Stimulation of actin synthesis in phalloidin-treated cells

Evidence for autoregulatory control

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We used the technique of scrape loading to introduce phalloidin into mouse embryo fibroblasts in mass culture. Phalloidin almost completely destroyed actin microfilament bundles, but the amount of polymerized cytoskeleton-associated actin was increased approximately two-fold and the amount of monomeric (Triton X-100 extractable) actin was significantly reduced. The major result of the present study is that the rate of actin synthesis in the phalloidin-treated cells was 2–3 times higher than in the control cells. Northern blot and translation in a cell-free system from rabbit reticulocytes showed that the actin mRNA level significantly increased as a result of phalloidin treatment.

Scrape loading; Phalloidin; Actin synthesis; Mouse fibroblast; Feedback control

1. INTRODUCTION

Actin cytoskeleton formation is a subject of complex multistep regulation. All eukaryotic cells contain a number of actin-binding proteins affecting different stages of the polymerisation process [1]. Activity of some of these proteins is in turn sensitive to the regulation by second messengers [2]. The actin monomers concentration is an important parameter driving the polymerization process. It is affected, however, not only by the polymerization and depolymerization, but also by the synthesis of actin de novo. Therefore the rate of actin synthesis should be precisely controlled.

In this paper we have shown that scrape loading is an effective technique to introduce phalloidin into mouse embryo fibroblasts. Phalloidin loading increased the level of polymerized and decreased the level of monomeric actin in the cells. These changes were accompanied by the specific stimulation of actin synthesis and an increase in the actin mRNA level.

2. MATERIALS AND METHODS

2.1. *Cell cultures, scrape loading and cell labelling*

Mouse embryo fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For scrape loading, cells were plated onto 14 cm Petri dishes at a density of 25 000 cells/cm^2. On the next day, culture medium was replaced by 1 ml of PBS with or without phalloidin (0.2–0.5 mg/ml Kemotex, Tallinn, USSR). Then cells were scraped from the substratum with a rubber policeman, carefully pipetted and the suspension was diluted 100-fold with ice-cold growth medium. Cells were pelleted by centrifugation, resuspended in the growth medium and replated. On the next day after scrape loading, cells were used for the estimation of the rate of protein synthesis or the amount of polymerized actin. Protein synthesis rate was measured using pulse labelling with [^{35}S]methionine (100 μCi/ml, Amersham) for 90 min in methionine-free medium with dialysed serum. Then cultures were washed with PBS and lysed in SDS-containing Laemmli sample buffer [3].

2.2. *Triton extraction and gel electrophoresis*

To estimate the amount of polymerized actin, we extracted an equal number of control and phalloidin-treated cells with 1% Triton X-100 in the cytoskeleton-stabilizing buffer (50 mM imidazole, pH 6.8, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 4% poly(ethylene glycol) 4000). Actin content was measured by densitometry of Coomassie-stained SDS-polyacrylamide gels of the whole-cell and Triton-insoluble fractions. Alternatively, we used cultures after 48 h metabolic labelling with 5 μCi/ml of [^{35}S]methionine. Actin and tubulin in both Triton-soluble and Triton-insoluble fractions were determined using radioimmunoprecipitation. Antibody to chicken gizzard actin, used in this experiment, was raised in rabbits and affinity purified. Monoclonal tubulin antibody TU-01 was kindly provided by Dr V. Viklicky (Institute of Molecular Genetics, Prague, Czechoslovakia).

Electrophoresis was performed in 7–15% polyacrylamide gels in Laemmli discontinuous buffer system [3]. Two-dimensional gel electrophoresis was performed by the procedure of O'Farrel [4].

2.3. *Translation of cellular DNA in a cell-free system*

Total cellular RNAs were purified by lithium chloride/urea extraction method [5]. Cell-free translation in rabbit reticulocyte lysate was as described in [6]. 10 μg of the total cellular RNA was translated at 34°C for 45 min, the system was stopped by the addition of the SDS-sample buffer, electrophoresed and subjected to autoradiography onto PMB film (Tasma, USSR).

