Acute Phalloidin Toxicity in Living Hepatocytes

Evidence for a Possible Disturbance in Membrane Flow and for Multiple Functions for Actin in the Liver Cell

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Actin filament based cell functions were examined in freshly isolated hepatocytes using phalloidin as an inhibitor. In particular, cell motility events, namely, surface bleb formation and canalicular contractile movements, were assessed and compared with morphologic changes in the cells. Phalloidin (in 0.1, 1.0, and 10.0 μg/ml dosages) was added to the culture medium 4 hours after isolation of the hepatocytes. Cell motility was recorded with time-lapse cinephotomicrography, and the morphologic changes were evaluated by phase-contrast optics and by transmission electron microscopy of serial sections. Membrane-bound pericanalicular cytoplasmic vacuoles appeared first, followed by cytoplasmic protrusions at the cell surface. Vacuolar membrane continuities with the canalicular membrane were noted, and later, with other regions of the cell surface, such that large tortuous irregular membrane-bound canals are seen on serial sectioning to link extracellular and canalicular spaces. These findings suggest a possible disturbance in membrane flow. Canalicular motility was greatly reduced and was dose-dependent. The time-based difference in the changes at the canalicular and sinusoidal surfaces may be indicative of different functions and/or sensitivities of the actin filaments within the liver cell. (Am J Pathol 1986, 122: 101–111)

PHALLOIDIN, a cytotoxin isolated from the mushroom *Amanita phalloides*, has a direct effect on filamentous actin (F-actin), causing irreversible polymerization of actin filaments.[1] Hepatocytes have a receptor for phalloidin; so these cells permit an excellent opportunity to evaluate actin-dependent cell functions with this agent.[2] Actin filaments are abundant in hepatocytes; they are numerous beneath the cell membrane, especially in the region of bile canaliculi.[3–9] It is known that repeated exposure to small doses of phalloidin results in the formation of cytoplasmic vacuoles and the filling of the cell with actin filaments. Bile canaliculi become encased by actin filaments, and bile flow is reduced.[10–13] Active contraction of bile canaliculi, an actin-filament based function,[14–19] is impaired in chronically phalloidin-treated cells.[16] When administered acutely to normal isolated hepatocytes, phalloidin causes blebs or protrusions to appear on the cell surface.[2,17] This has been ascribed to changes in membrane-associated actin filaments.[2–20] In this study, we examined the sequence of changes that occurred in living hepatocytes. By combining time lapse cinephotomicrography and the use of serial sections of the cell cultures taken at frequent intervals and examined by light and electron microscopy, we could make structural–functional correlations.

Materials and Methods

Animals and Phalloidin Regimen

Twenty female Wistar rats, each weighing approximately 250 g and fed *ad libitum* laboratory pellets and tap water, were divided into four groups of equal size. The first group served as controls. The remaining three

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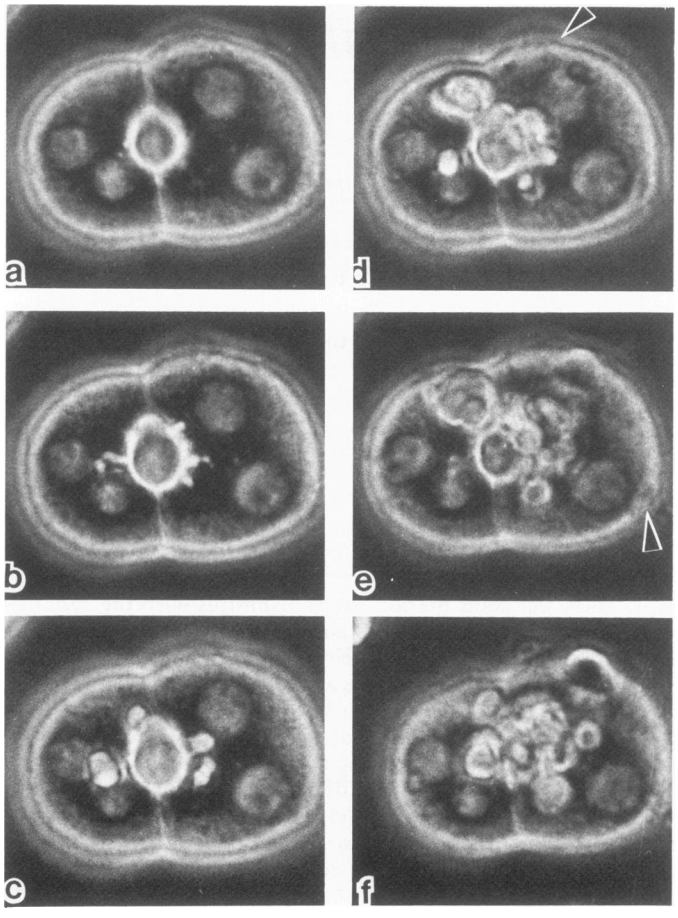


Figure 1—The process of vacuole formation after the treatment of 10 μg/ml phalloidin. **a**—Just prior to addition of phalloidin. Normal primary cultured hepatocytes. A slightly dilated bile canaliculus can be seen between coupled hepatocytes. Note the absence of protrusions and vacuoles. (×700) **b**—Twenty minutes after addition of phalloidin. The beginning of vacuole formation from the bile canalicular region can be seen. (vacuole formation time). (×700) **c**—Forty minutes after **a**. Large multiple vacuoles can be seen around the bile canaliculus. (×700) **d**—Sixty minutes after **a**. In addition to vacuoles, the beginning of protrusion formation from the free cell surface (*arrowhead*) can be seen (protrusion formation time). (×700) **e**—Eighty minutes after **a**. Another protrusion can be observed (*arrowhead*). (×700) **f**—One hundred minutes after **a**. Vacuoles and protrusions can be seen. (×700)

groups were administered 0.1, 1, or 10 μg/ml phalloidin, respectively, by addition of the phalloidin to the culture medium.

During the first 100 minutes of the experiment we examined the normal behavior of the cells in the culture; then the medium was changed. Aside from containing phalloidin, the experimental medium was the same as that used in the control period.

