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Effect of myosin phosphorylation on actomyosin ATPase activity: a flow microcalorimetric study

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Abstract

A flow microcalorimetric technique was used for the first time to evaluate the effect of myosin phosphorylation on actomyosin ATPase activity. Using an actomyosin system containing myosin light-chain kinase and phosphatase, myosin phosphorylation affects the actomyosin ATPase activity positively or negatively depending upon the molar myosin:actin ratio used in the assay. Our results show that under conditions near physiological ones the phosphorylation of myosin light chains decreases the actomyosin ATPase activity.

Keywords: ATPase; Microcalorimetry; Myosin; Phosphorylation

1. Introduction

Flow microcalorimetry, as predicted by Mönk and Wadsö [1], has been shown to be a powerful analytical tool in the study of several biological and biochemical systems [2–5], mainly because it can follow essential reactions that occur in crude biological homogenates such as the hydrolysis of ATP.

Myosin, a major protein involved in the process of muscle contraction, was first used in calorimetric studies due to its capacity to hydrolyse ATP [6]. Several different calorimetric approaches have been designed for studying the mechanism of the hydrolysis of ATP by myosin or by the complex actomyosin [7–9].

It is known that following muscle stimulation at least 50% of myosin light chains are phosphorylated [10]. However, the role myosin phosphorylation in the process of

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skeletal muscle contraction remains uncertain [11]. The effect of the phosphorylation of the regulatory myosin light chains, both in myosin–actin interactions and in the actomyosin ATPase activity, has been studied using several techniques. These include the measurement of the ATPase activity of the actomyosin complex by colorimetry [12], observation by electron microscopy of the interaction figures [13], analysis of the fragments produced by myosin proteolysis [14] and changes in turbidity [15].

In the present work, we used for the first time a flow microcalorimetric technique to evaluate the effect of myosin phosphorylation on actomyosin ATPase activity. Our results suggest that at the physiological myosin:actin molar ratio, the phosphorylation of myosin light chains decreases the actomyosin ATPase activity, thus affecting myosin–actin interaction.

2. Experimental

ATP, vanadium free, was purchased from Sigma. All other reagents were of the grade for biochemical analysis. Myosin, in a dephosphorylated state, was prepared as described by Pires et al. [16] and actin was prepared from muscle acetone powder according to Pardee and Spudich [17]. Both proteins were obtained from leg and dorsal white rabbit skeletal muscles. In order to obtain myosin preparation containing endogenous myosin light-chain kinase, phosphatase and calmodulin, myosin was precipitated only once (partially purified myosin).

The purity of the preparations was established by sodium dodecyl sulphate polyacrylamide gel electrophoresis [18] and the protein concentration was estimated by the biuret method [19] using bovine serum albumin as a standard. The state of phosphorylation of the myosin light chains was analysed by urea polyacrylamide gel electrophoresis [20] and measured using an LKB 2222-020 UltroScan XL laser scanning densitometer.

The calorimetric experiments were carried out in an LKB 2277 BioActivity Monitor (BAM) working in flow-through mode [21]. The calorimeter was electrically calibrated. The experiments were performed at 298 K in a medium containing 0.3 M KCl, 25 mM Tris pH 7.6, 5 mM $MgCl_2$, and additionally 1 mM EGTA or 0.1 mM Ca^{2+} when stated. When experiments were performed in the presence of actin, KCl was used in the medium at 0.1 M concentration instead of 0.3 M. Myosin concentrations ranging from 0.125 to 2 mg ml^{-1} and actin concentrations ranging from 0.125 to 0.6 mg ml^{-1} were used. Increasing amounts of actin were added to myosin in order to obtain myosin:actin molar ratios of 1:1.25, 1:2.25, 1:5 and 1:10. Myosin was used at a concentration of 1.9 mg ml^{-1} for a 1:1.25 myosin:actin ratio and at a concentration of 0.6 mg ml^{-1} for all the other experiments. After mixing the proteins, in a total volume of 20 ml, the reaction mixture was pumped through the calorimeter cell, using an LKB 2232 microperpex-S peristalic pump at a flow rate 28 ml h^{-1}. Before being pumped into the calorimetric cell, the medium solutions were filtered and degassed.

The ATPase reaction was initiated by addition of ATP to a final concentration of 1.6 mM. Under our experimental conditions, following the addition of ATP, only two minutes were required for the reaction mixture to reach the calorimetric cell and

a change in the heat flow was immediately observed. When a steady state for the heat evolved was reached, samples of 0.5 ml were collected at the outlet tube and mixed with 0.5 ml of a solution containing 8 M urea in order to analyse the phosphorylation state of the myosin light chains by electrophoresis.