2.4. *Blot analysis of RNA*

RNA was separated on 1.2% agarose gels containing 2.2 M for-

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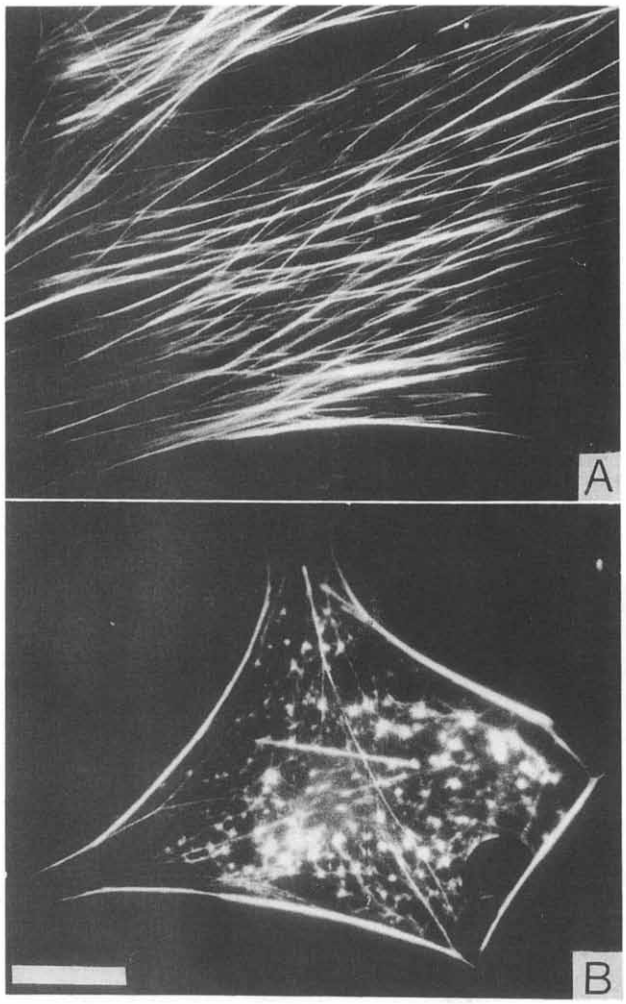

Fig. 1. Immunofluorescent staining of the control (A) and phalloidin-treated (B) cells with the antibody against actin. Note the replacement of the system of parallel actin cables in (A) by the disordered actin aggregates in (B). Bar = 20 μm.

maldehyde, then transferred to nitrocellulose [7]. Prehybridization and hybridization conditions were as described by Wahl et al. [8]. ^{32}P-labelled DNA probes were prepared by the random priming method of Feinberg and Vogelstein [9]. Cytoskeletal actin-specific mRNA sequences were detected with the pAct-1, the hamster β- or γ-actin plasmid [10]. The amount of RNA used for blot was 5 μg per lane as measured by E_{260}. All samples from control and phalloidin-treated cells contained equal amounts of mRNA for glyceraldehyde-3-phosphate dehydrogenase as measured by dot-hybridization with the genomic DNA probe (kindly provided by Drs A. Oleinikov and G. Dzhohadze, Institute of Protein Research, Pushchino).

3. RESULTS AND DISCUSSION

3.1. *Scrape loading of phalloidin into cultured cells*

To obtain a large population of cells loaded with phalloidin we used scrape loading [11]. Mouse embryo fibroblasts were scraped into 0.2–0.5 mg/ml phalloidin in PBS and allowed to spread on the substrate for 24 h. This treatment reduced the ability of cells to spread on the substratum as compared to the cells scraped into PBS. However, most of the cells were attached to the substrate 24 h after the plating. The phalloidin treatment significantly affected the distribution of actin in the cells (Fig. 1). Similar actin aggregates were described earlier in the cells after phalloidin injection [12]. More than 90% of the treated cells demonstrated typical changes in the actin distribution. These results

