Autoregulation of Actin Synthesis by Physiological Alterations of the G-Actin Level in Hepatocytes

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Summary: Hypotonic treatment of cultured rat hepatocytes significantly decreased the monomeric G-actin level by 18% after 120 min while the level of filamentous F-actin remained essentially unchanged. Simultaneously the level of cellular actin mRNA was increased by 53%.

Incubation of hepatocytes for 120 min with the F-actin stabilizing toxin phalloidin from *Amanita phalloides* led to a decrease of G-actin by 70% and an increase of F-actin by 55%. Although the toxin dependent decrease of G-actin was much more pronounced than the decrease after hypotonic treatment, the increase of actin mRNA was similar under both conditions. Simultaneous treatment with hypotonic medium did not result in a further decrease of the G-actin level. On the other hand, the G-actin elevating C2 toxin from *Clostridium botulinum* completely blocked the effects of osmotic stress on G-actin and actin-mRNA content.

The results demonstrate that already an essentially physiological decrease of G-actin without alterations of F-actin results in a substancial enhancement of the actin mRNA level, indicating the physiological significance of this autoregulation.

Introduction

Actin is a main component of the cytoskeletal framework in non-muscle cells. Moreover, it is involved in a variety of intracellular motile processes. Most of the functions of actin depend on the polymerization of monomeric G-actin[1]) and depolymerization of filamentous F-actin[1]), respectively. This dynamic process is controlled by a number of actin binding proteins (1−3).

The synthesis of actin, similar to other cytoskeletal proteins, appears to be under autoregulatory control on the basis of the equilibrium between the monomeric and the polymeric form (4−8). Under experimental conditions,

the equilibrium between G- and F-actin can be shifted to the monomeric or to the polymeric form by biological toxins, which arrest actin in one of the two forms. Phalloidin from *Amanita phalloides*, which is rapidly taken up by hepatocytes in culture, binds to F-actin and dramatically reduces actin depolymerization, resulting in a decreased pool of monomeric G-actin (9). On the other hand, C2 toxin from *Clostridium botulinum* specifically ADP-ribosylates non-muscle G-actin in cell free systems (10) as well as in intact cells (11). ADP-ribosylated G-actin acts as a F-actin capping protein (12), thereby increasing the cellular pool of monomeric G-actin (13).

In cultured rat hepatocytes, the stabilizing of F-actin by phalloidin is followed by an increase of actin mRNA, whereas the increase of G-actin by C2 toxin is followed by a decrease of actin mRNA, indicating an autoregulatory control of actin synthesis (14). However, the metabolic significance of this regulation is unclear, since the

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[1]) Abbreviations:
G-actin, globular actin; F-actin, filamentous actin; DNase I, deoxyribonuclease I; S.E. M., standard error of the mean; FITC, fluorescein isothiocyanate; SDS, sodium dodecylsulphate; EGTA, ethylene glycol bis(β-amino ethyl ether) tetraacetic acid; kb = 10^3 bases.

toxin dependent shifts of G- and F-actin are largely un-physiological. Moreover, a direct effect of the toxins on the actin mRNA level could not be excluded so far.

More physiological shifts of the G-/F-actin equilibrium are observed during hypotonic treatment of hepatocytes, followed by modulation of actin synthesis (15). In the present study, the effect of hypotonic treatment on actin synthesis was compared to the toxin-dependent regulation of actin synthesis. Phalloidin and hypotonic treatment, respectively, led to similar enhancements of actin mRNA although the decrease of G-actin was fourfold stronger in the presence of phalloidin. The effects of hypotonic stress plus phalloidin treatment were not additive. On the other hand, C2 toxin completely blocked the effects of osmotic stress on G-actin and actin mRNA content. It is concluded that the maximal effects on actin synthesis are already achieved by essentially physiological alterations of the G-actin level.

Materials and Methods

Materials

Skeletal α-actin (rabbit), DNA, DNase I[2]), phalloidin and rhodamine-labelled phalloidin were obtained from Sigma (Munich, Germany). Medium 199 was supplied by Gibco (Karlsruhe, Germany), collagenase was from Boehringer Mannheim (Germany). Hybond C hybridization membrane and [α-^{32}P]dCTP were purchased from Amersham Buchler (Braunschweig, Germany). All other chemicals were analytical grade and obtained from commercial sources.

