Pflügers Arch (1993) 424:30–38

Pflügers Archiv
European Journal of Physiology

Endogenous MLC2 phosphorylation and Ca^{2+}-activated force in mechanically skinned skeletal muscle fibres of the rat

Gabriela M. M. Stephenson, D. G. Stephenson

Department of Zoology, La Trobe University, Bundoora, Vic 3083, Australia

Received September 3, 1992/Received after revision February 1, 1993/Accepted February 2, 1993

Abstract. A method has been developed for measuring the level of phosphorylation of myosin regulatory light chains (MLC2) by the endogenous myosin light chain kinase in mechanically skinned skeletal muscle fibres. The method was used to characterize the endogeous MLC2 phosphorylation capacity of single fast-twitch fibres from the rat and to investigate the relationship between the endogenous MLC2 phosphorylation and the Ca^{2+}-activated force. The results show that (1) about 50% of MLC2 were ^{32}P-phosphorylated after activation of the skinned fibre preparation by 30 μM [Ca^{2+}] for longer than 30 s, but that there was variability between fibres; (2) most of the endogenous phosphorylating system diffused out of the skinned fibre preparation after 5 min exposure to an aqueous solution; (3) the MLC2 phosphorylation by the endogenous phosphorylating system followed with a delay of the order of 1–2 s after the sudden rise in [Ca^{2+}] from below 10 nM to 30 μM; and (4) the sensitivity of the contractile apparatus to Ca^{2+} was markedly increased when the MLC2 were phosphorylated by the endogenous phosphorylating system following a rise in [Ca^{2+}]. The K_d for MgATP of the endogenous MLC2 phosphorylating system was estimated to be less than 300 μM. These results unequivocally demonstrate that prolonged activation of the fast-twitch muscle fibre leads to increased Ca^{2+} sensitivity of the contractile apparatus and that mechanically skinned fibres can be successfully used to study the regulation of the endogenous MLC2 phosphorylation capacity at single muscle fibre level.

Key words: Myosin regulatory light chain – Myosin light chain kinase – Mechanical skinning – Skeletal muscle – Endogenous phosphorylation – Fast-twitch muscle – Myofibrillar proteins – Calcium activated force

Correspondence to: G. M. M. Stephenson

Introduction

The role of the regulatory myosin light chains (MLC2) in skeletal muscle contraction is still not well understood [9, 20]. However, it has been firmly established that skeletal muscle MLC2 can be monophosphorylated by a highly specific Ca^{2+}, calmodulin-dependent kinase activity (MLCK) [9], and intense efforts have been directed towards the purification [5], sequencing [33] and characterization [8] of the enzyme as well as towards identification of the role played by MLC2 phosphorylation in skeletal muscle contractility [30].

Earlier studies using intact muscle preparations have shown that in skeletal muscle, MLC2 phosphorylation is positively correlated with the post-tetanic potentiation of the isometric twitch [30]. However, intact muscles do not allow access or manipulation of the intracellular environment and therefore cannot be used to establish if there is a causal relationship between MLC2 phosphorylation and the tetanus-induced increase in isometric twitch force.

More recently, several studies using single muscle fibres permeabilized by chemical skinning suggested that MLC2 phosphorylation may play a modulatory role in skeletal muscle contractility, by showing that MLC2 phosphorylation is accompanied by an increase in isometric force response and rate of force production at submaximal levels of Ca^{2+} activation [15, 21, 32]. This view has been challenged by Godt and Nosek [12], who showed that the irreversible thiophosphorylation of MLC2 in chemically permeabilized fibres did not change the sensitivity to Ca^{2+} of the contractile apparatus.

In this study we have investigated the properties of the endogenous MLC2 phosphorylating system in single muscle fibres, skinned under paraffin oil [18, 25], which maintain the intracellular protein components while allowing access to the internal compartments. We have also examined for the first time the relationship between

endogenous MLC2 phosphorylation and Ca^{2+}-activated force.

Brief reports of these findings have been made to the Australian Physiological and Pharmacological Society [29] and to the British Physiological Society [28].

Materials and methods

Skinned fibre preparations and apparatus. Fast-twitch muscle fibres were obtained from the extensor digitorum longus (EDL) muscle of adult (approx. 3 months) male rats (Long Evans hooded) which were killed by ether overdose. The EDL muscles were blotted dry on Whatman 1 filter paper and placed in paraffin oil. Single fibres were isolated from the muscle by microdissection under a dissecting microscope and were mechanically skinned with fine forceps under paraffin oil as previously described [26]. For studying endogenous phosphorylation under various conditions, each skinned fibre was further divided into two or three segments (1−2.5 mm long). After dissection and skinning, different segments from one single muscle fibre were kept in paraffin oil and were used within less than 30 min of each other. Storing skinned muscle fibre preparations in oil for periods up to 1 h did not affect their ability to incorporate ^{32}P during contractile activation. Individual fibres and fibre segments were mounted with braided surgical silk (Deknatel, size 10) between a pair of jewellers forceps and a piezoresistive force transducer (AME 875, Horten, Norway) while still under oil. The length of the fibre segments was then adjusted under oil to just slack length (sarcomere length 2.6−2.7 μm, as measured by laser diffraction, [26], and the length and diameter of the fibre segments were carefully measured, while still under oil, with a dissecting microscope at 40× to 80× magnification. We wish to point out that due to surface tension forces which work to minimize the cross-sectional area of fibres under oil, the contour of fibres in such an environment is more regular than that of fibres in tissue or in an aqueous solution. The diameter was measured at least at two points along the fibre segment to allow accurate estimation of the segment volume assuming a circular cross-section. For fibre segments which did not appear approximately cylindrical during dissection, the cross-section was assumed to be ellipsoidal and both the large and the small diameter were measured. It is noteworthy that differences in fibre volumes measured in this manner were fully consistent with differences in the myofibrillar protein content, as estimated from the intensities of several major protein bands on the Coomassie Blue stained sodium dodecyl sulphate (SDS) gels. In some experiments, fibres were either stretched to 1.6 times slack length to decrease the overlap between actin and myosin filaments (average sarcomere length 4.2−4.3 μm) or were slackened to allow unloaded shortening down to very short sarcomere lengths (<1.4 μm). The mounted fibre preparation was rapidly (1−2 s) transferred between oil and various solutions contained in small Perspex cuvettes (volume 100 μl to 3 ml) by a simple mechanical system used in our laboratory [26].

