ACTIN MICROFILAMENTS, CELL SHAPE, AND SECRETORY PROCESSES IN ISOLATED RAT HEPATOCYTES

Effect of Phalloidin and Cytochalasin D

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ABSTRACT

The effects of phalloidin and cytochalasin D, drugs which, respectively, stabilize and destabilize actin microfilaments, have been tested on isolated rat hepatocytes. Both drugs produced a modification of cell shape, characterized by protrusions bulging from the cytoplasm. In phalloidin-treated hepatocytes, an accumulation of actin microfilamentous network was detectable at the base of each protrusion by electron microscopy, immunofluorescence, and HMM decoration. This accumulation of microfilaments was absent in cytochalasin D-treated cells. The release of triglycerides, an index of very low density lipoprotein secretion, was inhibited by phalloidin or cytochalasin D, and accompanied by an increase in cellular triglycerides. At the electron microscope examination, triglyceride accumulation was represented by fat droplets and vesicle-enclosed, very low density lipoprotein-like particles. Total protein and albumin secretion was only very slightly modified by either one of these drugs. With the use of various phalloidin analogs, a correlation was observed between their respective ability to stabilize F-actin in vitro, and their effects on cell shape and triglyceride secretion. In conclusion, phalloidin, and cytochalasin D: (a) modify the shape of isolated hepatocytes; (b) inhibit lipoprotein secretion. These effects possibly result from a modification of actin microfilament function.

KEY WORDS   isolated hepatocytes · phalloidin · cytochalasin D · microfilaments · antiactin antibodies · cell shape · secretory processes

In eucariotic cells, microtubular and microfilamentous proteins appear to be of importance in various physiological phenomena such as maintenance of cell shape (9, 10, 39) and secretory processes (4, 28, 35, 40, 43). In the liver, microtubules have been proposed to be implicated in the release of proteins (31, 48) and lipoproteins (30, 47, 48), possibly via a polymerization-depolymerization cycle of tubulin (41, 42). By analogy with the microtubular system, the existence of a polymerization-depolymerization cycle of actin could represent an additional system for controlling both shape and secretion of liver cells.

Two families of drugs appear to interfere with actin microfilaments. The first is the cytochalasins which destabilize the actin filament structure, although their precise mode of action is still disputed

(49). While cytochalasin B inhibits transmembrane glucose transport in various cell types (2, 33), cytochalasin D does not (2). Furthermore, cytochalasin D is 8-10 times more potent in altering cell shape of cultured fibroblasts than the B analog (2). The second is the cyclic peptide phalloidin from the genus *Amanita* (53), which has been shown in vitro to react stoichiometrically with muscle actin (for a review, see reference 54), to enhance the rate of actin polymerization, and to stabilize the F-actin structure (13, 32, 34). Liver membranes (1) and hepatocytes (16) obtained from rats treated in vivo with phalloidin, or membranes exposed to phalloidin in vitro (23, 32), show an increased amount of actin microfilaments. Furthermore, microinjection of this drug into cultured fibroblasts leads to a redistribution of the immunofluorescence pattern of actin (50).

In the present study, the effects of phalloidin and cytochalasin D on the cell shape and secretory processes of isolated rat hepatocytes were investigated.

MATERIALS AND METHODS

Animals

6-wk-old male albino rats derived from a Wistar strain bred in these laboratories were used. They weighed between 165 and 185 g, and were fed *ad libitum* with standard laboratory chow.

Preparation and Incubation of Rat Hepatocytes

Liver cells were isolated by a method described previously for mouse hepatocytes (38), with a few modifications. Livers were first perfused *in situ* at 37°C with a nonrecirculating calcium-free phosphate buffer (130 ml, pH 7.7) gassed with oxygen, then for 6 min with identical recirculating medium. Perfusion was continued for 20-25 min with a recirculating calcium-free phosphate buffer containing collagenase (50 mg/100 ml), bovine serum albumin (2 g/100 ml), glucose (150 mg/100 ml) and soybean trypsin inhibitor (14 mg/100 ml). Livers were removed, and, after gentle stirring in a Petri dish containing a calcium-free phosphate buffer with bovine serum albumin (0.5 g/100 ml, pH 7.4), isolated cells thus obtained were further incubated for 3 min at 37°C in a shaking incubator. The cells were filtered through a nylon gauze, centrifuged, and washed three times with this buffer. They were then preincubated under an atmosphere of O_2: CO_2 (95:5 vol/vol) for 45 min in a Krebs-Ringer bicarbonate buffer (pH 7.4, 37°C) containing glucose (15 mM), bovine charcoal-treated serum (25%) and bovine defatted albumin (1.5 g/100 ml) prepared as described elsewhere (30). After

preincubation, cells were centrifuged, resuspended in the same medium, and distributed as 1.5-ml aliquots into 10-ml Erlenmeyer flasks. Drugs were added at time zero of incubation. Phalloidin was stored at 4°C as a 100-fold concentrated solution in isotonic NaCl. Cytochalasin D was stored at 4°C as a 200-fold concentrated solution in dimethylsulfoxide. Dimethylsulfoxide (final concentration 0.5% in incubation vials) did not interfere with any of the parameters measured. Incubations were stopped by transferring cell suspensions into conical tubes placed in ice-water that were centrifuged at 4°C (3,000 *g*, 5 min). Pellets and supernates thus obtained were used for measurements of various metabolic indices. All results are expressed per g of wet weight of hepatocytes ± SEM, measured at time zero of incubation. Although figures and tables often show results of one particular series of experiments, all experiments reported have been repeated 2-4 times, and experimental vials were run in triplicate or quadruplicate.

Electron Microscopy

Isolated hepatocytes were fixed at room temperature for 30 min in 3% glutaraldehyde in cacodylate buffer (0.1 M) with 1% saccharose (55), pH 7.2-7.4. After rinsing in buffer, they were postfixed at 4°C with 2% OsO_4 in Millonig's phosphate buffer at pH 7.3-7.4 (36). Sections ~1 μm thick were cut with glass knives on an ultramicrotome (C. Reichert A. G., Vienna, Austria) and stained with methylene blue and Azur II. Thin sections were cut with diamond knives, mounted on noncoated grids, stained with uranyl acetate and lead-citrate, coated with a thin layer of carbon, and examined with a Philips EM 300 electron microscope. Heavy meromyosin (HMM) was prepared by tryptic digestion (56) from rabbit skeletal muscle myosin (46) and stored at −25°C in 25% glycerol. For the electron microscope study, isolated hepatocytes were placed in 50% glycerol standard salt solution (0.1 M KCl, 0.005 M $MgCl_2$, and 0.006 M phosphate buffer, pH 7.0) for 12-24 h, then for 12 h in 25% glycerol and 4 h in 5% glycerol. Hepatocytes were incubated for 40 h with HMM (5-6 mg/ml in 0.015 M KCl). As control, phosphate buffer (0.01 M, pH 7.0) containing KCl (0.015 M) was used instead of HMM (27). Hepatocytes were then fixed and processed for electron microscope examination as described above.

Immunofluorescence

Anti-actin antibodies (AAA) were obtained from the serum of two patients with chronic aggressive hepatitis (8, 15) having a titer of 1/1,280 and 1/640, respectively, when tested on rat intestinal smooth muscle. These sera were passed on columns of sepharose covalently linked (12) with rabbit skeletal muscle actin (8), followed by elution of the antibody at pH 2.7. The specificity of these antibodies was tested by immunodiffusion, immunoelectrophoresis and immunofluorescence (8, 18).