Cell culture and osmotic stress

Hepatocytes were isolated from Wistar rats by collagenase perfusion. Before experimental use, the cells were maintained on 6 cm Falcon® culture dishes for 18 hours in medium 199 (280 mosmol/l) as described (16). Later on, hypotonic treatment was performed in medium 199 adjusted to 220 mosmol/l by dilution. For normotonic treatment, this medium was adjusted to 280 mosmol/l by addition of sodium chloride to 280 mosmol/l.

Toxins

The two components I and II of C2 toxin were prepared and activated essentially as described (17). The final toxin concentration was 100 μg/l culture medium of component I and 200 μg/l of component II. Phalloidin was dissolved in H_2O and used in a final concentration of 2.5 mg/l.

Measurement of cellular G-actin, F-actin and of protein

The amount of cellular G-actin was determined by DNase I inhibition assay (18), essentially as described (13). Cells pretreated with or without osmotic stress or with toxin, respectively, were lysed by 500 μl of freshly prepared, ice-cold lysis buffer containing

5 mmol/l potassium phosphate, pH 7.6, 150 mmol/l NaCl, 2 mmol/l MgCl₂, 0.1 mmol/l dithiothreitol, 0.5 mmol/l ATP, 0.01 mmol/l phenylmethylsulphonyl fluoride, 2 mmol/l EGTA, 5 g/l Triton X-100 and 150 g/l glycerol. Cell lysates were scraped off, transferred to Eppendorf test tubes, mixed, placed on ice for 15 min, and homogenized using a syringe. Thereafter, the homogenates were spun for 45 min at 100 000 g and 4 °C. Small amounts (10−50 μl) of supernatants were mixed with 20 μl of DNase I solution (0.1 g/l in 50 mmol/l Tris-HCl, pH 7.5, 0.2 mmol/l, CaCl₂ and 0.01 mmol/l phenylmethylsulphonyl fluoride). For determination of DNase activity inhibition, 1 ml of prewarmed (25 °C) calf thymus DNA (40 mg/l in 100 mmol/l Tris-HCl, pH 7.5, 4 mmol/l MgSO₄, 1.8 mmol/l CaCl₂) was added, mixed for 3 s and immediately transferred into a cuvette for measuring the absorbance at 260 nm for 40 s after a total delay of 10 s. Increase of absorbance was measured with a photometer from Pharmacia, and plottet using the Pharmacia-enzyme-kinetik software. The slope of the linear part of the increase in absorbance is directly proportional to the amount of not inhibited DNase added (18). A standard curve for 30−70% inhibition was obtained by measuring the absorbance after addition of defined amounts of rabbit skeletal muscle G-actin to the reaction mixture instead of cell lysates. G-actin of hepatocytes was referred to cytosolic protein in the 100 000 g supernatants, determined according to Bradford (21), using bovine γ-globulin as standard. Data were expressed as μg G-actin per mg cytosolic protein.

F-actin content was measured by binding of rhodamine-labelled phalloidin to actin filaments (19) in permeabilized and formaldehyde fixed hepatocytes with some modifications (20). Cells were maintained on 6 cm Falcon® culture dishes, washed twice with stabilization buffer (75 mmol/l KCl, 3 mmol/l MgSO₄, 1 mmol/l EGTA, 0.2 mmol/l dithiothreitol, 0.1 mmol/l phenylmethylsulphonyl fluoride, 10 mmol/l imidazole, 10 mg/l aprotinin, pH 7.2) and permeabilized with 0.3 g/l saponin in stabilization buffer for 10 min at room temperature. Cell monolayers were fixed in freshly prepared 30 g/l formaldehyde in stabilization buffer for 20 min at room temperature, washed twice and stained in the dark with 175 μg/l rhodamine-phalloidin in stabilization buffer for 30 min. After washing thrice with stabilization buffer, extraction of rhodamine-labelled phalloidin was initiated by addition of ice cold HPLC-grade methanol for 30 min at −20 °C. Thereafter, the cells were scraped off with a rubber policeman and extraction was continued overnight at −20 °C. The suspension was centrifuged for 10 min at 10 000 g. Rhodamine in the supernatants was determined by means of a rhodamine-labelled phalloidin standard curve using an Aminco-Bowman spectrophotofluorometer (Colora, Lorch, Germany). Excitation and emission wavelengths were 542 and 563 nm. Cellular protein was determined according to Bradford (21) in parallely cultured hepatocytes lysed in the presence of 5 g/l Triton X-100. Data were expressed as ng rhodamine-phalloidin per mg cellular protein.

