Phosphorylation *in vivo* of the P light chain of myosin in rabbit fast and slow skeletal muscles

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1. The P light chain of myosin is partially phosphorylated in resting slow and fast twitch skeletal muscles of the rabbit *in vivo*. The extent of P light-chain phosphorylation increases in both muscles on stimulation. 2. Rabbit slow-twitch muscles contain two forms of the P light chain that migrate with the same electrophoretic mobilities as the two forms of P light chain in rabbit ventricular muscle. 3. The rate of phosphorylation of the P light chain in slow-twitch muscle is slower than its rate of phosphorylation in fast-twitch muscles during tetanus. 4. The rate of P light-chain dephosphorylation is slow after tetanic contraction of fast-twitch muscles *in vivo*. The time course of dephosphorylation does not correlate with the decline of post-tetanic potentiation of peak twitch tension in rabbit fast-twitch muscles. 5. The frequency of stimulation is an important factor in determining the extent of P light-chain phosphorylation in fast- and slow-twitch muscles.

Phosphorylation of the P {regulatory or DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)]} light chain of myosin from skeletal muscle occurs by the action of the highly specific enzyme myosin light-chain kinase (Pires & Perry, 1977). The enzyme requires calmodulin as an essential cofactor and is activated at Ca^{2+} concentrations similar to those that initiate contraction (Yagi *et al.*, 1978; Nairn & Perry, 1979). Although stimulation leads to light-chain phosphorylation, it is very unlikely in striated muscle that a phosphorylation–dephosphorylation cycle could occur synchronously with the cross-bridge cycle (Perry *et al.*, 1978). In fast skeletal muscle, the striated muscle in the rabbit with the highest myosin light-chain kinase activity, the activity per unit weight of tissue is only 1 or 2% of that of the Mg^{2+} -stimulated myofibrillar ATPase, and the light-chain phosphatase is much less active than the kinase. These facts, coupled with the highly specific nature of the two enzymes and their widespread distribution, imply that the role of phosphorylation of the P light chain in striated muscle is to modulate the contractile response rather than constituting an essential step in excitation–contraction coupling.

In resting skeletal muscle the P light chain is partially phosphorylated, although values reported in

the literature range from 10 to 50% (Barany & Barany, 1977; Stull & High, 1977; Barany *et al.*, 1979; Manning & Stull, 1979; Barany *et al.*, 1982; Kushmerick & Crow, 1982). There are several reports (Stull & High, 1977; Manning & Stull, 1979) that stimulation of fast skeletal muscle increases the phosphorylation up to close to 100% if prolonged, although Barany *et al.* (1982) found in chicken muscle that phosphorylation did not exceed 50% even after prolonged isometric contraction. The variation between the values for P-light-chain phosphorylation in apparently similar experimental conditions reported by different investigators may reflect muscle and species differences and whether the muscle is stimulated *in situ* or *in vitro*.

Manning & Stull (1979) consider that light-chain phosphorylation plays a role in post-tetanic potentiation in fast skeletal rat muscle, whereas Kushmerick & Crow (1982) suggest that it is responsible for the decreased velocity of shortening that occurs after continued activity in mouse fast-twitch muscle. On the other hand, Butler *et al.* (1983) do not report a correlation between P-light-chain phosphorylation and the velocity of shortening in this muscle.

Both of these physiological properties would be expected to be reflected in the effect of phosphorylation on the enzymic behaviour of Mg^{2+} -dependent ATPase of isolated actomyosin. Unfortu-

Abbreviations used: TA, tibialis anterior; EDL, extensor digitorum longus.

nately, here also the results are somewhat equivocal. At low ionic strength, slight or no activation of the ATPase on phosphorylation of the myosin has been reported (Morgan *et al.*, 1976; Cooke *et al.*, 1981), but Pemrick (1980) obtained much higher activation under certain conditions. On the other hand, it has been reported that, if myofibrils are lightly cross-linked with glutaraldehyde, phosphorylation of the P light chain leads to a fall in $V_{\max}$ (Cooke *et al.*, 1982). This would correlate well with the fall in maximum velocity of shortening observed after tetanus in mouse fast-twitch muscle (Kushmerick & Crow, 1982).