Anti-smooth muscle myosin antibodies (AMA) were obtained and their specificity was demonstrated as previously described (7, 14). Isolated hepatocytes were incubated with AAA or AMA as follows: 100 μl of hepatocytes in suspension ($8 \cdot 10^6$ cells/ml) were diluted in 10 ml of Hanks-FCS (5%) solution. One drop of the suspension was dried on a glass slide for 30 min, fixed with absolute ethanol for 30 s, rinsed three times in phosphate-buffered saline (PBS), and incubated for 15 min with AAA or AMA. After incubation, sections were washed in PBS and then stained for 15 min with either fluorescein-conjugated IgG fraction of goat antiserum to human IgG (Code No. 64.170, Miles Seravac, Lausanne, Switzerland) for AAA, or with fluorescein-conjugated IgG fraction of sheep antiserum to rabbit IgG (Behring Werke AG, Marburg Lahn, West Germany) for AMA. After rewashing in PBS and mounting in 90% glycerol in PBS, the level of fluorescence was compared with that found in control preparations treated with human γ-globulins or rabbit serum instead of AAA or AMA.

SDS Polyacrylamide Gel Electrophoresis

24×10^6 cells of control, phalloidin- or cytochalasin D-treated (1 h) hepatocytes were collected in Hanks-BBS (Gibco, Bio AG, Basel, Switzerland) and washed twice to eliminate albumin. After centrifugation at 300 g, pellets were suspended at 4°C in Tris-HCl-NP-40 buffer containing 0.05% Nonidet (Nonidet P-40, British Drug House, Poole, England), 3 mM $MgCl_2$, 10 mM NaCl, and 10 mM Tris-HCl, pH 7.4 (6). After sonication (twice 5 s) with a Branson sonifier (Branson Sonic Power Co., Danbury, Conn.), hepatocytes were centrifuged (20,000 g, 20 min). The resulting supernates (considered as total extract and containing 9.5 mg of proteins per ml) were centrifuged at 120,000 g for 60 min in a Beckman L5-50 ultracentrifuge (Beckman Co., Zurich, Switzerland), using a rotor type 50. Supernates obtained from ultracentrifugation contained 4.6 mg/ml protein (controls) and 4.0 mg/ml (phalloidin-treated cells); pellets contained 6.8 mg/ml protein (controls) and 9.6 mg/ml (phalloidin-treated cells). Total extracts, as well as supernates and pellets obtained after ultracentrifugation, were dissolved in 500 μl of Tris-HCl buffer boiled in sample buffer (29) and run on SDS-10% acrylamide tube gels (29), charging 50 μg of proteins per gel. Protein content of samples was measured with the biuret technique (22). The gels were stained with Coomassie blue, then planed by means of a Chromoscan (Joyce-Loebl Ltd., Goteshead, England).

Cell Shape Study

Cells were incubated in triplicate vials for 1 h, without addition (controls) or in the presence of various phallopeptides. Samples of each vial were then taken, placed on a slide under a light microscope (× 100), and pictures of each sample were taken. On each photograph, 120–300 cells could be counted and classified as follows: (a)

control-like (i.e., round shape) cells; (b) cells with abnormal shape (i.e., occurrence of several protrusions). Results were expressed as percent of total cells counted.

Biochemical Determinations

Intracellular water volume and total potassium content were determined as follows: cells were incubated for 2 h in a medium containing ^3H_2O (1.4 μCi/ml) as an extracellular plus intracellular marker and [hydroxy^{14}C methyl]-inulin (0.14 μCi/ml) as extracellular marker. At the end of incubation, cell suspensions were centrifuged (500 g, 2 min). Supernates were removed and the dpm/ml of ^{3}H and ^{14}C determined from supernates (0.5 ml), using quenching correction curves, and after substraction of the contamination of ^{14}C in the ^{3}H channel. 2 ml of distilled water were then added to the cell pellets. After sonication, cell debris were centrifuged (23,000 g, 5 min), and supernates (0.1 ml) were used for potassium measurement using flame photometry, and counted (0.5 ml) in a liquid scintillation spectrometer (Packard, Tri-Carb model 3880, Instrument Inc., Downers Grove, Ill.) so that the dpm of ^{3}H and of ^{14}C in the first centrifugation pellets could be known. Intracellular water volume was the difference between the ^{3}H space and the ^{14}C space, i.e.,

$$\frac{\text{dpm } ^3\text{H}}{\text{dpm } ^3\text{H/ml}} - \frac{\text{dpm } ^{14}\text{C}}{\text{dpm } ^{14}\text{C/ml}}.$$

Aliquots of supernates were used for the following measurements: 0.1 ml for urea (5), 0.2 ml for triglycerides (30), and 0.05 ml for lactate dehydrogenase (LDH) (5). ATP was determined with the luciferin-luciferase technique as described elsewhere (25). Labeled amino acid incorporation into cellular and released proteins was carried out according to a technique described previously (31).

Prelabeling Experiments

In experiments to investigate the secretion of prelabeled proteins and triglycerides, the following experimental design was adopted. Cells were preincubated (45 min) in a medium containing L-[U-^{14}C]amino acid mixture (0.21 mg/ml; 1.9 μCi/ml) and albumin-[9, 10-^{3}H]oleate (1.2 mM; 4.5 μCi/ml). After centrifugation, supernates were discarded and cells washed twice with 15 ml of Krebs-Ringer bicarbonate buffer (4°C). This temperature was used to minimize, due to our own previous observations, the exit of prelabeled compounds during cell washing and distribution. Cells were resuspended in a medium (4°C) containing unlabeled albumin-oleate (0.9 mM), unlabeled amino acid mixture (0.21 mg/ml), and cycloheximide (10^{-4} M), a concentration which totally blocks protein synthesis. Cells were then distributed and incubated at 37°C in different flasks containing either saline or drugs. At different time intervals, aliquots were taken to measure the appearance of prelabeled proteins and triglycerides in the medium.

Chemicals

All organic and inorganic chemicals were purchased from E. Merck (Darmstadt, Germany), Fluka AG (Buchs, Switzerland), Sigma Chemical Co. (St. Louis, Mo.), and were of analytical grade. Labeled compounds were secured from the Radiochemical Centre (Amersham, Buckinghamshire, England). Amino acid mixture (TC amino acids Hela × 100) was obtained from the Difco Laboratories (Detroit, Mich.), and collagenase (CLS IV) and soybean trypsin inhibitor from Worthington Biochemical Corporation (Freehold, N. Y.). Phalloidin, secophalloidin, phalloidin sulfoxid A and phalloidin sulfoxid B were a generous gift of Professor Th. Wieland (Max-Planck Institute, Heidelberg, Germany). Phalloidin was also obtained from Boehringer (Ingelheim/Rh., Germany). Virosin was a generous gift of Dr. T. Staron (I. M. B. A., Lucé, France).

RESULTS

Effects of Phalloidin and Cytochalasin D on Hepatocyte Shape

As shown by Fig. 1, control hepatocytes, incubated with or without fatty acids, had a round appearance. At high magnification a microfilamentous network could always be seen at the periphery of the cytoplasm and in the microvillous projections. The ultrastructure of hepatocytes incubated with phalloidin or cytochalasin D was similar to that of controls, with the remarkable exception of the cell contour. As shown by Fig. 2, phalloidin-treated cells were characterized by the presence of several protrusions bulging from the cytoplasm, at the base of which a clear band was observed. In cytochalasin D-treated cells (Fig. 3), numerous protrusions were also present but no clear band was seen at their base. In contrast to the lasting effect of phalloidin (from 5 min to >2 h), that of cytochalasin D on cell shape was transient, being maximal within 5 min and disappearing after ~25 min in presence of the drug. When observed at higher magnification (Figs. 4 and 5), the protrusions of both phalloidin- or cytochalasin D-treated cells contained the usual cytoplasmic organelles, and few microvilli when compared to control cells. In cytochalasin D-treated cells, protrusions and the inner part of the hepatocyte were in direct continuity (Fig. 4). In contrast, at the base of each protrusion of the phalloidin-treated cells, a characteristic filamentous network separated the extruded from the internal cytoplasm (Fig. 5). Single microfilaments were difficult to resolve even at high magnification. When visible (Fig. 5, inset) their diameter

measured 4–7 nm. After incubation in glycerol, the shape and the cytoplasmic topography of phalloidin-treated hepatocytes were no longer similar to that of phalloidin-treated, glutaraldehyde-fixed hepatocytes. In particular, microfilament bundles could not be precisely located at the base of the protrusions. However, the intracytoplasmic bundles of microfilaments were characteristically decorated by HMM (Fig. 6).