3. Results and discussion

Typical flow calorimeter thermograms obtained during the experiments of ATP hydrolysis by myosin or actomyosin can be observed in Fig. 1. After adding the enzyme, myosin, to the medium and pumping the solution through the calorimeter cell, a new baseline (b) was obtained before adding ATP to initiate the reaction. The heat produced due to the hydrolysis of ATP was measured when a new steady state heat liberation was reached (c). After all the ATP had been consumed, the level of heat liberated returned to the value that was observed before ATP addition (Fig. 1). Identical calorimetric profiles were obtained for three experiments and the heat produced due to the hydrolysis of ATP was 2.4 ± 0.1 μW (mean $\pm$ SD).

When saturating concentrations of substrate are used, a steady-state heat liberation during a longer period of time can be obtained [1]. Under these conditions, upon increasing the enzyme concentration we should expect a linear increase in the heat liberation, as a result of the enzymatic activity. In fact, this was observed, thus indicating that the heat flow is produced by the ATPase activity of the myosin (Fig. 2). However, the addition of monovanadate [22], known to be an inhibitor of the myosin ATPase [23], either after reaching a steady-state heat liberation or before the addition of ATP, resulted in a decrease in the heat flow produced during the myosin ATPase activity [24].

Parallel studies were performed, using mixtures of myosin and actin. As described above for the experiments performed with myosin, a very good correlation between the

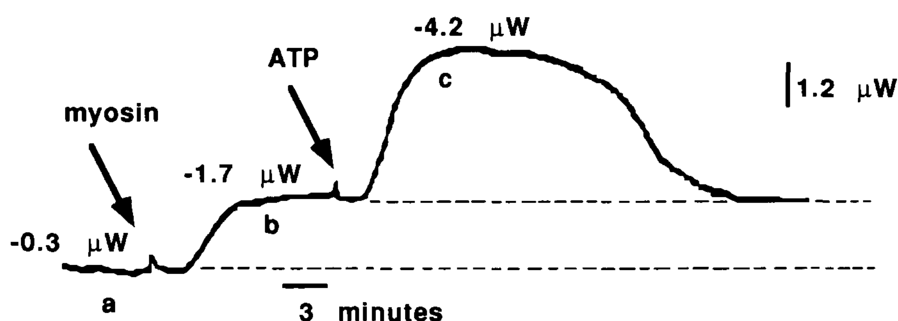

Fig. 1. Thermogram obtained during the hydrolysis of ATP by myosin in an LKB 2277 microcalorimeter working in flow through-mode: sensivity, 10 μW; flow rate, 28 ml hr^{-1}. Baselines a and b were established by pumping the reaction medium containing 0.3 M KCl, 5 mM $MgCl_2$ and 25 mM Tris/HCl, pH 7.6, in the absence or in the presence of 0.6 mg ml^{-1} of myosin, respectively. ATP to a final 0.5 mM concentration was added to initiate the reaction. Experiments were performed at 298 K.

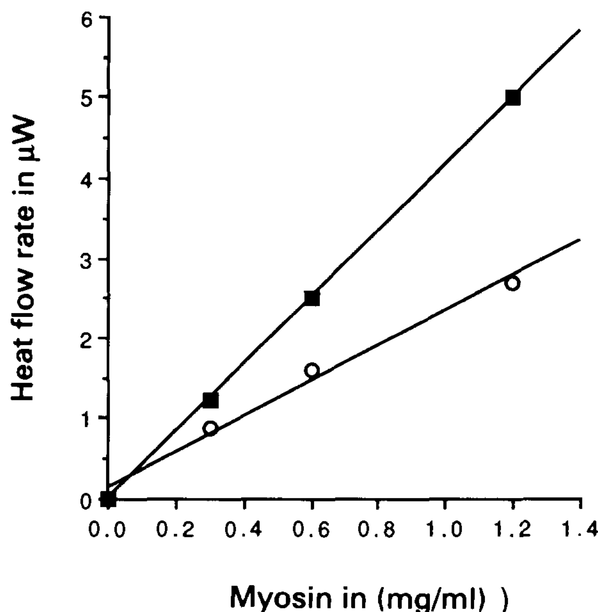

Fig. 2. Heat flow produced by myosin solutions in the absence (O) or presence of ATP (■). ATP to 1.6 mM final concentration was added to initiate the reaction. Other experimental conditions and procedures were identical to those described for Fig. 1.