These somewhat inconsistent findings may be a consequence of the differences in the prior treatment of the muscle and accurately measuring the extent of P-light-chain phosphorylation. The effect of stimulation *in situ* on the phosphorylation of the P light chain of fast and slow skeletal muscles of the rabbit *in vivo* has therefore been undertaken by using the high resolution of two-dimensional electrophoresis and procedures that avoid breakdown of P light chain during analysis. Some of the findings have been briefly reported elsewhere (Westwood *et al.*, 1982).

Methods

Preparation of muscles and stimulation

Experiments were performed on New Zealand White rabbits of either sex (2.5–3.5 kg body wt.) under fluothane anaesthesia. Drills inserted into the lower (distal) part of the femur and tibia ensured good fixation for recording of isometric tension of either fast (TA and EDL), or slow (soleus) muscles whose tendons were cut and connected to an Ether strain-gauge Dynamometer type UF2 (Pye Ether, Stevenage, Herts., U.K.). The tension was recorded on a Devices recorder. In all cases at least 45 min elapsed after anaesthesia before stimulation or resting extents of phosphorylation were determined. All muscles were stimulated via their nerves, and the optimal length was set with about five consecutive twitches. The period between the adjustment and the beginning of stimulation was about 10 min. Muscles were stimulated for different periods of time to produce either unfused tetanic contractions (60 Hz for fast, 30 Hz for slow muscles) or at different frequencies to produce single twitches, by using 0.1 ms pulse width and supramaximal voltage (usually 3–5 V), with a Grass S8 stimulator.

Preparation of muscle extracts for determination of P light-chain phosphorylation

Muscles were frozen *in situ* at the times and under the conditions described in the text by the application of large Wollenberg clamps previously

stored in liquid N₂. The tendons of the muscles frozen in the clamps were cut and the muscles immediately transferred to liquid N₂. Within 2 h approx. 3–5 g of frozen muscle was rapidly homogenized in 20 ml of ice-cold 15% (w/v) trichloroacetic acid at 0°C in a small high-speed Waring blender-type homogenizer. The precipitation with trichloroacetic acid was essential to destroy endogenous protein phosphatase activity (Westwood & Perry, 1981). The precipitated protein was dissolved in urea, and the solution was neutralized and prepared for two-dimensional electrophoresis by the procedure of Anderson & Anderson (1978a,b) as described by Westwood & Perry (1981).

The extent of phosphorylation of the P light chain was determined from two-dimensional gels by scanning with a densitometer and cutting out and weighing the traces (Westwood & Perry, 1981).

Results

Two-dimensional electrophoresis of skeletal-muscle extracts

When extracts prepared from the samples of resting skeletal muscle were examined by two-dimensional electrophoresis in a restricted gradient of pH 4–6, a good separation of the phosphorylated and non-phosphorylated forms of the P light chain of myosin was obtained (Fig. 1). The location of the spots corresponding to the P light chains was established by their mobilities in relation to other spots corresponding to known proteins on the two-dimensional electrophoretogram and by comparison with those observed on electrophoresis of samples of rabbit skeletal myosin that had been phosphorylated *in vitro* with myosin light-chain kinase (Westwood & Perry, 1981). In early control experiments, the muscles were removed separately for P-light-chain analysis without the surgery required to mount them for recording and without the initial stimulation required to set the optimal length. The values obtained for the individual EDL and TA muscles were not significantly different from those obtained when the EDL and TA muscles were frozen together. For most of the experiments the muscles were frozen together to speed up the procedure and to avoid any unnecessary manipulation of the two muscles that might lead to stimulation.

