CYCLIC AMP-INDUCED PHOSPHORYLATION IN *DICTYOSTELIUM* OF A POLYPEPTIDE COMIGRATING WITH MYOSIN HEAVY CHAINS

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1. Introduction

Local activation of cyclic AMP receptors at the cell surface of *Dictyostelium discoideum* results in a chemotactic response starting within the first few seconds after cyclic AMP addition [1,2]. The mechanism of coupling of cyclic AMP receptors to the contractile apparatus of the cells is unknown. Candidates for intermediary processes are an increase of the cyclic GMP concentration [3,4], an influx of Ca^{2+} [5] and a reaction which results in the decrease of extracellular pH [6]. Here we report on a protein, probably myosin, that acts as a phosphate acceptor in lysates from cyclic AMP-stimulated cells.

2. Methods

2.1. *Cell culture*

Dictyostelium discoideum strain Ax-2, clone 206, was grown in liquid medium as in [6]. Cell differentiation was initiated by washing the cells in 0.017 M Sörensen phosphate buffer, pH 6.0.

2.2. *Cyclic AMP stimulation*

For stimulation the cells were used after 4–5 h development at 23°C. They were washed 4 times in an unbuffered solution of 10 mM KCl and 10 mM NaCl and incubated in the same medium for about 1 h at 23°C, and another hour at 11°C. 2×10^7 cells/ml were stimulated at 11°C by pulses of 3 nM cyclic AMP. Samples of 20–40 μl were withdrawn from the suspension for the assay of phosphorylation, and cellular responses were monitored by recording extracellular pH [6].

2.3. *Assay of protein phosphorylation*

Phosphorylation was assayed either in intact cells or lysates for 1 min at ~20°C. The medium for cell lysates consisted of 2 mM $MgCl_2$, 7.5 mM Tris/HCl, pH 7.5, 20 μM [γ-^{32}P]ATP (500 Ci/mol ATP) and 0.2% Triton X-100 in total vol. 200 μl. The reaction was started by adding 10^6 cells. The Triton caused immediate lysis of all cells. For phosphorylation in intact cells Triton was omitted and the suspension was shaken. Further specifications are given in the figure legends. After termination of the reaction with 1 ml cold 10% (w/v) trichloroacetic acid, the samples were incubated for 10 min in a boiling water bath. For the determination of total ^{32}P-incorporation the protein was collected on millipore HAWP filters and washed 3 times with 10 ml 10% trichloroacetic acid. The filters were counted with Instagel.

2.4. *SDS–gel electrophoresis and autoradiography*

The trichloroacetic acid precipitates were centrifuged on a Beckman Microfuge, washed once with 1 ml buffer containing 50 mM Tris/HCl and 1 mM dithiothreitol, pH 7.5, then resuspended in 50 μl 3-fold concentrated sample buffer. After boiling for 2 min, the samples were incubated with RNase (Worthington, 250 U/ml) for 15 min at 23°C. Polyacrylamide gel electrophoresis was as in [7] using a linear acrylamide gradient from 5–15%. About 40 μg cellular protein was applied per sample. The gels were stained with Coomassie Blue, dried and subjected to autoradiography using Kodak RP Royal X-Omat film. Molecular weight markers were human spectrin (mol. wt 240 000 and mol. wt 220 000), β-galactosidase (130 000), phosphorylase (92 000), bovine

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serum albumin (67 000), ovalbumin (43 000) and cytochrome *c* (12 000).

2.5. *Phosphorylation of purified actomyosin*

Actomyosin was prepared from *D. discoideum* by solubilization in sucrose and precipitation by dialysis against 0.1 M KCl as in [8]. For phosphorylation of myosin (fig.3) about 150 μg actomyosin was incubated in 200 μl assay medium with the lysate of 2×10^5 cells for 1 min at ~20°C. About 10% of these quantities were applied per sample to SDS–gel electrophoresis.

