Volume 286, number 1,2, 100–104 FEBS 09924 July 1991

ADONIS 001457939100634F

Autoregulatory control of actin synthesis in cultured rat hepatocytes

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Received 21 February 1991; revised version received 16 May 1991

ADP-ribosylation of actin by *Clostridium botulinum* C2 toxin resulted in a depolymerization of filamentous F-actin and an increase of monomeric G-actin in cultured hepatocytes. Simultaneously the de novo synthesis of actin was largely reduced, while the synthesis of albumin and of other proteins was not significantly impaired. The specific decrease of actin mRNA to 30% of the control indicates a down-regulation of actin synthesis at a pretranslational level. On the other hand, treatment with the mycotoxin phalloidin resulted in an increase of F-actin and a decrease of monomeric G-actin. Under this condition the de novo synthesis of actin was specifically enhanced and the level of actin mRNA was increased to 600% of the control. The data suggest an autoregulatory control of the actin synthesis.

Actin; Botulinum C2 toxin; Phalloidin; Hepatocyte

1. INTRODUCTION

In non-muscle cells, actin participates in motile processes. Furthermore, the cytoskeletal actin plays a key role in the cellular architecture. The functions of actin depend on the reversible polymerization of monomeric G-actin forming filamentous F-actin. The dynamic process of polymerization and depolymerization is apparently controlled by a number of actin-binding proteins [1,2]. On the other hand, the ratio of monomeric to polymeric actin might influence the actin synthesis [3]. Using toxins like *Clostridium botulinum* C2 toxin and phalloidin which specifically shift the cellular G-/F-actin state to the monomeric and to the polymeric form, respectively, we studied the influence of the relative G- and F-actin levels on the synthesis of actin in cultured hepatocytes. C2 toxin specifically ADP-ribosylates non-muscle G-actin in vitro and in intact cells [4,5]. ADP-ribosylated G-actin acts like a capping protein at the fast growing end of F-actin which inhibits further polymerization [6]. Since depolymerization, on the other hand, is not affected, C2 toxin increases the pool of monomeric G-actin in intact cells [7]. In contrast, binding of the mycotoxin phalloidin to F-actin dramatically reduces actin depolymerization resulting in a decreased pool of G-actin [8]. Studies with phalloidin are hampered by the fact that the mycotoxin is not able to enter most cells. Therefore, in the present study rat hepatocytes in primary cultures were used, which selectively take up phalloidin [9].

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The present investigation demonstrates that the level of actin mRNA and the synthesis of actin are under autoregulatory control by the G-/F-actin ratio in rat hepatocytes similar to the autoregulation of tubulin synthesis by tubulin monomers [10–14].

2. MATERIALS AND METHODS

2.1. *Cell cultures, protein distribution and gel electrophoresis*

Hepatocytes isolated from Wistar rats were cultured as described previously [15]. All experiments were performed with hepatocytes maintained in primary culture for 24–48 h. The cells were treated up to 20 h without or with C2 toxin (1 μg/ml) or with phalloidin (1–2.5 μg/ml), respectively. Thereafter, [^{14}C]leucine (0.5 Ci/mol) was added for further 4 h. For separation of cytoskeletal and cytosolic proteins, the cells were lysed by addition of buffer containing Triton X-100 (2% v/v) as described [16]. The insoluble cytoskeletal proteins including F-actin were separated from soluble proteins including G-actin by centrifugation for 10 min at 10000 × *g*. The supernatant proteins were concentrated by precipitation with methanol and chloroform. The pellets of cytoskeletal proteins and of cytosolic proteins were dissolved in SDS sample buffer [17] and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) [17]. Gels were stained with Coomassie blue, dried and subjected to autoradiography up to 14 days at −70°C (Fig. 1A). The quantitation of stained gels and of autoradiographs was performed by densitometry. In addition, the 43 kDa protein band was excised for determination of the radioactivity in a liquid scintillation counter.

Moreover the cellular concentration of G-actin was measured by determination of the selective inhibition of DNase I by monomeric actin [18] using a modified lysis buffer [19].

The degradation of total cellular actin was determined in a pulse-chase experiment: after 12 h of incubation in the presence of 0.45 mM [^{14}C]leucine (0.9 Ci/mol) the medium 199 was removed and the cell monolayers were washed 3 times with medium containing 2.5 mM unlabelled leucine. The decrease of radioactivity in the 43 kDa protein of cell lysates was monitored up to 48 h in the presence of medium 199 containing 2.5 mM leucine.