In addition, the ratio of filamentous to non-filamentous actin was determined by separation of proteins insoluble or soluble in Triton X-100, respectively (22). After treatment of hepatocytes with or without osmotic stress or with toxins, respectively, the cells were lysed by addition of an ice cold Triton solution containing 20 g/l Triton X-100, 160 mmol/l KCl, 20 mmol/l EGTA, 8 mmol/l sodium azide, and 40 mmol/l imidazole HCl, pH 7.0. The resulting lysates of hepatocytes were scraped off, transferred to test tubes, vortexed and placed on ice for 15 min. Thereafter, the tubes were centrifuged for 15 min at 3000 g. The resulting pellets, containing cytoskeletal proteins, were washed once with the Triton solution and dissolved essentially as described (11) in a solution containing 50 g/l SDS, 50 g/l saccharose, 50 g/l glycerol, 200 mmol/l dithiothreitol, and 50 mmol/l Tris-HCl, pH 7.5. The cytosolic proteins of supernatants were precipitated as described (11) and dissolved as described for cytoskeletal pellet proteins. Both pellet- and supernatant-proteins were analyzed by SDS gel electrophoresis. Filamentous cytoskeletal and non-filamentous cytosolic actin were identified by their relative molecular mass. Quantification was performed by scanning the M_r 43 000 protein actin using an Epson GT 6000 scanner and the Gel-Image programme from Pharmacia (Freiburg, Germany).

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[2]) Enzymes:
Alanine aminotransferase (EC 2.6.1.2); aspartate aminotransferase (EC 2.6.1.1); deoxyribonuclease I (EC 3.1.4.5); lactate dehydrogenase (EC 1.1.1.27).

Detection and quantitation of actin and albumin mRNA

RNA of rat hepatocytes was isolated according to *Chomczynski & Sacchi* (23). Northern- and dot blot hybridization was performed as described previously (14). In brief, total RNA (10 µg) was subjected to electrophoresis on agarose gel and transferred to a nitrocellulose filter, or was directly dotted onto nitrocellulose, respectively. Hybridization was performed at 42 °C in a mixture containing formamide (500 g/l) using ^{32}P-labelled random primed cDNA. As probes were used a 1.3 kb *Pst* I fragment of mouse β-actin cDNA and a 0.8 kb *Pst* I fragment of rat albumin cDNA, both cloned into standard bluescript vector (Stratagene, La Jolla, USA). The filters were washed once in SSPE-buffer (150 mmol/l NaCl, 1 mmol/l EDTA and 10 mmol/l sodium phosphate, pH 7.4) containing SDS (1 g/l) at room temperature followed by washing three times in 1 : 10 diluted SSPE containing SDS (1 g/l) each for 20 min at 60 °C. The filters were subjected to autoradiography for 12−24 h and the hybridization signals were quantified by counting in a liquid scintillation counter or by scanning densitometry. The β-actin mRNA levels were normalized to the levels of albumin mRNA, which were essentially unchanged.

Statistics

Statistical analysis was performed using *Student's* t-test.

Measurement of cellular enzyme activities

The enzymes alanine aminotransferase[2], aspartate aminotransferase[2] and lactate dehydrogenase[2] were measured in culture medium according to the recommendations of the German Society for Clinical Chemistry (24), using reagents from Boehringer (Mannheim, Germany).