Liver Cell Culture

Liver cells were isolated according to a modification of the procedures of Seglen[21] and Laishes and Williams.[22] The details of the procedure of cell culture have been reported in previous papers.[14-16] Briefly, the procedure was as follows. Normal rat liver was perfused *in vivo* with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 0.5 mM EGTA for 4 minutes at 40 ml/min and then with L-15 medium containing 0.5% Type IV collagenase (Sigma Chemical Co., St. Louis, Mo) for 8 minutes at 25 ml/min. One million cells were inoculated into 60-mm Corning culture dishes (Corning Glass Works, Corning, NY) and maintained with L-15 medium which contained 10% fetal bovine serum, 10 mM HEPES, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were allowed to settle out in the culture dishes for a period of 4 hours before the experiments began.

The viability of the hepatocytes was examined both at the start and end of the experimental period with

Table 1—Vacuole Formation Time

Phalloidin	Mean ± SD per rat (3 BC)	Statistics		
		10 μg/ml	1 μg/ml	0.1 μg/ml
10 μg/ml	53.4 ± 11.3 min			
1 μg/ml	217.2 ± 55.9 min	$P < 0.05$		
0.1 μg/ml	642.2 ± 134.4 min	$P < 0.001$	$P < 0.001$	

Table 2—Protrusion Formation Time

Phalloidin	Mean ± SD per rat (3 BC)	Statistics		
		10 μg/ml	1 μg/ml	0.1 μg/ml
10 μg/ml	121.5 ± 24.0 min			
1 μg/ml	344.7 ± 56.8	$P < 0.05$		
0.1 μg/ml	1202.9 ± 313.7 min	$P < 0.001$	$P < 0.001$	

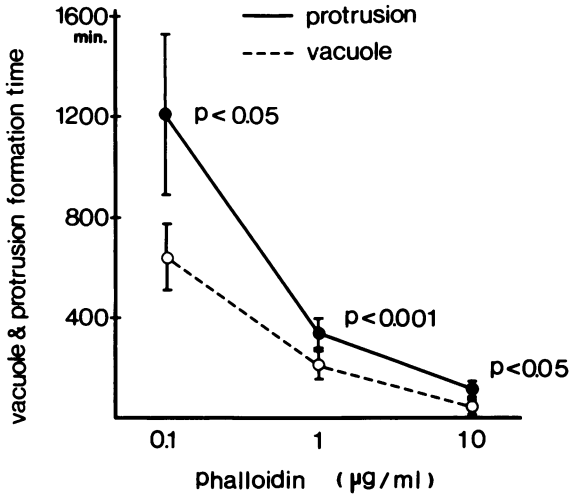


Figure 2—Graphic illustration to show the time lag between "vacuole formation time" and "protrusion formation time." Effects of phalloidin to hepatocytes in a dose-response manner. Vacuole formation precedes protrusion formation.

the trypan blue exclusion test and by observations of cell motility with the use of time-lapse motion pictures, as reported previously.[16] Viability ranged from 85% to 95% at the start of the experimental period and from 76% to 88% at the end of the experiments. The difference in viability between controls and phalloidin-treated groups was not significant statistically.

Time-Lapse Cinephotomicrography

An inverted camera microscope (ICM 405, Carl Zeiss) with phase-contrast optics, a 16-mm movie camera (H16, RX-5 Bolex), 16-mm reversal film (Plus-X 7276 Kodak), and a motor drive system with a time-lapse controller (Nikon) were used. All motion pictures were taken at the speed of 1 frame per 15 seconds.

Analysis of Motion Pictures

Analysis of motion pictures was performed with an analytic motion picture projector (Photo-Optical Data Analyzer 2240A, MKV, L-W International, Calif) in a frame-by-frame fashion. Paired hepatocytes were selected so that canaliculi could be examined. We observed 15 bile canaliculi from 5 rats (3 bile canaliculi from each rat) in each group. The experimental period comprised 2400 frames (ie, 10 hours of real time). Special attention was given to bile-canalicular activity, and the hepatocytes were observed closely with respect to decrease in diameter of the canalicular lumen and the formation of protrusions and vacuoles. The coupled hepatocytes selected in the present study had no protrusions and vacuoles before the treatment with phalloidin.

Electron Microscopy

Representative samples of treated and normal isolated hepatocytes were examined by electron microscopy. Standard methods of tissue processing were used.[23,24] Serial sections cut with a diamond knife were examined in a Philips EM 400 electron microscope.

Statistical Analysis

The statistical assessment was performed with analysis of variance.

Results

Time-Lapse Films of Cultured Hepatocytes

As expected, in normal hepatocytes several types of motile activity were observed throughout the cytoplasm of hepatocytes. Some of these movements were random Brownian-like movements, and spontaneous bile-canalicular contractile movements were noted. In phalloidin-treated hepatocytes, we observed the formation of vacuoles from bile-canalicular regions and of protrusions from cell surfaces (Figure 1). The vacuoles observed in phalloidin-treated hepatocytes were usually large and frequently cavernous and tortous.

We recorded the time interval from the addition of

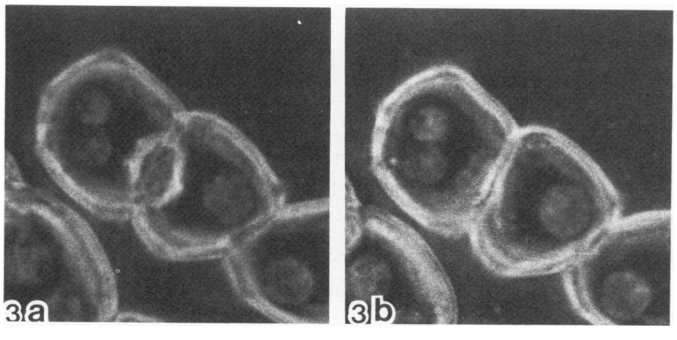


Figure 3—Normal bile canalicular contraction from controls. **a**—Slightly dilated bile canaliculus can be seen. (×700) **b**—Two minutes after **a**. The bile canaliculus has contracted. (×700)

