IS SMOOTH MUSCLE MYOSIN A SUBSTRATE FOR THE cAMP-DEPENDENT PROTEIN KINASE?

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1. Introduction

The most popular theory to account for the regulation by Ca^{2+} of smooth muscle actomyosin is based on the phosphorylation–dephosphorylation of the myosin molecule [1–3]. In the presence of Ca^{2+} the M_r 20 000 light chains of myosin are phosphorylated by a calmodulin-dependent myosin light chain kinase [4] and this event is thought to be a prerequisite for contraction. At lower Ca^{2+} levels the light chains are dephosphorylated by one or more phosphatases [5], the actomyosin complex ceases to hydrolyze ATP and relaxation follows. This basic hypothesis was subsequently modified and it was suggested that cAMP is also involved in the regulatory process via phosphorylation of the myosin light chain kinase by the cAMP-dependent protein kinase [6]. The phosphorylated form of the myosin light chain kinase was found to have a reduced affinity for calmodulin [7]. Thus at limiting concentrations of calmodulin the myosin light chain kinase activity correspondingly would be inhibited. In support of this theory it was shown that a process related to the cAMP-dependent protein kinase caused an inhibition of the Mg^{2+}-ATPase activity of smooth muscle actomyosin [8,9] and a reduction of tension development in functionally skinned smooth muscle fibers [10].

In [11] it was reported that the cAMP-dependent protein kinase also phosphorylated the isolated light chains of chicken gizzard myosin. This raised the obvious possibility that myosin itself might be a substrate for the cAMP-dependent protein kinase and could therefore constitute an additional regulatory mechanism in smooth muscle.

During a study with chicken gizzard actomyosin to identify the possible sites of phosphorylation for the cAMP-dependent protein kinase we found that the M_r 20 000 light chain of intact myosin was not phosphorylated (M. P. W., D. J. H., in preparation). Therefore, we undertook this study to compare the phosphorylation by the cAMP-dependent protein kinase of partially purified light chains and intact myosin to evaluate whether or not this process might be of physiological significance. The results show clearly that although the isolated light chains can act as a substrate for the cAMP-dependent protein kinase, intact myosin is not phosphorylated by this enzyme.

2. Materials and methods

The following chicken gizzard proteins were prepared as described: myosin [12]; mixed light chains [13]; myosin light chain kinase [14]. Calmodulin was purified from bovine testes according to [15] with the addition of a final chromatographic step on a fluphenazine–Sepharose affinity colum [16]. The purified catalytic subunit of bovine heart cAMP-dependent protein kinase was purchased from Sigma Chemical Co. (St Louis MO). Protein concentrations were determined by the biuret method [17] or the dye-binding assay [18]. 7.5–20% Polyacrylamide gradient slab gel electrophoresis in the presence of 0.1% SDS was carried out at 30 mA [19]. Urea–polyacrylamide gel electrophoresis was performed according to [13].

Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether) N,N^1-tetraacetic acid; SDS, sodium dodecyl sulfate

Coomassie blue-stained gels were scanned at 550 nm using a Zeiss Spectralphotometer PM 6 attached to a Spectra-Physics SP 4050 printer/plotter, an SP 4020 data interface and an SP 4000 central processor.

3. Results

Densitometric scans of Coomassie blue-stained gels of the myosin and mixed light chains used in this study indicated that the myosin was >97% pure, and that the M_r 20 000 light chain represented 39% of the total protein in the mixed light chain preparation.

The effect of incubation of the catalytic subunit of cAMP-dependent protein kinase with mixed light chains and intact myosin is shown in fig.1. In agreement with the data in [11], the M_r 20 000 light chain is phosphorylated by cAMP-dependent protein kinase; up to 1 mol ^{32}P/mol light chain was incorporated (fig.1A). The slower rate of light chain phosphorylation observed in the presence of Ca^{2+} was due probably to a slight inhibition of the protein kinase catalytic subunit by Ca^{2+}. However, from the data presented in fig.1B it is clear that intact myosin is not phosphorylated by the cAMP-dependent protein kinase. The low level of myosin phosphorylation observed in the presence of Ca^{2+} was a result of contamination of the myosin with traces of myosin light chain kinase and calmodulin (see below).

It was important to show the extent to which the M_r 20 000 light chain could be phosphorylated in this intact myosin preparation (fig.2). This was done by measuring the phosphorylation upon addition of calmodulin and purified chicken gizzard myosin light chain kinase. Up to 0.8 mol ^{32}P/mol light chain were incorporated in a Ca^{2+}-dependent manner. Also as shown in fig.2, addition of myosin light chain kinase without calmodulin did not enhance myosin phosphorylation. Therefore, the myosin preparation contained a limiting amount of calmodulin, making it ideally suited for the experiment in fig.1B, since it was then possible to dissociate clearly phosphorylation catalyzed by cAMP-dependent protein kinase from that catalyzed by myosin light chain kinase.