production of heat and actomyosin concentration was obtained (Fig. 3). Under the experimental conditions used, the heat flow produced during the ATPase activity of actomyosin was greater than the heat flow produced during the ATPase activity of myosin, which is consistent with the stimulating effect of actin on the myosin ATPase activity [25]. In fact, the addition of actin to myosin, at a myosin:actin molar ratio of 1:1.7, increases the heat flow produced during the ATPase activity of myosin from $4.0 \pm 0.2\,\mu W\ (\text{mg myosin})^{-1}$ to $18.0 \pm 1\,\mu W\ (\text{mg myosin})^{-1}$. However, the heat flow values obtained for actomyosin in the absence of ATP are lower than those observed for myosin.

In order to study the effect of the phosphorylation of regulatory myosin light chains on the actomyosin ATPase activity, we used a myosin sample containing endogenous myosin light-chain kinase and phosphatase. The presence of Ca^{2+} in the medium induced the phosphorylation of the light chains whereas the presence of EGTA in the medium induced a dephosphorylated state. In fact, densitometric analysis of the gels indicated that when EGTA was present, 100% of the myosin light chains were not phosphorylated whereas in the presence of Ca^{2+}, 100% of the myosin light chains were phosphorylated (not shown). In these conditions, the heat flow produced during the hydrolysis of ATP by myosin, when myosin light chains were phosphorylated, was always greater than the heat flow produced when myosin was not phosphorylated (dephosphorylated state). Using three different myosin preparations, the results obtained in eleven experiments were (means $\pm$ SD) 8.9 ± 0.5 and $6.5 \pm 0.5\,\mu W$ for

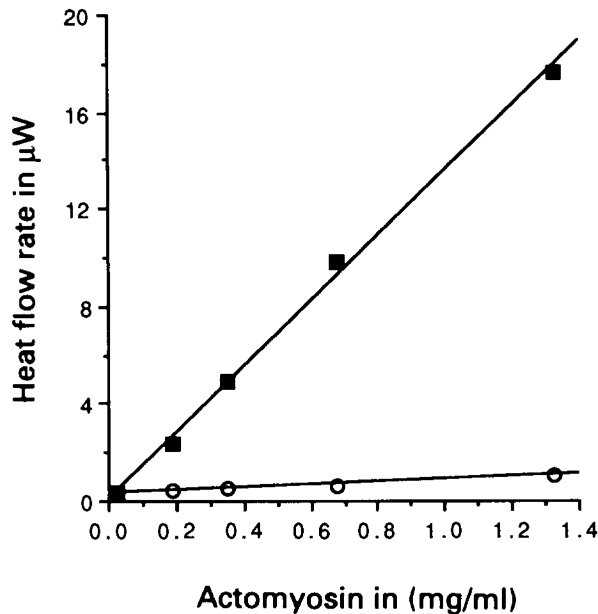

Fig. 3. Heat flow produced by actomyosin solutions in the absence (O) or presence of ATP (■) (mg actomyosin = mg myosin + mg actin). Actin was added to myosin at a myosin:actin molar ratio of 1:1.7. The experimental medium contains 0.1 M KCl instead of 0.3 M. Other experimental conditions and procedures were identical to those described for Fig. 1.

phosphorylated and dephosporylated myosin, respectively. All of these values are greater than the value of heat flow obtained with a purified sample of myosin. It is possible that the myosin sample obtained after being precipitated only once also contains small amounts of actomyosin, thus contributing to the increased observed heat flow. However, lower heat flow values were observed upon addition of actin to this myosin sample (Fig. 4). Thus, Fig. 4 shows that the heat flow produced during the ATPase activity increased from 6.5 μW (mg myosin)$^{-1}$ to 8.6 μW (mg myosin)$^{-1}$ upon actin addition. When increasing amounts of actin were added to myosin, resulting in myosin:actin molar ratios from 1:1.25 to 1:10, different results were obtained upon myosin phosphorylation, depending on myosin:actin molar ratio used in the assay. At myosin:actin molar ratios of 1:1.25 and 1:10, the heat flow produced during the hydrolysis of ATP by the complex actomyosin was higher when myosin was phosphorylated than the heat flow produced by the dephosphorylated form (Fig. 5). Conversely, the myosin:actin molar ratios of 1:2.5 and 1:5, the heat flow produced during the hydrolysis of ATP by the complex actomyosin was lower when myosin was phosphorylated than the heat liberation produced by the dephosphorylated form. Furthermore, because it was observed that the heat flow produced due to the ATPase activity of phosphorylated myosin is always greater than that for the dephosphorylated form, the effect reported upon addition of actin at 1:2.5 and 1:5 myosin:actin ratios is even more significant.

