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The cytoskeleton of digitonin-treated rat hepatocytes

(membrane permeability/microfilaments/10-nm filaments/membrane skeleton)

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ABSTRACT **Treatment of isolated rat hepatocytes with low concentrations of digitonin increases the permeability of the plasma membrane to cytosolic proteins without causing release of organelles such as mitochondria into the surrounding medium. Electron microscopy showed that treatment of the cells with increasing concentrations of digitonin results in a progressive loss in the continuity of the plasma membrane, while most other aspects of cellular morphology remain normal. Depletion of background staining material from the cytosol by digitonin treatment of the cells greatly enhances the visualization of the cytoskeleton. The use of this technique, together with immunofluorescent light microscopy, has verified the presence of an actin-containing filamentous network at the hepatocyte cortex as well as intermediate filaments distributed throughout the cell. Digitonin is thus useful both for selectively permeabilizing the plasma membrane and for intensifying the appearance of intracellular structures such as microfilaments that are normally difficult to observe in cells such as hepatocytes.**

Digitonin, a steroid glycoside, increases the permeability of different types of cells to inorganic ions (1, 2, *), metabolites (1–4, *), and enzymes (3, 5, 6). This effect is believed to be the result of the binding of digitonin to cholesterol and other β-hydroxysterols present in the plasma membrane (7). Because the molar ratio of cholesterol to phospholipid in eukaryotic plasma membranes is severalfold or manyfold greater than in intracellular membranes (8), treatment of intact cells with low concentrations of digitonin can be expected to bring about a selective increase in the permeability of the plasma membrane. This property has been used in this and other laboratories to make measurements of transport activities by mitochondria (1, 2, 9, *) and endoplasmic reticulum (9) in cells *in situ*. Digitonin offers a major advantage over other commonly used agents, such as Triton, glycerol, or toluene, because the latter are relatively nonspecific in their effects on different types of cell membranes.

Experiments described in this paper demonstrate another useful consequence of the treatment of cells with digitonin—namely, greatly enhanced electron microscopic visualization of certain intracellular structures, particularly the cytoskeletal network of filaments, which are normally difficult to observe due to the presence of dark-staining cytosolic protein. Such pretreatment of hepatocytes with low levels of digitonin causes no loss or impairment of the normal morphology of membrane-limited organelles, such as mitochondria, endoplasmic reticulum, and lysosomes. The enhanced visualization of intracellular structures as described here reveals some hitherto unreported associations of microfilaments and intermediate filaments within isolated normal hepatocytes, supplementing earlier reports on the role of microfilaments in maintenance of cell shape (10), in endocytosis (11), and in secretion of lipoproteins (10).

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MATERIALS AND METHODS

Isolation of Hepatocytes. Hepatocytes were isolated from large male Charles River CD rats by the collagenase/hyaluronidase perfusion method of Cornell *et al.* (12). The cells were washed several times and resuspended in sucrose/KCl medium (125 mM sucrose/60 mM KCl/3 mM K-Hepes, pH 7.1) to a final stock concentration of 40×10^6 cells per ml (approximately 50 mg of protein per ml) and kept at 4°C. Consistently over 95% of the isolated cells excluded Trypan Blue.

Electron Microscopy. Primary fixation was carried out by transferring 0.05-ml aliquots (0.1 mg of protein) of cells to Microfuge tubes containing 0.3 ml of sucrose/KCl medium supplemented with 3.0% (vol/vol) glutaraldehyde and immediately centrifuging the samples at $500 \times g$ for 30 sec in a Coleman Microfuge. These micropellets were stored on ice for 1–2 hr, rinsed with medium to remove excess glutaraldehyde, and stored overnight at 4°C. The cells were postfixed at 4°C for 30 min with sucrose/KCl medium containing 1.0% OsO_4, then dehydrated, embedded, sectioned, and stained as described (13). Micrograph plates were obtained from a Zeiss EM10 electron microscope operated at 60 kV with a 60-μm objective aperture.

Immunofluorescent Detection of Cellular Actin. Digitonin-treated rat hepatocytes (1.3 mg of protein) were pelleted at $60 \times g$ for 5 min and resuspended in 50 μl of 150 mM NaCl/10 mM phosphate (pH 7.5) containing antibody against actin (120 μg/ml), normal rabbit IgG (140 μg/ml), or rhodamine-labeled goat antibody against rabbit IgG (1 mg/ml). Cells were incubated at 4°C for 30 min, then washed twice by centrifugation at $60 \times g$ for 5 min and resuspended with 5 ml of sucrose/KCl medium.