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2.2. *Identification of actin*

Cultured hepatocytes were treated with or without botulinum C2 toxin for 24 h. Thereafter, [^{14}C]leucine (0.5 Ci/mol) was added for 6 h. The cells were lysed in the presence of F-actin depolymerizing buffer as described [20]. After homogenizing and centrifugation for 1 h at 100000 × *g*, the supernatants were applied to columns of DNase I-coupled Sepharose 4B which specifically bind G-actin [20,21]. The columns were washed and actin was eluted with formamide as described [20]. The eluates were collected and precipitated by trichloroacetic acid for separation on SDS-PAGE as described above. The gel was stained with Coomassie blue and subjected to autoradiography for about 6 weeks at −70°C (Fig. 1B). For immunoblot analysis the radio-labelled cytosolic proteins were subjected to SDS-PAGE. Thereafter, immunoblotting was performed according to Towbin et al. [22] using monoclonal mouse anti-actin antibodies (ICN), peroxidase-coupled rabbit anti-mouse IgG antibodies (Sigma) and 4-chloro-1-naphthol as peroxidase substrate. The immunoblot was subjected to autoradiography for 20 days at −70°C (Fig. 1C).

2.3. *Detection and quantitation of actin and albumin mRNA*

RNA of cultured hepatocytes was isolated according to Chomczynski and Sacchi [23]. For Northern blot analysis total RNA (10 μg per lane) was subjected to electrophoresis on agarose gel (1% w/v), containing 2.2 M formaldehyde [24]. RNA was transferred to a nitrocellulose filter (Hybond-C extra, Amersham); hybridization was performed at 42°C in a mix containing formamide (50% v/v) [24] using ^{32}P-labelled random primed cDNA [25]. As probes, a 1.3 kb *Pst*I fragment of mouse β-actin cDNA [26] cloned into standard bluescript vector (Stratagene, La Jolla, CA) and a 0.8 kb *Pst*I fragment of rat albumin cDNA [27] cloned into pBR322 were used. The filters were washed once in 1× SSPE-buffer (150 mM NaCl, 1 mM EDTA and 10 mM sodium phosphate, pH 7.4) containing SDS (0.1% w/v) at room temperature followed by washing three times in 0.1× SSPE containing SDS (0.1%) each for 20 min at 60°C. The filters were subjected to autoradiography for 12–24 h and the hybridization signals were quantified by scanning densitometry.

In order to test a transcriptional regulation, cultured hepatocytes were incubated for up to 24 h in the presence of phalloidin (2.5 μg/ml) with or without actinomycin D (6 μM), which prevented the transcription by more than 95% without significant alterations in cell morphology. The quantitation of actin mRNA was performed by dot blot analysis; the dots were localized by autoradiography and excised for determination of the radioactivity in a liquid scintillation counter. All experiments were performed with triplicates; mean values are given. Statistical analysis was performed using Student's *t*-test.

3. RESULTS

3.1. *Influence of C2 toxin and of phalloidin on the cellular concentration and synthesis of actin*

The cellular concentrations of monomeric G-actin and of polymeric F-actin were studied after separation of the two forms of actin using Triton X-100 [16]. Treatment with C2 toxin increased the cellular concentration of a 43 kDa protein soluble in Triton X-100 containing buffer, which was identified later on to be monomeric actin; it decreased the amount of a Triton X-100-insoluble 43 kDa protein, which represented filamentous actin (Fig. 1A, left side). A similar shift to G-actin was described previously in cultures of chicken fibroblasts after treatment with C2 toxin [5,7]. An inverse distribution of actin was observed after phalloidin treatment, which resulted in an increase of cytoskeleton-associated F-actin and a relative decrease of the cytosolic G-actin pool (Fig. 1A). As determined by inhibition of DNase I activity [18], the cellular concentrations of monomeric G actin per mg cytosolic protein were 10.3 μg in untreated hepatocytes, 19.3 μg after 14 h of treatment with C2 toxin and 0.7 μg after 14 h of treatment with phalloidin.