Table 3—Number of Normal Contractions

Phalloidin	Mean ± SD per rat (3 BC)	Statistics			
		10 μg/ml	1 μg/ml	0.1 μg/ml	Control
10 μg/ml	4.0 ± 0.6				
1 μg/ml	12.4 ± 2.1	$P < 0.001$			
0.1 μg/ml	35.6 ± 4.4	$P < 0.001$	$P < 0.001$		
Control	80.8 ± 16.8	$P < 0.001$	$P < 0.001$	$P < 0.001$	

phalloidin to the start of formation of cytoplasmic vacuoles (lag time for vacuole formation) and to the start of the formation of protrusions from cell membrane surface (lag time for protrusion formation). The mean "lag" time of vacuoles in 5 rats (15 canaliculi) was 53.44 ± 11.3 (mean ± SD) minutes in the 10 μg/ml phalloidin-treated group, 217.2 ± 55.9 minutes in the 1 μg/ml group, and 642.2 ± 134.4 minutes in the 0.1 μg/ml group (Table 1). Vacuole formation was always accompanied by abnormal motility movements, including sustained contractile movements, further vacuole formation, and hemicanalicular contractions, which continued to the end of the experiments. The mean lag time for protrusion formation was 121.5 ± 24.0 minutes in the 10 μg/ml phalloidin-treated group, 344.7 ± 56.8 minutes in the 1 μg/ml group, and 1202.19 ± 313.7

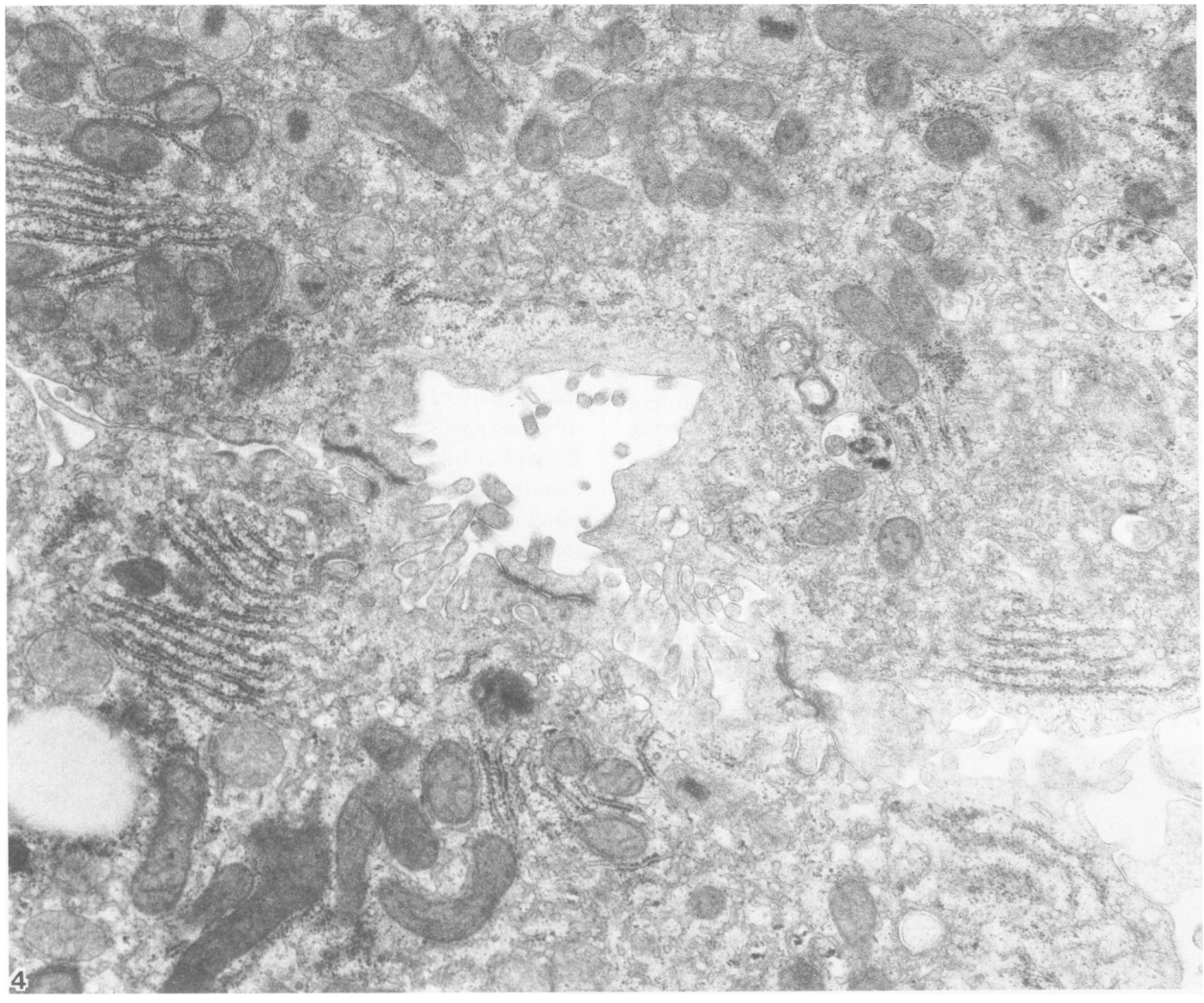


Figure 4—Electron micrograph of cultured hepatocytes. Normal cultured hepatocytes. Slightly dilated bile canaliculus with microvilli and tight junctions can be seen. Pericanalicular actin filaments are not prominent. (Original magnification, × 10,200)