Simultaneous measurement of endogenous MLC2 phosphorylation and force response in skinned fibre preparations. [^{32}P] Phosphorylation of the myosin light chains was obtained by transferring the skinned fibre segments from oil to a 100-μl trough containing 54 μl of the Ca/[^{32}P]ATP solution overlaid with a small volume of paraffin oil to avoid evaporation. Unless otherwise specified, the Ca/[^{32}P]ATP solution contained (mM): K^+, 120; Na^+, 14; free Mg^{2+}, 1; [^{32}P]ATP, 7 [3.7−7.4 MBq/ml (100−200 μCi/ml)]; MgATP, 6; $CaEGTA^{2-}$ + $EGTA^{2-}$, 44 ([Ca^{2+}] approx. 30 μM); $HDTA^{2-}$, 8.8; NaN_3, 0,9; HEPES, 52; MOPS, 0.4; TRIS, 0.9; pH = 7.10; where EGTA is ethylenebis(oxonitrilo)tetraacetate, HDTA is hexamethylenediamine *N,N,N′,N′*-tetraacetate, HEPES is 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, MOPS is 4-morpholinopropanesulphonic acid, TRIS is tris(hydroxymethyl)aminomethane. The force developed by the fibre preparation was measured continuously on a chart recorder. At the end of an experiment, the fibre segment was rapidly (within 2 s) relaxed in a solution containing (mM): K^+, 137; Na^+, 16; free Mg^{2+}, 1; ATP, 8; MgATP, 7; $EGTA^{2-}$, 50 ([Ca^{2+}]<1 nM); $HDTA^{2-}$, 10; NaN_3, 1; HEPES, 60 (pH 7.10), and after another 30 s was transferred to a 0.5-ml Eppendorf tube containing 8 μl SDS sample buffer (62.5 mM TRIS, 25% glycerol, 2.3% SDS, 5% β-mercaptoethanol, 0.1 mM phenyl methyl sulphonyl fluoride (PMSF), 2 μM leupeptin, 1 μM pepstatin and 0.01% bromphenol blue).

SDS-gel electrophoresis. The myofibrillar proteins solubilized by incubation of the fibre preparations in SDS sample buffer at room temperature, overnight [23], were resolved by SDS-polyacrylamide gel electrophoresis with an acrylamide concentration of 18% [11] using the Bio-rad minigel apparatus. At the end of the electrophoretic run, gels were fixed and then stained either with Coomassie blue or with the Bio-rad silver staining kit, and the ^{32}P-labelled proteins were visualized by autoradiography.

Quantitation of endogenous MLC2 [^{32}P]phosphorylation. The levels of MLC2-bound radioactivity in individual fibres and fibre segments were measured densitometrically with a Molecular Dynamics scanning densitometer (Model 300A) and were expressed as arbitrary density units (ADU). Given the intrinsic limitations of the method (e. g. inter-experimental differences in the amount of label used or in the exposure time to X-ray film) comparisons were made only between fibres or fibre segments studied under identical conditions of phosphorylation and of post-experimental processing. Single fibre preparations or segments from the same fibre also differed in volume. Therefore, to compare the MLC2 [^{32}P]-phosphorylation in different fibres or in different segments from the same fibre, each individual value of MLC2-bound radioactivity was further divided by the respective fibre volume and the result was expressed as ADU/nl. In several experiments, an estimation of the actual ratio between [^{32}P]MLC2 and total MLC2 in individual fibre segments was carried out as follows. First, the amount of [^{32}P]MLC2 was estimated by comparing the densitometric value of the MLC2 band on the autoradiogram with that of a known amount of [^{32}P]ATP, sampled from the phosphorylating solution and spotted on a thin strip of filter paper similar in size to the MLC2 electrophoretic band (see Fig. 1). The result was then divided by the total amount of MLC2 in each fibre segement, a value which was obtained by multiplying the segment volume, measured at slack length, by 200 μM (the concentration of MLC2 found in mammalian fibres) [34]. The use of this method was based on the assumption that all MLC2 had been extracted from the fibre segment and that no MLC2 losses had occurred during the electrophoresis, fixation and staining of the sample. Therefore, the values obtained tend to be underestimates of the true proportion of [^{32}P]MLC2. All results are given as mean ± SEM (n).

Materials. [$\tau^{32}P$]ATP [>1.48 MBq/mmol (>4,000 Ci/mmol)] was purchased from Bresatec (Australia). Lyophilized inhibitor-2 (rabbit skeletal muscle) was a gift from James Maller, University of Colorado, USA and purified MLC2 was a gift from Terry Walsh, Queensland Institute of Technology, Australia. All reagents used for electrophoresis were from Bio-rad and all other reagents were purchased from Sigma (St. Louis, Mo., USA).

Results

Simultaneous measurement of endogenous MLC2 phosphorylation and maximal Ca^{2+}-activated force

Endogenous phosphorylation of the regulatory MLC2 and Ca^{2+}-activated force responses were measured simultaneously in single, fast-twitch muscle fibres of the rat. The fibres, mechanically skinned under paraffin oil, were attached to a force transducer while still under oil, and then were bathed in Ca^{2+}/[τ-^{32}P]ATP-containing solutions (see Materials and methods). All studies reported here were conducted at 22 °C, since it has been reported that this temperature favours the phosphory-