The binding of unlabeled antibodies was detected by incubating the cells again with rhodamine-labeled goat anti-rabbit IgG by the same procedure. Final pellets were suspended in a drop of sucrose/KCl medium, and 7 μl was spread on a glass slide under a coverslip. Specimens were examined with a Leitz Ortholux 11 equipped with a Ploem vertical illuminator and a Phaco 63× oil immersion objective (n.a. = 1.3).

Antibody to chicken gizzard actin was raised in rabbits, affinity-purified on a column of rabbit skeletal muscle actin, covalently coupled to Sepharose 2B, and characterized as described (14). The preparation of rhodamine-labeled goat anti-rabbit IgG (Fc fragment) has been described (15).

* Fiskum, G., Villalobo, A. & Lehninger, A. L. (1979) *XIth International Congress Biochemistry* (Toronto, Canada).

RESULTS AND DISCUSSION

Permeability of digitonin-treated cells

During the experiments designed to measure mitochondrial and endoplasmic reticulum ion-transport activities within whole cells (9, *) an effort was undertaken to determine the optimal level of digitonin that would make the plasma membrane freely permeable to small ions and proteins without affecting the permeability of the organelle membranes. Zuurendonk and Tager (3) and, more recently, Mackall *et al.* (5) have found that treatment of hepatocytes, either in suspension or as cultured monolayers, with digitonin at a concentration of 0.04–0.10% causes rapid and efficient release of cytosolic enzymes without significant release of mitochondrial enzymes. Electron microscopic analysis has indicated that at these high concentrations of digitonin the plasma membrane (4, 16), as well as the endoplasmic reticulum (4), is heavily damaged. We have recently reported* that even at a digitonin concentration as low as 0.005% there is a very rapid equilibration of ions and small molecules between the hepatocyte interior and the suspending medium. Ion-transport measurements carried out under these conditions have indicated that little or no change in the permeability of the endoplasmic reticulum (9) or the mitochondrial inner membrane (9, *) occurs. The results shown in Table 1 demonstrate the change in permeability of isolated hepatocytes treated with low levels of digitonin. Cells suspended for 2 min at 25°C in control medium not containing digitonin released a small fraction of their total protein. The lack of succinate dehydrogenase activity in the supernatant after low-speed centrifugation of the untreated cells indicates that mitochondria were not released during the incubation; however, the appearance of lactate dehydrogenase in the supernatant does indicate that some cytosolic protein was lost. Upon treatment of the hepatocytes with 0.005% digitonin, essentially all of the cell lactate dehydrogenase and approximately 30% of the total protein were released, but virtually none of the succinate dehydrogenase. These results strongly indicate that this treatment causes release of most or all of the cytosolic protein but does not cause a loss of mitochondria from the cells. The same results were obtained when the cells were treated with 0.015% digitonin; however, treatment with 0.001% digitonin had no effect on the release of protein, lactate dehydrogenase, or succinate dehydrogenase. The use of digitonin at these relatively low concentrations was also effective in increasing the permeability of Ehrlich ascites tumor cells to cytosolic proteins as well as small molecules.*

Table 1. Effect of digitonin on plasma membrane permeability of isolated hepatocytes*

	Protein	Lactate dehydrogenase	Succinate dehydrogenase
Control			
Pellet	85	68	100
Supernatant fluid	15	32	0
+ Digitonin			
Pellet	73	7	97
Supernatant fluid	27	93	3

* A 0.25-ml sample (10×10^6 cells) was diluted with sucrose/KCl medium to a concentration of 2×10^6 cells per ml in the absence or presence of 0.005% digitonin. After slow magnetic stirring for 3 min at 25°C, an aliquot of the cell suspension was withdrawn and centrifuged for 2 min at $500 \times g$; measurements of lactate dehydrogenase (17), succinate dehydrogenase (18), and protein (19) were made on the pellet and the supernatant. Values are expressed as percent of total cell activity in both fractions.

