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Contraction in intact pig aortic strips is not always associated with phosphorylation of myosin light chains

Kenneth J. MURRAY and Paul J. ENGLAND
Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K.

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Intact pig aortic strips were incubated in medium containing [^{32}P]P_i and various Ca^{2+} concentrations. The ^{32}P content of the myosin P-light chain was determined by radioautography after electrophoresis in the presence of sodium dodecyl sulphate. Although treatment of the strips with noradrenaline always caused a rise in tension, this was not necessarily accompanied by increased phosphorylation of the P-light chain. These results indicate that, in aortic smooth muscle, phosphorylation of the P-light chain is not obligatory for contraction.

Mammalian myosin contains a P-light chain (Frearson & Perry, 1975), which can be phosphorylated by a specific Ca^{2+}-dependent myosin light-chain kinase in all muscle (Bárány & Bárány, 1980) and also in some non-muscle cells (Adelstein, 1978). In smooth muscle the hypothesis has been put forward that phosphorylation of the P-light chain is obligatory for contraction (Adelstein, 1978). This proposes that an increase in cytoplasmic Ca^{2+} activates the myosin light-chain kinase, which leads to phosphorylation of the P-light chain and permits interaction of myosin and actin. Relaxation is envisaged as occurring by dephosphorylation of P-light chain, catalysed by a specific light-chain phosphatase (Morgan *et al.*, 1976). This is based on the observation that with actomyosin prepared from gizzard (Aksoy *et al.*, 1976; Sobieszek, 1977) and arterial (DiSalvo *et al.*, 1978; Mrwa *et al.*, 1980) muscle, phosphorylation of the P-light chain was correlated with ATPase activity. However, in contrast with these results, Mikawa *et al.* (1977) reported that with gizzard actomyosin, actin–myosin interaction could occur in the absence of phosphorylation of P-light chain.

In striated muscle, control of contraction occurs through the interaction of Ca^{2+} with troponin, which is located on the thin filament. It has been proposed that regulation via the thin filament could also occur in smooth muscle. Ebashi (1980) has reported the presence in various smooth muscles of a protein complex, leotonin, which appears to function in a similar manner to troponin. In contrast with troponin, however, leotonin is composed of only two proteins, with mol.wts. of 80000 and 18000. Marston *et al.* (1980) have also reported thin-filament regulation in smooth muscle, and the presence of striated-muscle troponin-like proteins. At present, there appears to be conflicting evidence from studies of isolated contractile proteins from smooth muscle as to the relative importance of thick- and thin-filament regulation.

Studies with intact smooth muscle (Barron *et al.*, 1979; Driska & Murphy, 1979) have shown an increased phosphorylation of P-light chain on contraction. Our approach has been to study the phosphorylation of P-light chain in intact aortic strips under conditions that could dissociate contraction and phosphorylation. A preliminary report of some of these findings has been presented previously (Murray & England, 1980).

Materials and methods

Aortas were obtained from freshly killed pigs and placed in ice-cold Krebs bicarbonate medium (Krebs & Henseleit, 1932). Transverse strips (25 mm × 3 mm) were cut from the thoracic aorta, stripped of superficial fat and incubated in 25 ml of modified Krebs bicarbonate medium containing 0.234 mM-[^{32}P]P_i (specific radioactivity 10–20 MBq/μmol) and 22 mM-glucose. $CaCl_2$ or EGTA were added as described in the Results and discussion section. The medium was bubbled with O_2/CO_2 (19:1) and maintained at 37°C throughout the preincubation period of 2 h. For measurement of tension the aortic strips were attached to force-displacement transducers (UF-1; Ormed Engineering, Welwyn Garden City, Herts., U.K.) and subjected to a tension of 5 g. The radioactive medium was then replaced by fresh Krebs bicarbonate medium containing either 5 mM-

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EGTA or 5 μM-noradrenaline in the presence of 5 mM-$CaCl_2$ or 5 mM-EGTA. After 15 min, by which time the noradrenaline-treated strips had reached and maintained a maximal increase in isometric tension, the strips were rapidly removed and frozen in liquid N_2. Parallel incubations were also performed without the strips being subjected to the resting tension of 5 g (see the Results and discussion section).

Each aortic strip was homogenized in 1 ml of 0.6 M-trichloroacetic acid, and the precipitated protein redissolved in 2 ml of 8 M-urea. Undissolved protein, which consisted of mainly connective and elastic tissue, was removed by centrifugation, and the dissolved protein re-precipitated by addition of 8 ml of 0.6 M-trichloroacetic acid. The protein was finally dissolved in 10% (w/v) sodium dodecyl sulphate/125 mM-Tris/HCl (pH 6.8)/20% (w/v) sucrose/150 mM-2-mercaptoethanol at 100°C for 5 min. The concentration of protein in this solution was measured by the method of Zaman & Verwilghen (1979), and, after addition of 0.001% (w/v) Bromophenol Blue, 200 μg was subjected to electrophoresis on 11.25% (w/v) polyacrylamide gels by the method of Laemmli (1970). Protein was detected by staining with Coomassie Brilliant Blue, and protein-bound ^{32}P was measured by densitometric scanning after radioautography of the gel. The radioautographs were shown to be within the range of proportionality between absorbance and radioactivity by exposing the radioautographs for increasing periods of time.

