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CALCIUM BINDING AND THE ACTIVATION OF FIBRILLAR INSECT FLIGHT MUSCLE

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SUMMARY

A new method has been used to measure calcium binding in intact glycerol extracted muscle fibres; results with rabbit psoas muscle are in agreement with previous work. *Lethocerus cordofanus* flight muscle bound up to 140 μM calcium at high affinity in the presence of ATP; removal of the ATP increased the maximum amount bound to 210 μM and the affinity approx. 3-fold. Calcium binding in the presence of ATP correlated with calcium activation of the ATPase but no changes in calcium binding occurred when the muscle was further activated by stretching.

INTRODUCTION

It has recently been shown that arthropod muscle contains two calcium sensitive regulatory proteins, one on the myosin and the other on the thin filaments [1, 2]. In addition to calcium activation certain insect flight muscles, the so-called fibrillar muscles [3], show the phenomenon of stretch activation in which a small extension of the muscle increases the myosin ATPase within it [4].

Studies on the regulatory system in vertebrate-striated muscle, which contains regulatory proteins on the thin filaments only [5] have revealed the existence of cooperation between calcium binding to troponin and myosin head binding to actin. Both appear to move the tropomyosin in relation to the thin filament [6] and hence to affect each other [7].

In this work we have measured calcium binding in glycerol-extracted fibres from the much studied fibrillar flight muscle of the water bug *Lethocerus cordofanus* [8]. We have attempted to find whether the principle of cooperativity demonstrated in vertebrate-striated muscle applies to the different regulatory system of the insect muscle and whether changes in calcium binding are associated with stretch activation. In the only previous study of calcium binding by this preparation Chaplain [9] showed a great increase of calcium binding on extension.

Abbreviations: EGTA, ethyleneglycolbis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid. PIPES, piperazine-*N*,*N*′-bis-2-ethanesulphonic acid.

METHODS AND MATERIALS

Muscle fibres

Dorsal longitudinal muscles of *L. cordofanus* were glycerol extracted according to the procedure established in this laboratory [10].

Single muscle fibres were dissected out and stored at 1 °C overnight in a non-ionic detergent solution (0.5% polyoxyethylene (2) sorbitan monooleate (Tween 80, Koch–Light Laboratories Ltd) 5 mM $MgCl_2$, 5 mM ethyleneglycolbis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), 5 mM piperazine-*N,N'*-bis-2-ethane sulphonic acid (PIPES) at pH 7.1, 50 mM KCl) in order to wash out mitochondria, as described by Abbott and Chaplain [11]. Samples from each set of fibres so treated were tested mechanically before use; only if they were found to relax on adding 10 mM MgATP in the absence of calcium, and to perform mechanical work on being forcibly oscillated in length in the presence of ATP and 2 μM calcium was the set of fibres used for further experiments (cf. Jewell and Rüegg [10] for details of the mechanics of fibrillar flight muscle).

Rabbit psoas fibres were also used in certain experiments; they were glycerol extracted as described previously [12].

Single fibres were either used unrestrained or mounted between needles; in the latter case they could be extended by small amounts using a micromanipulator. Unrestrained fibres were totally immersed in the incubating solution whilst only the central section of the mounted fibres was immersed, as previously described [12]. When stretch activation was studied the fibre was either incubated first stretched and then unstretched or vice versa. The procedure for following the time-course of elution of bound calcium was the same as that used by Marston [12].

Calcium binding

The principle of the method was the same as that employed to measure the binding of nucleotides to glycerol-extracted muscle fibres [12]. Solutions were prepared containing a known, buffered free calcium concentration (105–108 μM $^{45}CaCl_2$, 1 mM [^{14}C]mannitol, 0–2 μM EGTA, 0 or 5 mM Na_2ATP, 5 mM $MgCl_2$, 50 mM KCl, 5 mM PIPES at pH 7.1). The absolute concentration of ^{45}calcium was calculated from the manufacturer's data, non-radioactive calcium contaminant in the incubating solutions, measured by atomic absorption spectroscopy prior to the addition of ^{45}calcium, was 5–8 μM and this was allowed for in the calculation of calcium specific radioactivity and free calcium concentrations. The concentration of free calcium was varied from 0.01 to 100 μM by varying the concentration of EGTA [13] and the free calcium concentrations were calculated using a computer programme [14].

