μM $^{45}CaCl_2$, and sufficient EGTA to adjust the free [Ca^{2+}] to the desired value. Free [Ca^{2+}] was calculated in the basis of a CaEGTA binding constant (at

pH 7.0) of $5 \cdot 10^6$ M^{-1} and an MgEGTA binding constant (at pH 7.0) of 40 M^{-1}. In these studies no correction was made for Ca^{2+} binding to ATP, since, on the basis of calculations, this was considered to be negligible in the presence of a 2-fold excess of Mg^{2+} over ATP. The pH changes due to mixing of EGTA buffers during the releaxation phase of the experimental protocol was in all cases less than 0.03 pH units.

Results

A tracing of an original record which illustrates the basic experimental protocol is shown in Fig. 1. Maximum force was attained within 10 s after the addition of MgATP. Partial relaxation was induced by adding EGTA 30 s after MgATP addition. Relaxation was generally complete 15–20 s after EGTA addition. Variation in the amount of time spent at maximal or reduced force had no influence on the bound Ca^{2+}. Each bundle of two fibers contained 5–10 μg protein. Single fiber measurements are also feasible; two fibers were used to enhance the precision of the protein analysis.

The data obtained are listed in Table I. Free [Ca^{2+}] was varied from $6 \cdot 10^{-8}$ to $1.2 \cdot 10^{-5}$ M and over this range bound Ca^{2+} increased from 0.25 μmol/g at the lowest [Ca^{2+}] to 1.65 μmol/g at the highest [Ca^{2+}]. The binding sites were saturated at slightly greater than 10^{-6} M Ca^{2+}. Half-maximal binding was at approx. $2 \cdot 10^{-7}$ M Ca^{2+}.

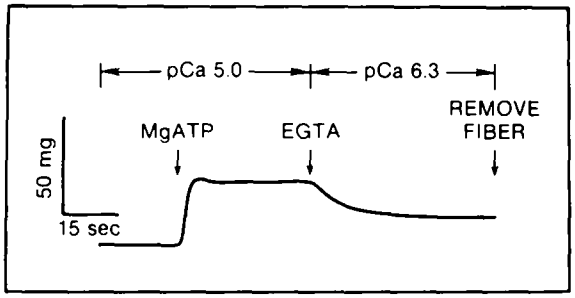

Fig. 1. Tracing of an original record of force as a function of time, illustrating experimental protocol.

To estimate Ca^{2+}-troponin stoichiometry it was assumed, on the basis of previous work from this laboratory [15], that the fibers contained about 0.5 μmol troponin-C/g fiber protein and that in the presence of 5 mM Mg^{2+} the troponin-C was the only significant Ca^{2+}-binding species. Hence, at saturation the bound Ca^{2+} was equivalent to 3 mol Ca^{2+}/mol troponin. One would have expected the ratio to be 4, given the fact that each troponin-C subunit has four Ca^{2+}-binding sites [11]. If there was some residual binding of Ca^{2+} to myosin in the presence of 5 mM Mg^{2+} then the discrepancy with regard to Ca^{2+}-troponin stoichiometry is even greater. To make sure that this discrepancy was not due to some systematic error a series of measurements was made on fiber bundles mounted isometrically in the muscle chamber and exposed to buffer solutions containing 10^{-5} M

TABLE I

BOUND Ca^{2+} AND RELATIVE FORCE AT VARYING LEVELS OF FREE Ca^{2+}

Solutions contained 80 mM KCl, 20 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 100 μM $^{45}CaCl_2$, 5 mM [^{3}H]glucose, 5 mM MgATP, and sufficient EGTA to adjust free [Ca^{2+}] to the desired concentration. The molar ratio of bound Ca^{2+} to troponin was calculated on the assumption that the fibers contained 0.5 μmol troponin/g protein. Relative force and bound Ca^{2+} are expressed as mean ± S.E.