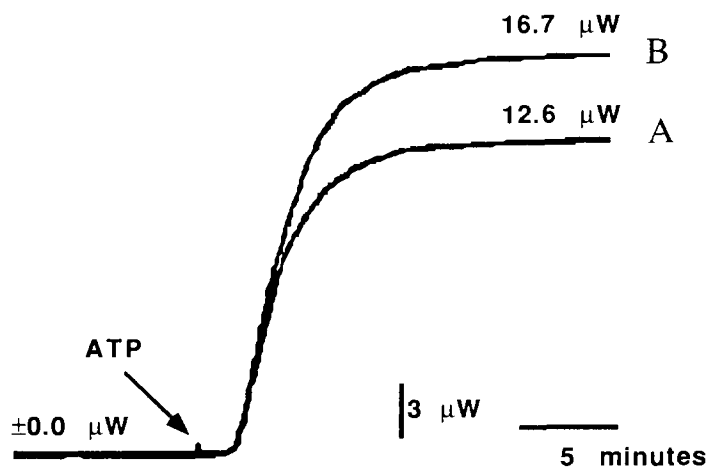

Fig. 4. Calorimetric profiles obtained for the ATPase activity of a partially purified myosin sample in the absence (A) or presence (B) of actin. Actin at a final concentration of 0.25 mg ml^{-1} was added to a 1.9 mg ml^{-1} myosin solution and the mixture pumped to the calorimeter cell at a flow rate of 28 ml h^{-1}. After a baseline was established, ATP was added at a final concentration of 1.6 mM. Experiments were performed at 298 K in a medium containing 0.1 M KCl, 5 mM $MgCl_2$, 1 mM EGTA and 25 mM Tris/HCl, pH 7.6.

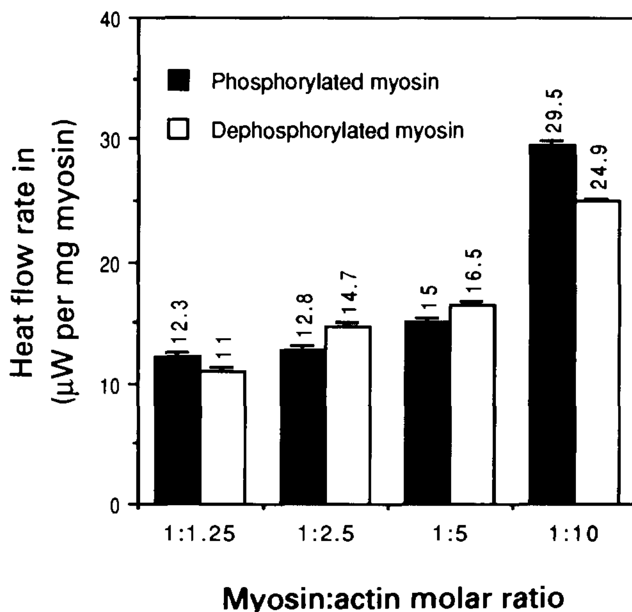

Fig. 5. Comparison of the effect of myosin phosphorylation on the heat flow produced during the ATPase activity of actomyosin at different myosin:actin molar ratios. Myosin light chains are phosphorylated (■) or dephosphorylated (□) when 0.1 mM $CaCl_2$ or 1 mM EGTA are present in the medium, respectively. Other experimental conditions and procedures were identical to those described for Fig. 4.

Therefore, under conditions very close to physiological ones and also for a myosin:actin ratio near the physiological ratio, 1:5 in the skeletal muscle [26], the phosphorylation of myosin light chains decreases the actomyosin ATPase activity. These results are in agreement with those reported using other techniques [12, 15] and suggest that the phosphorylation of the regulatory myosin light chains of skeletal muscle modulates the interaction of myosin with actin [13–15, 27]. However, the effect of myosin phosphorylation on actomyosin ATPase activity appears to depend on the conditions used for measuring this enzymatic activity: it has been reported that either it decreases [12, 15], does not change [28] or increases [29, 30] the actomyosin activity.

