Phosphorylation of tropomyosin during development in mammalian striated muscle

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α-Tropomyosin from rat cardiac muscle was shown by two-dimensional gel electrophoresis to become phosphorylated when tissue slices were incubated in Eagle's medium supplemented with $^{32}P_i$. In the adult rat and mouse heart the level of phosphorylation was ~30%, but the level was much higher in the foetal heart (60–70%). A similar developmental trend was observed in skeletal muscle from the rat and mouse, where phosphorylated forms of both α- and β-tropomyosins were observed. When rat cardiac cells were grown in tissue culture in the presence of $^{32}P_i$, radioactivity was incorporated into the region of the gel containing tropomyosin.

Mammalian muscle *Tropomyosin* *Isoelectric focusing* *Phosphorylation* *Development* *Tissue culture*

1. INTRODUCTION

Evidence now exists for the phosphorylation of all the proteins of the I filament of striated muscle with the exception of troponin C. Apart from troponin I in cardiac muscle, the phosphorylation of which changes the sensitivity of the Mg^{2+}-stimulated actomyosin ATPase to Ca^{2+} (review [1]) no clear function has yet emerged for the phosphorylation of the other proteins of the I filament.

In the case of tropomyosin, which in the rabbit is phosphorylated at the penultimate residue serine 283, it has been suggested that phosphorylation could play a role in the head-to-tail interaction between tropomyosin molecules [2]. Evidence for phosphorylation of α-tropomyosin has been obtained with frog and rabbit skeletal muscle [2] and for what are described as minor acidic variants of α and β tropomyosin in chicken skeletal muscle [3]. In mammalian skeletal muscle the extent of phosphorylation in adult tissue appears to be low, ~10% of the total tropomyosin present, although somewhat higher values have been reported in the rabbit heart [2]. Such evidence as is available suggests that the level of phosphorylation of tropomyosin is not closely correlated with contractile activity in an adult skeletal muscle such as the frog sartorius [4], but incorporation of ^{32}P into the tropomyosin has been reported over long incubation periods both in tissue culture and tissue slices [3]. To throw further light on the possible role of tropomyosin phosphorylation during myofibrillogenesis we have investigated the extent of phosphorylation of tropomyosin during foetal and early post-natal development in mammalian muscle.

2. MATERIALS AND METHODS

2.1. *Preparation of muscle samples*

Rats were killed by either stunning or by anaesthesia followed by decapitation. Muscle was immediately removed and homogenized in 10 vol. 9 M urea and the solution then made 15 mM and 2% (v/v) with respect to mercaptoethanol and the detergent NP40, respectively. The homogenate was subjected to electrophoresis either immediately or frozen and stored at −30°C for subsequent study. In some cases the tissue was homogenized in 15% (w/v) trichloroacetic acid and processed for electrophoresis as in [5].

2.2. *In vitro phosphorylation of tropomyosin in muscle samples*

Rat muscle slices (5–10 mg) were incubated in phosphate-free Eagles medium (200–500 μl) containing carrier-free $^{32}P_i$ (200–500 μCi) for 4 h at

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30°C. The tissue was then homogenized as above and loaded onto gels for electrophoresis.

2.3. *Phosphorylation of tropomyosin in cells growing in tissue culture*

Rat cardiac cells from 7 day-old animals were grown in tissue culture using Eagles medium. After 4–6 days, plates containing ~10^6 cells were exposed to carrier-free $^{32}P_i$ (100–500 μCi) in 5 ml medium for periods of 20–40 h. Cells were harvested in 9 M urea/0.5% NP40 (100–200 μl) and applied directly to the first-dimension gel.

2.4. *Electrophoresis*

Two-dimensional electrophoresis was done following [6] using the procedures in [7,8]. The pH ranged from 4–6, using 2% ampholines (LKB Instruments, Croydon) to produce an expanded isoelectric focusing dimension. In some cases an extended pH range from 3–11 was employed using a mixture of pH 3–10 and pH 9–11 ampholines (LKB Instruments, Croydon).