Electron microscopy

Low-magnification electron micrographs of thin sections taken from normal and digitonin-treated rat hepatocytes are shown in Fig. 1. In contrast to untreated hepatocytes (Fig. 1*A*), cells treated with either 0.005% digitonin (Fig. 1*B*) or 0.015% digitonin (Fig. 1*C*) lack the normal background staining material present in the cytoplasmic compartment. This is consistent with the loss of cytosolic protein that occurs upon digitonin treatment (Table 1). Although there is a striking difference in the intensity of background staining between normal and treated cells, most other aspects of gross cell morphology are comparable. These findings are in contrast with those previously reported for hepatocytes treated with a much higher concentration (0.40%) of digitonin (4). Under the latter conditions, the mitochondrial outer membrane was lost and only small vesicular remnants of endoplasmic reticulum were observed (4). At the lower levels of digitonin used here the cells retain normal-appearing smooth and rough endoplasmic reticulum and the mitochondria possess both their inner and outer membranes. The change in appearance of the mitochondria after treatment of the cells with digitonin is simply a change in conformation from orthodox to condensed (20) that occurs upon deenergization, in this case due to release of ATP and oxidizable substrates from the cells (G. Fiskum, unpublished results). The functional integrity of the mitochondria present within digitonin-treated cells is as good as or better than that of isolated mitochondria.*

Further information on the ultrastructure of digitonin-treated hepatocytes was obtained from observations made at higher magnification (Fig. 1 *D–F*). When the hepatocytes were treated with digitonin at a concentration of 0.001%, a level that did not increase their permeability to cytosolic proteins, little change in the cell morphology took place (Fig. 1*D*). When the concentration of digitonin was raised to 0.005%, the loss of cytosolic staining material enabled visualization of an extensive cytoskeletal network of filaments (Fig. 1*E*). The cytoskeleton of hepatocytes consists of two distinct populations of filaments. Small filaments, approximately 5 nm thick, are found around the entire periphery of the cells within a cortical zone of less than 0.5 μm (enclosed by large arrows); thicker filaments, approximately 10 nm wide, were observed throughout the cytosolic compartment. These larger filaments are often found in bundles (enclosed by small arrows) and traverse the cell in a random fashion.

The smaller filaments typically appear to be in contact with each other and with the inner surface of the plasma membrane. Their appearance is very similar to that of actin-containing microfilaments that have been observed near the surface of other types of cells (see, e.g., refs. 21 and 22). Such filaments have also previously been observed in hepatocytes (10, 11, 23, 24); however, in intact cells from livers of normal animals visualization of cytoplasmic filaments has been limited due to the presence of surrounding background staining material. Treatment of cells with low levels of digitonin is thus a useful technique for intensifying the appearance of cytoplasmic filaments, particularly those present in cells such as hepatocytes that do not normally contain an abundance of these structures.

The cortical network of microfilaments observed in hepatocytes treated with 0.015% digitonin (enclosed by large arrows in Fig. 1*F*) appears to be more tightly packed than in cells treated with 0.005% digitonin. This difference may be related to the disruption of the plasma membrane that occurs at high digitonin concentrations (4, 16). As can be seen in Fig. 1*F* (treatment at 0.015% digitonin), the area that is normally occupied by the plasma membrane is replaced by a zone (less than