In order to study the de novo synthesis of actin, incorporation of [^{14}C]leucine into newly synthesized cellular proteins was measured. The incorporation of [^{14}C]leucine into the 43 kDa protein was clearly altered by treatment with C2 toxin and with phalloidin, respectively, while the labelling of the total precipitable protein (not shown) and of other single proteins was essentially unchanged. The incorporation of [^{14}C]leucine into the 43 kDa protein was largely enhanced after phalloidin treatment with predominance of the label in the Triton-insoluble cytoskeletal fraction as compared to the control (Fig. 1A, right side). On the other hand, the incorporation was drastically reduced after C2 toxin treatment in the cytosolic as well as in the cytoskeletal fractions of hepatocytes. The intracellular degradation of actin was slightly modulated by C2 toxin and by phalloidin. In the absence of toxins, 24% of the 43 kDa protein was lost within 24 h; the degradation was increased to 34% in the presence of C2 toxin while it was decreased to 17% in the presence of phalloidin. Due to the long half-life of actin, these alterations may contribute only slightly to the observed changes of cellular actin observed in the presence of C2 toxin or phalloidin.

In order to identify the 43 kDa protein as actin, lysates of [^{14}C]leucine labelled cells were applied onto DNase I columns, which specifically bind G-actin and ADP-ribosylated G-actin [6,20]. Actin eluted from the DNase I affinity columns by formamide was identified as 43 kDa protein on SDS-PAGE (Fig. 1B). As shown by autoradiography of the gel, incorporation of [^{14}C]leucine into actin was largely reduced after C2 toxin treatment indicating an inhibition of actin synthesis. Furthermore, radioactively labelled actin of control and toxin-treated cells was identified by immunoblotting using a monoclonal anti-actin antibody (Fig. 1C). Again, the autoradiography of the labelled proteins indicates that the actin synthesis was decreased after C2 toxin treatment while it was increased after treatment with phalloidin.

Enhancement of G-actin in the cytosolic pool and inhibition of actin synthesis by botulinum C2 toxin was accompanied by a dose-dependent ADP-ribosylation of intracellular actin. Neither C2 toxin nor phalloidin treatment of hepatocytes resulted in a significant increase of the release of typical hepatocellular enzymes indicating the cellular integrity (not shown).

3.2. *Molecular mechanism of the toxin-dependent modulation of actin synthesis*

The molecular mechanism of the described toxin-dependent modulation of actin synthesis was studied by