3. Results

3.1. *Overall protein phosphorylation*

Cells were stimulated by pulses of cyclic AMP and protein phosphorylation was determined in lysates prepared at various intervals before and after stimulation. Samples of the cell suspension were incubated for 1 min with [^{32}P]ATP in a solution containing Triton X-100. The results shown in fig.1A are con-

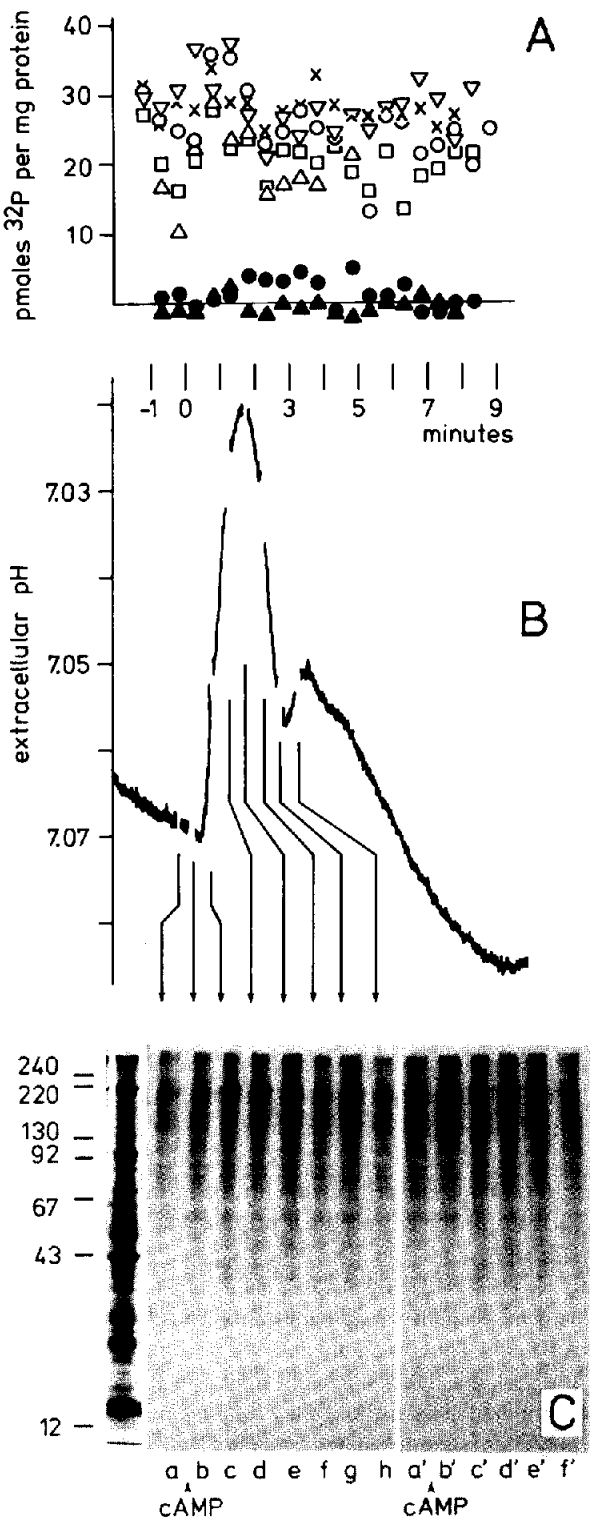

Fig.1. Protein phosphorylation related to responses of cells to cyclic AMP. Suspended cells were stimulated at 11°C with pulses of 3 nM cyclic AMP.

A: Total ^{32}P-incorporation into proteins (ordinate). The cyclic AMP was added at 0-time (abscissa). At the times indicated samples were transferred into the assay medium. Open symbols: Incorporation of ^{32}P into cell lysates (5 expt.). Closed symbols: Intact cells tested under the same conditions except that no detergent was added (2 expt.). Background incorporation was determined by the addition of trichloroacetic acid immediately after mixing of the cells with the assay medium. It corresponded to about 5 pmol ^{32}P/mg protein and was substracted.

B: The time course of cellular responses was followed by recording pH [6]. Abscissa as in A.