Single muscle fibres were incubated in 20 μl of the solutions for 2 min at 21 °C and transferred immediately to 10 μl of solution containing a high concentration of non-radioactive free calcium (1 mM $CaCl_2$, 5 mM $MgCl_2$, 50 mM KCl, 5 mM PIPES at pH 7.1) to wash out radioactive calcium and mannitol. Mounted fibres were given a $\frac{1}{4}$–$\frac{1}{2}$-s preliminary wash to remove surface liquid; it was estimated (see Fig. 3) that such a short wash eluted less than 4% of the material in the fibre. After 3 min the fibre was removed and found to contain a negligible amount of radioactivity.

The entire 10 μl of eluting solution was transferred to carboxymethyl cellulose thin-layer chromatography sheets and the ^{45}calcium and [^{14}C]mannitol were separated, by eluting the chromatogram with water, and assayed in a liquid scintillation counter. The total calcium concentration in the fibre was calculated from the relative ^{45}calcium and ^{14}C eluted, assuming that mannitol distributed completely throughout the fibre and was not bound. The bound calcium was calculated from the difference between this and the concentration in incubating solution; this process has been described in detail for nucleotide binding [12].

Where appropriate the binding data were fitted to a hyperbolic function by means of the non-linear regression procedure devised by Wilkinson [15]. For this purpose the individual estimations were employed and not the means for each calcium concentration shown in Figs 1 and 2.

ATPase

The ATP hydrolysis by muscle fibres was measured by incubating single fibres in 10 μl of a radioactive ATP solution of the same constitution as that used for the calcium binding measurements, for 10 min at 21 °C. The ADP formed was separated on polyethyleneimine-impregnated cellulose [12] and assayed by liquid scintillation counting; results are expressed as picomoles ADP formed $\cdot$ min^{-1} $\cdot$ (cm fibre)$^{-1}$, a unit in common use in such studies; 500 pmoles $\cdot$ cm^{-1} $\cdot$ min^{-1} is approximately equivalent to a catalytic site activity of 1 s^{-1}, since there are 8 pmoles myosin enzymic sites per cm fibre [16].

RESULTS

Rabbit muscle

Individual estimates of the amount of calcium bound by fibres under the same conditions varied widely, sometimes by as much as a factor of two; large numbers of estimates were therefore necessary. On occasion a whole set of experiments showed extremely low or zero calcium binding; when this occurred the entire set was discarded and fresh solutions and fibres prepared, on the grounds that contamination with non-radioactive calcium had probably occurred. Apart from this the results were not selected.

Rabbit psoas fibres, mounted at rest length (2.6 μm sarcomere length), were studied to see if the results by this method were compatible with those obtained by others on isolated myofibrils [7]. A regular relation between the concentration of bound and free calcium was obtained over the range 0.01–10 μM free calcium. The data fitted reasonably well to a calculated hyperbolic relation and showed no systematic deviation from the line in any one region of the binding curve (Fig. 1A). However the Scatchard plot of the same data (Fig. 1B) revealed considerable scatter of the points about the calculated line, and it is therefore impossible to exclude the degree of binding heterogeneity reported by Bremel and Weber [7]. The calculated dissociation constant for the fibres (0.18 μM) was slightly higher than the calcium concentration required to half-activate the fibre ATP (arrow, Fig. 1A; cf. Marston [12]). The calculated maximal binding (270 μM) is similar to that found by Bremel and Weber [7] and is equivalent to between 3 and 4 calcium per troponin molecule (1 troponin per 7 actin monomers assumed [18]; actin concentration lies between

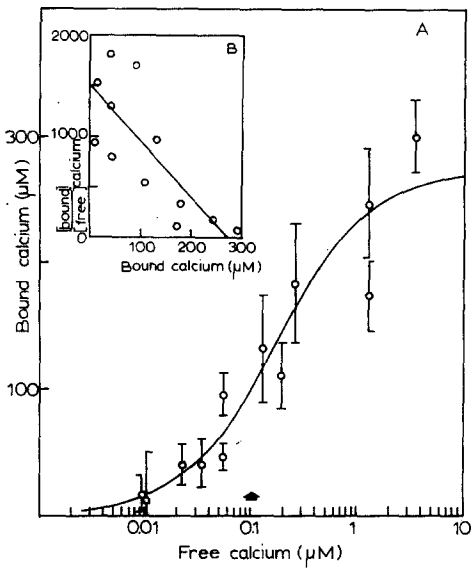

Fig. 1. The binding of calcium to single glycerol-extracted rabbit psoas fibres mounted at rest length in the presence of 5 mM MgATP. A, direct plot (note logarithmic abscissa); B, Scatchard plot. Each point represents eight estimations; the error bars extend to the 5 % fiducial limits of the mean. The solid lines were calculated as a hyperbolic fit to all points by a non-linear regression technique [15]. The arrow indicates 50 % activation of fibre ATPase [12].