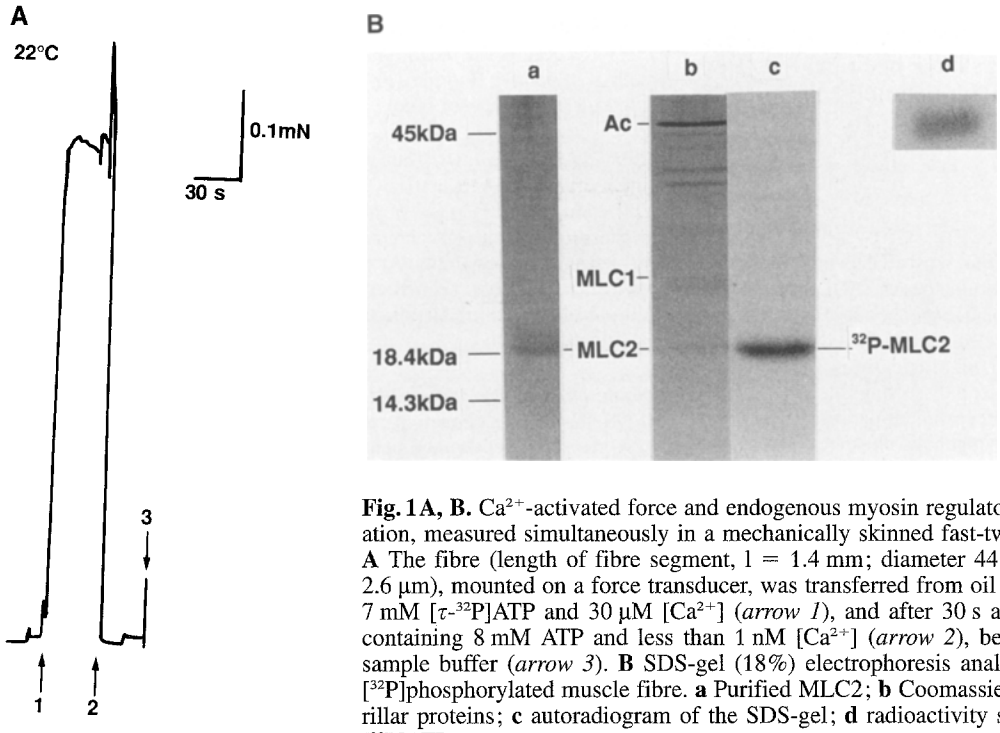


Fig. 1A, B. Ca^{2+}-activated force and endogenous myosin regulatory light chain (MLC2) phosphorylation, measured simultaneously in a mechanically skinned fast-twitch (EDL) muscle fibre of the rat. **A** The fibre (length of fibre segment, l = 1.4 mm; diameter 44 µm; sarcomere length, SL approx. 2.6 µm), mounted on a force transducer, was transferred from oil to an activating solution containing 7 mM [τ-^{32}P]ATP and 30 µM [Ca^{2+}] (*arrow 1*), and after 30 s activation was relaxed in a solution containing 8 mM ATP and less than 1 nM [Ca^{2+}] (*arrow 2*), before being transferred to the SDS-sample buffer (*arrow 3*). **B** SDS-gel (18%) electrophoresis analysis of myofibrillar proteins in the [^{32}P]phosphorylated muscle fibre. **a** Purified MLC2; **b** Coomassie blue stained profile of the myofibrillar proteins; **c** autoradiogram of the SDS-gel; **d** radioactivity standard corresponding to 175 fmol [^{32}P]ATP

lation of MLC2 and minimizes their dephosphorylation [17].

A representative result of an experiment carried out with a fast-twitch fibre is presented in Fig. 1. Figure 1 A shows the force response developed by the skinning fibre when rapidly activated in 30 µM Ca^{2+}, 7 mM [τ-^{32}P]ATP (6 mM MgATP) and Fig. 1 B shows side by side the SDS-gel pattern of the myofibrillar proteins and the autoradiogram of the SDS-gel. As seen in Fig. 1 B (lane c), during maximal activation by Ca^{2+}, mechanically skinned fibres incorporate ^{32}P into one major electrophoretic species (M_r approx. 19,000) which has the same electrophoretic mobility as purified MLC2 (rabbit skeletal muscle) (Fig.1 B, lane a). A minor radioactive band ($M_r > 50{,}000$) of unknown identity was also observed occasionally (data not shown). Using the quantitation procedure described in Materials and methods it was estimated that about 40% of the MLC2 in the fibre shown in Fig. 1 was in the [^{32}P]-phosphorylated form.

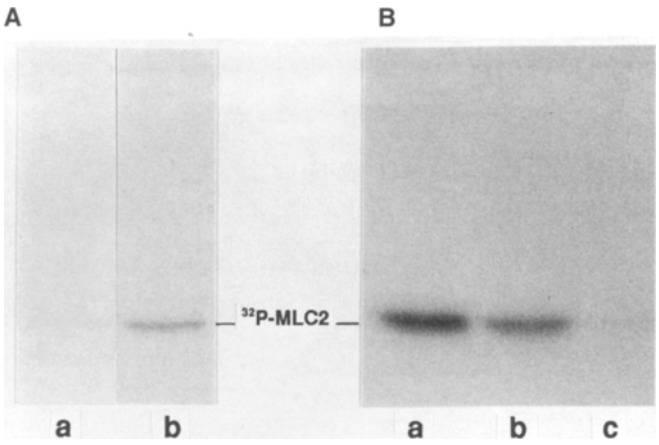
Results obtained with seven single, mechanically skinned fibres have shown that after 2 min of maximum contractile activation at pH 7.10 in solutions containing 7 mM ATP (6 mM MgATP) and 30 µM Ca^{2+}, 48.3 ± 7.3% ($n = 7$) of the MLC2 was in the [^{32}P]phosphorylated form. The level of endogenous MLC2 [^{32}P]phosphorylation was found to vary between fibres which otherwise displayed similar electrophoretic profiles of the myofibrillar proteins. Thus, the proportion of [^{32}P]MLC2 in some of the seven fibres studied was about 3.5-fold higher than in others (range 0.23–0.81 mol $^{32}P_i$/mol MLC2). Therefore, in most experiments, an individual fibre was further divided after skinning, into two or three segments, which were then used to examine the effect of changing various parameters on the endogenous phosphorylating capacity of skinned muscle fibres.

Table 1. The level of endogenous MLC2 phosphorylation in mechanically skinned muscle fibre segments pre-equilibrated in a relaxing solution (see text for experimental details and composition of solutions) expressed as % of MLC2 phosphorylation without pre-incubation

Pre-equilibration conditions	% [^{32}P]MLC2 [a]
5 s, [τ-^{32}P]ATP-relaxing solution	76.0 ± 10.6 ($n = 8$)
5 s, ATP-relaxing solution	73.2 ± 13.4 ($n = 8$)
No preincubation	100 ($n = 8$)

[a] [^{32}P]phosphorylation was carried out for 30 s at 22°C in 30 µM Ca^{2+}, 7 mM [τ-^{32}P]ATP (6 mM MgATP), pH 7.10 and the proportion of [^{32}P]MLC2 in skinned fibre segments was estimated as described in Materials and methods

To determine the time course of endogenous MLC2 [^{32}P]-phosphorylation, three segments from one fibre were activated for 30 s, 2 min and 10 min, respectively in the Ca^{2+}/^{32}P solution. In two different experiments, it was found that the average level of [^{32}P]MLC2 detected after 30 s and 10 min activation was 103% and 119%, respectively, of that observed after 2 min activation in the [^{32}P]phosphorylating solution. This indicates that MLC2 phosphorylation by the endogenous system occurs relatively fast during the rapid activation of a skinned fibre in 30 µM Ca^{2+}. At Ca^{2+} concentrations of 10 nM and lower, when the fibre was relaxed, less than 3% of MLC2 were in the [^{32}P]-phosphorylated form after 10 min incubation in the [τ-^{32}P]ATP-containing solution. This clearly shows that the endogenous MLC2 phosphorylation observed in mechanically skinned muscle fibres is Ca^{2+} dependent.