Whereas only two spots corresponding to the P light chain and its phosphorylated form were observed with the EDL/TA muscle extracts, with soleus muscle four spots were invariably present (Fig. 1). In resting soleus muscle the two most basic spots represented the major forms of the P light chain, and the two fainter, more acidic, spots rep-

Fig. 1. Two-dimensional electrophoresis of the light chains of myosin in resting and stimulated rabbit skeletal muscles. The Figure shows regions of the two-dimensional electrophoretogram, obtained as described in the Materials and methods section, that contain the myosin light chains. They represent regions containing proteins with M_r in the range 15000–30000 and with pI values in the range 4.8–5.5. The P light chains are enclosed in dashed ellipsoids, and arrows indicate the phosphorylated forms of the P light chains. P1 and P2 are the two forms of the P light chains in soleus, and P3 and P4 are respectively their phosphorylated derivatives. (a) Resting EDL/TA muscles; (b) EDL/TA muscles after 13 s at 60 Hz; (c) resting soleus muscle; (d) soleus muscle after 24 s at 30 Hz.

resented less than 20% of the total P light-chain fraction. The two more acidic spots were presumed to represent the phosphorylated forms by the similarity of the P-light-chain pattern of soleus muscle to that of rabbit heart, in which tissue the P light chain exists in two isotypic forms. This view was confirmed by the fact that the amounts of the two acidic forms increased at the expense of the basic forms on stimulation (Fig. 1). When rabbit cardiac extracts were added to the soleus-muscle extracts, the cardiac P light chains P1, P2, P3 and P4 (Westwood & Perry, 1981, 1982) migrated with mobilities identical with those of the four P light-chain spots present in the soleus-muscle extracts. A similar nomenclature to that employed for the heart was therefore adopted for the P light chain in soleus muscle (Fig. 1). In adult rabbit soleus muscle the P2 light chain and its phosphorylated form P4 represented $43 \pm 0.8\%$ (S.E.M., $n = 17$) of the total light-chain fraction.

The results in Table 1 indicate that the extent of phosphorylation of the P light chain of myosin in resting fast-twitch muscles was significantly higher than in the soleus. As the extent of phosphorylation roughly paralleled the myosin light-chain kinase activities of the two types of muscle, it was considered that the difference might be caused in part by the activation of the myosin light-chain kinase owing to a rise in sarcoplasmic Ca^{2+} concentration as a consequence of the freeze-clamping proce-

Table 1. Extents of phosphorylation of the P light chains of myosin in resting rabbit striated muscle *in vivo*

Results are percentages, expressed as means $\pm$ S.E.M. for the numbers of different experiments shown in parentheses. Values for soleus and cardiac muscles include values for both forms of the P light chain.

Muscle	Type	Phosphorylation (%)
Skeletal		
Combined EDL/TA	Fast	50.4 ± 3.4 (11)
Rectus abdominis	Fast	54.8 ± 2.6 (4)
Soleus	Slow	17.2 ± 1.0 (6)
Cardiac*		23.0 ± 3.0 (4)

* Normal beating heart in the anaesthetized animal (Westwood & Perry, 1981).

dures. This effect must be small, as clamps chilled in liquid N_2 were applied to the intact muscle before removal from the animal and the frozen muscle was immediately immersed and stored in liquid N_2 before processing for electrophoresis.

Effect of the duration of tetanus on the phosphorylation of the P light chain in rabbit fast-twitch and slow-twitch skeletal muscles

In combined EDL and TA muscles the maximum extent of phosphorylation (98%) was reached after 5 s tetanus at 60 Hz (Fig. 2b). This extent of phosphorylation was maintained if the tetanus was prolonged to 50 s. The maximum extent of P-light-

Fig. 2. Effect of duration of stimulus on the phosphorylation of the P light chain of rabbit skeletal muscles. Muscles were stimulated isometrically and freeze-clamped at times indicated. (a) Soleus muscle stimulated at 30 Hz; (b) combined EDL/TA muscles stimulated at 60 Hz.

chain phosphorylation in soleus muscle (80%) was obtained after 30 s tetanus at 30 Hz (Fig. 2a). Further stimulation at that frequency caused the soleus muscle to become fatigued, and tension fell, as did the extent of P-light-chain phosphorylation.

The rate of P-light-chain phosphorylation in response to stimulation was slower in the soleus than in the combined EDL/TA muscles. A 15 s tetanus at 30 Hz of the soleus muscle resulted in 50% phosphorylation of P light chains, whereas a similar period of stimulation at 20 Hz, a frequency at which the contractions were not fused, resulted in 90% phosphorylation in fast muscle.