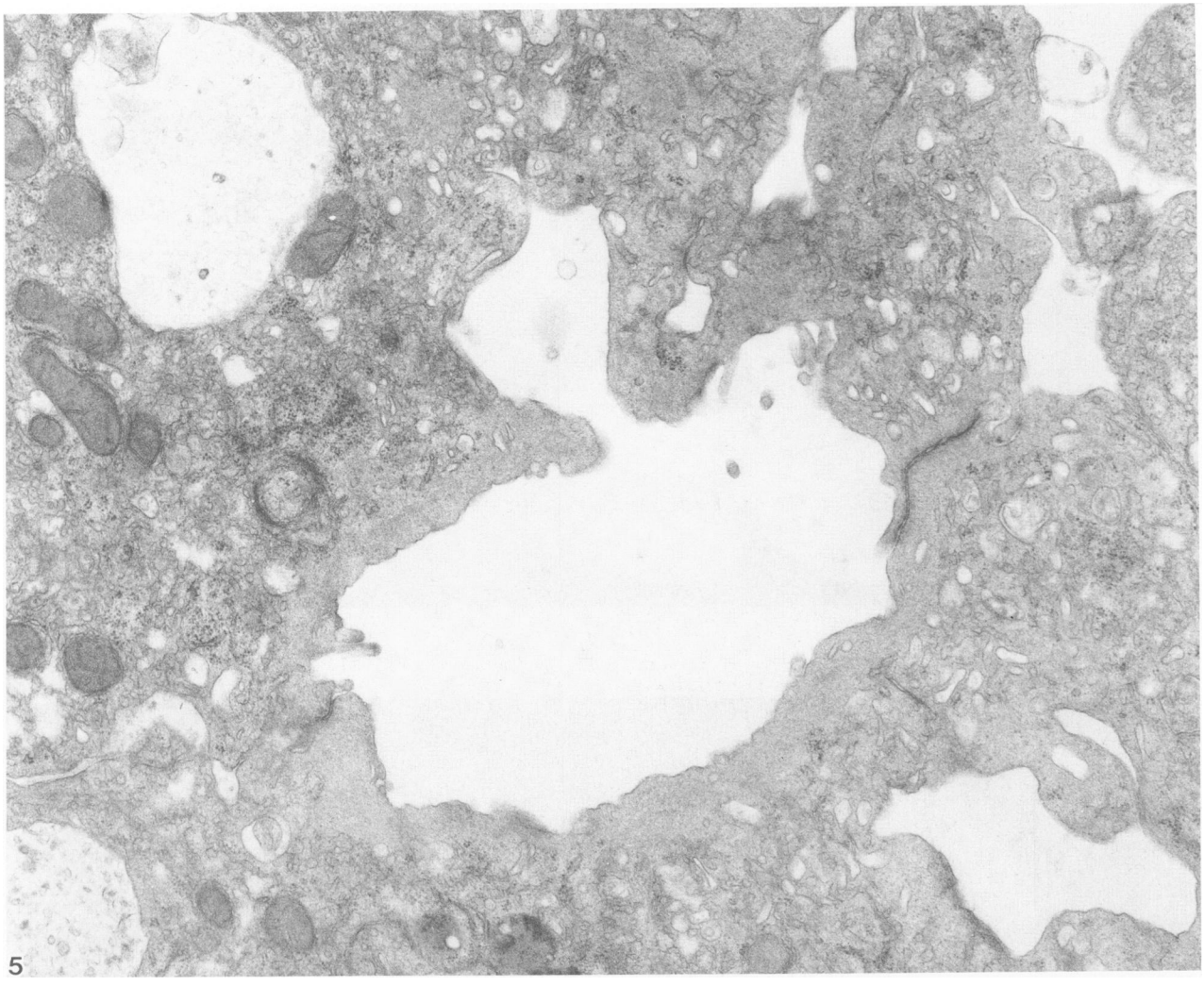
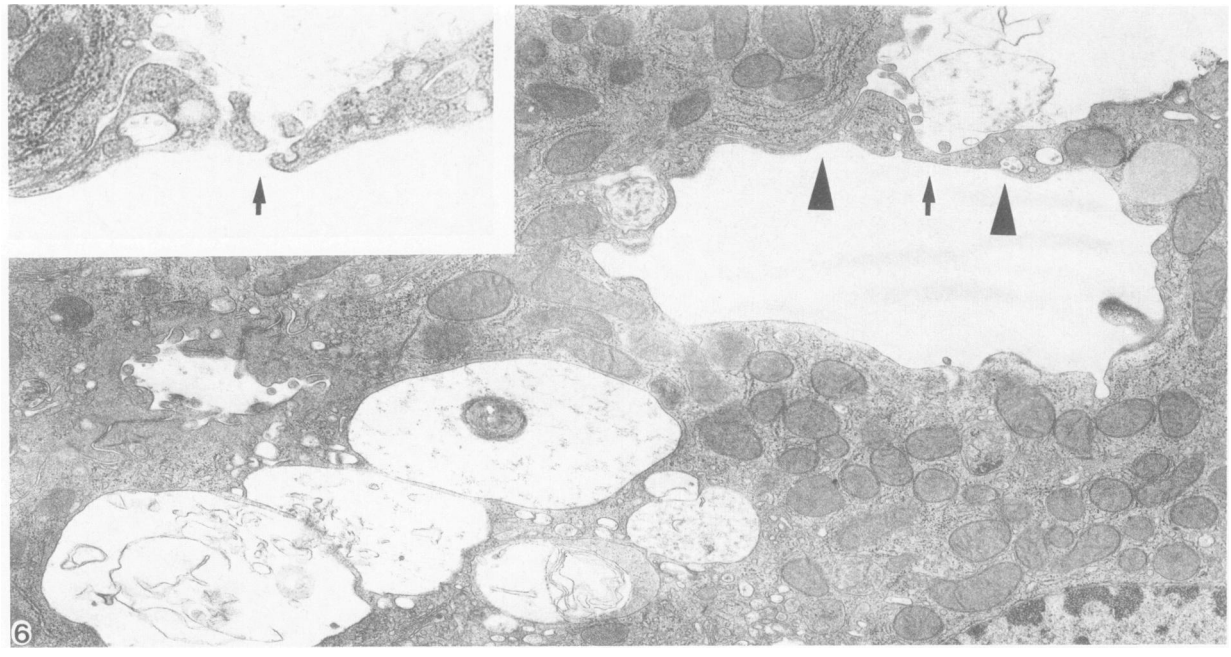


Figure 5—Electron micrograph of cultured hepatocytes 60 minutes after adding 10 μg/ml phalloidin. Multiple vacuoles are observed around the bile canaliculus. Actin filaments are recognized easily around the bile canaliculus. The involvement of the bile canalicular membrane in the process of vacuole formation is observed. (Original magnification, × 10,200)

minutes in the 0.1 μg/ml group (Table 2). The effects of phalloidin on both vacuole and protrusion formation were dose-dependent (Tables 1 and 2). The difference between the onset of vacuole formation and the onset of protrusion formation in each group was statistically significant (Figure 2). In controls, there were no vacuoles or protrusions.