After immunofluorescent staining with anti-actin antibodies, control hepatocytes were diffusely positive (Fig. 7b). In hepatocytes treated with phalloidin, anti-actin antibody staining was localized principally as bright bands at the cell periphery, corresponding to the base of cell protrusions (Fig. 7c). In hepatocytes treated with cytochalasin D, the intensity and distribution of staining with AAA were analogous to that of controls, and no immunofluorescent positive bands were seen in relation to protrusions (Fig. 7d). Staining with anti-myosin was not modified in phalloidin-treated hepatocytes when compared to controls (data not shown). Control sections treated with normal human γ globulins (Fig. 7a) or rabbit serum were negative.

The amount of actin present in total homogenates, as well as in ultracentrifugation supernates and pellets of control and phalloidin-treated hepatocytes, was semiquantified using SDS polyacrylamide gel electrophoresis. Planimetric evaluation of gel scannings (Fig. 8) showed that the total extract of control and phalloidin-treated cells contained similar amounts of a protein having the same electrophoretic mobility as actin. The pellet obtained after ultracentrifugation of extracts from phalloidin-treated cells was richer in such protein (co-migrating with actin) than pellets from control hepatocytes (Fig. 8). Concurrently, in phalloidin-treated cells the supernate obtained after ultracentrifugation was poorer in this protein than the control supernates (data not shown). The amounts of actin-like protein present in total homogenates, in supernates or ultracentrifugation pellets of control or cytochalasin D-treated cells were similar.

To control the action of microtubules on cell shape, hepatocytes were incubated with colchicine (10⁻⁵ M) from 5 min to 2 h. No changes in cell shape were observed at any time.

Effects of Phalloidin and Cytochalasin D on Hepatic Secretory Processes

Despite the morphological changes, it should be noted that intracellular water volume, potassium
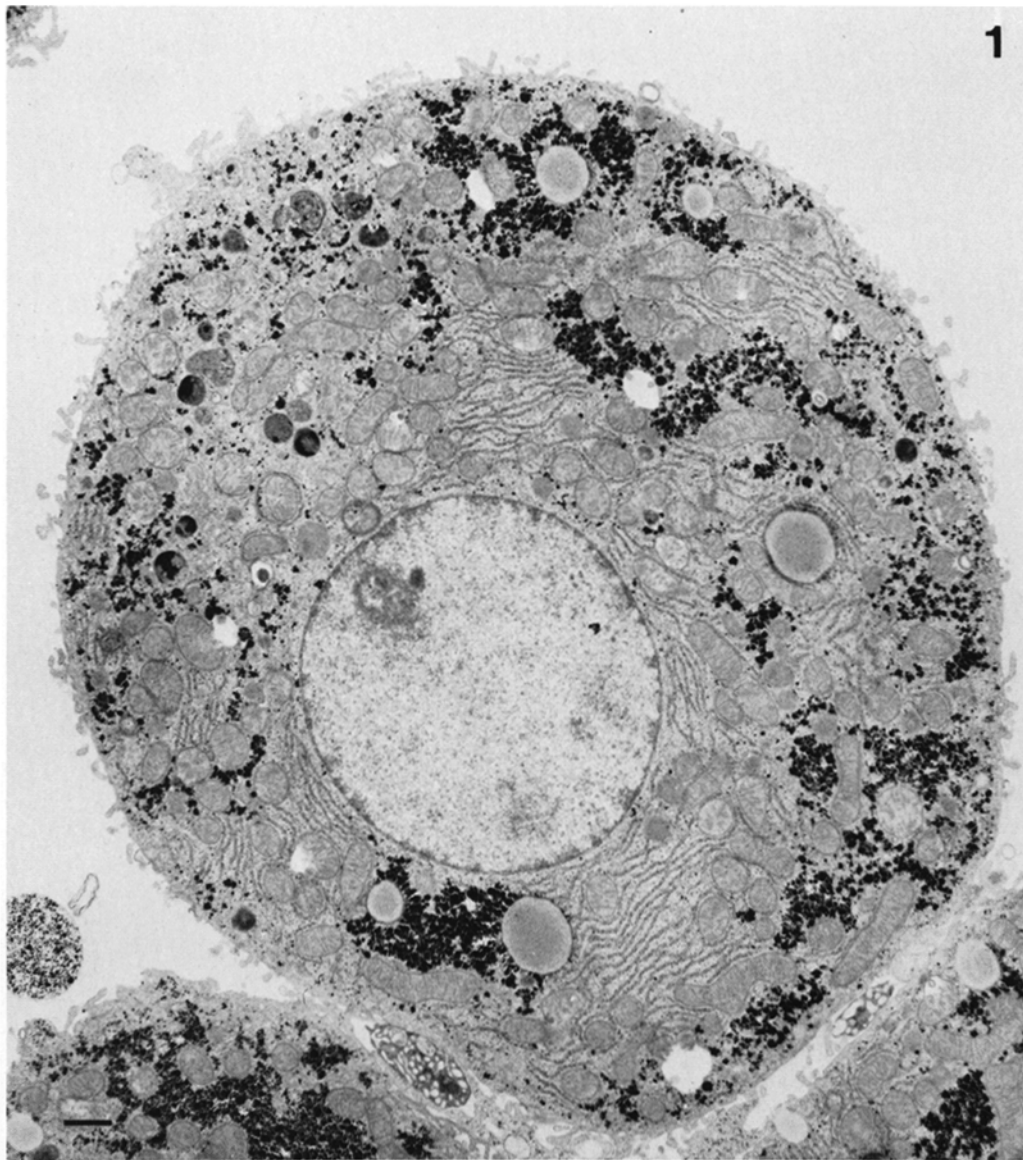
FIGURE 1 Control isolated hepatocyte. The cell shape is round, and the most conspicuous cytoplasmic organelles are rough endoplasmic reticulum, mitochondria, liposomes, and glycogen granules. Microvilli are present at the cell periphery. Bar, 1 μm. × 6,500.

and ATP content, and urea output of phalloidin-treated cells remained unmodified, while lactic acid dehydrogenase output was kept at a minimal value (Table I). Similarly, ATP content remained normal in cytochalasin D-treated cells (controls: 3.30 ± 0.08; cytochalasin, 3 μg/ml: 3.38 ± 0.01 μmol/g wet weight). This, together with the data summarized below, indicates that phalloidin- or cytochalasin D-treated hepatocytes were functionally well preserved.

As illustrated by Fig. 9, the triglyceride output (an index of very low density lipoprotein [VLDL] secretion by isolated hepatocytes [30]) was linear for 2 h. In the presence of phalloidin (10^{-5} M) or cytochalasin D (3 μg/ml), the output of triglycerides into the medium was markedly curtailed from