Actomyosin was prepared from pig aorta by the method of Sparrow *et al.* (1970), and a fraction containing essentially pure A- and P-light chains prepared from this by the method of Perrie & Perry (1970). Treatment of 8 M-urea and sources of chemicals were as described previously (Jeacocke & England, 1980).

Results and discussion

Incubation of aortic strips in medium containing [^{32}P]P_i caused ^{32}P to be associated with a number of protein bands after polyacrylamide-gel electrophoresis. There are three major phosphoproteins of high mobility, and these have apparent mol.wts. of 27000, 20000 and 16000. The protein of mol.wt. 20000 co-migrated with the P-light chain of a purified light-chain fraction (see Fig. 1). In addition, this band contained ^{32}P when a light-chain fraction was prepared from aortic strips that had been incubated with [^{32}P]P_i. However, no ^{32}P was associated with the light chain of mol.wt. 16000. The phosphoprotein of apparent mol.wt. 16000 was not therefore the A-light chain, but a protein that co-migrated with it. To exclude the possibility that the ^{32}P associated with the band of mol.wt. 20000

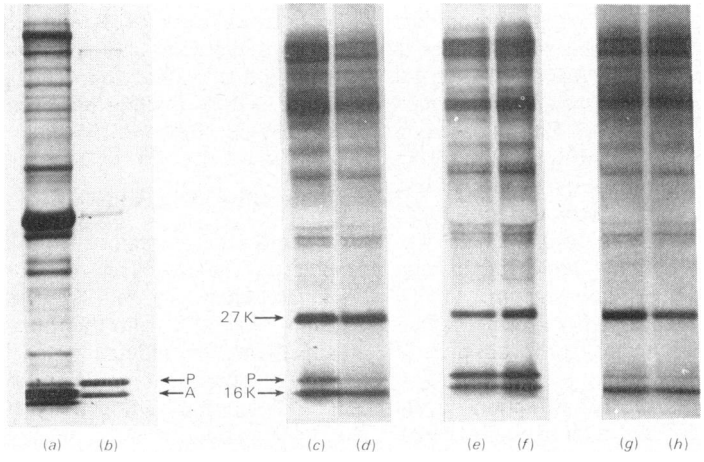

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and radioautography of aortic proteins*
Protein, after being subjected to electrophoresis on 11.25% polyacrylamide gels in the presence of sodium dodecyl sulphate, was detected by staining with Coomassie Brilliant Blue (*a* and *b*) and its ^{32}P content by radioautography (*c–h*) as described in the text. (*a*) 200 μg of whole aortic protein; (*b*) light-chain fraction prepared from pig aorta; (*c* and *d*) strips preincubated with 20 μM-$CaCl_2$, and treated with noradrenaline (*c*) or EGTA (*d*); (*e* and *f*) strips preincubated with 5 mM-$CaCl_2$, and treated with noradrenaline (*e*) or EGTA (*f*); (*g* and *h*) strips preincubated with 5 mM-EGTA, and treated with noradrenaline (*g*) or EGTA (*h*). The position of myosin P- and A-light chains are shown by P and A respectively. The ^{32}P associated with the proteins of apparent mol.wt. 27000 and 16000 are shown as 27 K an 16 K respectively.

was due to a phosphoprotein other than the P-light chain, tissue extracts were subjected to electrophoresis in 8M-urea (Perrie & Perry, 1970), and subsequently in a second dimension in the presence of sodium dodecyl sulphate (Laemmli, 1970). This showed the presence of only one phosphoprotein of mol.wt. 20000, which co-migrated in both dimensions with purified P-light chain.

Control experiments measuring the tissue [γ-^{32}P]ATP specific radioactivity (England & Walsh, 1976) showed that the ^{32}P content of the 27000-mol.wt. protein remained constant relative to the tissue [γ-^{32}P]ATP specific radioactivity under all the conditions used. The ^{32}P content of the P-light chain is therefore expressed relative to that of the 27000-mol.wt. protein, which, as well as correcting for the tissue [γ-^{32}P]ATP specific radioactivity, also has the advantage of internally correcting for a number of other variable factors (e.g. different exposure times of the radioautographs). The only assumption made is that the relative extractions of the two proteins from the tissue remain constant.

The effect of subjecting the strips to a passive tension of 5g during the preincubation period was also studied in control experiments. As, under all conditions used, this treatment was found to have no effect on the ^{32}P content of either the P-light chain or the 27000-mol.wt. protein, not all the strips described in Table 1 were subject to passive tension.