To assess the bile-canalicular motility function, we analyzed the number of normal contractions of bile canaliculi (Figure 3). As we reported previously, normal contractions ended within 60 ± 30 seconds.[15] In more than 95% of instances, the canalicular contraction time was less than 120 seconds. Using this baseline, we analyzed the number of normal contractions of bile canaliculi. The mean number of observed contractions in 10 hours was 80.8 ± 16.8 (mean ± SD) per rat (15 bile canaliculi) in controls, 35.6 ± 4.4 in the 0.1 μg/ml phalloidin-treated group, 12.4 ± 2.1 in the 1 μg/ml group, and 4.0 ± 0.6 in the 10 μg/ml group (Table 3). The canalicular contractions in the phalloidin-treated groups were also greatly prolonged.

The cells before treatment showed normal or slightly dilated bile canaliculi (Figure 4) and showed no blebbing or vacuoles. The surface microvilli of the hepatocytes were well preserved. In a previous report, we documented by morphometric analysis the increase in the pericanalicular actin filament zone after phalloidin treatment in experiments of a design essentially similar to that of these and showed that the increase was dose-dependent.[16] With acute phalloidin treatment, bile canaliculi showed irregular shapes, and actin filaments were prominent around the canaliculi. We observed large vacuoles in the cytoplasm of hepatocytes and cytoplasmic protrusions (blebs) at the cell surfaces.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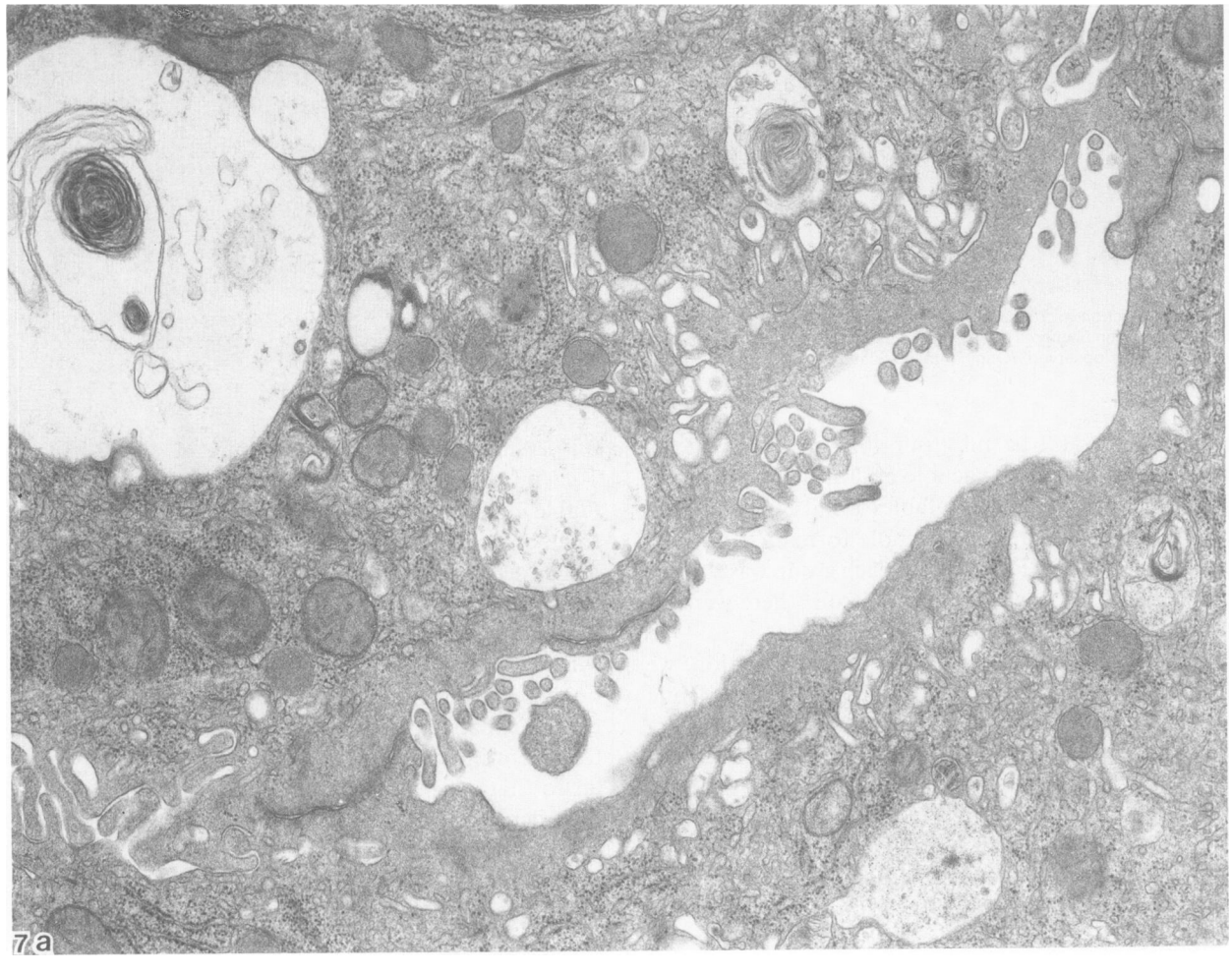
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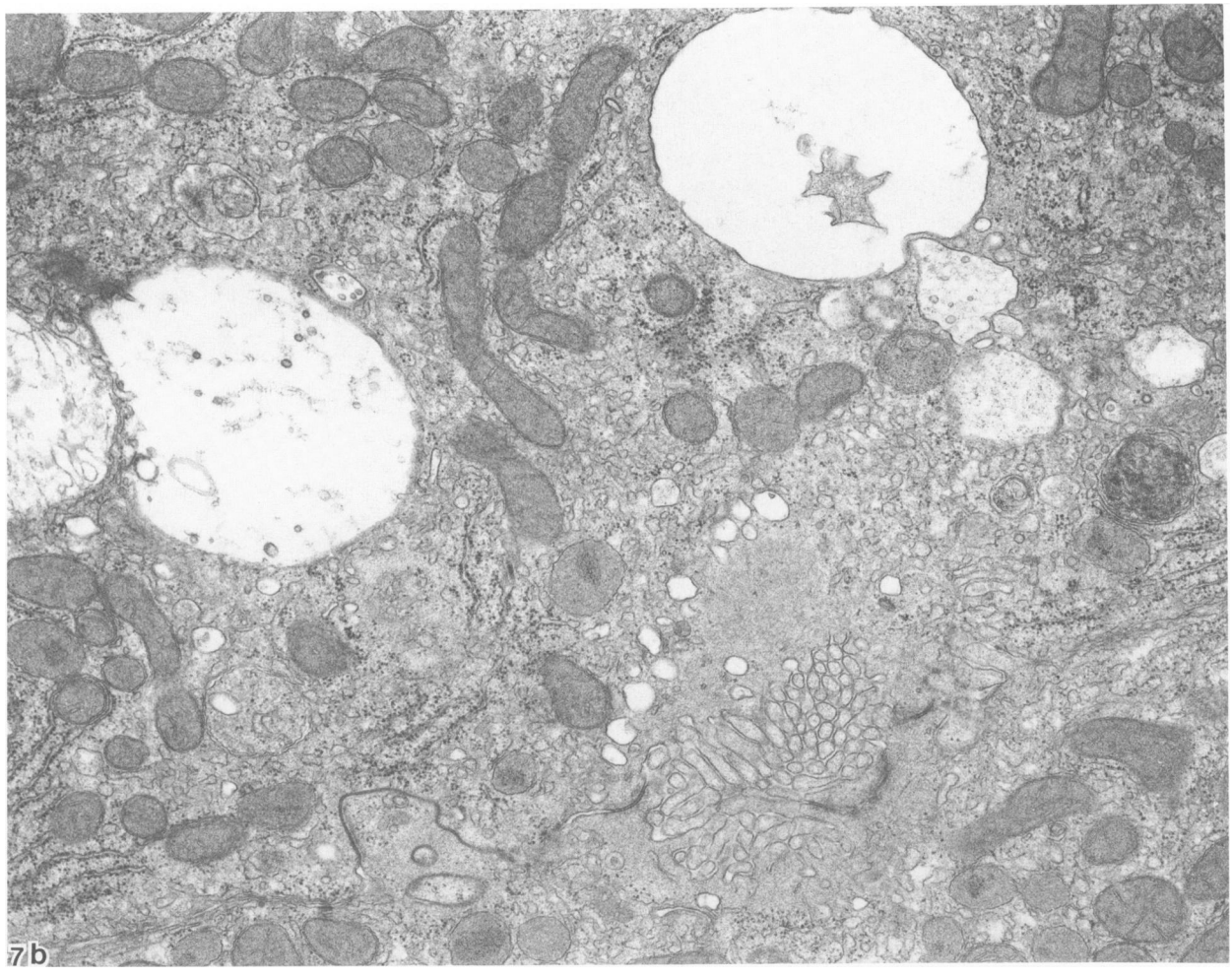