30 min of incubation onwards. The effects of phalloidin and cytochalasin D on triglyceride secretion were dose-dependent. When reaching maximal inhibitory concentrations, phalloidin used at 10^{-4} M and cytochalasin D at 20 μg/ml decreased triglyceride output to the same extent as phalloidin 10^{-5} M and cytochalasin D 3 μg/ml (data not shown). As a result of this, all subsequent experiments were carried out with 10^{-5} M phalloidin or 3 μg/ml cytochalasin D. Of further interest was the observation, summarized in Table II, that the decrease in triglyceride output brought about by phalloidin was accompanied by an accumulation of triglycerides within the hepatocytes, such that total triglycerides (i.e., cellular plus medium triglycerides) were identical in control
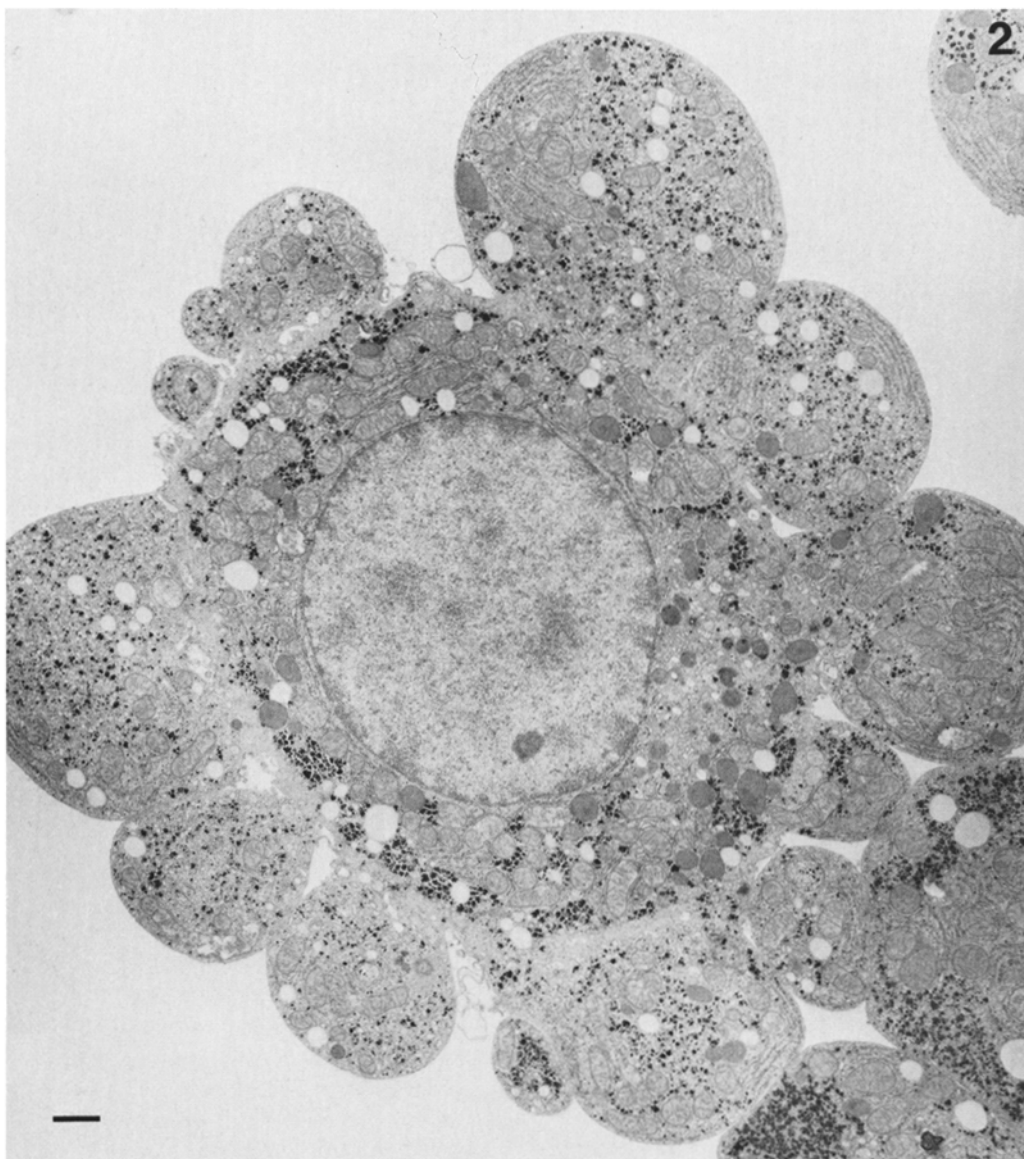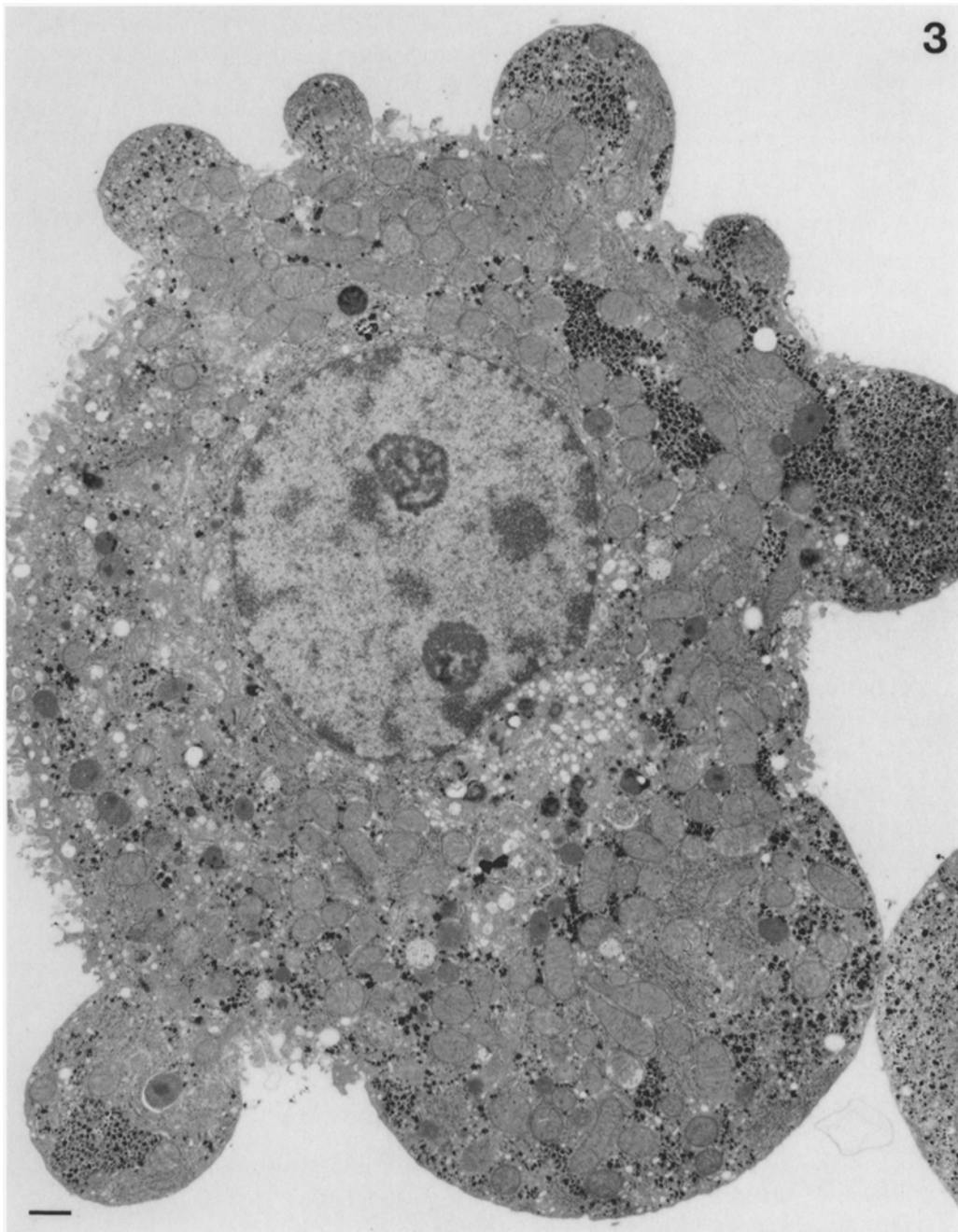

FIGURE 2   Isolated hepatocyte incubated with oleate (1 mM) and phalloidin (10^{-5} M, 1 h). The cell periphery shows several bubble-like protrusions. The cytoplasmic content of protrusions is similar to that of the perinuclear cytoplasm. Very few microvilli are visible at the cell periphery. Bar, 1 μm. $\times$ 6,200.

FIGURE 3   Isolated hepatocyte incubated with cytochalasin D (3 μg/ml, 5 min). The cell periphery shows several bubble-like protrusions similar to those present in phalloidin-treated hepatocytes. Bar, 1 μm. × 6,200.

and phalloidin-treated cells. In the electron microscope, such an accumulation of triglycerides in the phalloidin-treated cells corresponded to the presence (never seen to that extent in control cells) of both lipid droplets and vesicles containing VLDL-like particles (Fig. 10), in the protrusions as well

as in the remaining cytoplasm. Lipid droplets were conspicuously more numerous than vesicles containing VLDL.