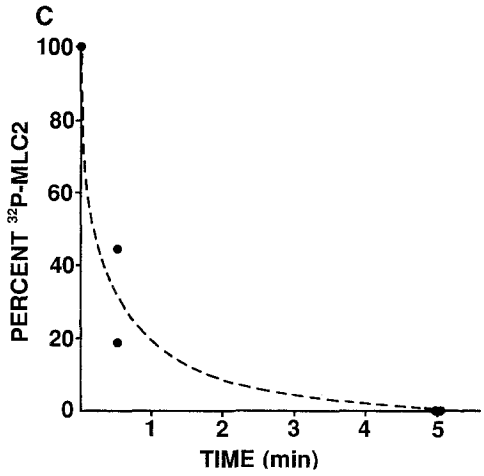


Fig. 2A–C. Effect of exogenous calmodulin on the endogenous MLC2 phosphorylation in skinned muscle fibre preparations. **A** Two fibres (3.4 and 4.3 nl) were washed for 5 min in a low-[Ca^{2+}] solution, before being phosphorylated in the absence (**a**) and in the presence (**b**) of calmodulin (5 μM). This result has been obtained with three different fibres. **B** The level of MLC2-bound radioactivity in three fibre segments phosphorylated in the presence of 5 μM calmodulin, after being washed for 0 min (**a**), 30 s (**b**) and 5 min (**c**). The volumes of the three segments, obtained from the same skinned fibre, were 1.7 nl, 2.0 nl and 2.6 nl, respectively. **C** The time course of the wash-induced decrease in endogenous MLC2 phosphorylating capacity of the fibre segments shown in **B** and from another fibre. The proportion of [^{32}P]MLC2 in the control (unwashed) segments of the two fibres was 67% and 42% respectively

Endogenous dephosphorylation of MLC2 [^{32}P]phosphorylated by the endogenous phosphorylating system in mechanically skinned muscle fibres

The amount of radioactivity bound to MLC2 during the 2-min activation of mechanically skinned muscle fibres was not increased in the presence of inhibitor 2, a specific inhibitor of type-1 phosphatases believed to dephosphorylate the MLC2 [24]. This result suggests that at 22°C no significant dephosphorylation of MLC2 takes place during the rapid activation of mechanically skinned muscle fibres in high [Ca^{2+}]/[^{32}P]ATP solutions. Also, the proportion of [^{32}P]MLC2 in two fibres segments (obtained from two different fibres), which were incubated in a relaxing solution for 5 min post-phosphorylation, was only marginally lower than that in their controls (average 88%). This observation suggests that only little dephosphorylation of [^{32}P]MLC2 occurs in fibres which are briefly washed in relaxing solution (≤5 min) post-phosphorylation.

Endogenous MLC2 phosphorylation in skinned fibres pre-equilibrated in a relaxing solution

The level of endogenous protein radiolabelling is usually increased by pre-equilibrating the intracellular ATP pool with extracellular ^{32}P [10]. To investigate the temporal relationship between endogenous MLC2 phosphorylation in mechanically skinned muscle fibres and the inward diffusion of [τ-^{32}P]ATP, a fibre was cut after skinning into three segments; segments 1 and 2 were incubated for 5 s in the pre-equilibrating containing radioactive (segment 1) or non-radioactive ATP (segment 2), before being phosphorylated as described in Materials and methods, while segment 3 (control) was transferred directly from oil to the phosphorylating solution. The composition of the pre-equilibrating solution was as follows (mM): K^+, 120; Na^+, 14; free Mg^{2+}, 1; ATP or [^{32}P]ATP, 7; MgATP, 6; $EGTA^{2-}$, 0.13 ([Ca^{2+}]<10 nM); $HDTA^{2-}$, 52.4; NaN_3, 0.9; HEPES, 52 (pH 7.10). Results obtained with eight fibres (diameters ranging between 23 and 43 μm) show (Table 1) that the values of MLC2-bound radioactivity in the fibre segments pre-equilibrated in solutions containing labelled or unlabelled ATP are similar and do not significantly differ from each other ($P > 0.1$, paired t-test). This finding indicates that in skinned muscle preparations, activated as described in Materials and methods, the endogenous phosphorylation of MLC2 starts only after the diffusion of radioactive ATP from the external activating solution into the middle of the skinned fibre, despite the rapid rise in [Ca^{2+}] (estimated to occur within a fraction of 1 s, [26]). Using a published value of the diffusion coefficient for ATP in a muscle fibre of $1.2 \cdot 10^{-10}$ m^2 s^{-1} [13], we estimated that in a typical skinned fibre segment of about 40 μm in diameter the radioactive ATP would reach 90% of the steady value in about 1.1 s. Results presented in Table 1 show also that a 5-s preincubation of the skinned muscle fibre preparations in low-[Ca^{2+}] solutions caused a 20−30% decrease in the MLC2-bound radioactivity. This finding suggests that important components of the endogenous phosphorylating system may have been "washed away" during the 5-s pre-equilibration step.

Effect of calmodulin on endogenous MLC2 phosphorylation in "washed" skinned fibres

The MLC2-bound radioactivity in unwashed fibres (i. e. fibres which were transferred directly from oil to the phosphorylating solution) was not significantly affected by the presence of exogenous calmodulin (5 μM) during

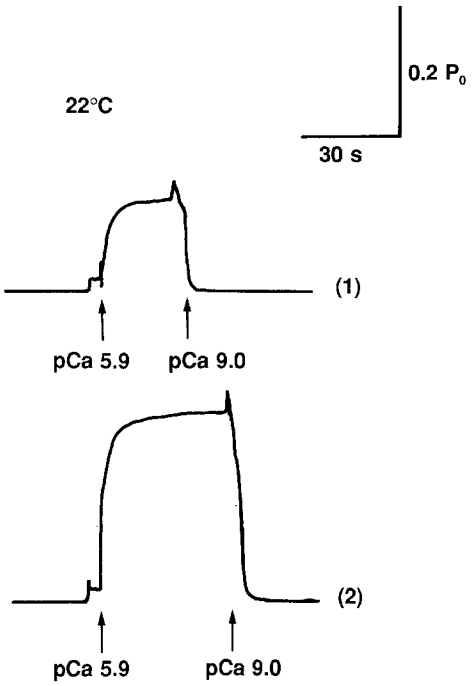


Fig. 3. The effect of endogenous MLC2 phosphorylation on the sensitivity to Ca^{2+} of mechanically skinned muscle fibres. After skinning, the fibre was cut into two segments. One segment was washed for 5 min in a relaxing solution, then was maximally activated for 30 s in a solution containing 30 μM Ca^{2+} and 7 mM ATP, and finally was activated at pCa 5.9 (washed fibre); the other segment was not washed, but was otherwise handled in an identical manner (unwashed fibre). Adjustments were made to ensure that the sarcomere lengths of the two fibre segments (measured by laser diffraction) were identical (2.7 μm). *Trace 1* shows the isometric force response developed by the washed fibre segment at pCa 5.9. *Trace 2* shows the isometric force response developed by the unwashed fibre segment at pCa 5.9. The magnitude of the sub-maximal force responses developed by unwashed and washed segments from this and six other fibres (processed identically) is given in Table 2. *Vertical calibration bar* corresponds to 20% of maximum force response (P_O) or 0.067 mN

Table 2. Effect of endogenous MLC2 phosphorylation on the isometric force response of mechanically skinned muscle fibre segments at a sub-maximal level of Ca^{2+} activation (pCa 5.9 for experiment 1 and pCa 6.18 for experiment 2).