Using serial sections, it was possible to show continuity between membranes of bile canaliculi and cytoplasmic vacuoles and between cytoplasmic vacuoles and the lateral cell membranes (Figures 5 and 6). Hence, by examining serial sections, it was found that some of these same membrane-bound vacuoles, which appeared circular or irregular in shape in single sections, were in fact tortuous canals, some of which connected the lateral and canalicular cell surfaces. Smaller vacuoles (diameter > 1000 Å) were frequently disposed around the margins of canaliculi abutting on the zone of increased pericanalicular actin filaments in the phalloidin-treated cells (Figure 7).

Protrusion formation occurred in treated cells (compare Figures 8a, b, and c). Cytoplasmic components abutted directly on the plasma membrane because of an apparent change in distribution of cytoskeletal components (Figure 8b). The fully formed protrusion had a slender stalk, and aggregated microfilaments were often seen in the neck region (Figure 8c).

Discussion

It is known that phalloidin causes enhanced polymerization and stabilization of filamentous actin[1] and that the administration of phalloidin impairs motility functions in living unicellular organisms[25] and in cultured cells.[26] It is also established that in liver, the great

Figure 6—Electron micrograph of cultured hepatocytes 90 minutes after adding 10 μg/ml phalloidin. Multiple vacuoles are observed in the cytoplasm. One of the vacuoles (*arrows*) is located just beneath the sinusoidal membrane. (Original magnification, × 10,200) **Inset**—Serial sectioning of the area indicated by the *large arrowheads*. Note the connection between the vacuole and extracellular space (*arrow*) (Original magnification, × 23,000)
Figure 7—Cultured hepatocytes 90 minutes after adding 10 μg/ml phalloidin. **a**—Note the slightly dilated bile canaliculus and the presence of multiple vacuoles. Smaller vesicles (diameter > 1000 Å) are seen around the increased actin filament zone. (Original magnification, × 10,200) **b**—Closed bile canaliculus with vacuoles. Small vesicles are observed in the pericanalicular area, around the thick actin filament zone. (Original magnification, × 10,200)