Post-tetanic potentiation and P-light-chain phosphorylation in rabbit fast skeletal muscle

Repeated stimulation of fast-twitch muscle leads to post-tetanic potentiation, i.e. the increase in tension developed in a single twitch after a short period of tetanus. In the combined rabbit EDL/TA muscle the peak twitch tension increased by over 100% within 10 s of the tetanus, but the single-twitch response had returned to the pre-tetanic value within 5 min. Tetanic stimulation for 5 s at 60 Hz resulted in approx. 100% phosphorylation of the P light chain. After tetanus ceased, dephosphorylation proceeded relatively slowly (Fig. 3). Although there was some correlation between the fall in post-tetanic potentiation and P-light-chain phosphorylation for the first 2 min after tetanus, this was not the case throughout recovery. In the later stages the extent of phosphorylation fell much more slowly than post-tetanic potentiation and was still 130–140% of the resting value after 15 min, when the tension had returned to the pre-tetanic value. Extrapolation from the rate of dephos-

Fig. 3. Phosphorylation of the P light chain and post-tetanic potentiation after isometric tetanus of rabbit fast skeletal muscle

Combined EDL/TA muscles were stimulated for 5 s at 60 Hz. The peak tension of single twitch was measured at different times after tetanus, and muscles were immediately freeze-clamped. Tensions are expressed as percentages of the average value for a single twitch before tetanus, and extents of phosphorylation as a percentage of the resting value (Table 1). ●, P-light-chain phosphorylation; ○, post-tetanic potentiation.

phorylation occurring 5–15 min after tetanus by using the data shown in Fig. 3 suggests that it would take 45 min for the P light chain to return to the resting value of phosphorylation.

Effect of frequency of stimulation on P-light-chain phosphorylation of rabbit fast- and slow-twitch muscles

Phosphorylation of the P light chain of EDL/TA muscles was obtained with stimulation at lower

frequencies producing single twitches. The extent of phosphorylation was dependent not on the total number of impulses received by the motor end-plate, but on the frequency at which they were applied (Fig. 4). Electrical stimulation below 1 Hz failed to produce a marked increase in the extent of P-light-chain phosphorylation in fast muscles even after 30 min of stimulation, e.g. stimulation at 0.5 Hz for 30 min, equivalent to 900 impulses, increased the extent of phosphorylation to 10% above the unstimulated values. In contrast, stimulation at 4 Hz for 30 s, producing only 120 impulses, increased the extent of phosphorylation to 80% in EDL/TA muscles.

It is well known that the repetitive stimulation of fast-twitch muscle leads to a gradual increase in the peak tension of the isometric twitch. Also the time taken to reach peak tension is in inverse relationship to the frequency of stimulation. In view of the results of the effect of frequency of stimulation on P-light-chain phosphorylation described above, a similar relationship should exist between the extent of P-light-chain phosphorylation and the time required to develop peak tension when muscle is stimulated at different frequencies. The results illustrated in Fig. 5 indicate that this is the case. In these experiments, the muscles were freeze-clamped at the time at which peak tension was developed in response to electrical stimulation.

If the soleus muscle was stimulated at the natural frequency (10 Hz) for 30 s, it was 50% phosphorylated, and little change in phosphorylation occurred

if stimulation was maintained for 4 min. This suggests that the frequency of stimulation rather than the duration of stimulation was important in determining the extent of P-light-chain phosphorylation in slow muscle.

Discussion

The resting values for the extents of phosphorylation of the P light chain of myosin from rabbit fast skeletal muscle *in vivo* are high compared with those reported for isolated rat and chicken muscles (Stull *et al.*, 1983; Barany *et al.*, 1982), but similar to that reported for the rabbit by Stull & High (1977). The different values may indicate species differences, but the possibility exists that some stimulation of the kinase occurs on fixing the muscle for analysis. It is considered that this effect must be minimal in the experiments reported here, for the muscles were rapidly cooled to liquid-N₂ temperatures in the present investigation. Also the stimulation experiments indicate that the response of phosphorylation to stimulation is relatively slow. In addition to inactivating the kinase, it is equally important to ensure that the light-chain phosphatase is completely destroyed before extracts are processed for electrophoresis. The phosphatase has been shown to be active in 8M-urea (Westwood & Perry, 1981), and it is possible that some of the low values reported in the literature are due to dephosphorylation by this enzyme during processing the light chains for analysis.