Fibre	Relative force	
	Washed segment	Unwashed segment
	Experiment 1	
1	0.06	0.24
2	0.09	0.23
3	0.14	0.27
4	0.12	0.24
	Experiment 2	
5	0.28	0.54
6	0.19	0.63
7	0.23	0.42

The sarcomere lengths of paired fibre segments were carefully matched. Force is expressed as a fraction of maximum Ca^{2+}-activated force. Experimental details are given in the legend to Fig. 3 and in the text

the phosphorylating step (percentage incorporation 50.8 ± 5.7%, $n = 6$, with calmodulin versus 48.3 ± 7.3%, $n = 7$, without calmodulin). In contrast, as seen in Fig. 2 A, the MLC2-bound radioactivity in a fibre washed for 5 min in the pre-equilibrating/washing solution and phosphorylated in a Ca^{2+}/[^{32}P]phosphorylating solution containing 5 μM calmodulin (lane b) was higher than that in another fibre, of similar size, which was also washed for 5 min, but was phosphorylated in the absence of added calmodulin (lane a). This result, representative of three experiments, indicates that calmodulin is one of the components of the endogenous MLC2 phosphorylating system which diffuses out of the myofibrillar space during the brief washing of skinned fibres.

It should be pointed out, however, that the level of endogenous MLC2 phosphorylation in skinned fibres washed for 5 min was substantially lower than that in the unwashed controls, even when calmodulin was added to the phosphorylating solution. This can be seen from the results of an experiment in which we compared the level of MLC2-bound radioactivity in three skinned fibre segments, phosphorylated in the presence of 5 μM exogenous calmodulin without prior washing (segment a, control), or after being washed for 30 s (segment b) and 5 min (segment c) (Fig. 2 B). As shown in Fig. 2 B and C, despite the presence of calmodulin in the phosphorylating solution, the endogenous MLC2 phosphorylating capacity of the fibre segment washed for 30 s was markedly decreased compared with the control, and was almost undetectable in the fibre segment washed for 5 min. This result, obtained with two different fibres and additional data obtained with paired segments (controls versus "washed" for various lengths of time) from other four fibres, suggest that not only calmodulin but also MLCK diffuses out relatively quickly when mechanically skinned fibres are washed in an aqueous solution.

We have used a published curve describing the course of desorption of a solute from a cylinder into the surrounding solution [6], the measured values for the percentage of endogenous MLC2 phosphorylating activity lost by skinned fibres after various washing times, and the diameters of individual fibre segments to estimate the apparent outward diffusion coefficient for the endogenous phosphorylating system in the absence and in the presence of added calmodulin. These values are: $5.75 \pm 1.81 \cdot 10^{-12}$ m^2/s ($n = 8$) (no added calmodulin) and $12.0 \pm 3.7 \cdot 10^{-13}$ m^2/s ($n = 6$) (with added calmodulin) and are significantly different from each other ($P < 0.001$, t-test).

Effect of endogenous MLC2 phosphorylation on the steady-state isometric tension

Results presented in Fig. 3 and Table 2 show that the Ca^{2+} sensitivity of the contractile apparatus in skinned fibre segments, which have lost their endogenous phosphorylating capacity prior to exposure to the maximal Ca^{2+}-activating (30 μM Ca^{2+}, 7 mM ATP, 6 mM MgATP) solution, was lower than that in skinned fibres

Table 3. Effects of [ATP] and [Ca^{2+}] on endogenous MLC2 phosphorylation and Ca^{2+}-activated force in paired segments of mechanically skinned muscle fibres.

	Tension	Ratio of MLC2-bound radioactivity at 0.8 μM Ca^{2+} to that at 30 μM Ca^{2+}	Ratio of force at 0.8 μM Ca^{2+} to that at 30 μM Ca^{2+}
[ATP] = 3.5 mM [a]	Isometric (n = 6)	1.24 ± 0.13	0.62 ± 0.06
	Unloaded (n = 6)	2.09 ± 0.11	n. a.
[ATP] = 1.8 mM [a]	Isometric (n = 6)	1.82 ± 0.22	0.73 ± 0.05
	Unloaded (n = 6)	3.02 ± 0.73	n. a.

Segments from the same fibre were activated and [^{32}P]phosphorylated for 2 min in solutions containing the indicated concentrations of [Ca^{2+}] and [τ-^{32}P]ATP. Myofibrillar proteins were resolved by SDS-gel electrophoresis, and the MLC2-bound radioactivity was measured densitometrically and then was expressed per fibre segment volume.

[a] In these solutions, the concentrations of MgATP and $HDTA^{2-}$ were 3 mM and 12.5 mM respectively at 3.5 mM ATP, and 1.5 mM and 14.2 mM respectively at 1.8 mM ATP. The concentration of other ionic species was the same as in the standard activating solution (see Materials and methods)

which had an operational MLC2 phosphorylating system. In this experiment, half of each skinned fibre was phosphorylated immediately after skinning (unwashed fibre segment) in maximal Ca^{2+}-activating solution, while the other half was bathed for 5 min in a relaxing solution prior to exposure to the high-Ca^{2+} solution (washed fibre segment). Each of the two segments was then sub-optimally activated and their isometric force responses were recorded and compared. As shown in Fig. 3 and Table 2, unwashed fibres developed considerably more force than their washed counterparts when sub-maximally activated. In all these experiments, the loss of endogenous phosphorylating activity caused by the washing of mechanically skinned fibres had no effect on the maximal force response.

Effect of high [Ca^{2+}] on endogenous MLC2 phosphorylation in skinned fibres at low [ATP]