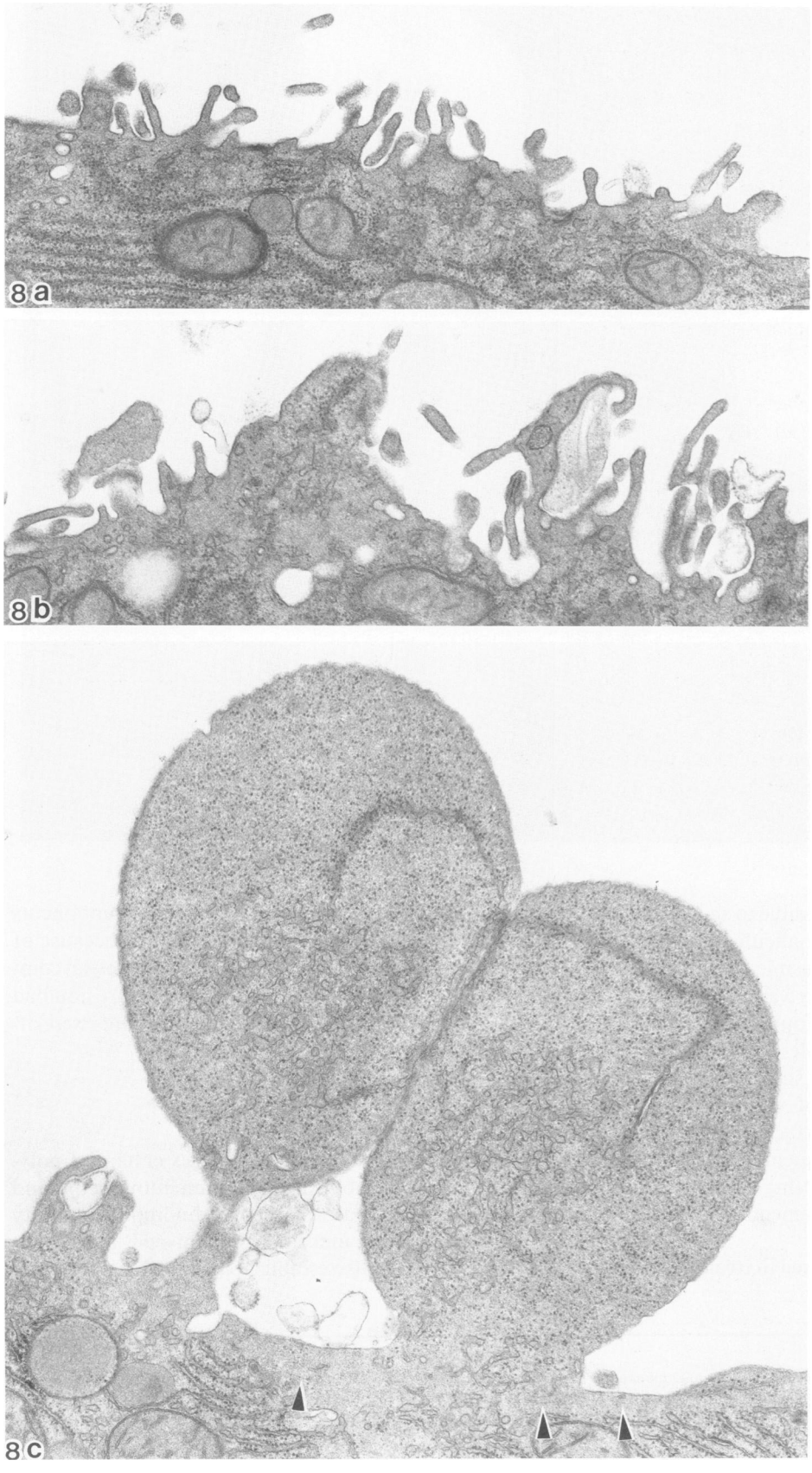
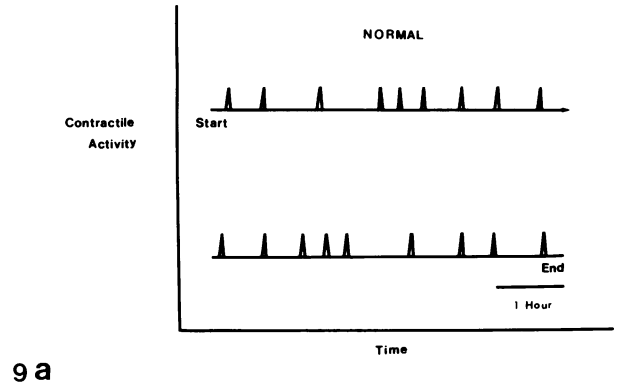


Figure 8a—Normal cultured hepatocytes. Note the presence of microvilli on the cell surface. (×23,000) **b**—Cultured hepatocytes 40 minutes after adding 10 μg/ml phalloidin. Protrusion formation has started. (×23,000) **c**—Cultured hepatocyte 120 minutes after adding 10 μg/ml phalloidin. Protrusions are fully formed; aggregated actin filaments (*arrowheads*) can be seen in the basal region of these protrusions. (×23,000)

increase in pericanalicular actin filaments that occur are functionally abnormal: bile flow is decreased[12]; tight junction permeability is altered,[10] and bile-canalicular contractions are impaired.[16] Since phalloidin induces visible effects on isolated hepatocytes, we have been able to correlate the functional motility effects with the morphologic changes induced by this toxin. In essence, the actin-based motility functions of the cell are greatly reduced and are associated with the appearance of large vacuoles in the cytoplasm, and later protrusions appear on the cell surface. The vacuoles begin as canalicular diverticula, narrow connections between pericanalicular vacuoles and the canalicular membrane and can be shown on serial sections. Presumably, as more vesicles fuse together, large cytoplasmic vacuoles develop. Serial sections show that, in the more advanced stages, what appear as round or irregular-shaped vacuoles in routine sections are in fact long tortuous canals connecting distant regions of the cell. According to the membrane flow hypothesis, actin filaments are required for the directional transport of vesicles, and cytoskeletal components, especially actin, are thought to drive the membrane flow process.[27] The molecular mechanisms involved, however, are completetly unknown.