It would be interesting to use flow microcalorimetry in this biological system to study the effect of several other natural ligands of myosin, such as ADP, Mg^{2+}, Ca^{2+} or even vanadate, which mimics the behaviour of phosphate.

4. Conclusions

Important conclusions can be drawn from this flow calorimetric study. By using a myosin preparation containing endogenous myosin light-chain kinase, phosphatase and calmodulin, i.e. under conditions near physiological ones, we have shown that the phosphorylation of the myosin increases the myosin ATPase activity. This was not always true for the actomyosin complex because it depends on the myosin:actin ratio used in the assay. At the physiological myosin:actin ratio, the calorimeteric results indicate that the phosphorylation of myosin modulates the interaction between these proteins, thus decreasing the heat flow produced during the hydrolysis of ATP. Therefore, isothermal microcalorimetry working in a flow-through mode seems to be a very useful tool to study the effects of myosin phosphorylation on actomyosin ATPase activity.

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References

[1] P. Mönk and I. Wadsö, Acta Chem. Scand., 23 (1969) 29.
[2] C.M.P. Silva, M.C.P. De Lima, C.R. Oliveira and A.P. Carvalho, Thermochim. Acta, 179 (1991) 221.
[3] A. Schon and I. Wadsö, Cytobios, 55 (1988) 33.
[4] X. Chang-Li, T. Hou-Kuan, S. Zhau-Hua, Q. Song-Sheng, L. Yao-Ting and L. Hai-Shui, Thermochim. Acta, 123 (1988) 33.
[5] J.C. Sari, R. Gilli, V. Peyrot and C. Briano, Thermochim. Acta, 147 (1989) 119.
[6] R.J. Podolsky and J. M. Sturtevant, J. Biol. Chem., 217 (1955) 603.
[7] T. Yamada, H. Shimizu and H. Suga, Biochim. Biophys. Acta, 305 (1973) 642.

[8] T. Kodama, Physiol. Rev., 65 (1985) 467.
[9] N.C. Millar, J.V. Howarth and H. Gutfreund, Biochem. J., 248 (1987) 677.
[10] J.T. Barron, M. Barany and K. Barany, J. Biol. Chem., 254 (1979) 495.
[11] S.V. Perry, H. A. Cole, O. Hudlicka, V.B. Patched and S.A. Westood, Federation Proc., 43 (1984) 3015.
[12] D. Stepkowski, D. Szczesna, M. Wrotek and I. Kakol, Biochim. Biophys. Acta, 831 (1985) 321.
[13] D. Stepkowski, H. Osinska, D. Szczesna, M. Wrotek and I. Kakol, Biochim. Biophys. Acta, 830 (1985) 337.
[14] A. Mrakovcic-Zenic and E. Reisler, Biochemistry, 22 (1983) 525.
[15] I. Kakol, K. Kasman and M. Michinicka, Biochim. Biophys. Acta, 704 (1982) 437.
[16] E.M.V. Pires, S.V. Perry and M.A.W. Thomas, FEBS Lett., 41 (1974) 292.
[17] J.D. Pardee and J.A. Spudich, Meth. Enzymol., 85 (1982) 164.
[18] K. Weber and M. Osborn, J. Biol. Chem., 244 (1969) 4406.
[19] A.G. Gornall, C.J. Baldawill and M.M. Davis, J. Biol. Chem., 177 (1949) 751.
[20] W.T. Perrie and S.V. Perry, Biochem. J., 119 (1970) 31.
[21] J. Suurkuusk and I. Wadsö, Chem Scr., 20 (1982) 155.
[22] M. Aureliano and V.M.C. Madeira, Biochim. Biophys. Acta, 1221 (1994) 259.
[23] C.C. Goodno, Proc. Natl. Acad. Sci., 76 (1982) 2620.
[24] M. Aureliano, MSc. Thesis, Coimbra University, 1991.
[25] R.W. Lynn and E. W. Taylor, Biochemistry, 10 (1971) 4617.
[26] C.R. Bagshaw, Muscle Contraction, Chapman and Hall, London, 1982.
[27] D. Szczesna, A. Sobieszek and I. Kakol, FEBS Lett., 210 (1987) 177.
[28] M. Morgan, S.V. Perry and J. Ottaway, Biochem. J., 157 (1976) 687.
[29] S.M. Pemrick, J. Biol. Chem., 255 (1980) 8836.
[30] D.I. Levitsky, L.A. Shuvalova and B.F. Poglazov, FEBS Lett., 221 (1987) 77.