Since VLDL secretion requires protein synthesis (3), it was important to assess the integrity of this process by measuring the incorporation of labeled amino acids into proteins. As shown in Table III, phalloidin decreased the labeled protein output but also total protein synthesis (~25%), a value in accord with previous results (24), while cytochalasin D barely changed protein secretion or synthesis. Similar data were obtained when albumin output was measured (data not shown). To decide whether the inhibitory effect of phalloidin upon protein and triglyceride secretion was secondary to decreased protein synthesis, it was necessary to dissociate protein synthesis from protein and triglyceride secretion. To do this, the release, in the presence of cycloheximide, of triglycerides and proteins previously labeled during a preincubation of hepatocytes with labeled precursors (see Materials and Methods) was investi-

gated. As shown by Fig. 11, phalloidin reduced only slightly the release of prelabeled proteins into the medium, while that of prelabeled triglycerides was markedly curtailed.

Specificity of Phalloidin for Microfilaments

As phalloidin has been well documented to interact with and bind to muscle actin in vitro (for a review, see reference 54), the following experiments were carried out to suggest similar specificity in the liver. It was observed (Table IV) that the monocyclic secophalloidin that does not react with F-actin (34, 54) and, therefore, does not protect it from heat denaturation, had no effect either on the shape of the hepatocyte or on triglyceride secretion. For the other phallopeptides including phalloidin, a reasonable fit between protective effect from heat denaturation of F-actin, change in cell shape, and inhibitory effect upon triglyceride secretion was observed (Table IV). It should be pointed out that the in vitro protection of F-actin from heat denaturation was
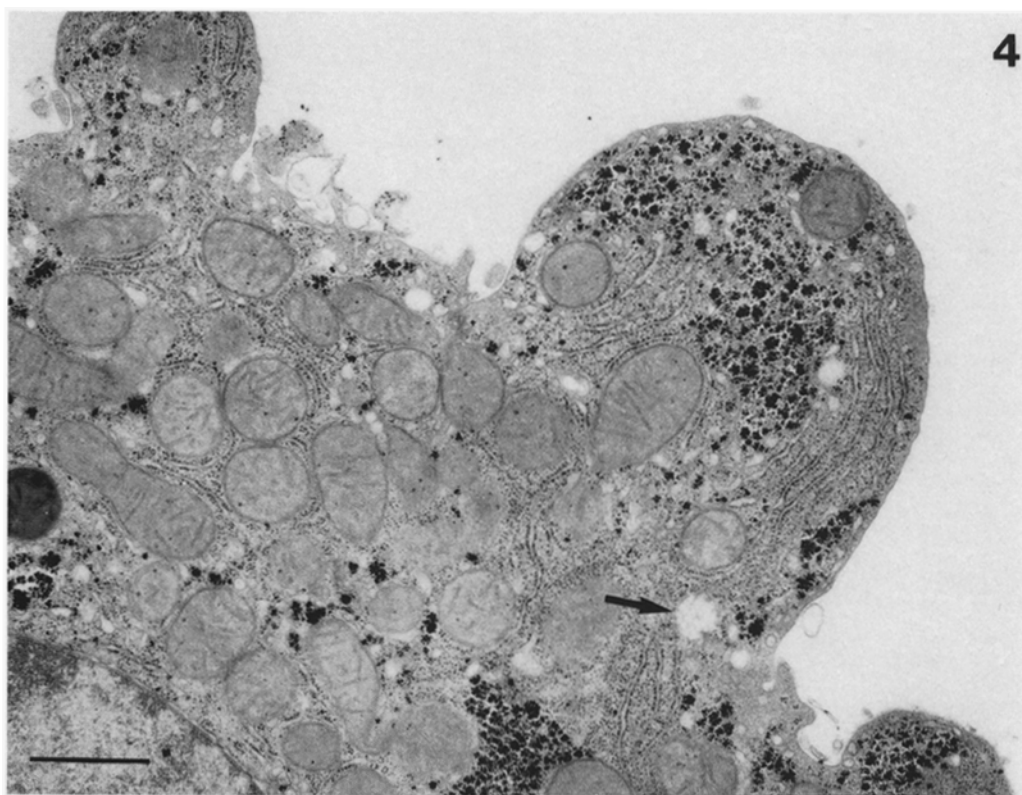