C: Phosphorylation of specific proteins in cell lysates determined by autoradiography of SDS–gels. Same experiment as in B. The cells were stimulated twice, as indicated, with an interval of 35 min. Effects of Ca^{2+} were tested by the assay of phosphorylation in the presence of 5×10^{-4} M EGTA (a–h), or in its absence (a'–f'). Samples a and a' were taken from the cell suspension before stimulation, b and b' at 15 s after cyclic AMP addition, the subsequent samples at intervals of 30 s thereafter. The marker proteins are indicated in kilodaltons. In the protein staining pattern (left) the strong band at 14 kilodaltons is added ribonuclease.

sistent with, although do not prove, a transient increase of ^{32}P-incorporation by 30% in lysates prepared during the first 2 min after stimulation of the cells. This increase, if it does exist, is superimposed on a high background of phosphorylation in lysates of both cyclic AMP-stimulated and unstimulated cells. When instead of lysates intact cells were incubated with [γ-^{32}P]ATP, no phosphorylation was found (fig.1A).

3.2. *Phosphorylation of a specific protein in lysates of stimulated cells*

SDS–polyacrylamide gel electrophoresis showed ^{32}P-incorporation into at least 28 bands. With the exception of one major protein and perhaps a few minor ones, all these proteins were similarly phosphorylated in homogenates from stimulated and unstimulated cells (fig.1C). The major exception was a protein of mol. wt 210 000. This protein comigrated with the heavy chain of myosin from *D. discoideum* which, according to [8], has mol. wt 210 000.

At 11°C cellular responses to cyclic AMP are slowed down by a factor of about 3 as compared to the optimal temperature, 23°C. When the cells were kept at 11°C, ^{32}P-incorporation into the mol. wt 210 000 protein was increased in lysates prepared at 45 s and 75 s after cyclic AMP addition. This increase was not yet detectable at 15 s, and it was no longer clearly observed after 105 s (fig.1C). No label was detected in the mol. wt 210 000 or any other band when intact cells were incubated in the same way with [γ-^{32}P]ATP.

Cyclic AMP stimulation of *D. discoideum* cells in an unbuffered medium leads to a biphasic decrease of pH [6]. The pH pattern was recorded in order to relate ^{32}P-incorporation to known responses of the cells. The increased incorporation into the mol. wt 210 000 protein coincided with the initial sharp increase of the extracellular protein concentration (fig.1B).

Cyclic GMP, cyclic AMP or Ca^{2+}, the 3 factors known to change after stimulation of cells by cyclic AMP, did not detectably activate phosphorylation of the mol. wt 210 000 protein in lysates from unstimulated cells, nor did they inhibit phosphorylation in lysates from stimulated cells (fig.2). Also 5×10^{-4} M EGTA did not have an effect (fig.1C).

The presence of a myosinkinase in *D. discoideum*

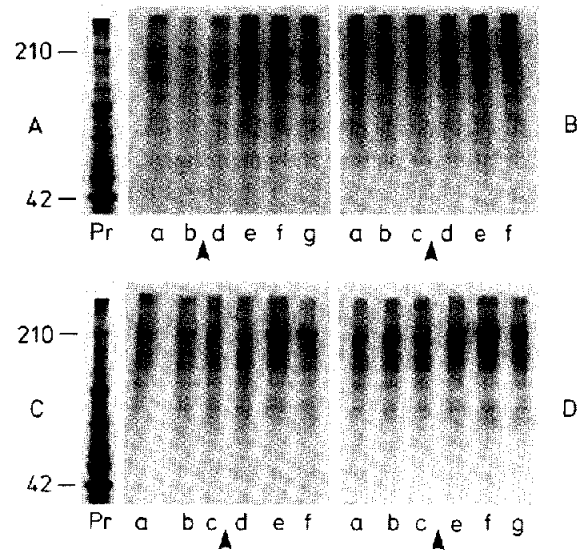

Fig.2. Protein phosphorylation in the presence of cyclic AMP (B), cyclic GMP (C), Ca^{2+} (D) and without these additions (A). Samples on the left of the arrows were lysed before stimulation: d at 15 s, e at 45, f at 75, g at 105 s, after stimulation. Additions to the assay medium for phosphorylation: (A) none; (B), (a) none; (b–f) 10^{-5} M cyclic AMP; (C), (a) none; (b–f) 10^{-5} M cyclic GMP; (D), (a) none; (b–g) 5×10^{-4} M $CaCl_2$. A–D correspond to consecutive stimulations of the same cell suspension. Similar results were obtained with additions of 2×10^{-4} M cyclic AMP or cyclic GMP. Molecular weights of myosin and actin [12] are indicated at the gel stained for protein (Pr).

has been established by incubating purified actomyosin with a dilute cell lysate and [γ-^{32}P]ATP. The myosin heavy chains were strongly phosphorylated (fig.3).