[Ca^{2+}] (M)	Relative force (% maximum)	Bound Ca^{2+} (μmol/g protein)	Molar ratio (mol Ca^{2+}/mol troponin)	n
$6 \cdot 10^{-8}$	15.5 ± 3.8	0.25 ± 0.05	0.5	14
$1.2 \cdot 10^{-7}$	24 ± 3.0	0.59 ± 0.07	1.2	9
$2.4 \cdot 10^{-7}$	23 ± 4.2	0.74 ± 0.11	1.5	14
$6 \cdot 10^{-7}$	66 ± 4.6	1.23 ± 0.16	2.5	13
$2.4 \cdot 10^{-6}$	94 ± 1.5	1.52 ± 0.09	3.0	14
$6 \cdot 10^{-6}$	98 ± 1.2	1.53 ± 0.09	3.0	20
$1.2 \cdot 10^{-5}$	100	1.65 ± 0.14	3.3	38

TABLE II

EFFECTIVE OF MgATP ON Ca^{2+} BOUND TO ISOMETRICALLY HELD FIBERS AT 10^{-5} M FREE Ca^{2+} CONCENTRATION

See Table I for experimental conditions. Bound Ca^{2+} is expressed as mean ± S.E. Number of measurements is given in parenthesis.

[MgATP] (mM)	Bound Ca^{2+} (μmol/g protein)	Molar ratio (mol Ca^{2+}/ mol troponin)
0	2.03 ± 0.09 (7)	4.1
5	1.34 ± 0.11 (5)	2.7
	$P < 0.001$	

Ca^{2+}, with and without MgATP. A shown in Table II, fibers developing active force in the presence of MgATP bound significantly less Ca^{2+} than fibers in rigor. As in previous work from this laboratory [13,14], fibers in rigor bound an equivalent of 4 mol Ca^{2+}/mol troponin; in contrast, fibers developing active force bound about 3 mol Ca^{2+}/mol troponin. It should be noted that 10^{-5} M Ca^{2+} is a saturating concentration both in the presence of MgATP (see Fig. 2) and in rigor [15].

Normalized curves relating Ca^{2+} saturation and relative force to free [Ca^{2+}] are shown in Fig. 2. Half-maximal force was observed at a free [Ca^{2+}] of $5 \cdot 10^{-7}$ M, as compared to $2 \cdot 10^{-7}$ M free [Ca^{2+}] at

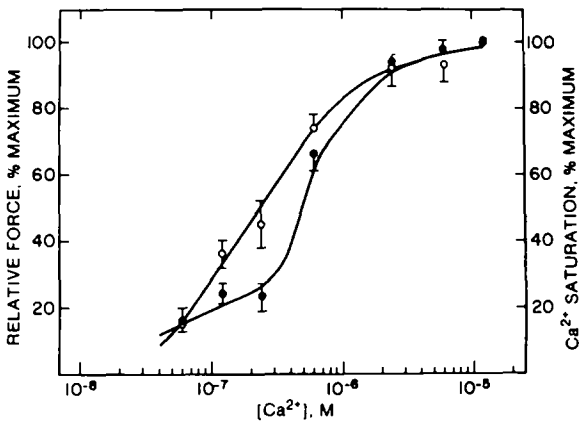

Fig. 2. Normalized plots of Ca^{2+} saturation (○) and relative force (●) as a function of free [Ca^{2+}].

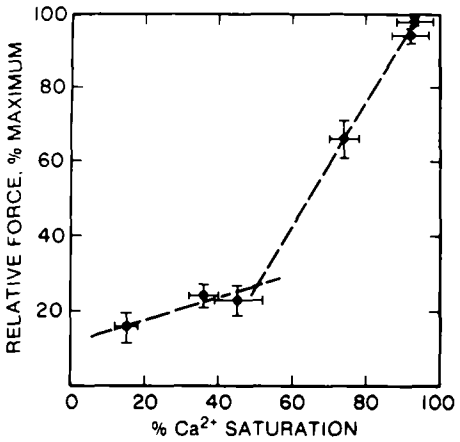

Fig. 3. Plot of relative force as a function of Ca^{2+} saturation, based on data in Table I.

half-maximal Ca^{2+} saturation. Half-maximal Ca^{2+} saturation was associated with 20% maximal force. When relative force is plotted as a function of Ca^{2+} saturation (Fig. 3) it is evident that there is a steep rise in developed force as Ca^{2+} saturation exceeds 50%.

Discussion

There are now substantial data available on both the Ca^{2+}-binding properties of purified troponin-C [11] and the Ca^{2+} dependence of the tension response in skinned fiber preparations [2–5]. Recent work in this laboratory is designed to integrate these two approaches through studies on Ca^{2+} binding in organized myofilament systems. This approach seems justified by several lines of evidence showing that Ca^{2+}-receptor interaction may be modulated through cooperative interactions between myofibrillar proteins [17–19].