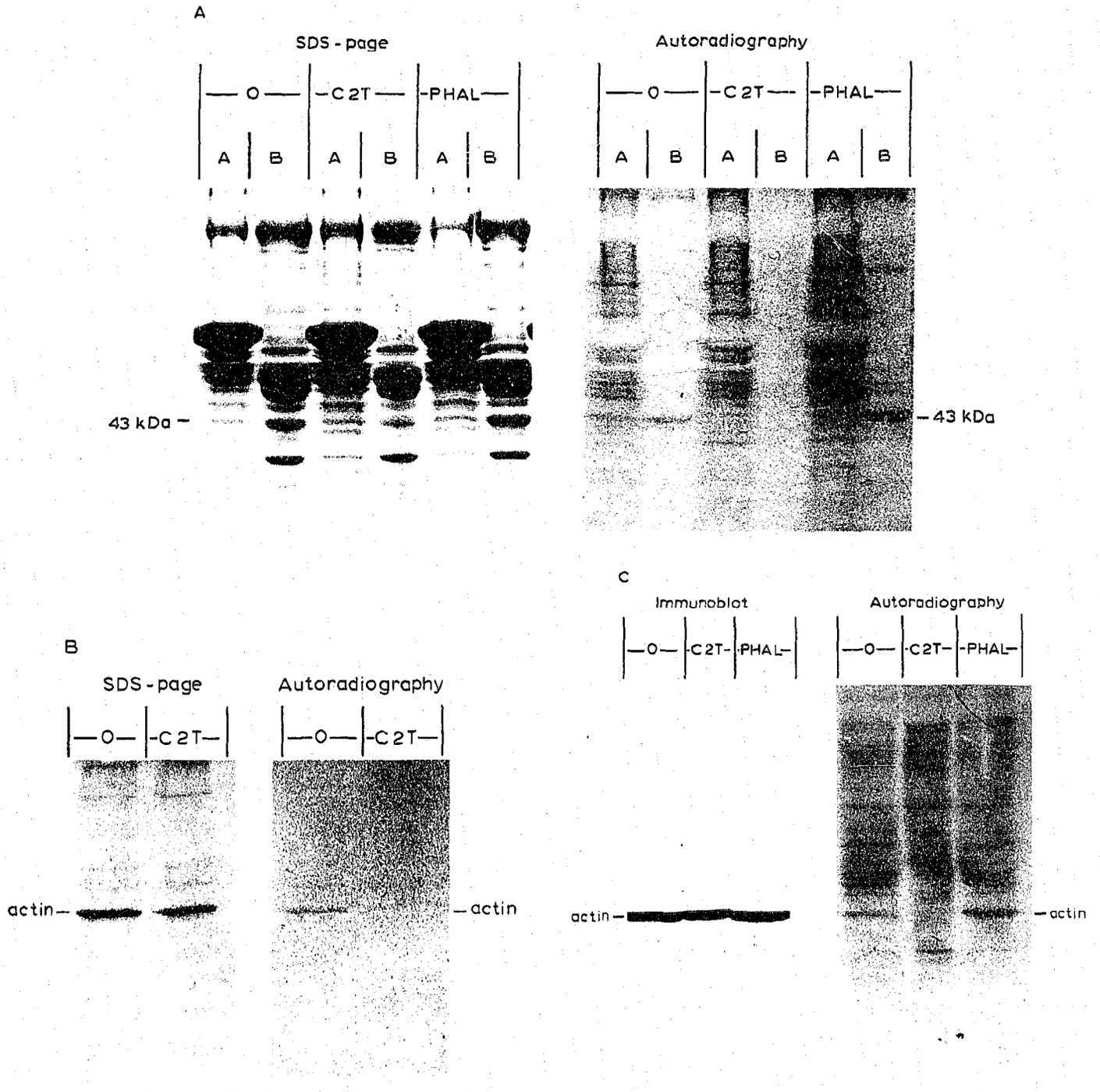

Fig. 1. Influence of botulinum C2 toxin (C2T; 1 μg/ml) and of phalloidin (PHAL; 1 μg/ml) on the amount of cytosolic and cytoskeletal proteins and on protein synthesis in cultured rat hepatocytes (ϕ = control). (A) Distribution of cytosolic and cytoskeletal proteins in Triton X-100 soluble (A) and insoluble (B) fractions. Left side: SDS-PAGE. Right side: autoradiography after labelling with [^{14}C]leucine. The position of actin on the SDS-PAGE is indicated by its molecular mass of 43 kDa. (B) Isolation of cellular actin by affinity chromatography on DNase I. (C) Identification of cellular actin by immunoblotting.

determination of the actin mRNA levels. Northern blot analysis using [^{32}P]cDNA shows that treatment of hepatocytes with C2 toxin decreased the actin mRNA level to about 30% whereas phalloidin largely increased the amount of actin mRNA to about 600% of the control (Fig. 2). In order to distinguish whether the increase of specific mRNA was due to an enhanced transcription or to a decreased degradation of mRNA, the effect of phalloidin was studied in the presence of actinomycin D. As demonstrated by dot blot analysis,