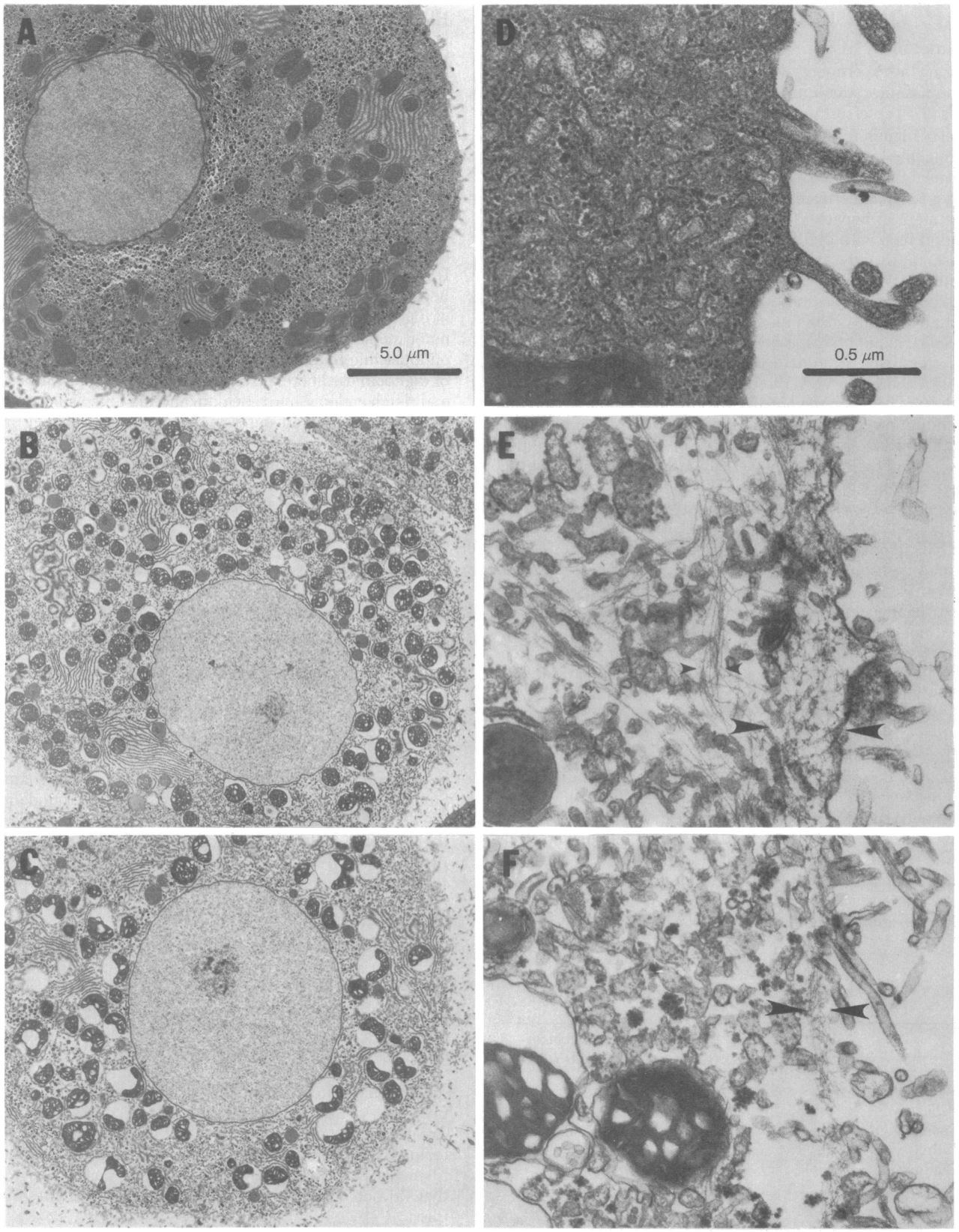

FIG. 1. Electron micrographs of normal and digitonin-treated rat hepatocytes. Micrographs are of cells fixed after dilution as described in the legend to Table 1 in medium containing either no digitonin (*A*) or 0.001% (*D*), 0.005% (*B* and *E*), or 0.015% (*C* and *F*) digitonin.

1 μm wide) containing vesicular and tubular structures. These structures may represent either a severely altered form of the plasma membrane or, as previously suggested (16), tubules composed primarily of digitonin and cholesterol. The observation that there is a high degree of discontinuity in the membranes of these structures located at the cell surface poses the question of how the cells stay "intact" and retain their normal spherical shape after treatment with high levels of digitonin.