In a contracting muscle fibre containing an operational enzyme system for MLC2 phosphorylation, both the actomyosin ATPase and the MLCK are controlled by Ca^{2+} and depend on [MgATP]. The interplay between the two enzymes, [Ca^{2+}], and [MgATP] has been the object of a series of experiments, the results of which are presented in Table 3. Paired experiments with fibre segments activated isometrically at 30 μM Ca^{2+} and 3.0 or 6.0 mM MgATP, showed that the level of MLC2-bound radioactivity at 3.0 mM MgATP was 75.8 ± 16.7% (n = 5) of that at 6.0 mM [MgATP], while the force responses were not significantly different. At 3.0 mM MgATP, a decrease in [Ca^{2+}] from 30 μM to 0.8 μM caused on average a 38% decrease in the isometric force response but increased by 24% the MLC2 [^{32}P]-phosphorylation by the endogenous enzyme system. The percentage $^{32}P_i$-MLC2 in fibre segments contracted isometrically at 0.8 μM Ca^{2+} and 3.0 mM MgATP was 47.0 ± 5.7% (n = 4), which was similar to the corresponding value obtained at 30 μM Ca^{2+} and 6 mM MgATP (48.3 ± 7.3%, n = 7). When [ATP] was lowered to 1.8 mM ([MgATP], 1.5 mM) the same change in [Ca^{2+}] (i. e. a drop from 30 to 0.8 μM) caused, again, a 27% decrease in the force developed by the fibre, but this decrease was accompanied by an 80% increase in the MLC2-bound radioactivity. The percentage $^{32}P_i$ incorporation in MLC2 of fibre segments contracted isometrically at 0.8 μM Ca^{2+} and 1.5 mM MgATP was 12.6 ± 1.6% (n = 3), which is about 4 times lower than the corresponding value at 3.0 mM MgATP. The Ca^{2+}-induced change in the level of endogenous MLC2 phosphorylation was even more marked in free shortening (unloaded) fibres both at 3.0 and 1.5 mM MgATP, suggesting that the process responsible for lowering the MLC2-bound radioactivity at high [Ca^{2+}] and low [MgATP] is the same in unloaded and isometrically contracted fibres. The values for the [^{32}P]MLC2 incorporation in the unloaded fibre segments activated at 0.8 μM Ca^{2+} were 35.3 ± 3.1% (n = 6) at 3.0 mM MgATP and 13.2 ± 0.6% (n = 4) at 1.5 mM MgATP.

Table 4. The effect of [Ca^{2+}] on endogenous MLC2 phosphorylation, at low [ATP] (1.8 mM), in skinned muscle fibre segments is sarcomere length (SL)-dependent

	R [a]
SL ≈ 2.6–2.7 μm	
Fibre 1	0.95
Fibre 2	0.94
Fibre 3	0.72
SL ≈ 4.2–4.3 μm	
Fibre 4	3.50
Fibre 5	8.30
Fibre 6	1.94

[a] R = [MLC2-bound radioactivity at 4 μM Ca^{2+} in segment 1 (ADU/nl)]/[MLC2-bound radioactivity at 0.8 μM Ca^{2+} in segment 2 (ADU/nl)]

Segments 1 and 2 were obtained from the same skinned fibre

Endogenous MLC2 phosphorylation in skinned fibres at long sarcomere length, high [Ca^{2+}] and low [ATP]

The inhibitory effect of high [Ca^{2+}] on endogenous MLC2 phosphorylation at low [ATP] may be due to the rapid depletion of the available [τ-^{32}P]ATP pool by the Ca^{2+}-activated myofibrillar ATPase. To further investi-

gate this possibility, we have determined the effect of high [Ca^{2+}] on endogenous MLC2 phosphorylation at low [ATP] in mechanically skinned muscle fibres which had been stretched to an average sarcomere length of approximately 4.2−4.3 μm (beyond the filament overlap), prior to activation in the Ca^{2+}/[^{32}P]ATP containing solution. Stretching muscle fibres to such sarcomere length has been shown previously [27] to decrease substantially their myofibrillar ATPase.

At 1.8 mM ATP (1.5 mM MgATP), 2.6−2.7 μm average sarcomere length and 0.8 μM [Ca^{2+}], the percentage [^{32}P]MLC2 incorporation was 9.1 ± 1.9% (n = 3), which is about 5 times lower than that measured under optimal conditions (MgATP, 6; Ca^{2+}, 30 μM). An increase in [Ca^{2+}] from 0.8 to 4 μM without changing other parameters caused a slight decrease in MLC2-bound radioactivity (ratio MLC2-bound radioactivity at 4 μM Ca^{2+} in segment 1/MLC2-bound radioactivity at 0.8 μM Ca^{2+} in segment 2 of the same fibre = 0.87 ± 0.08, n = 3).

However, in stretched fibres (average sarcomere length 4.2−4.3 μm) an increase in [Ca^{2+}] from 0.8 μM to 4 μM at 1.8 mM MgATP caused a large increase in MLC2-bound radioactivity (ratio MLC2-bound radioactivity at 4 μM Ca^{2+} in stretched segment 1/MLC2-bound radioactivity at 0.8 μM Ca^{2+} in stretched segment 2 from the same fibre = 4.58 ± 1.91, n = 3), strongly suggesting that myofibrillar ATPase interferes markedly with the MLC2 phosphorylation at high levels of force activation when [ATP] in the bathing solutions was 1.8 mM. An additional experiment with two segments from the same fibre further supports this suggestion. In this experiment the stretched fibre segment (average sarcomere length 4.2−4.3 μm) activated at 4 μM Ca^{2+} and 1.8 mM ATP had a 2.8-fold higher percentage [$^{32}P_i$]MLC2 incorporation than its paired segment (average sarcomere length 2.6−2.7 μm), which was activated under the same conditions. No significant difference in MLC2-bound radioactivity was, however, observed between two fibre segments (one stretched and one non-stretched) which were activated/[^{32}P]phosphorylated at 7 mM ATP and 30 μM Ca^{2+}.

Taken together, the aforementioned results indicate that: 1. At 1.8 mM [ATP] and relatively high [Ca^{2+}] (≥4 μM), the Ca^{2+}-activated myofibrillar ATPase depletes rapidly the available [τ-^{32}P]ATP pool, thereby greatly reducing the activity of MLCK. 2. At 7 mM [ATP] the influence of the myofibrillar ATPase on MLCK activity is rather small. This information is important when selecting the optimum [^{32}P]ATP concentration in the solutions used to study endogenous MLC2 phosphorylation in mechanically skinned muscle fibres.

Discussion

This is the first study of endogenous protein phosphorylation in permeabilized skeletal muscle fibres. Single fibres of the rat fast-twitch muscle (EDL), carefully dissected under paraffin oil, were shown to incorporate a radioactive label in one major protein species (identified electrophoretically as the regulatory myosin light chain) while isometrically activated in solutions containing [τ-^{32}P]ATP and Ca^{2+}.

No significant (less than 3%) [^{32}P]phosphorylation of MLC2 could be detected at low [Ca^{2+}] (10 nM). Furthermore, the level of MLC2 [^{32}P]phosphorylation decreased rapidly when fibres were washed briefly in a low-[Ca^{2+}] solution prior to the [^{32}P]phosphorylation step, but was partly restored when calmodulin was included in the phosphorylating solution. These data strongly suggest: (1) that the endogenous phosphorylation of MLC2 in mechanically skinned muscle fibres rapidly activated by Ca^{2+} is due to the Ca^{2+}/calmodulin-dependent MLCK, the highly specific enzyme known to be present and active in skeletal muscle [22], and (2) that the endogenous calmodulin diffuses out rapidly when the skinned fibres are washed in aqueous solutions. The calmodulin dependence of the endogenous MLC2 phosphorylating system indicates also that mechanical skinning does not trigger proteolytic events, since proteolytic digestion has been shown [7] to render MLCK calmodulin independent.