FIGURE 4   Cytoplasmic protrusion of an isolated hepatocyte incubated with cytochalasin D (3 µg/ml, 5 min). No microfilaments are present between the inner cytoplasm and the protrusion. Note a VLDL-like containing vesicle (arrow). Bar, 1 µm. × 15,700.
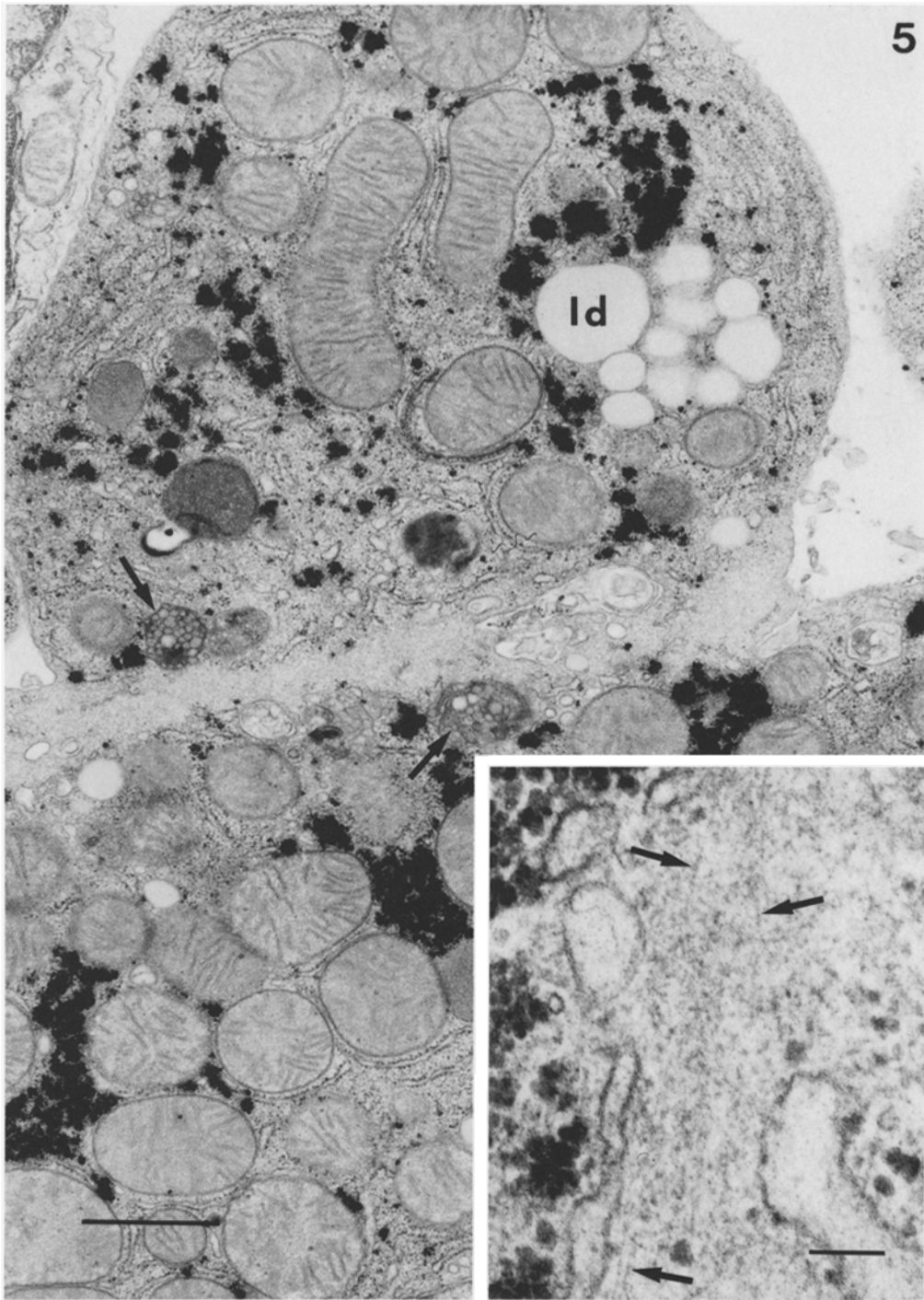
FIGURE 5 Cytoplasmic protrusion from an isolated hepatocyte incubated with oleate (1 mM) and phalloidin (10^{-5} M, 1 h). The protrusion is separated from the inner cytoplasm by a clear band. Cytoplasmic organelles in the protrusion and in the inner cytoplasm appear normal. Note VLDL-like containing vesicles (arrows) and lipid droplets (*l d*). The inset shows a detail of the band separating a protrusion from the inner cytoplasm. This is composed of a network where single microfilaments (4–7 nm in diameter) are difficult to resolve (arrows). Bar, 1 μm. × 20,800; (*inset*) Bar, 0.1 μm. × 108,000.
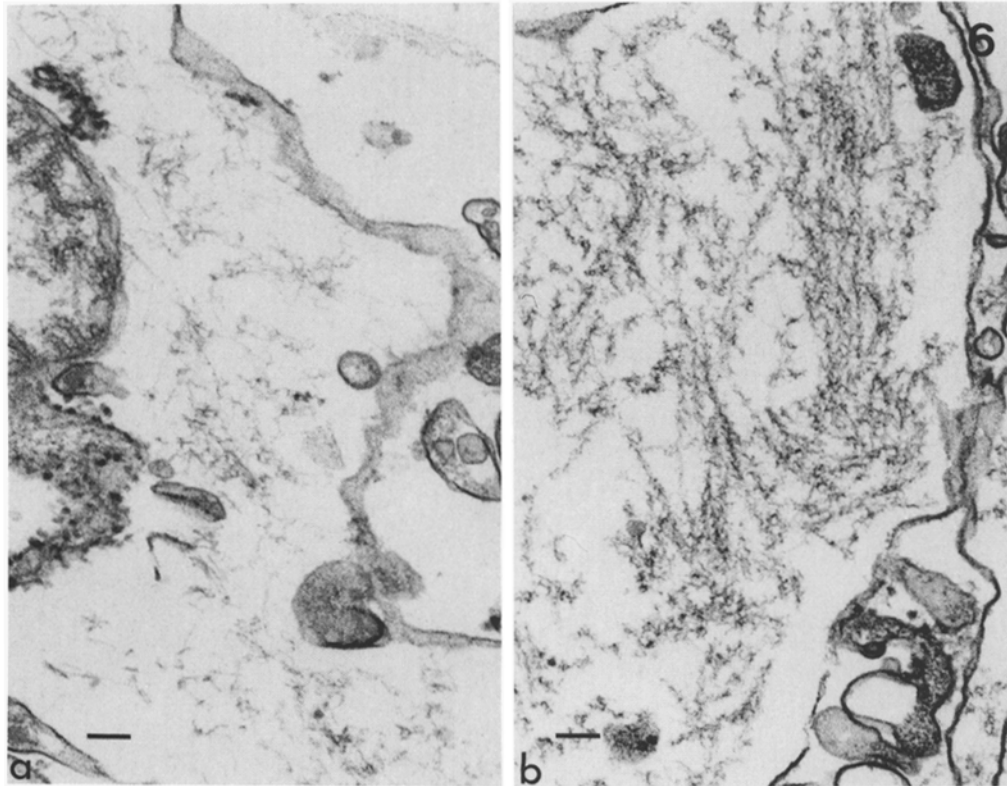
Figure 6 Decoration with HMM of actin microfilaments in phalloidin-treated (10^{-5} M, 1 h) isolated rat hepatocytes. (a) Periphery of an isolated hepatocyte incubated with 0.01 M phosphate buffer (pH 7.0), containing 0.015 M KCl. (b) Periphery of an isolated hepatocyte decorated after incubation with HMM. Bar, 0.1 μm. × 55,000.

achieved in the presence of both actin and phalloidin at equimolar concentration (10^{-5} M), a concentration of the drug used throughout the present study.
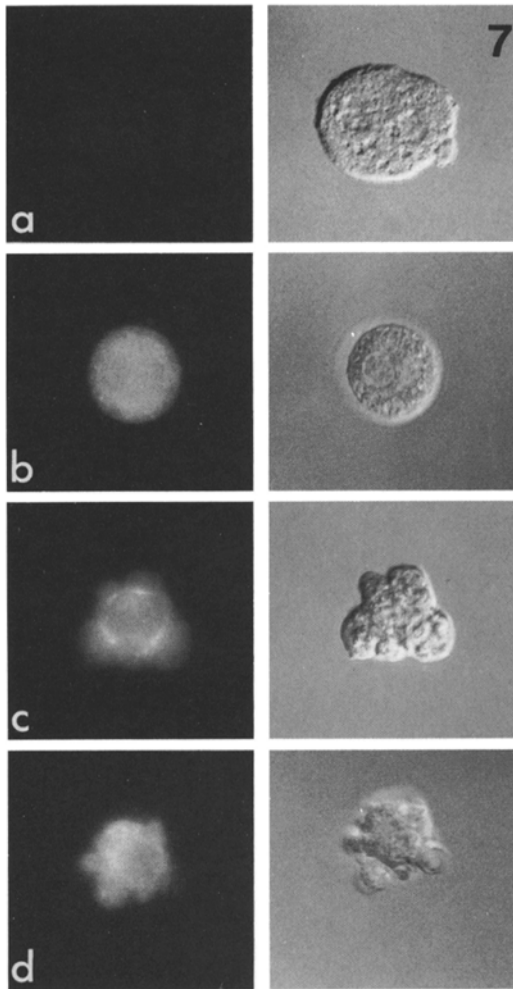
Effects of Virosin

A few experiments were carried out to characterize the effects of virosin, a toxin isolated from *Amanita virosa* (11) which also produces a marked accumulation of actin filaments in hepatocytes following in vivo administration for several days (G. Gabbiani, unpublished observations). The morphological observations and the immunofluorescence studies done in the presence of either virosin (16 μg/ml) or phalloidin (8 μg/ml = 10^{-5} M) were identical. Various metabolic indices, such as ATP content, triglyceride secretion, labeled protein synthesis and secretion, gave results similar to those recorded with phalloidin.

DISCUSSION

Morphological Changes Produced by Phalloidin and Cytochalasin D

Several factors have been implicated in the regulation of cell shape, for instance: (a) the cytoskeletal protein tubulin (44) which, under our experimental conditions, does not seem to play a structural role in hepatocytes, since no cell shape change in the presence of colchicine was observed; (b) interaction of actin and myosin via a system analogous to that described for smooth and striated muscles (21); (c) microfilament formation and disappearance through polymerization and depolymerization of actin (19, 20, 26). Other factors, which have not been taken into consideration here, such as 10-nm filaments, are probably important as well.
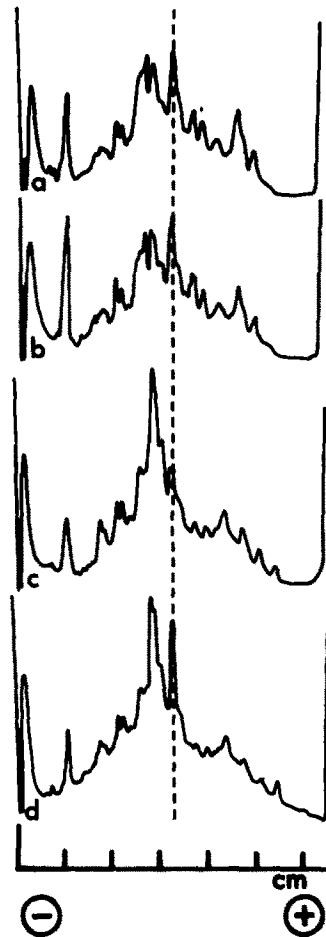
In the present experiments, both phalloidin and cytochalasin D were found to produce a dramatic

previously reported, although microfilament organization in phalloidin-treated cell was not investigated (52). The main differences found during the present study between the effects of phalloidin and cytochalasin D were that the effect of cytochalasin D upon cell shape was reversible and that few microfilaments could be observed in hepatocytes treated with cytochalasin D, the protrusions and the inner cell body being in direct continuity. In contrast, the protrusions produced by phalloidin were separated from the rest of the hepato-