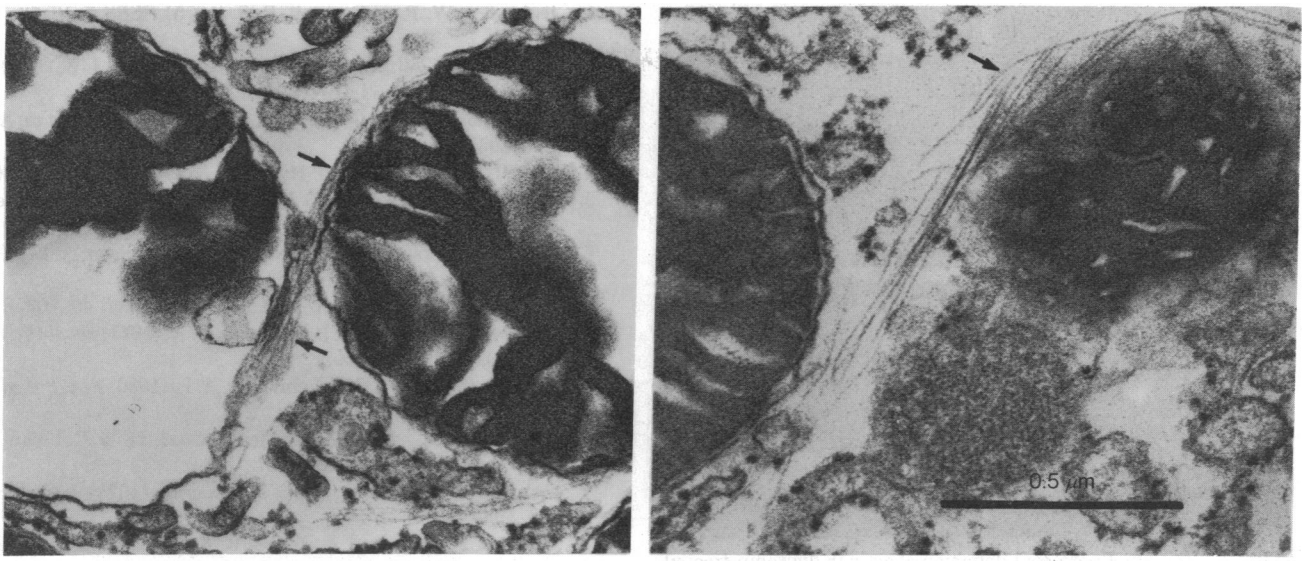

FIG. 2. Electron micrographs showing apparent association of intermediate filaments (arrows) with the mitochondrial outer membrane.

It is speculated that the tightly knit network of microfilaments that is located beneath the vesicular zone at the cell surface but outside the cell contents may hold the internal structures in place, as seen in Fig. 1*F*. The cortical filamentous network of hepatocytes may thus function in a manner similar to the membrane skeleton of erythrocytes (25), which plays an important role in maintaining their structure.

The intracellular organization of hepatocytes may also be influenced by the presence of intermediate filaments. As seen in Fig. 1*F* and more clearly in Fig. 2, 10-nm filaments are often found in apparent contact with the outer membrane of mitochondria. In many instances these filaments appear to form connections between two or more mitochondria (Fig. 2 *Left*). The existence of such attachments is supported by previous observations (24, 26) suggesting linkage between hepatocyte mitochondria and desmosomes via 10-nm "tonofilaments." We have not observed such an intimate association between intermediate filaments and other cellular structures, although an apparent binding of similar filaments to smooth Golgi vesicles located near bile canaliculi has been described (23).

Identification of actin

The presence of actin in mammalian liver has been reported (27). Although it may constitute less than 5% of the total content of hepatocyte protein, it can be detected *in situ* by the use of immunofluorescent microscopy (10, 24). The results of one such study (24) have suggested that actin is localized mainly near the cortex of hepatocytes. These findings are consistent with the interpretation presented here and elsewhere (6, 10, 23, 24) that the 4- to 7-nm filaments found near the hepatocyte plasma membrane are actin microfilaments. This is supported in the present studies by the following experiments using both slab-gel electrophoresis and immunofluorescent light microscopy.

The results of an experiment in which rat hepatocyte proteins were separated by slab-gel electrophoresis are shown in Fig. 3. The Coomassie blue-stained profiles (lanes a and b) of the total protein fractions from untreated and digitonin-treated hepatocytes are similar. The electrophoretic profile of proteins released from digitonin-treated hepatocytes (lane c) is also similar; however, several protein bands are not as pronounced as in the samples from normal or treated cells. The actin protein band present in these three samples was identified by the antibody overlay technique (30). The electrophoresed proteins were initially exposed to anti-actin, then to ^{125}I-labeled protein A (which binds to immunoglobulins). The autoradiographs of protein samples so treated from normal hepatocytes, digitonin-treated hepatocytes, proteins released by digitonin treatment, and a sample of pure actin are shown in lanes e–h, respectively. A single band was observed in all samples which corresponded to the Coomassie blue-stained band of pure actin (lane d). The "antibody staining" was specific for actin, as indicated by the absence of bands in samples treated with normal immunoglobulins (lanes i–l) rather than anti-actin. These results thus indicate that both untreated and digitonin-treated rat hepatocytes contain actin. It is also noteworthy that actin is released from the cells upon treatment with digitonin (lane g). Further studies are necessary to establish whether this represents loss of monomeric actin that is normally present in rat hepatocytes or whether a polymerized or filamentous form of actin is lost upon treatment of these cells with digitonin.