The apparent diffusion coefficient for the endogenous MLC2 phosphorylating system (i. e. without added calmodulin) was estimated to be about $5.75 \cdot 10^{-12}$ m^2/s. Since this value is similar to that of the diffusion coefficient for aequorin [1], a Ca^{2+} luminescent protein whose molecular weight is twice as high as that of calmodulin, it suggests that in rat skeletal muscle, calmodulin is bound weakly to various myofibrillar proteins.

It has been reported that MLC2 [^{32}P]phosphorylated in vitro by purified MLCK is dephosphorylated by a type-1 myosin-bound phosphatase activity [4]. The inclusion of inhibitor-2 in the phosphorylating solution was not found to increase convincingly the level of MLC2 [^{32}P]phosphorylation in mechanically skinned muscle fibres, during the 2-min activation/[^{32}P]phosphorylation at 22 °C. Also, the level of MLC2-bound radioactivity did not decrease appreciably after 5 min incubation of the [^{32}P]phosphorylated fibre preparations in relaxing solutions. Our inability to detect endogenous MLC2 dephosphorylation may be due to the documented low MLC2 phosphatase activity in fast skeletal muscle at 22 °C [17, 30]. Alternatively, a rapid diffusional loss of phosphatase activity may have taken place during the incubation of the preparation in the aqueous solution.

Prolonged incubation (≥5 min) of mechanically skinned muscle fibres in a relaxing solution prior to the phosphorylating step practically abolished their endogenous MLC2 phosphorylating capacity, and this effect was only partially reversed by adding excess calmodulin to the phosphorylating solution. This finding suggests that, like calmodulin, MLCK also diffuses away from the substrate (MLC2) during the washing of skinned fibres. This result is not surprising if one assumes that MLCK is located either in the cytoplasm or is distributed between the cytoplasm and myofibrils, being only weakly associated with the filament structures. Such an assumption is in agreement with the intracelluar location of MLCK proposed by Stull et al. [31] and Cavadore et al. [3]. The diffusion coefficient for the MLC2 phos-

phorylating system with added calmodulin was estimated to be about $1.20 \cdot 10^{-12}$ m^2/s, a value consistent with the larger molecular weight of MLCK (approx. 70 kDa) [31] compared with calmodulin (17 kDa) [2] and with the weak binding of MLCK to the myofibrillar structure.

Moore and Stull [16] have shown previously that repetitive neural stimulation of fast glycolytic (FG) and fast oxidative-glycolytic (FOG) muscles at 37°C is accompanied by an increase in the MLC2 phosphate content from 0.12 mol P_i/mol MLC2 to 0.59 mol P_i/mol MLC2 (FG) and from 0.02 mol P_i/mol MLC2 to 0.22 mol P_i/mol MLC2 (FOG). We estimated that, under conditions of maximal Ca^{2+} activation (7 mM ATP, 30 μM Ca^{2+}), mechanically skinned fibres examined in the present study incorporated between 0.23 and 0.81 mol $^{32}P_i$/mol MLC2. All these fibres were of the fast-twitch type, as shown by the electrophoretic pattern of their myofibrillar proteins, but they were not identified in terms of their metabolic properties. Thus, we can only suggest that the fibre-linked differences in endogenous phosphorylating capacity observed by us may be due, in part, to the different MLCK activities reported for FG and FOG fibres by Moore and Stull [16]. However, one should bear in mind that errors in estimating fibre volume could have also contributed to the scatter of the results.

In vivo, the tetanus associated MLC2 phosphorylation peaks at 10−20 s after stimulation [16]. It appears that the endogenous protein phosphorylating system associated with mechanically skinned muscle fibres acts also moderately fast. Indeed, if the MLC2 phosphorylation would have taken place immediately after the influx of Ca^{2+} ions into the interfibrillar space (approx. 0.15 s, [26] and before the equilibration of the intracellular ATP pool with the external [τ-^{23}P]ATP, then no MLC2-bound radioactivity should have been detected. Moreover, the level of MLC2 [^{32}P]-phosphorylation should have been increased by pre-equilibration of the skinned muscle fibre preparations with labelled ATP prior to the phosphorylation step, but this was not found to be the case. Taken together, these data suggest that the endogenous MLCK activity operating in mechanically skinned muscle fibre preparations compares well functionally with the MLCK activity in intact muscle.

At high [Ca^{2+}] (30 μM), a decrease in [ATP] from 7 to 1.8 mM caused a substantial drop in the level of endogenous MLC2 [^{32}P]phosphorylation, without decreasing the isometric force response. The simplest explanation for this finding is that at high [Ca^{2+}], the Ca^{2+}-dependent myofibrillar MgATPase in the skinned fibre (about 0.5 mM ATP/s, when expressed per unit volume of fibre) [27] depletes rapidly the available myofibrillar [τ-^{32}P]ATP pool. This explanation is supported by our experiments in which mechanically skinned fibres were stretched beyond filament overlap, a strategy known to decrease markedly the actomyosin MgATPase activity [27]. This strategy abolished the high-[Ca^{2+}]-induced decline in MLC2 phosphorylation observed at low [ATP]. The inhibitory effect of [Ca^{2+}] at 1.8 mM ATP was more pronounced in fibres allowed to shorten freely than in isometrically contracting fibres. This was to be expected because the rate of ATPase activity is higher in fast-twitch rat fibres which are allowed to shorten to very short sarcomere lengths [27] and because at very short sarcomere lengths the fibre diameter increases considerably, thus amplyfing effects caused by diffusional processes.

A decrease in [MgATP] in the bathing solution from 6 mM to 3 mM did not produce a significant change in the percentage of [^{32}P]MLC2 incorporation at optimal [Ca^{2+}] (48.3 ± 7.3% (n = 7) at 6 mM ATP versus 47.0 ± 5.7% (n = 4) at 3.0 mM ATP) which means that the dissociation constant of the endogenous MLCK for MgATP must be at least 10-fold lower than 3.0 mM (i. e. <300 μM). Such a K_d value is also consistent with the marked effect caused by stretch at high levels of Ca^{2+} activation in solutions of low [ATP] (1.8 mM). Similar values for K_d^{MgATP} have been reported for the purified skeletal muscle MLCK [14, 19].

Earlier studies using intact skeletal muscle fibres have established a positive correlation between post-tetanic potentiation of isometric twitch and MLC2 phosphorylation (for review, see [31]). This correlation has been strengthened by several reports that in chemically skinned fibres, MLC2 phosphorylation by exogenous MLCK and calmodulin increases isometric force responses at submaximal levels of Ca^{2+} activation [15, 21, 32]. However, thiophosphorylation of MLC2 in chemically skinned fibres was not accompanied by any clear change in the force sensitivity to Ca^{2+} [12]. In this study we showed unequivocally that rapid phosphorylation of MLC2 by the endogenous enzyme system markedly increased the magnitude of the force response developed by mechanically skinned muscle fibres activated submaximally. This finding also confirms that the level of endogenous MLC2 phosphorylation obtained in these fibres is sufficient to markedly alter their contractile properties.