The intact myosin used in these studies was freshly prepared since we found that it was highly susceptible to proteolysis by an endogenous protease(s) when stored at 4°C. For example, after 10 days, 31% of the myosin heavy chain was proteolysed, as evidenced by densitometric scanning of a polyacrylamide gradient

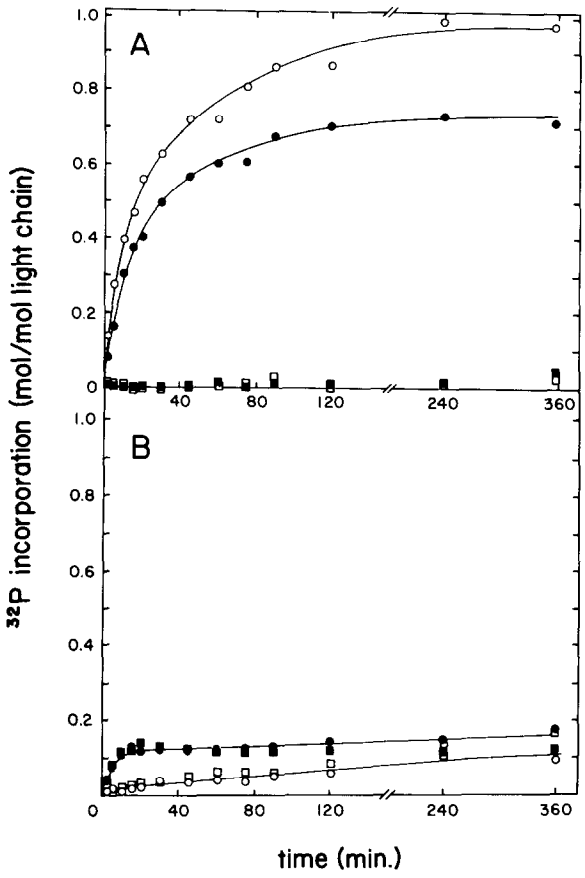

Fig.1. Phosphorylation of mixed light chains and intact myosin by cAMP-dependent protein kinase. Smooth muscle mixed light chains (A, 62 μg/ml) or intact myosin (B, 1 mg/ml) were incubated at 37°C in 25 mM Tris–HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (~3000 cpm/nmol), with either 1 mM EGTA (○,□) or 0.1 mM $CaCl_2$ (●,■), in the presence (○,●) or absence (□,■) of the catalytic subunit of cAMP-dependent protein kinase (E:S = 1:100). Aliquots (0.5 ml) were withdrawn at selected time intervals and reactions quenched by addition to 0.5 ml 25% trichloroacetic acid, 2% sodium pyrophosphate. Protein-bound ^{32}P was quantitated as in [20].

slab-gel of the preparation. Such proteolysis, which did not significantly affect the M_r 20 000 light chain, resulted in myosin which could be slightly phosphorylated by the cAMP-dependent protein kinase: 0.03 mol ^{32}P were incorporated/mol M_r 20 000 light chain after incubation for 2 h at 37°C. Thus it is possible that if the conformation of the myosin molecule is altered, some phosphorylation of the light chain might occur. The latter does not occur, however, with myosin in its native state.

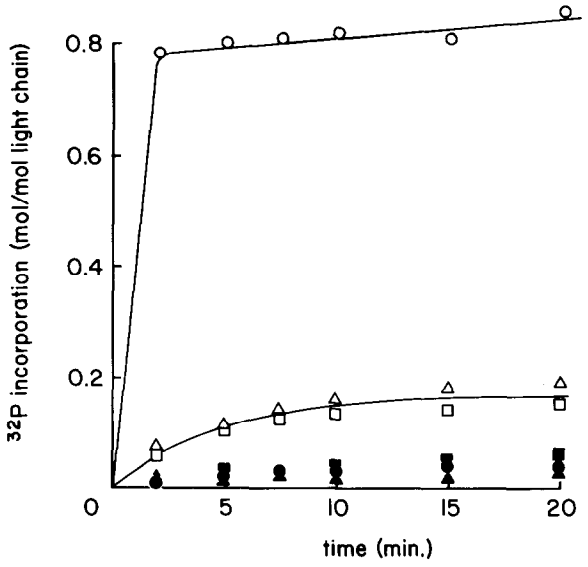

Fig.2. Phosphorylation of intact myosin by calmodulin-dependent myosin light chain kinase. Smooth muscle myosin (1 mg/ml) was incubated at 37°C in 25 mM Tris–HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (~3000 cpm/nmol), with either 0.1 mM $CaCl_2$ (○,□) or 1 mM EGTA (●,■), in the presence of myosin light chain kinase and calmodulin (○,●), myosin light chain kinase alone (□,■), or no further addition (△,▲). Final concentrations of myosin light chain kinase and calmodulin were 4×10^{-8} M and 10^{-6} M, respectively. Aliquots (0.5 ml) were withdrawn at selected time intervals and treated as in fig.1.