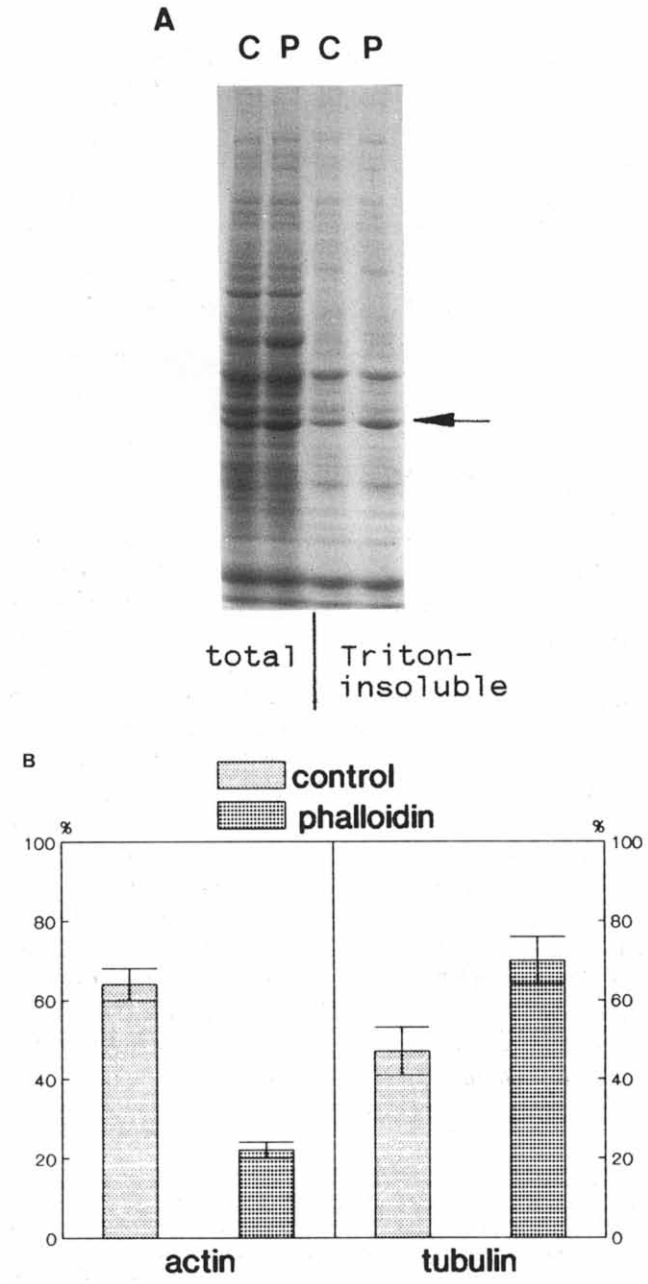

Fig. 2. Effect of phalloidin treatment on the amount of actin in the (A) cytoskeletal and (B) soluble fractions. (A) Cellular proteins were resolved by SDS-gel electrophoresis. Equal amounts of solubilized cells were applied on each of the lanes. Actin position (arrow) is shown on the right; c, control cells; p, phalloidin-loaded cells. (B) The relative amount of soluble actin and tubulin in control and phalloidin-treated cells, as measured by radioimmunoprecipitation and liquid scintillation counting.

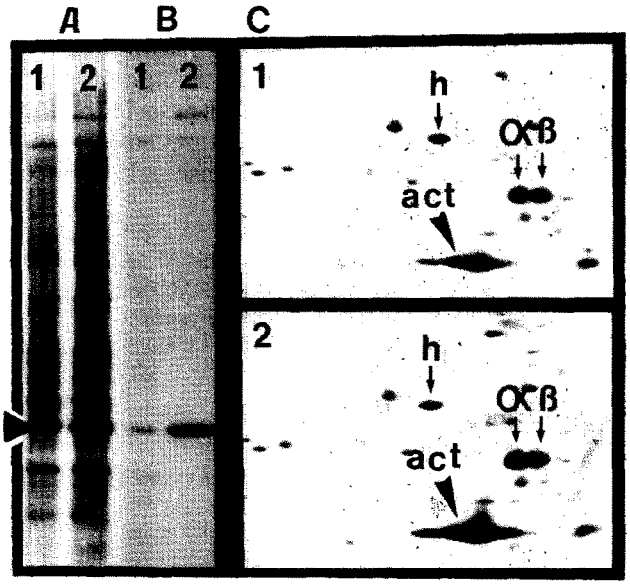

Fig. 3. The rates of actin synthesis in the control (1) and phalloidin-treated (2) cells with [^{35}S]methionine. (A) One-dimensional electrophoresis of total proteins. (B) Radioimmunoprecipitation of actin from the cell lysates with the actin antibody. (C) Fragments of the two-dimensional gels. Positions of individual proteins are marked by arrows: act, actin; α, α-tubulin; β, β-tubulin; h, HSC72.

show that the scrape loading procedure leads to the effective penetration of phalloidin into cultured fibroblasts.

3.2. *Phalloidin increased the amount of non-extractable (polymerized) actin in the cells*

To compare the pools of polymerized actin in the control and phalloidin-treated cells we extracted plasma membrane and soluble cytoplasmic proteins by Triton X-100 in the buffer stabilizing cytoskeletal structures and determined the amount of actin in the Triton-insoluble residue by SDS-gel electrophoresis and densitometry of the gels. The difference between the amount of total and non-extractable actin represented an estimation of the quantity of monomeric actin. According to these data, non-extractable actin represented about 55% of the total actin pool in the control cultures but after phalloidin treatment its amount was significantly increased (Fig. 2A) and, within the experimental error, was equal to the amount of total actin. Therefore the monomer actin pool was dramatically decreased in the phalloidin-treated cells.