FIGURE 7 Indirect immunofluorescent staining with AAA of control, phalloidin-treated and cytochalasin D-treated isolated rat hepatocytes. On the left, immunofluorescent staining; on the right, interference contrast optics of the same hepatocytes. (a) Control hepatocyte stained with human γ-globulins instead of AAA; no staining. (b) Control hepatocyte stained with AAA; the staining is diffusely cytoplasmic. (c) Hepatocyte incubated with phalloidin for 1 h, then stained with AAA. In addition to a diffuse cytoplasmic staining, immunofluorescent bands are present at the base of each protrusion. (d) Hepatocyte incubated with cytochalasin D for 5 min, then stained with AAA. The intensity and distribution of the staining is similar to that of control. × 400.

alteration of hepatocyte shape, characterized by the appearance of numerous protrusions, while cellular organelles remained unaltered. Changes in hepatocyte shape due to phalloidin have been

FIGURE 8 Profile of SDS polyacrylamide gels from: (a) total extract of control hepatocytes; (b) total extract of phalloidin-treated (10⁻⁵ M, 1 h) hepatocytes; (c) ultracentrifugation pellet of control hepatocytes; (d) ultracentrifugation pellet of phalloidin-treated hepatocytes. The dotted line corresponds to the actin peaks. The surfaces of the material co-migrating with actin in a and b are similar; but the surface of the material co-migrating with actin in d is larger than that in c.

Table I

Assessment of Cellular Integrity of Isolated Rat Hepatocytes in the Presence of Phalloidin

Addition	A. Intracellular water volume	B. Intracellular potassium content	C. ATP content	D. Urea output into the medium	E. LDH output into the medium
	ml/g wet wt	meq/l	μmol/g wet wt	μmol/g wet wt	U/g wet wt
None	0.48 ± 0.03	145.8 ± 3.9	2.77 ± 0.25	14.94 ± 1.15	6.11 ± 2.46
Phalloidin	0.49 ± 0.03	147.1 ± 4.0	2.75 ± 0.38	15.92 ± 2.34	6.03 ± 1.53

Hepatocytes from fed rats were incubated for 2 h (A–D) or 1 h (E) with and without phalloidin (10^{-5} M), in 1.5 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing bovine charcoal-treated serum (25%), bovine serum albumin (1.5 g/100 ml) and glucose (15 mM). Each figure is the mean ± SEM of three different experiments carried out in triplicate. LDH = lactate dehydrogenase.
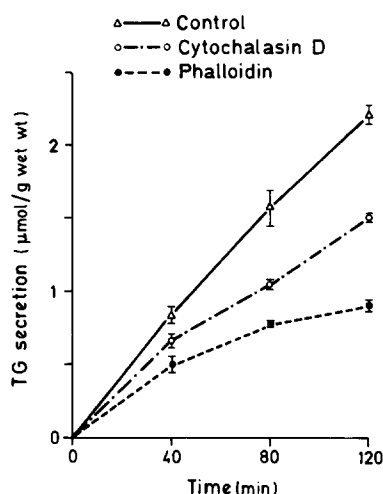

Figure 9   Effect of phalloidin and cytochalasin D on triglyceride (TG) secretion by isolated rat hepatocytes. Experimental conditions were those described in Table I, with albumin-oleate (0.9 mM). Phalloidin: 10^{-5} M; cytochalasin D: 3 μg/ml. Each point is the mean ± SEM of four values.

cyte by an accumulation of actin microfilaments detectable by electron microscopy, immunofluorescence and HMM binding. Furthermore, SDS polyacrylamide gel electrophoresis revealed that the total content of actin from phalloidin-treated hepatocytes was unchanged when compared to controls, whereas polymerized actin was recovered in greater amount in the ultracentrifugation pellets of phalloidin-treated hepatocyte extracts than in controls. This is in accordance with previous in vivo and in vitro data showing that phalloidin promotes actin polymerization and stabilizes polymerized actin (13, 23, 32, 50). In the presence of cytochalasin D, the total content of actin in hepatocytes was unchanged. Since cytochalasin D appears to destabilize F-actin, a de-

crease of actin recovered in the pellets obtained from cytochalasin-treated cells, compared to controls, was anticipated. This, however, was not the case, due either to the limited sensitivity of the gel scanning technique or to the fact that cytochalasin D may destabilize F-actin by a mechanism other than simple depolymerization. It is also possible that cytochalasin D destabilizes bundles but does not depolymerize individual filaments. Unorganized bundles are probably difficult to see in the electron microscope but could nevertheless sediment during ultracentrifugation.

Taken together, these observations suggest that, in isolated hepatocytes, a normal equilibrium between G- and F-actin is necessary for the maintenance of cell shape. When this equilibrium is altered by either phalloidin (stabilizing F-actin) or cytochalasin D (destabilizing F-actin), a change in hepatocyte shape results. The changes in cell shape produced by either one of these drugs suggest that actin is connected to the plasmalemma of the hepatocyte, as previously proposed for other cell types (17, 37, 51).

Effects of Phalloidin and Cytochalasin D upon Secretory Processes

Although the effects of phalloidin upon secretory processes were studied in more detail than those of cytochalasin D, the most prominent finding was that either one of these drugs caused a marked, rapid decrease in triglyceride (VLDL) secretion. At maximal inhibitory concentration, the effect of phalloidin was more pronounced than that of cytochalasin D. In phalloidin-treated cells, the diminution of triglyceride secretion was accompanied by intracellular triglyceride accumulation, resulting in unimpairment of total triglycerides (cell plus medium). This accumulation of lipids was represented in the electron microscope by an accumulation (never seen to such an extent

TABLE II

Effect of Phalloidin on Triglyceride Secretion by Isolated Rat Hepatocytes

Addition	Triglycerides secreted into the medium (A)	Cellular triglycerides (B)	Total triglycerides (A + B)
	μmol/g wet wt		
Exp. 1			
None	2.51 ± 0.13	8.05 ± 0.42	10.52 ± 0.29
Phalloidin	1.16 ± 0.07*	10.26 ± 0.38‡	11.41 ± 0.34§
Exp. 2			
None	3.14 ± 0.24	11.62 ± 0.46	14.76 ± 0.58
Phalloidin	1.27 ± 0.07*	13.27 ± 0.22 ‖	14.65 ± 0.26§