4. Discussion

Our data show clearly that, while the M_r 20 000 light chain of chicken gizzard myosin is phosphorylated by cAMP-dependent protein kinase when the light chain is partially purified, in agreement with [11], it is not a substrate of the protein kinase in intact myosin. It is reasonable to conclude, therefore, that cAMP-dependent phosphorylation of myosin does not occur in vivo and is not of physiological significance. Indeed, it would be difficult to reconcile observations of cAMP-dependent phosphorylation of myosin light chain kinase, which is thought to favor muscle relaxation [6,7], with direct phosphorylation of the light chain by cAMP-dependent protein kinase, which would lead to muscle contraction.

Why the isolated light chain is phosphorylated by the cAMP-dependent protein kinase and the light chain in intact myosin is not phosphorylated is not known. With the myosin light chain kinase there is a tendency in heterologous systems (i.e., kinase and myosin from different myscle types) for the light chains to be phosphorylated more easily than the parent myosin, and it is known for myosin and light chains from a given muscle that the patterns of phosphorylation differ [20]. Thus there is some evidence to suggest that the conformation around the phosphorylated serine is changed when the light chain is removed from the myosin molecule and this alteration is manifest by the activities of both the myosin light chain kinase and the cAMP-dependent protein kinase. Steric hindrance due to the other myosin subunits or the masking of the essential arginine residue (the third amino acid to the N-terminal side of the phosphorylated serine) are possible contributors to this effect. The slight extent of phosphorylation by the cAMP-dependent protein kinase which was observed with the proteolysed myosin may also be rationalized by assuming a slight alteration of structure due to peptide bond cleavage.

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References

[1] Gorecka, A., Aksoy, M. O. and Hartshorne, D. J. (1976) Biochem. Biophys. Res. Commun. 71, 325–331.
[2] Sobieszek, A. (1977) Eur. J. Biochem. 73, 477–483.
[3] Chacko, S., Conti, M. A. and Adelstein, R. S. (1977) Proc. Natl. Acad. Sci. USA 74, 129–133.
[4] Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K. and Hartshorne, D. J. (1978) Biochemistry 17, 253–258.
[5] Pato, M. D. and Adelstein, R. S. (1980) J. Biol. Chem. 255, 6535–6538.
[6] Adelstein, R. S., Conti, M. A., Hathaway, D. R. and Klee, C. B. (1978) J. Biol. Chem. 253, 8347–8350.
[7] Conti, M. A. and Adelstein, R. S. (1980) Fed. Proc. FASEB 39, 1569–1573.
[8] Mrwa, U., Troschka, M. and Rüegg, J. C. (1979) FEBS Lett. 107, 371–374.
[9] Silver, P. J. and DiSalvo, J. (1979) J. Biol. Chem. 254, 9951–9954.
[10] Hoar, P. E. and Kerrick, W. G. L. (1980) Fed. Proc. FASEB 39, 1817.
[11] Noiman, E. S. (1980) J. Biol. Chem. 255, 11067–11070.

[12] Persechini, A., Mrwa, U. and Hartshorne, D. J. (1981) Biochem. Biophys. Res. Commun. in press.
[13] Perrie, W. T. and Perry, S. V. (1970) Biochem. J. 119, 31–38.
[14] Walsh, M. P., Cavadore, J.-C., Vallet, B. and Demaille, J. G. (1980) Can. J. Biochem. 58, 299–308.
[15] Teo, T. S., Wang, T. H. and Wang, J. H. (1973) J. Biol. Chem. 248, 588–595.
[16] Charbonneau, H. and Cormier, M. J. (1979) Biochem. Biophys. Res. Commun. 90, 1039–1047.
[17] Itzhaki, R. F. and Gill, D. M. (1964) Anal. Biochem. 9, 401–410.
[18] Spector, T. (1978) Anal. Biochem. 86, 142–146.
[19] Laemmli, U. K. (1970) Nature 227, 680–685.
[20] Mrwa, U. and Hartshorne, D. J. (1980) Fed. Proc. FASEB 39, 1564–1568.