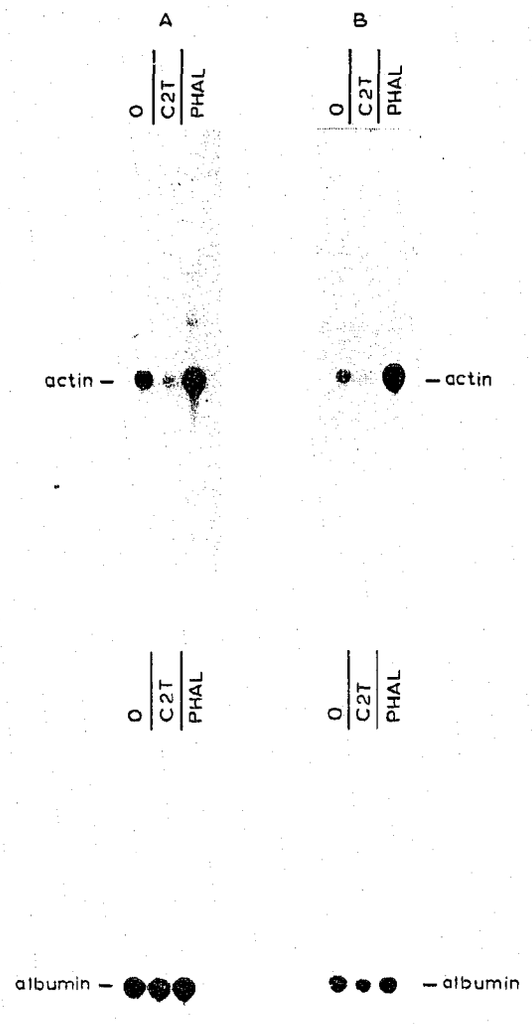

Fig. 2. Northern blot analysis of actin mRNA and albumin mRNA from cultured rat hepatocytes. (A) After 9 h incubation and (B) after 17 h incubation. The cells were treated without (ϕ) or with C2 toxin (C2T; 1 μg/ml) or with phalloidin (PHAL; 2.5 μg/ml), respectively.

the phalloidin-dependent increase of actin mRNA was completely prevented in the presence of actinomycin D indicating a transcriptional regulation (not shown). In contrast, treatment of hepatocytes with either toxin did not significantly affect the albumin mRNA, a finding which is in agreement with the selectivity of the toxin's effects on actin synthesis.

4. DISCUSSION

The present study demonstrates that botulinum C2 toxin and phalloidin exhibit antagonistic effects on the G-/F-actin equilibrium and on actin synthesis in intact cultured hepatocytes. C2 toxin increased the cytosolic monomeric G-actin pool; it decreased actin synthesis and the amount of actin mRNA specifically. On the other hand, phalloidin shifted the G-/F-actin ratio to the cytoskeletal F-actin; actin synthesis and the amount of actin mRNA were specifically enhanced at the transcriptional level.

Quite recently, it has been reported that in phalloidin-treated fibroblasts G-actin was decreased whereas actin synthesis was increased; an autoregulatory control of actin synthesis by G-actin was proposed [3]. However, the influence of enhanced cellular concentrations of G-actin on actin synthesis was not tested, a direct modulation of actin synthesis by phalloidin was not excluded and the specificity of the observed increase of actin mRNA was not documented. Since in the present study C2 toxin and phalloidin exhibited specific reciprocal effects on the intracellular level of G-actin and on actin synthesis, it is most likely that actin synthesis was modulated by the cellular concentrations of G-actin rather than by direct effects of the two structurally different toxins. Thus, an autoregulatory control of actin synthesis by the G-/F-actin ratio can be assumed.

A similar regulation was previously described for the synthesis of tubulin. Agents like colchicine, nocodazole or colcemid which cause microtubule depolymerization and an increase in unpolymerized tubulin subunits, decrease tubulin synthesis [10,13]. Furthermore, microinjection of purified tubulin selectively inhibits tubulin production [12]. These effects are accompanied by a decrease in the tubulin mRNA [11].

Interactions between protein synthesis and the cytoskeleton apparently exist. It has been reported that mRNA coding for proteins of the cytoskeleton is associated with cytoskeletal structures [28,29]. The release of mRNA from the cytoskeleton reduced the total protein synthesis by a decrease in the translation of the mRNA [30]. In this study high concentrations of cytochalasin D were used, which effected rearrangements of the cytoplasmic architecture without diminishing or disaggregating the cytoskeletal network or changing the G-/F-actin ratio [30]. The effects of C2 toxin and phalloidin observed in the present study are apparently not mediated by this mechanism, since overall protein synthesis was not significantly impaired, the level of actin mRNA was specifically modulated and inhibition of the transcription prevented the phalloidin dependent enhancement of actin mRNA. Thus, the data suggest that the actin synthesis in intact hepatocytes is under autoregulatory control of the G-/F-actin ratio similar to the autoregulation of tubulin synthesis by a shift of polymerized to unpolymerized tubulin.

In vitro studies recently demonstrated that not only F-actin but also G-actin bind to isolated hepatocellular membranes with similar affinities [31]. Although the

physiological significance of the G-actin binding is not clear, it cannot be excluded that the equilibrium between unbound and membrane-bound G-actin is involved in the observed autoregulation of actin synthesis. Further studies will be necessary to answer this question.

Acknowledgements: We thank Dr. W. Birchmeier and Dr. G. Ryffel (D-4300 Essen) for providing actin and albumin cDNA probes, respectively, and Dr. M. Kröger (D-6300 Giessen) for valuable advice on molecular biological techniques. The excellent technical assistance of Monika Philipp and Karin Klapettek is gratefully acknowledged. The study was supported by the Fonds der Chemischen Industrie.

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