400 μM [19] and 700 μM [20]).

The agreement between the present results and those on rabbit myofibrils was considered sufficient for the method to be pursued.

Insect muscle

Lethocerus flight muscle contains a large mass of mitochondria; the detergent treatment used here was intended to remove the mitochondria [11] and it certainly reduced the calcium binding. The concentration bound at 4 μM free calcium in the presence of ATP before Tween treatment was 210 ± 30 μM (10 experiments) whilst after Tween treatment it was 117 ± 8 μM (19 experiments). Ruthenium red which inhibits calcium binding by mitochondria [21, 22] did not further reduce the calcium binding (concentration bound by Tween-treated fibres at 4 μM free calcium in the absence of ATP: before ruthenium red treatment 210 ± 15 μM (8 experiments), after treatment with 20 μg/ml ruthenium red 240 ± 30 μM (13 experiments)). It is therefore probable that most of the binding observed was on the myofibrillar proteins.

The calcium bound by Tween-treated *Lethocerus* fibres was regularly related to the free calcium concentration (Fig. 2). At all concentrations more calcium was bound in the absence of ATP (●) than in its presence (○). Although the relations between free and bound concentrations appeared regular they did not fit a hyperbolic function (solid lines, Fig. 2), even when the high calcium point was excluded (this exclusion is justified by the probable non specific binding, for instance to myosin [7]). The direction of misfit to a hyperbolic relation is that expected from positive cooperativity, rather than from heterogeneous binding (Scatchard plot, Fig. 2); thus the values of the

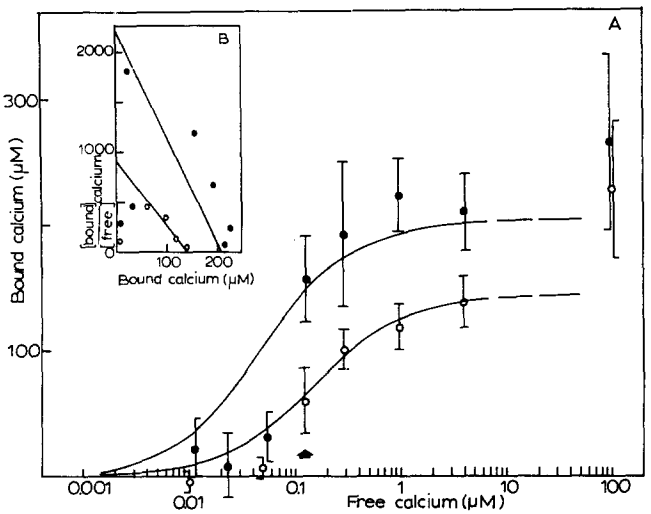

Fig. 2. The binding of calcium to single unrestrained glycerol-extracted *Lethocerus* flight muscle fibres, in the presence (○) and absence (●) of 5 mM MgATP. A, direct plot; B, Scatchard plot. Each point represents 4–23 estimations. The solid lines are hyperbolic fits calculated with the 100-μM points excluded. The arrow indicates 50 % activation of fibre ATPase [23]. Other details as Fig. 1.

dissociation constant calculated from the hyperbolic fit, 0.16 ± 0.02 μM in the presence and 0.04 ± 0.01 μM in the absence of ATP, are underestimates of the calcium concentration necessary to produce 50% binding. The calculated values of maximal binding, 142 ± 7 μM in the presence and 208 ± 15 μM in the absence of ATP, are probably also less than the concentration of binding sites.

TABLE I

THE EFFECT OF 5 % EXTENSION ON ATPase AND CALCIUM BINDING AT 1 μM CALCIUM IN THE PRESENCE OF ATP BY *LETHOCERUS* FIBRES

Mean$\pm$S.E.; number of experiments in brackets.

Condition	ATPase (moles · cm^{-1} · min^{-1})	Calcium binding (μM)
1 μM calcium		
Unstretched	$98\pm9(5)$	$86\pm10(10)$
Stretched	131 ± 9	74 ± 4
Difference*	32 ± 5	-12 ± 8
0.07 μM calcium		
Unstretched	60,70**	$11\pm5(4)$
Stretched	110,80	4 ± 5
Difference	50,10	7 ± 6

* Each experiment contained one measurement with the fibre stretched and one with it unstretched; the order in which the two measurements were made was randomised. The differences were obtained for each experiment.