The Ca^{2+} content of intact smooth-muscle preparations may be changed by varying the external Ca^{2+} concentration (Kuriyama *et al.*, 1976), and the effect of this on the ^{32}P content of the P-light chain, the resting tension and the response to noradrenaline of the aortic strips was examined (see Table 1).

Preincubation of the aortic strips in medium without added $CaCl_2$ (estimated concentration 20μM) had no effect on the resting tension, with only a relatively low ^{32}P content of the P-light chain being observed (Fig. 1*c*). However, when this was replaced with medium containing 5μM-noradrenaline and 5mM-$CaCl_2$, an increase in isometric tension of approx. 3g was recorded. This increase in tension was accompanied by a 3-fold increase in the ^{32}P content of the P-light chain (cf. Figs. 1*d* and 1*c*).

When the aortic strips were preincubated in medium containing 5mM-$CaCl_2$ an increase in resting tension of approx. 2g was observed, and this was associated with a high ^{32}P content of the P-light chain (Fig. 1*f*). The ^{32}P content of the P-light chain was not changed by additions of 5μM-noradrenaline, which did, however, cause a further increase in tension of approx. 1g (Fig. 1*a*).

Incubation of arterial strips in EGTA is known to decrease their Ca^{2+} content (Andersson, 1973) and, in agreement with this, strips preincubated with 5mM-EGTA showed a slight decrease in resting tension and consequently a diminished response to noradrenaline. A contraction of approx. 1g was, however, observed when these strips were challenged with 5μM-noradrenaline in the presence of 5mM-EGTA, showing the importance of intracellular stored Ca^{2+} in smooth-muscle contraction (Deth & van Breemen; 1974). The ^{32}P content of the P-light chain remained low throughout the preincubation period (Fig. 1*h*) and was not increased by the action of noradrenaline (Fig. 1*g*), which did, however, elicit a contractile response.

These results show that in intact aortic strips an increase in contractile force is accompanied by either no change or an increase in the ^{32}P content of the P-light chain. Although hormonal stimulation may increase the ^{32}P content of a protein without increasing its total phosphate (Rudolph *et al.*, 1978), it is not, however, possible under the experimental conditions described here for the P-light chain to increase its phosphate content without a consequent increase in its ^{32}P content. For an increase in the

Table 1. *Tension and relative ^{32}P content of myosin P-light chain in incubated aortic strips*

Aortic strips were preincubated in modified Krebs bicarbonate medium for 2h (column A) and then subjected to various treatment conditions for 15min (column B). Column C shows the total change in isometric tension after the entire 2.25h preincubation and treatment periods. The ^{32}P content of the P-light chain was corrected as described in the text, and the strips preincubated in 20μM-$CaCl_2$ and treated with 5mM-EGTA were given a defined content of 1.00. All other ^{32}P contents are shown relative to this (column D). All results are quoted as means ± S.D. for the numbers of observations given in parentheses. $^{**}P < 0.01$; $^{*}P < 0.05$ [tested against values for treatment with EGTA (Student's *t* test)].

Pre-incubation condition (A)	Treatment condition (B)	Change in tension (g) (C)	Relative ^{32}P content of the P-light chain (D)
20μM-$CaCl_2$	5mM-EGTA	0.04 ± 0.14 (4)	1.00 ± 0.23 (5)
	5μM-Noradrenaline, 5mM-$CaCl_2$	3.02 ± 0.75 (9)**	3.01 ± 0.80 (4)*
5mM-$CaCl_2$	5mM-EGTA	2.02 ± 0.15 (5)	3.62 ± 0.81 (5)
	5μM-Noradrenaline, 5mM-$CaCl_2$	2.98 ± 0.22 (4)**	3.57 ± 1.50 (5)
5mM-EGTA	5mM-EGTA	−0.27 ± 0.22 (4)	1.39 ± 0.30 (5)
	5μM-Noradrenaline, 5mM-EGTA	1.16 ± 0.19 (5)**	1.12 ± 0.11 (5)

P-light-chain ^{32}P content to be interpreted as an increase in its total phosphate, it must be shown that the P-light-chain-bound phosphate and that of the γ-P_i of the ATP are in isotopic equilibrium. Barron *et al.* (1979) have reported that this indeed is the case in arterial smooth muscle, and so the increases in the ^{32}P content of the P-light chain described here may be tentatively interpreted as reflecting a genuine increase in its phosphorylation.

These results do not therefore support the hypothesis that the phosphorylation of the P-light chain is obligatory for contraction in smooth muscle, and it would appear that there must be another mechanism capable of regulating contractile activity. However, as the aortic strips only developed maximum tension under conditions that also showed increased P-light-chain phosphorylation, these results do not exclude P-light-chain phosphorylation as a controlling factor. These results could possibly provide evidence *in vivo* for the suggestion that contraction in smooth muscle is subject to dual regulation (Marston *et al.*, 1980).

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