In summary, this study has presented convincing evidence that mechanically skinned muscle fibre preparation can be used to gain further insight into the regulation of skeletal muscle MLCK and into the role of MLC2 phosphorylation in muscle contraction.

Acknowledgement. We thank Mrs. R. Cafarella and the Reprography Unit at La Trobe University for technical assistance and NH & MRC (Australia) for financial support.

References

1. Ashley CC, Moisescu DG (1975) The part played by Ca^{2+} in the contraction of isolated bundles of myofibrils. In: Carafoli E, et al. (eds) Calcium transport in contraction and secretion. North Holland, Amsterdam, pp 517−525
2. Blumenthal DK, Stull JT (1980) Activation of skeletal muscle myosin light chain kinase by calcium (2+) and calmodulin. Biochemistry 19:5608−5614
3. Cavadore JC, Molla A, Harricane M-C, Gabrion J, Benyamin Y, Demaille JG (1982) Subcellular localization of myosin light chain kinase in skeletal, cardiac, and smooth muscles. Proc Natl Acad Sci USA 79:3475−3479
4. Chisholm AAK, Cohen P (1988) The myosin-bound form of protein phosphatase 1 (PP-1_M) is the enzyme that dephos-

phorylates native myosin in skeletal and cardiac muscles. Biochim Biophys Acta 971:163–169
5. Conti MA, Adelstein RS (1991) Purification and properties of myosin light chain kinases. Methods Enzymol 196:34–47
6. Crank J (1967) The mathematics of diffusion. Clarendon Press, Oxford
7. Edelman AM, Takio K, Blumenthal DK, Hansen RS, Walsh KA, Titani K, Krebs EG (1985) Characterization of the calmodulin-binding and catalytic domains in skeletal muscle myosin light chain kinase. J Biol Chem 260:11 275–11 285
8. Edelman AM, Blumenthal DK, Krebs EG (1987) Protein serine/threonine kinases. Annu Rev Biochem 56:576–613
9. England P (1980) Protein phosphorylation in the regulation of muscle contraction. In: Cohen P (ed) Molecular aspects of cellular regulation, vol 1. Elsevier, Amsterdam, pp 153–173
10. Franco R, Rosenfeld MG (1990) Hormonally inducible phosphorylation of a nuclear pool of ribosomal protein S6. J Biol Chem 265:4321–4325
11. Giulian GG, Moss RL, Greaser ML (1983) Improved methodology for analysis and quantitation of proteins on one-dimensional silver-stained slab gels. Anal Biochem 129:277–287
12. Godt RE, Nosek TM (1989) Changes of intracellular milieu with fatigue or hypoxia depress contraction of skinned rabbit skeletal and cardiac muscle. J Physiol (Lond) 412:155–180
13. Kushmerick MJ, Podolsky RJ (1969) Ionic mobility in muscle cells. Science 166:1297–1298
14. Mayr GW, Heilmeyer MG (1983) Shape and substructure of skeletal muscle myosin light chain kinase. Biochemistry 22:4316–4326
15. Metzger JM, Greaser ML, Moss RL (1989) Variations in cross-bridge attachment rate and tension with phosphorylation of myosin in mammalian skinned skeletal muscle fibres. J Gen Physiol 93:855–883
16. Moore R, Stull JT (1984) Myosin light chain phosphorylation in fast and slow skeletal muscles in situ. Am J Physiol 247:C462–C471
17. Moore RL, Palmer BM, Williams SL, Tanabe H, Grange RW, Houston ME (1990) Effect of temperature on myosin phosphorylation in mouse skeletal muscle. A J Physiol 259:C432–C438
18. Natori R (1954) The property and contraction process of isolated myofibrils. Jikeika Med J 1:119–126
19. Nunnaly MH, Rybicki SB, Stull JT (1985) Characterization of chicken skeletal muscle myosin light chain kinase. J Biol Chem 260:1020–1026
20. Payne MR, Rudnick SE (1989) Regulation of vertebrate striated muscle contraction. Trends Biochem Sci 14:357–360
21. Persechini A, Stull JT, Cooke R (1985) The effect of myosin phosphorylation on the contractile properties of skinned rabbit skeletal muscle fibres. J Biol Chem 260:7951–7954
22. Pires EM, Perry SV (1977) Purification and properties of myosin light-chain kinase from skeletal muscle. Biochem J 167:137–146
23. Salviati G, Betto R, Danielli Betto D (1982) Polymorphism of myofibrillar proteins in rabbit skeletal-muscle fibres. Biochem J 207:261–272
24. Shenolikar S, Nairn AC (1991) Protein phosphatases: recent progress. Adv Second Messengers Phosphoprotein Res 23:3–121
25. Stephenson DG (1988) Ca^{2+}-activation of contraction in skeletal and cardiac muscle. Proc Austr Physiol Pharmacol Soc 19:108–122
26. Stephenson DG, Williams DA (1981) Calcium-activated force responses in fast and slow-twitch skinned muscle fibres of the rat at different temperatures. J Physiol (Lond) 317:281–302
27. Stephenson DG, Stewart AW, Wilson GJ (1989) Dissociation of force from myofibrillar MgATPase and stiffness at short sarcomere lengths in rat and toad skeletal muscle. J Physiol (Lond) 410:351–366
28. Stephenson GMM (1993) Studies of myosin light chain phosphorylation by the endogenous enzyme system in mechanically skinned muscle fibres of the rat. J Physiol (Lond) 459:17P
29. Stephenson GMM, Stephenson DG (1991) Simultaneous measurement of protein phosphorylation and contractile activity in mechanically skinned skeletal muscle fibres of the rat. Proc Austr Physiol Pharmacol Soc 22:70P
30. Stull JT, Sanford CF, Manning DR, Blumenthal D, High C (1981) Phosphorylation of myofibrillar proteins in striated muscles. Cold Spring Harbor Conf Cell Proliferation (Protein Phosphorylation) 18:823–839
31. Stull JT, Nunnaly MH, Moore RL, Blumenthal DK (1985) Myosin light chain kinases and myosin phosphorylation in skeletal muscle. Adv Enzyme Regul 23:123–140
32. Sweeney HL, Stull JT (1990) Alteration of cross-bridge kinetics by myosin light chain phosphorylation in rabbit skeletal muscle: Implications for regulation of actin-myosin interaction. Proc Natl Acad Sci USA 87:414–418
33. Takio K, Blumenthal DK, Walsh KA, Titani K, Krebs EG (1986) Amino acid sequence of rabbit skeletal muscle myosin light chain kinase. Biochemistry 25:8049–8057
34. Yates LD, Greaser ML (1983) Quantitative determination of myosin and actin in rabbit skeletal muscle. J Mol Biol 168:123–141