For more precise estimation of the ratio between monomeric and polymerized actin we extracted metabolically labelled cultures with Triton X-100 and measured the amount of actin in the Triton-soluble and Triton-insoluble fractions by radioimmunoprecipitation using rabbit antibody to actin. The amount of tubulin in the cytoskeletal and soluble fractions was determined simultaneously using monoclonal tubulin antibody TU-01. These data are shown in Fig. 2B. After phalloidin treatment the amount of tubulin in the Triton-soluble fraction increased approximately 1.5-fold, but we suspect that this result at least partially can be explained by the presence of the detached cells in the Triton-soluble fraction. However, the amount of actin in the same Triton-soluble fraction of phalloidin-treated cells was significantly less than in the control cells (the apparent content of soluble actin was 3 times less in the phalloidin-treated than in the control cells). We consider the apparent amount of depolymerized actin determined in this experiment as the upper boundary of the estimation (due to the contribution from the detached cells) and the amount determined in the previous experiment as the lower boundary, and conclude that the monomeric actin in the control cells is about half of the total actin, and after phalloidin treatment it is between 0% and 20%.

3.3. *Stimulation of actin synthesis in the phalloidin-treated cells*

We used pulse-labelling of control and phalloidin-treated cultures with [^{35}S]methionine to study the rate of protein synthesis. Autographs of the gels after electrophoretic separation of the total proteins are presented in Fig. 3. It is evident that the rate of methionine incorporation into the band with the electrophoretic mobility of actin in the phalloidin-treated cultures was significantly higher than in the control ones (Fig. 3A). Densitometric scanning of the autographs showed that

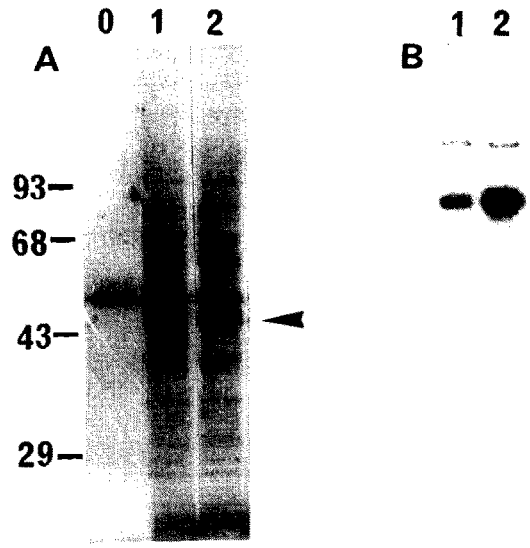

Fig. 4. Analysis of the actin mRNA content in the control (1) and phalloidin-treated (2) cells. (A) Total RNA preparations from both cultures were translated in a rabbit reticulocyte cell-free system. Lane 0, no RNA in the system. The band in this lane was labelled due to the non-translational incorporation of methionine in the endogenous reticulocyte protein. The actin position is marked by the arrow at the right. (B) The same RNA preparations were electrophoresed, transferred to nitrocellulose and hybridized with labelled actin DNA probe. 5 μg of RNA was loaded on each lane.

the rate of synthesis of this component after phalloidin treatment increased 2–3 times.

To show that this component was indeed actin, we precipitated actin from the control and phalloidin-treated cells using the actin antibody. The data presented in Fig. 3B demonstrate that actin isolated from the control culture contained significantly less label than the actin from the phalloidin-treated cells. Similar results were obtained using two-dimensional gel electrophoresis (Fig. 3C). Determination of the label in individual spots of the two-dimensional gels demonstrates that actin synthesis in the phalloidin-treated cells was stimulated 2.5-fold while synthesis of tubulin and constitutive heat shock protein HSC72 was not changed. Thus, the present data clearly show that polymerization of actin in the cells correlates with increase in actin synthesis.

Translation of total cellular RNA in the rabbit reticulocyte cell-free system was used to compare the amounts of actin mRNAs in control and phalloidin-treated cells. The results presented in Fig. 4A show that the incorporation of the label into actin in the system supplemented with RNA from the phalloidin-treated cells is 2–3 times higher than in the control sample. Incorporation of the label into all other components is not changed significantly.

Northern blot analysis also demonstrated a marked increase in actin mRNA level in phalloidin-loaded cells (Fig. 4B). Therefore, the phalloidin treatment increased not only the rate of actin synthesis, but also the amount of actin mRNA in these cells.

The general result obtained in this study is that cells exposed to phalloidin by scrape loading start to synthesize actin more intensively. It correlates with the increase in the amount of actin mRNA in the cells.

The most plausible explanation is that the observed stimulation of actin synthesis after phalloidin treatment was the result of the decrease in G-actin concentration in the cytoplasm through the mechanism of autoregulatory control, similar to that reported for tubulin synthesis [13–17]. In fact, we have shown here that phalloidin significantly reduced the amount of G-actin (e.g. actin extracted from the cells by mild Triton treatment). It is also possible that the stimulation of actin synthesis was the result of the increase in F-actin concentration.

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