2.5. *Densitometry*

The relative amounts of phosphorylated-and dephosphorylated tropomyosin were determined by scanning two-dimensional gels with a Zeineh soft-laser densitometer (supplied by T. and J. Crump, Rayleigh, Essex). Areas under the peaks were determined by cutting out the traces and weighing.

2.6. *Autoradiography*

Gels for autoradiography were sealed into polythene bags and exposed to Kodak Regilux Blue Brand Medical X-ray films for 2–3 weeks at room temperature.

3. RESULTS AND DISCUSSION

When the extracts of adult rat and rabbit heart were examined by two-dimensional gel electrophoresis immediately after homogenization in 9 M urea, the tropomyosin migrated as 2 spots, the major of which was identified as α-tropomyosin (fig.1a). The minor spot was more acidic and could be identified as the phosphorylated form of α-tropomyosin since it, but not the major spot, became radioactive when heart slices were incubated with $^{32}P_i$ (fig.1b). The spots migrated with the same mobilities as the 2 spots observed when samples of purified α-tropomyosin known to contain the phosphorylated form were examined by two-dimensional electrophoresis. If fresh hearts were homogenized in 15% trichloroacetic acid rather than 9 M urea before preparation for electrophoresis [5], a slight increase (<5%) in the amount of the phosphorylated form was observed indicating that very little phosphatase action occurred in the strong urea solution during preparation of the samples for electrophoresis. It is worthy of note that on some occasions each of the spots corresponding to α-tropomyosin and its phosphorylated form could be further resolved into a poorly separated doublet.

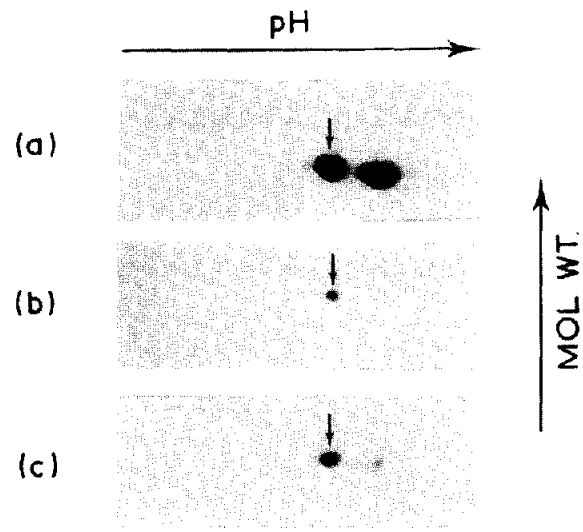


Fig.1. Identification of phosphorylated form of α-tropomyosin in rat heart. Figures are regions of two-dimensional electrophoretograms of 2 homogenates of rat heart prepared as in section 2. Phosphorylated form of α-tropomyosin indicated by small arrows: (a) 11-days old; (b) autoradiograms of (a); (c) 7 days before birth.

In the adult rat heart ~30% of the α-tropomyosin was consistently phosphorylated. The extent of phosphorylation was greater in the foetal rat heart reaching values of ~70%, 10 days before birth (fig.1c). With development, the level of phosphorylation fell reaching the adult level of phosphorylation 30 days after birth (fig.2). Similar elevated levels of phosphorylation of α-tropomyosin were also observed in foetal hearts of the mouse (table 1).