** Individual experiments shown.

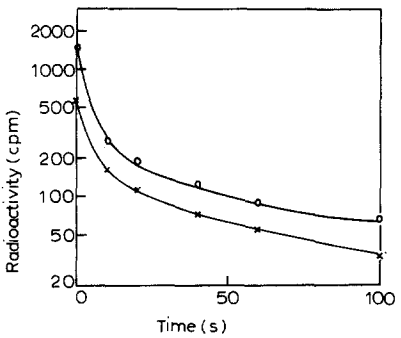

Fig. 3. The time-course of release of mannitol (×) and calcium (○) from a single mounted *Lethocerus* fibre, on repeated washing after incubation at 4 μM free calcium plus 5 mM MgATP.

These *Lethocerus* fibres showed stretch activation, in that their ATPase increased some 30% when the mounted muscle was extended by 5% (Table I). The degree of activation, and its absolute amount, were smaller than that achieved in previous experiments [23], but were present in every experiment performed. This degree of stretch activation was not accompanied by any increase in calcium binding; at 1 μM calcium there was a slight indication of a decrease in binding while at 0.07 μM calcium there was an equally slight indication of an increase (Table I).

These data are completely at odds with those of Chaplain [9], who found a very large increase of calcium binding on stretching fibres in 0.05 μM calcium. We investigated the time-course of elution of the calcium from the fibres, since Chaplain washed the fibres for 15 s in a non-radioactive solution before eluting the calcium in order to differentially remove the free calcium from the fibres. We found that all the calcium present washed out rapidly; the rate of washout was apparently limited by diffusion from the fibres rather than by the dissociation of calcium from the binding protein, since the rate for calcium was the same as that for the unbound mannitol (Fig. 3).

DISCUSSION

Lethocerus flight muscle binds calcium at approximately the same concentration as that required to activate the ATPase of the fibres; 50% calcium binding was obtained at about 0.15 μM free calcium (Fig. 2) whilst 50% activation of the ATPase requires 0.11 μM calcium [23]. It is therefore probable that the calcium binding causes activation.

It has been shown that in the locust flight muscle both the thin filaments and myosin are calcium sensitive and it is probable that this conclusion extends to *Lethocerus* flight muscle [2]. If so both systems should bind calcium and indeed the maximum amount of calcium found here in the presence of ATP (142 μM) is probably too great to be accounted for by the troponin binding alone: *Lethocerus* flight muscle contains 40% of myofibrils by volume [24] leading to an overall actin concentration of approx. 300 μM [20] and the published calcium binding capacity of arthopod thin filaments [1] is less than one per seven actin monomers, equivalent to less than the 40-μM binding sites in the fibres. Since the concentration of myosin

in the fibres is 100 μM [12, 16, 17] the remaining bound calcium could be accounted for if one calcium was bound per myosin molecule.

The thin and thick filament-linked calcium sensitivities are known to be synergistic in vitro [2] and the apparent cooperation seen in calcium binding in our experiments may be a further expression of this interaction.

The large increase in calcium binding induced by the removal of ATP from *Lethocerus* fibres parallels the observation of Bremel and Weber [7] on rabbit myofibrils. It differs from their effect, however, in that it appears to represent the exposure of further calcium binding sites rather than an increase of affinity of those already available in the presence of ATP. The function of the extra binding sites remains unknown since only the 140-μM sites are occupied under conditions where the ATPase is fully activated (cf. ref. 23 and Fig. 2).

When the *Lethocerus* muscle fibres were extended by 5% the calcium binding did not change significantly, whilst the ATPase was increased (Table I). It is therefore unlikely that stretch activation is due to increased calcium binding [4, 9] although changes may still occur at greater degrees of stretch activation than we were able to achieve. Invariance of calcium binding with extension is also indicated indirectly both by the lack of effect of stretch on the free calcium concentration required for half activation of the ATPase [23, 24] and the independance of the effect of calcium and extension of the fibres [25]. The direct measurements of calcium binding by Chaplain [9] remain unexplained. It is improbable that his technique enabled him to differentiate between free and bound calcium since we found both elute together (Fig. 3).

The present evidence shows that the presence of ATP affects calcium binding in the same way in insect flight and rabbit skeletal muscle, despite the different control systems in the two muscles. This encourages the speculation that the effect has a functional significance.

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