Hepatocytes from fed rats were incubated with 1.0 mM oleate bound to albumin for 2 h under experimental conditions described in Table I. Phalloidin: 10^{-5} M. Each figure is the mean ± SEM of 3-4 values.
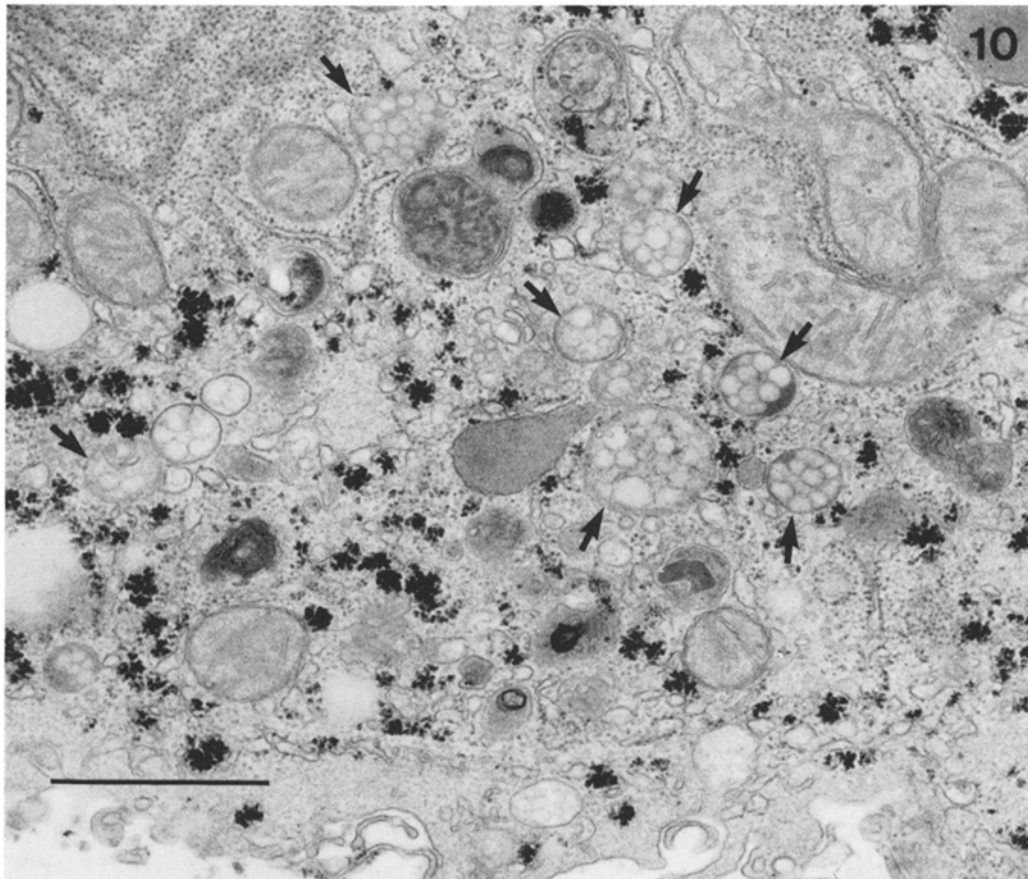
* $p < 0.0025$.
‡ $p < 0.01$.
§ NS.
‖ $p < 0.025$.

FIGURE 10   Cytoplasm of an isolated hepatocyte incubated with oleate (1 mM) and phalloidin (10^{-5} M, 1 h). The cytoplasm contains many VLDL-like-containing vesicles (arrows) and is otherwise normal. Bar, 1 μm. × 29,500.

TABLE III

Effect of Phalloidin and Cytochalasin D on the Incorporation of Labeled Amino Acids into Proteins by Isolated
Rat Hepatocytes

	Proteins secreted into the medium (A)	Cellular Proteins (B)	Total Proteins (A + B)
	dpm × 10⁻⁵/g wet wt		
	$dpm \times 10^{-5}/g$ wet wt		
Control	6.29 ± 0.07	12.75 ± 0.34	19.03 ± 0.42
Phalloidin	3.69 ± 0.08	10.52 ± 0.60	14.20 ± 0.72
Cytochalasin D	5.89 ± 0.06	13.73 ± 0.26	19.59 ± 0.30

Hepatocytes were incubated for 2 h, as described in Table I, with amino acid mixture (21.2 mg/100 ml) and randomly labelled ^{14}C-amino acids (0.5 μCi/ml). Phalloidin: 10^{-5} M. Cytochalasin D: 3 μg/ml. Each figure is the mean ± SEM of three values.

in control cells) of vesicles containing VLDL-like particles as well as free lipid droplets, the latter being more conspicuous than the former. Lipid droplets, and vesicles containing VLDL-like particles, were present in both protrusions and the remaining cytoplasm of phalloidin-treated cells. This indicates that the intracellular migration of VLDL had been, at some ill-defined step(s), interfered with by these drugs.

Cytochalasin D (Table III) or phalloidin (Fig. 11) had only a very slight inhibitory effect upon protein secretion. This is in marked contrast with previous results obtained with colchicine (an inhibitor of the microtubular system), in which inhibition of triglyceride as well as total protein secretion was observed in the presence of the drug (30, 31, 45, 48). The observation that phalloidin or cytochalasin D inhibits triglyceride secretion but only very slightly protein secretion suggests that the intracellular routes are, at least in part, different for the two types of secretory products, and that in the case of proteins a step or a series of steps of the intracellular migration are unaffected by drugs that appear to interfere with actin microfilaments.

Specificity of Phalloidin Action

The specificity of phalloidin for actin was suggested by the use of phallopeptide analogs. Thus, secophalloidin which has been shown not to react with actin, and therefore does not protect F-actin from heat denaturation or from depolymerization by potassium iodide (34, 54), did not change the shape of hepatocytes or affect triglyceride secretion. Moreover, a satisfactory relationship was obtained between the ability of phallopeptides to react with actin in vitro and their effect on hepa-
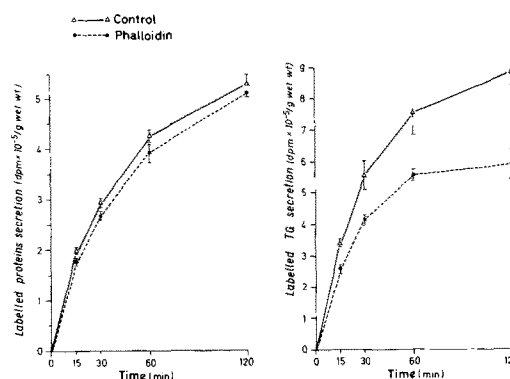

FIGURE 11   Effect of phalloidin on the secretion of prelabeled proteins (left panel) and prelabeled triglycerides, TG, (right panel), in the presence of cycloheximide, by isolated rat hepatocytes. For experimental design, see Materials and Methods. Phalloidin: 10^{-5} M. Each point is the mean ± SEM of three values.

tocyte shape and triglyceride secretion. These observations do not exclude the possibility that phallopeptides interact, in addition, with structures other than microfilaments. However, the fact that similar cell shape and secretory changes were observed with phalloidin or cytochalasin D treatment suggests that the effects of these drugs are mediated, at least in part, through perturbation of microfilaments.

In conclusion, phalloidin and cytochalasin D change the cell shape and inhibit lipoprotein secretion in isolated hepatocytes. Both drugs have been shown to interfere with the organization of actin microfilaments. Hence, it is conceivable that, in isolated rat hepatocytes, microfilaments play a role in the control of cell shape and lipoprotein secretion.

TABLE IV

Relationships between Stabilization of F-Actin by Various Phallopeptides and their Respective Effect on Cell Shape and Triglyceride Secretion of Isolated Rat Hepatocytes

Addition	A. Relative turbidity of F-actin solution	B. Cell with protrusions	C. Triglyceride secretion
	% from control	*% of total*	*µmol/g wet wt*
None	100	0	3.24 ± 0.11
Secophalloidin	96	0	2.97 ± 0.14
Phalloidin Sulfoxid A	67	8.1 ± 0.7	2.80 ± 0.17
Phalloidin Sulfoxid B	24	90.0 ± 2.0	1.54 ± 0.22
Phalloidin	12	98.3 ± 0.5	1.34 ± 0.02

Exp. A: Protective effect of phallopeptides (10^{-5} M) on F-actin (10^{-5} M) from denaturation by heating to 70°C for 3 min in 0.1 M KCl, 1 mM Tris, pH 7.4. Data from J. X. de Vries, A. J. Schäfer, H. Faulstich, and Th. Wieland, *Hoppe-Seyler's Z. Physiol. Chem.* **357**:1139-1143, 1976, used for comparison with exps. B and C, with authors' authorization.

Exps. B and C: Hepatocytes from fed rats were incubated as described in Table I. All drugs (5.10^{-5} M), except phalloidin (10^{-5}). Exp. B: experimental conditions as described in Materials and Methods. Incubation time: 1 h. Exp. C: 1.0 mM oleate bound to albumin. Incubation time: 2 h. Each figure is the mean ± SEM of three values.

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