In this report, a fairly simple method is described for making simultaneous measurements of bound Ca^{2+} and relative force and, with this method, data have been obtained relating the Ca^{2+}-saturation curve to the force-[Ca^{2+}] curve. As might have been expected from the steep relationship between force and [Ca^{2+}], force is not a linear function of Ca^{2+} saturation. Rather, at greater than half saturation there is an increase in slope of the force-saturation curve. More data will be needed for a rigorous quantitative analysis of this relationship but the results are

consistent with an activation scheme which requires multiple occupancy of binding sites on individual troponin-C subunits for initiation of cross-bridge interaction [8,17]. Fig. 3 bears a qualitative resemblance to the data published by Murray et al. [18] on the relationship between Ca^{2+} saturation and myofibrillar ATPase activity.

Interpretation of the Ca^{2+}-troponin-C interaction in an organized myofilament system is dependent upon assumptions about the troponin-C content and the location and specificity of Ca^{2+}-binding sites. The mean troponin-C content of our fibers has been determined by gel electrophoresis and found to be about 0.5 μmol/g fiber protein [15]. This value is in reasonably good agreement with an earlier estimate [20] obtained with a different technique. Evidence has been published elsewhere [15] showing that in glycerinated psoas fibers in the presence of 5 mM Mg^{2+}, troponin-C is the only significant Ca^{2+}-binding species. We assume, of course, that in the presence of MgATP there is no dissociation of troponin-C from the myofilaments. Indirect evidence has already been published showing that preexposure to MgATP does not alter the Ca^{2+}-binding capacity of psoas fibers in rigor [14]. In interpreting binding data it should be kept in mind that small errors in troponin-C determination and ^{45}Ca-binding measurements could lead to significant errors in the calculated Ca^{2+}-troponin stoichiometry. Further work is needed before binding data obtained with glycerinated fibers can be interpreted with confidence.

The results reported here are strongly reminiscent of earlier data from this laboratory showing that fibers (in rigor) with optimal filament overlap bound 25–30% more Ca^{2+} than fibers stretched to lengths at which filament overlap was lost [13,14]. Our original interpretation [13,14], based on earlier work of Bremel and Weber [17], was that there were conformational differences between attached and unattached thin filaments which were somehow transmitted to the Ca^{2+}-binding sites. If MgATP causes cross-bridge detachment and if a large fraction of the cross-bridges are detached even when the muscle is developing maximal force [21], then the effect of MgATP on Ca^{2+} binding might have the same basis as that due to stretch. Earlier X-ray diffraction measurements indicated that even in a muscle developing maximal force, less than half of the cross-bridges are attached to the thin filaments at any given instant [22]. However, more recent X-ray [23] and mechanical [24] measurements suggest that the number of attached cross-bridges during maximum isometric contraction is not markedly different from that in rigor. It may be that the important factor is not the number of attached cross-bridges but the lifetime of a particular conformational state relative to the duration of the cross-bridge cycle.

As far as we can determine, the only other work in the literature which is related to that reported here is the paper of Marston and Tregear [25]. These workers measured the Ca^{2+} bound by glycerinated rabbit psoas fibers in the presence of MgATP and by detergent-treated insect flight muscle fibers in both the presence and absence of MgATP. Unfortunately, they did not record the associated mechanical responses and, probably because of the differences in technique, their data had a much greater scatter. For the psoas fibers in the presence of MgATP they reported an apparent Ca^{2+}-binding constant of $5 \cdot 10^6$ M^{-1} and a maximum bound Ca^{2+} of 3–4 mol Ca^{2+}/mol troponin. The insect flight muscle fibers were shown to bind less Ca^{2+} in the presence of MgATP then in rigor over the entire range of free $[Ca^{2+}]$; at saturation there was 30% difference in the amount of bound Ca^{2+} between the two groups of fibers. Thus, there is a general consistency between their data and the results reported here. Further work will be directed to obtaining a more precise relationship between Ca^{2+}-receptor occupancy and mechanical output and to determining the effect of mechanical parameters on Ca^{2+}-receptor properties.

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