Results

Influence of osmotic stress on cellular G-actin/F-actin and on actin mRNA

Rat hepatocytes were cultured for 18 hours as described in Materials and Methods. Thereafter the medium 199 was replaced by hypotonic (220 mosmol/l) or normotonic (280 mosmol/l) media for 120 min, respectively. Under hypotonic conditions the amount of cytosolic G-actin was significantly decreased by 18% after 60 min without further alteration during the next 60 min, indicating that the cells adapted to the hypotonic condition (fig. 1). The decrease of monomeric G-actin was not accompanied by a substantial alteration in the cellular F-actin content as measured by histochemical staining of F-actin using rhodamine-labelled phalloidin (tab. 1) as well as by direct quantification of filamentous and non-filamentous actin separated by Triton X-100 extraction and gel electrophoresis (not shown). Reciprocally to the decreased amounts of cytosolic G-actin the cellular level of actin mRNA was enhanced by 53% after 120 min of incubation with hypotonic medium (fig. 1). The effect of osmotic stress on actin mRNA was specific, since albumin mRNA for control was essentially unchanged as demonstrated by Northern blot analysis (fig. 2).
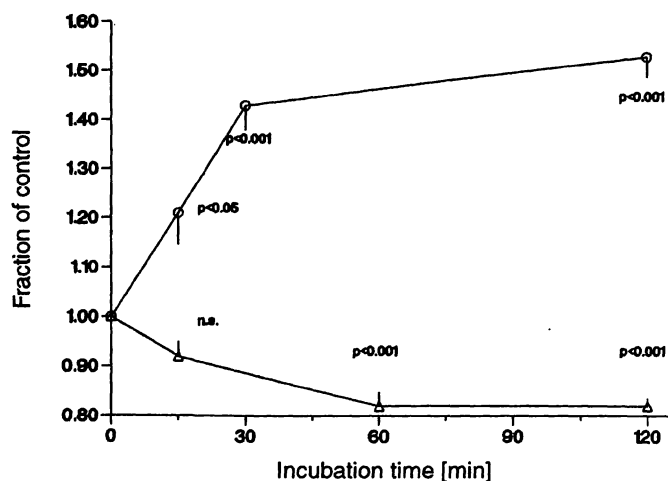
Fig. 1 Influence of osmotic stress on G-actin and on actin mRNA levels in cultured rat hepatocytes.

After culture for 18 hours the normotonic medium was removed and normotonic or hypotonic media were added up to 120 min. Thereafter, monomeric G-actin (△) and actin mRNA (○) were determined as described in Materials and Methods. The initial concentration of G-actin, as measured by DNase inhibition, was 13 µg per mg of cytosolic protein, which was determined as protein soluble in Triton X-100 in the 100 000 g supernatants of hepatocytes. Specific mRNA was measured by Northern- or dot-blot hybridizations using specific β-actin- and albumin-probes. Actin mRNA levels were normalized to the levels of albumin mRNA, which were essentially unchanged. Data presented are means ± S. E. M. of 6 separate determinations. The P values were obtained by *Student's* t-test for unpaired data in comparison with control cells under normotonic conditions (280 mosmol/l). n.s., not significant.

Modulation of osmotic effects by C2 toxin and phalloidin

After primary culture for 18 h, hepatocytes were incubated with normotonic or hypotonic medium with or without phalloidin or C2 toxin, respectively. Under normotonic conditions, phalloidin dramatically decreased cellular G-actin by 70% after incubation for 120 min, which largely exceeded the decrease by hypotonic treatment (fig. 3a). Simultaneously, the level of F-actin was increased by 55% (tab. 1). Hypotonic incubation during phalloidin treatment did not result in an additional decrease of G-actin (fig. 3a). On the other hand, treatment with C2 toxin increased G-actin by about 50% (fig. 3a) and decreased the F-actin content by 45% within 120 min (tab. 1). This effect was identical under normotonic as well as under hypotonic conditions (fig. 3a), indicating that C2 toxin overcomes the decrease of cellular G-actin by osmotic stress.

The dramatic decrease of G-actin in the presence of phalloidin resulted in an increase of actin mRNA by 39%, which was even less pronounced than the increase of actin mRNA during the moderate decrease of G-actin observed under hypotonic conditions (fig. 3b). This indicates that a physiological decrease of G-actin might be sufficient for a maximal up regulation of actin mRNA. On the other hand, the increase of actin mRNA under

Tab. 1 Influence of osmotic stress and of toxin treatment on filamentous F-actin in cultured rat hepatocytes.
Treatment of cells with osmotic stress was performed as described in Materials and Methods. Incubation with C2 toxin (100 µg/l component I and 200 µg/l component II) or with phalloidin (2.5 mg/l) was performed under normotonic conditions. F-actin was determined by quantitative binding of rhodamine-labelled phalloidin to actin filaments. After preincubation with unlabelled phalloidin, which disturbs quantitative binding of rhodamine-labelled phalloidin by competition, F-actin was measured by Triton X-100 extraction as described in Materials and Methods. Data are means ± S.E.M. of 9 single determinations. No significant differences compared to normotonic controls were detected, except after treatment of hepatocytes with C2 toxin or with phalloidin (p < 0.001). n.m., not measured.