Of further interest, of the two major morphologic parameters that could be assessed — formation of vacuoles and surface protrusions — vacuole formation always occurred first. There did not appear to be any other special correlation between these two parameters either functionally or morphologically. It was also evident that the cytoplasmic vacuoles began in continuity with canaliculi. This fact might indicate that the canaliculus-associated actin has higher sensitivity to phalloidin than the actin beneath other regions of the cell membrane. Alternatively, it may reflect a mass action effect, because there is more actin in the pericanalicular region of the hepatocyte, and it is the area most affected by phalloidin administration when given in repeated small doses as suggested by Gabbiani et al.[11] Another distinct possibility is that the actin around canaliculi may be physiologically and functionally different in its reponse to phalloidin. This view, that the actin filaments in the liver might subserve different functions, is supported by the fact that bile canaliculi have a distinctive motility behavior in the form of spontaneous active contractions.[14,15] These contractions are sensitive to cytochalasin B[19] and appear independent of other motility



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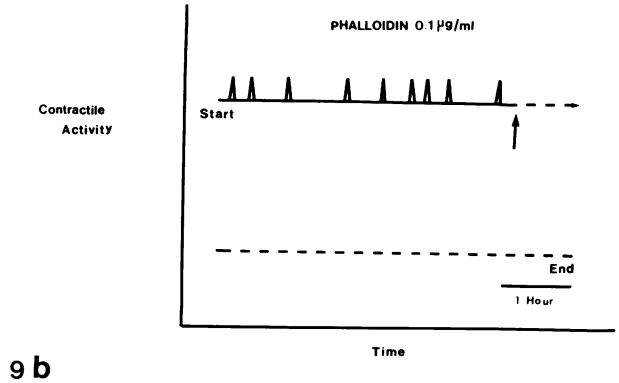

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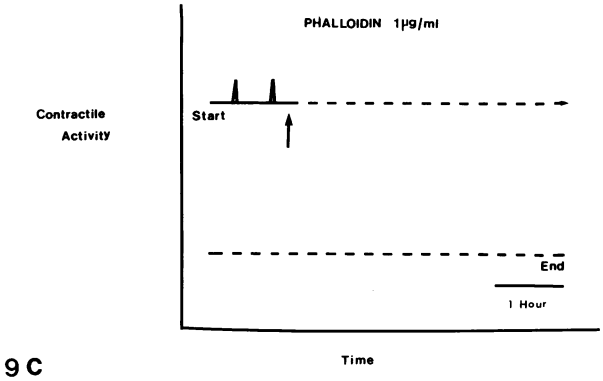

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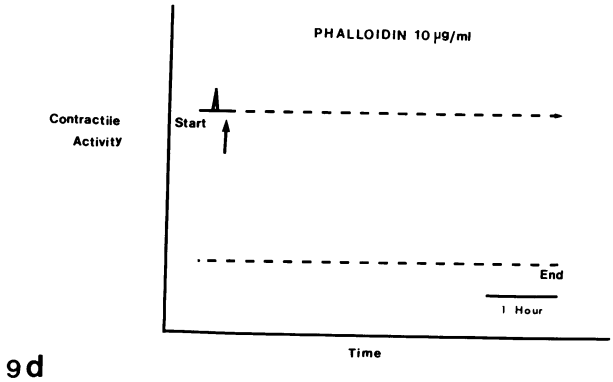

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Figure 9—Diagram of canalicular contractile activity. Graphic representation of bile-canalicular contractions from the control (**a**), 0.1 µg/ml phalloidin group (**b**), 1 µg/ml phalloidin group (**c**), and 10 µg/ml phalloidin group (**d**). The tracing begins at the upper left and ends at the lower right, with each strip representing 5 hours. *Arrows* indicate the start of vacuole formation from the bile canaliculi; bile-canalicular contractions (*spikes*) were rarely observed after the beginning of vacuole formation (*broken line*).

activities such as the Brownian-like movements, which are continuous.[14]

Concerning bile-canalicular movements, the results reported here confirm previous findings that there is a significant decrease in the number of contractions in phalloidin-treated hepatocytes.[16] Figure 9 is a graphic representation of one of the canaliculi from the control group and one from each of the treated groups. Normal contractions occurred until the beginning of vacuole formation, after which there were few normal contractions. The incidence of normal contractions was more than 95% prior to the beginning of vacuole formation and less than 5% after that. The decrease of contractility depended also on the dose of phalloidin administered. An increase in actin filaments was noted after phalloidin treatment and occurred rapidly. The uptake of phalloidin into hepatocytes has been well investigated by others[28-30]; their reports show that the uptake of phalloidin is very rapid and that its effect also appears rapidly. The decrease in contractility of bile canaliculi in phalloidin-treated hepatocytes is interpreted as functional impairment, a consequence of the increase in polymerized actin. Normally, canalicular contractions in isolated hepatic cell pairs is accompanied by extrusion of the canalicular luminal content (canalicular bile).[15] It is of interest, in this regard, that canalicular lumens did not widen progressively after phalloidin treatment in spite of the diminished contractions and may reflect a decrease in bile secretion by the hepatocytes. This is in contrast to what is observed with cytochalasin B, where secretion continues with progressive distension of bile canaliculi.[19] From the observation of electron micrographs, the transport of endocytic vesicles (diameter > 1000 Å) appeared to be blocked mechanically by the increased investment of actin filaments around canaliculi. This finding may be an added factor in explaining the diminished secretion, because vesicles of this size are believed to play an important role in the formation of bile.[31]

Death of cultured hepatocytes treated with phalloidin has been discussed by others[32,33] and needs brief comment. It is notable that the viability of cultured hepatocytes after the treatment with phalloidin for 10 hours was relatively high in this study (76–88%). The culture medium used contained a low concentration of ionized calcium, 0.92 ± 0.01 mM,[34] and the concentration of phalloidin was 10 μg/ml or less. These experimental conditions might possibly explain the high viability of the hepatocytes in the present study.

Cytoplasmic protrusions on the hepatocyte surface are cited as manifestations of hepatocellular injury and cell death.[32,33,35] Godman et al[36] reported that the cytoplasmic protrusions associated with cytochalasin D were caused by disruption of actin filaments beneath the cell membrane, resulting in weakening of the membrane and cytoplasmic herniation. The mechanism by which phalloidin causes protrusions is unknown but is undoubtedly related to a disturbance in actin filament function.

In summary, the results of this study add new information on the functions of actin in hepatocytes. Impaired actin-associated motility not only inhibits canalicular contractions but appears to be associated with major disturbances in membranes with the formation of large vacuoles and even canals that traverse broad regions of the cytoplasm. The results also provide an indication that the functions of actin in hepatocytes are multiple.

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