4. Discussion

In platelets and other non-muscle cellls phosphorylation of myosin by a specific kinase enhances actin-activated myosin ATPase activity [9]. The substrate of the myosinkinase is in this case a mol. wt 20 000 myosin light chain whereas it is a heavy chain in *D. discoideum*. A simple device for the control of motility by external signals would be a cyclic AMP-dependent protein kinase oriented in the membrane with its regulatory subunit towards the extracellular space where it senses cyclic AMP; and

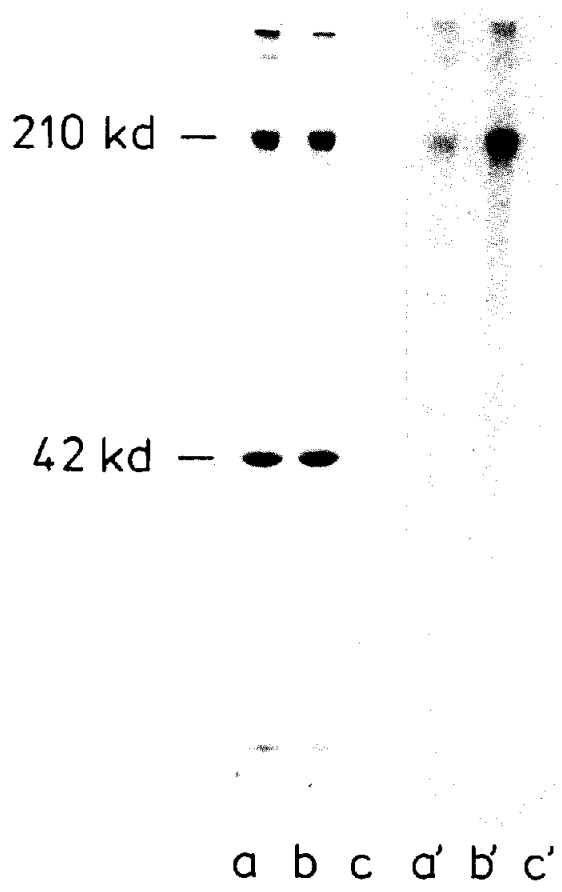

Fig.3. Phosphorylation of myosin heavy chains by cell lysates. Protein staining patterns (a–c) and autoradiograms (a'–c') of actomyosin (a, a'), actomyosin plus cell lysate (b,b'), and cell lysate alone (c,c'). The actomyosin, prepared as in [8] contained some myosin kinase activity as indicated by the background phosphorylation in a'. High myosinkinase activity was found in the cell lysate (b') which was diluted such that phosphorylation of its own myosin became undetectable (c'). The protein band on the bottom is the added RNase (see section 2).

with its catalytic subunit oriented towards the cytoplasmic surface where it regulates contractility by the phosphorylation of myosin. The fact that phosphorylation of the mol. wt 210 000 protein is not the earliest response observed after cyclic AMP stimulation argues against this simple mechanism. Cyclic GMP concentration and Ca^{2+} influx both increase before phosphorylation becomes detectable [4,5]. There are two possibilities: either phosphorylation of the mol. wt 210 000 protein is not directly coupled to receptor activation, or signal transduction occurs via multiple pathways. Activation of one type of cyclic AMP receptor could lead to myosin phosphorylation, activation of others to the regulation of cyclic GMP and Ca^{2+} channels.

It remains to be clarified whether the mol. wt 210 000 protein from cyclic AMP-stimulated cells is a better phosphate acceptor because it is in the dephosphorylated state, in contrast to the protein from non-stimulated cells. Alternatively, the stronger incorporation of ^{32}P into the mol. wt 210 000 protein from stimulated cells could be due to the presence of an activated protein kinase in the cell lysates, or to inhibition of a phosphatase.

In contrast to [10,11] no endogenous phosphorylation was obtained in intact cells with extracellular ATP as substrate (fig.1A). Particularly the mol. wt 210 000 protein was phosphorylated only after lysis of the cells. These results indicate that the kinase which phosphorylates the mol. wt 210 000 protein is located either in the cytosol or at an inner membrane surface where it is accessible to intracellular ATP.

Acknowledgements

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