Incubation time	F-actin levels			
	Normotonic	Hypotonic	C2 toxin	Phalloidin
15 min	100 ± 1.8%	103 ± 2.8%	n.m.	n.m.
30 min	100 ± 3.8%	99 ± 5.6%	n.m.	n.m.
60 min	100 ± 2.8%	109 ± 2.5%	66 ± 1%	n.m.
120 min	100 ± 3.8%	98 ± 2.4%	55 ± 2.8%	155 ± 3%

hypotonic stress was completely abolished by incubation with C2 toxin, which decreased actin mRNA under normotonic condition by about 20% (fig. 3b).

Effects on the hepatocellular integrity

Hypotonic treatment of hepatocytes did not result in a remarkable release of the cellular enzyme activities, e. g. alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase, respectively. Also incubation with C2 toxin or with phalloidin under normotonic conditions did not enhance enzymes in the culture medium compared to the control. Exclusively simultaneous treatment with C2 toxin or phalloidin as well as with hypotonic medium led to a slight increase of alanine aminotransferase and aspartate aminotransferase activities to 125 to 140% of control cultures, indicating a moderate injury of hepatocytes.
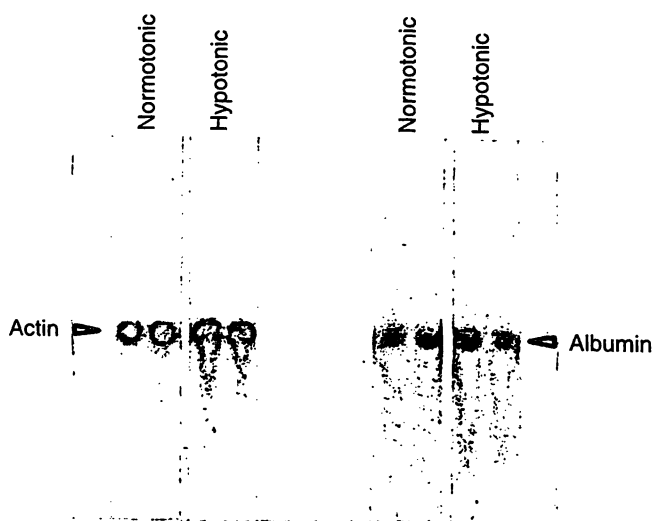

Fig. 2 Northern blot analysis of actin mRNA and albumin mRNA from cultured rat hepatocytes
Cells were treated with normotonic (280 mosmol/l) or hypotonic (220 mosmol/l) medium for 2 h, respectively. Thereafter, RNA was prepared, and Northern blot hybridization was performed as described in Materials and Methods. The autoradiography of a typical experiment is shown.

Discussion

The present study demonstrates that in primary cultures of rat hepatocytes a moderate hypotonic stress resulted in a mild decrease of cytosolic G-actin, while F-actin was essentially unchanged, as demonstrated by two different methods. Simultaneously, the amount of actin mRNA was specifically increased. The results strongly support the hypothesis that in cultured hepatocytes the actin level is under autoregulatory control (14). This is in line with a previous study, which demonstrated an increase of actin mRNA after exposure of cultured hepatocytes to hypotonic or hypertonic medium, respectively (15). However, this study did not present data on the level of filamentous F-actin. The present results suggest that the autoregulation of actin synthesis is due to a specific modulation by the level of monomeric G-actin rather than by the level of filamentous F-actin. The lack of an alteration of F-actin levels during cytoskeletal rearrangement by osmotic stress might be explained by interaction with actin binding proteins or by formation of small actin oligomers, which might not be detected as filamentous actin (25). Moreover, the C2 toxin dependent inhibition of the osmotic decrease of G-actin and increase of actin mRNA argues against a direct osmotic effect on actin synthesis.