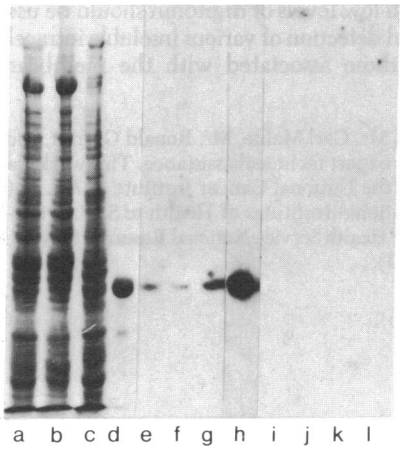

FIG. 3. Reactivity of antibody against actin with rat hepatocyte proteins. Proteins were separated on 7.5% polyacrylamide mini-slab gels (8 × 10 × 0.5 cm) (28) with the Laemmli buffer system (29). Binding of anti-actin was detected by the antibody gel overlay technique described by Adair *et al.* (30). Lanes a–d are photographs of Coomassie blue-stained proteins; lanes e–l, are autoradiographs of proteins exposed to ^{125}I-labeled protein A after exposure to either anti-actin (lanes e–h) or normal IgG (lanes i–l). The analyzed proteins were from untreated (lanes a, e, and i) and digitonin-treated (lanes b, f, and j) hepatocytes or were those released upon digitonin treatment (lanes c, g, and k). An actin standard (lanes d, h, and l) was also included.

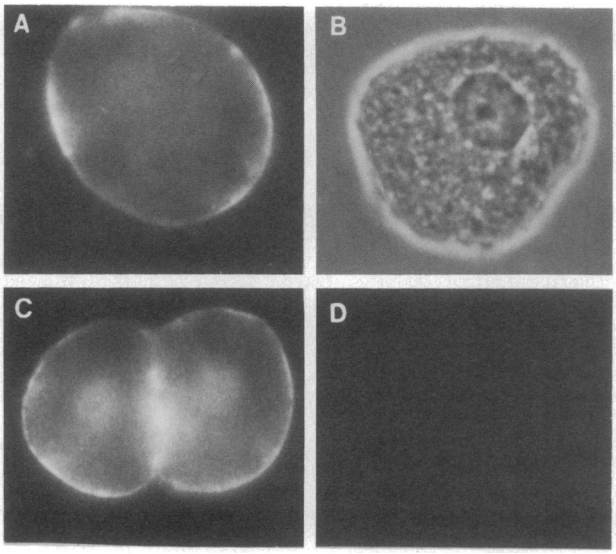

FIG. 4. Immunofluorescent detection of actin in digitonin-treated hepatocytes. Cells treated with 0.005% digitonin were exposed to either anti-actin (*A* and *C*) or normal IgG (*D*) followed by rhodamine-labeled anti-IgG. Both fluorescent (*A*, *C*, and *D*) and phase-contrast (*B*) light micrographs are shown.

The actin present within digitonin-treated hepatocytes is localized mainly near the cortex of the cells, as demonstrated by the immunofluorescent light micrographs shown in Figs. 4 *A* and *C*. The hepatocytes, which were incubated with unlabeled anti-actin followed by rhodamine-labeled anti-IgG, also exhibited some fluorescence at the nucleus and, as seen in Fig. 4*C*, bright fluorescence at regions of cell–cell contact. Detection of actin near the plasma membrane supports the conclusion that the filaments observed in the same area with the electron microscope are actin microfilaments.

These results also indicate that, in addition to causing the release of cytosolic proteins and intensifying the appearance of intracellular structures, digitonin can be used to increase the permeability of cells to various exogenous agents, including molecules as large as antibodies. This suggests that treatment of cells with low levels of digitonin should be useful in the immunological detection of various insoluble intracellular proteins, including those associated with the membranes of organelles.

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