In contrast to cardiac muscle, the combined leg

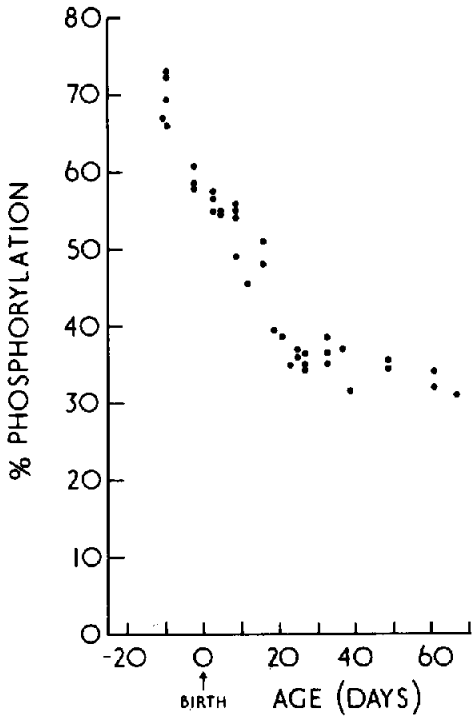


Fig.2. Change in phosphorylation of α-tropomyosin in the developing rat heart. Level of phosphorylation determined from two-dimensional electrophoretograms of homogenates of whole hearts as in section 2.

muscles in the rat contains both α- and β-tropomyosin. Phosphorylated forms of α- and β-tropomyosin were observed in homogenates when rat leg muscle slices had been incubated with $^{32}P_i$. The level of phosphorylation of α-tropomyosin in the combined leg muscles of the rat was found to change from 50% in the foetal stage to ~20% in the adult. In the combined leg muscle of the rat, β-tropomyosin was found to be 45% phosphorylated in the foetus, decreasing to 14% in the adult (table 1).

Two-dimensional gel electrophoresis over pH 3–11 demonstrated that a number of proteins incorporated radioactivity when rat cardiac cells were grown in tissue culture in the presence of $^{32}P_i$. When extracts of cardiac myocytes were examined by two-dimensional gel electrophoresis using a pH gradient of 3–5 it was found that in the region of the gel where tropomyosin normally migrated there was a cluster of 6–8 spots, 2 of which were labelled with $^{32}P_i$. One of the radioactively labelled spots had a mobility that was identical with that of adult cardiac phosphorylated α-tropomyosin. The addition of urea to the second-dimension gel, normally run in SDS alone, selectively retarded the mobility of all these spots in the manner characteristic of tropomyosin [9,10].

Table 1

Phosphorylation of α and β tropomyosin in developing mammalian striated muscles

Stage of development	Tropomyosin subunit	Phosphorylation (% total subunit)			
		Rat		Mouse	
		Cardiac	Skeletal	Cardiac	Skeletal
Foetal	α	70 (−7)	50 (−7)	50 (−3)	48 (−3)
	β	ND (−7)	46 (−7)	ND (−3)	32 (−3)
Post-natal	α	45 (+12)	40 (+12)	29 (+7)	–
	β	ND	28 (+12)	ND (+7)	–
	α	35 (+38)	20 (+28)		
	β	ND (+28)	14 (+28)		

ND = Not detectable

Phosphorylation determined by densitometric measurement of two-dimensional electrophoretograms of homogenates of whole muscle. Values are average of ≥2 determinations on 2–4 separate muscle samples. Figures in parentheses indicate the number of days before (−) or after birth (+)

These studies confirm that when tropomyosin in whole adult muscle is analysed under conditions that prevent the action of endogenous phosphatase the extent of phosphorylation is low, particularly in skeletal muscle. Nevertheless, the phosphate covalently bound to the tropomyosin is in dynamic equilibrium with the intracellular phosphate pool.

Much higher levels of phosphorylation were observed in conditions where rat and mouse cardiac and skeletal muscle were undergoing rapid development and when active myofibrillogenesis was taking place. Similar high levels of phosphorylation have been reported in chicken embryonic muscle [11,12]. The high level of phosphorylation appears to be associated with development in whole muscle rather than in cells in tissue culture. In the latter case, phosphorylated forms can also be identified but more forms of tropomyosin appear to be present than in the foetal or adult tissue, many of which are in the unphosphorylated form.

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