On the other hand, treatment of hepatocytes with phalloidin led to much stronger decrease of G-actin, which was not further enhanced by the simultaneous presence of hypotonic medium. This might be due to the extensive disturbance of the actin cytoskeleton observed in the presence of phalloidin. Nevertheless, this fourfold stronger decrease of G-actin did not effect a higher increase of actin mRNA within 2 h than osmotic stress did. Thus, it can be concluded that a moderate and essentially physiological decrease of G-actin by about 20%, as observed during hypotonic stress, results in a maximal rate of increase of actin mRNA. Higher levels of actin mRNA observed during prolonged treatment
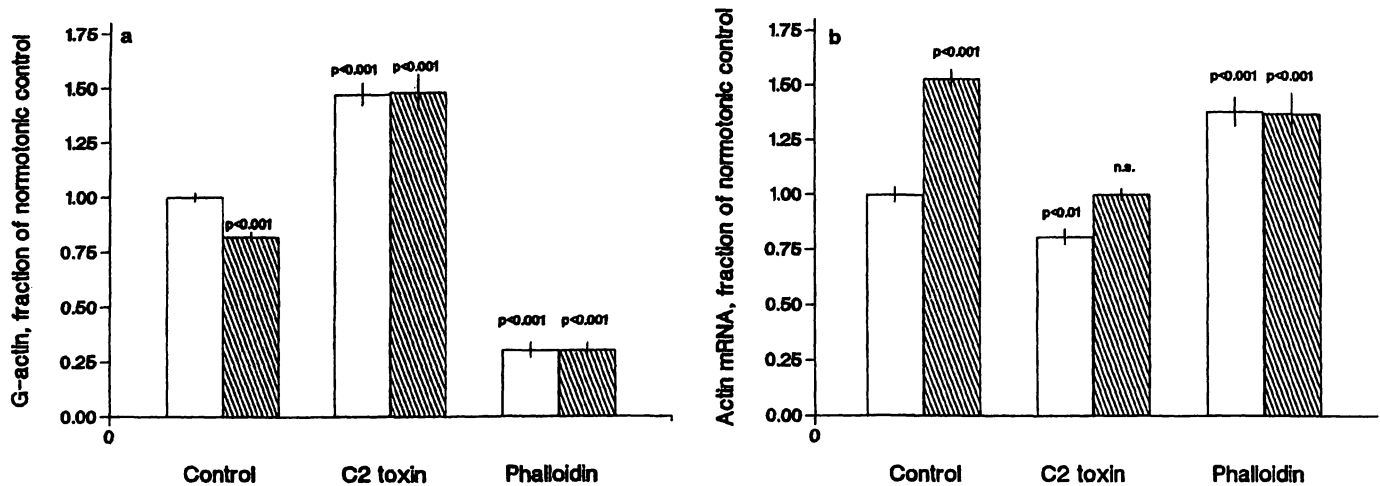
Fig. 3 Influence of C2 toxin and of phalloidin on the osmotic effect on G-actin (a) and actin mRNA (b) in rat hepatocytes. G-actin (a) and actin mRNA (b) was determined as described in figure 1 after 120 min of incubation with normotonic (□) or hypotonic (⊠) medium in the presence of phalloidin or C2 toxin. Means

± S.E.M. of 7 separate determinations, compared to normotonic controls are given. The P values were obtained by *Student*'s t-test for unpaired data in comparison with control cells under normotonic conditions without toxins. n.s., not significant.

with phalloidin (14) are apparently due to the longer lasting decrease of G-actin rather than to the stronger extent of the decrease. For that reason moderate physiological changes of the G-actin level or of the G-/F-actin equilibrium, as observed after osmotic stress or after exposure to hormones (26) are apparently sufficient for an effective regulation of actin synthesis.

The autoregulatory increase of actin mRNA in the presence of phalloidin is due to a specific transcriptional regulation, as recently demonstrated by nuclear run-on transcription studies (27). This might be true also for the autoregulation of actin synthesis observed during moderate alterations of the G-actin level by osmotic stress.

In spite of the cellular rearrangement during hypotonic stress, integrity of hepatocytes was not impaired by hypotonic medium, as indicated by lack of enzyme release

into the culture medium. Exclusively, when C2 toxin or phalloidin were added to hypotonic medium, the activities of alanine aminotransferase and aspartate aminotransferase in the culture medium were slightly increased. Possibly, the arrest of the cytosolic G-actin by C2 toxin or of the cytoskeletal F-actin by phalloidin hinders the cells to react to osmotic stress by swelling. Permeabilization of membranes may occur, since interactions between actin and liver cell membranes exist (28). Thus, hepatocyte culture in the presence of moderate hypoosmolarity can be regarded as a physiological model to study autoregulation of actin synthesis.

Acknowledgements

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