Fig. 4. Effect of frequency of stimulation on the phosphorylation of the P light chain of myosin in rabbit fast skeletal muscles

Combined EDL/TA muscles were stimulated isometrically at frequencies indicated for 60 s and immediately freeze-clamped.

Fig. 5. Effect of frequency of stimulation on the time to reach maximum tension and the extent of phosphorylation of the P light chain

Combined EDL/TA muscles were contracting isometrically at frequencies indicated until maximum tension was developed. Muscles were then immediately freeze-clamped. ○, % increase in P-light-chain phosphorylation; ●, time to reach maximum twitch tension.

In contrast with the report of Barany *et al.* (1982) that phosphorylation does not exceed 50% in isolated chicken skeletal muscles, despite repeated stimulation, phosphorylation approaching 100% was obtained with rabbit fast muscles. In view of the fact that in studies of isolated myosin and actomyosin systems 100% phosphorylation of the P light chain is readily obtained, it seems surprising that, provided that adequate stimulation is given, 100% phosphorylation of the light chains should not occur *in vivo*. Indeed our results and those of Stull & High (1977) indicate that, in the rabbit at least, this is the case.

In view of the failure of some workers (Kushmerick & Crow, 1982) to report the phosphorylation after stimulation of the P light chains of slow mammalian skeletal muscle, but not in the chicken (Barany *et al.*, 1982), the results with rabbit soleus are of interest. The polymorphism of the P light chain appears to be a characteristic of slow skeletal muscle, for no evidence was obtained for the existence of isotypes of this light chain in the EDL or TA muscles. In this respect the soleus muscle resembles the heart, and it may be significant that polymorphism of the P light chain is a feature of the slower types of striated muscle. The relatively high proportion of the total light-chain fraction represented by the P2 form indicates that it is not solely located in the different cell types, i.e. true type II representing 4.5% (Gray & Renkin, 1978), or 'intermediate' type, representing 18.5% of the total (Dhoot *et al.*, 1981), that have been identified in the adult rabbit soleus muscle. It seems likely that both P1 and P2 light chains are present in all type-I cells of the soleus.

It appears that in both fast and slow muscles a certain minimum frequency of stimulation is required to produce significant phosphorylation of the P light chain. This is presumably related to the frequency of stimulation required to maintain the steady-state Ca^{2+} concentration of the sarcoplasm at a high enough value to activate the myosin light-chain kinase so that the rate of phosphorylation is much greater than the rate of dephosphorylation catalysed by the myosin light-chain phosphatase. This follows from the close relationship that exists between the extent of phosphorylation and time taken to reach peak twitch tension in fast muscle and frequency of stimulation (Fig. 5). Similar observations have been reported by Stull *et al.* (1983).

The role of P-light-chain phosphorylation in skeletal muscle still remains uncertain. Although the correlation between phosphorylation and post-tetanic potentiation was quite good at higher degrees of phosphorylation, the potentiation had disappeared within 6 min, whereas the phosphorylation was still 40% above the resting value 16 min after the tetanus. Stull *et al.* (1983) have described

a similar discrepancy in isolated rat EDL with potentiation ending at 100 s, at which time there was 50% P-light-chain phosphorylation. The faster return of tension to pre-tetanic values in their experiments is probably due to the fact that they worked with isolated muscle at 23°C.

The observation that light-chain phosphorylation occurred in soleus muscle, which does not exhibit post-tetanic potentiation (Close & Hoh, 1969), makes it very unlikely that the latter effect is a direct consequence of phosphorylation of myosin, as has been implied by Manning & Stull (1979). Indeed, the observation that P-light-chain phosphorylation occurred in both fast and slow muscle types would suggest that any physiological property that is unique to either fast or slow muscle is not a consequence of P-light-chain phosphorylation.

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