Effects of Cytochalasin B on Membrane-associated Microfilaments in a Cell-free System

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ABSTRACT The mode of action of cytochalasin B was examined in vitro using bile canaliculus-enriched plasma membrane fractions isolated from rat liver. The pericanalicular microfilaments, which are mainly actin filaments and which are normally attached to the canalicular membranes, were dissociated from the membranes by cytochalasin B treatment. A microfilamentous network was found in the supernate of the cytochalasin-treated specimens and a number of polypeptides, of which a polypeptide corresponding in molecular weight to actin was a notable member. These results suggest that actin filaments become detached from the canaliculus membranes by cytochalasin B.

The cytochalasins are widely used in biology and pathology as cytopharmacological agents because of their effects on the contractile functions of actin filaments (34). Alteration of microfilament contractility is a general effect that has been observed in many cell types, but the functional sequelae vary with the specialized nature of the cell involved (4, 18, 36). Accompanying these functional changes in cell motility, structural changes have been observed in the electron microscope. The actin filaments (F-actin), which are ~5 nm in diameter, disappear and the regions normally occupied by the filaments contain granules and short filaments. It has generally been held that this structural change is a manifestation of disassembled or altered microfilaments (19, 30).

Isolated bile canaliculus-enriched plasma membrane fractions of rat liver provide a useful experimental tool for the study of microfilament-membrane association. It has been shown by heavy meromyosin binding (7, 24), by immunofluorescent staining, and by immunoelectron microscopic techniques (10, 12) that the pericanalicular microfilaments are mainly actin filaments, and that they remain attached to the canalicular membranes even after the homogenization and centrifugation procedures involved in the isolation of these fractions (6, 21). This model lends itself to an examination of the direct effects of cytochalasin B (CB) on plasma membrane–associated filaments in a cell-free system, and hence gets away from difficulties inherent in the interpretation of results in a more complex cellular system.

MATERIALS AND METHODS

The bile canaliculus-enriched fractions of liver cell plasma membranes (BCM) were isolated from the livers of male Wistar rats, weighing ~150 g, according to a modification (6) of the method reported by Song et al. (29). CB (Aldrich Chemical Co. Inc., Milwaukee, Wisc.) was dissolved in dimethyl sulfoxide (DMSO) (3 mg of CB/0.1 ml of DMSO) and was diluted with 3 mM phosphate-buffered saline (PBS) (pH 7.4) at an appropriate concentration. Each BCM fraction (2.2. mg average protein content) determined by the method of Lowry et al. (16) was divided into two parts of equal weight. One part was incubated at room temperature for 90 min with 1.5 ml of PBS containing CB and 0.2% DMSO. Studies were carried out with final CB concentrations of 2.2×10^{-5} M and 2.2×10^{-6} M. The other part of the BCM was treated similarly but without CB. After incubation, the suspensions were centrifuged at 3,000 rpm for 10 min at 4°C. The resultant pellets were processed for electron microscopy by the ruthenium red (RR) staining technique as described previously (21). Ultrathin sections were cut with a diamond knife on an LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.) and were counterstained with lead citrate. A part of the supernate was centrifuged using an ultrafiltration membrane cone (2100 CF 50; Amicon Corp. Lexington, Mass.) and was negatively stained on a Formvar-carbon-coated grid with 2% aqueous uranyl acetate. The specimens were examined in a Philips 300 electron microscope with 60 kV acceleration voltage, using a cooling device to prevent contamination.

The molecular weights of proteins from the BCM fractions before CB treatment and from the supernates obtained after CB treatment were determined electrophoretically on 9% polyacrylamide gels containing 0.1% SDS at pH 8.8 (13). The technique for the SDS PAGE on these rat liver membrane preparations was performed as previously described (37). The micrograms of protein shown in the tables were calculated from scans obtained using a Gilford densitometer and standard methods (37). Actin was purified from rabbit striated muscle according to the method of Spudich and Watt (31) and was used as a standard protein. The gels were stained with 0.1% Coomassie Brilliant Blue R (Edward Gurr Ltd., London, England). A comparison was made of the two CB preparations (see Tables I–III).

RESULTS

The electron microscope findings of the RR-stained preparations of untreated control and DMSO-only-treated control samples showed no significant differences, as expected (21). The isolated bile canaliculi were entirely surrounded by a

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TABLE I

Comparison of Polypeptides in Canalicular Membrane Pellets and Supernatants Combined

Molecular mass	Control	CB (10^{-6}) treatment	CB (10^{-5}) treatment
kdaltons			
170	3.61	2.91	3.36
160	1.24	0.50	0.64
150	8.59	9.78	7.39
140	7.37	7.46	7.24
120	6.50	4.20	5.27
110	1.68	0.42	0.59
100	9.64	8.18	6.99
90	1.37	2.04	1.60
80	6.25	5.90	4.11
70	5.31	2.79	5.71
60	4.48	1.51	3.25
50	6.91	4.63	4.11
40	25.36	30.33	33.24
30	12.03	19.33	16.50

The results are expressed as μg protein/kdalton region, before (*control*) and after two regimens of CB treatment. Generally, there is reduction in peptides higher than 40 kdaltons (i.e., 160, 120, 110, 100, 80, 50), but there is an increase in the 40- and 30-kdalton regions. This increase may represent a breakage in the peptides (160, 120, 110, 100, 80, 50) that is solely the result of the CB treatment and of the release of actin (see Tables II and III). There was no measurable protein in the 130-kdalton region.

TABLE II

Comparison of Polypeptides in Canalicular Membrane Pellets Only

Molecular mass	Control	CB (10^{-6}) treatment	CB (10^{-5}) treatment
kdaltons			
170	2.84	2.50	2.04
160	0.54	0.39	0.55
150	5.68	8.26	6.38
140	6.60	2.67	2.45
120	4.68	3.51	4.48
110	0.54	0.39	0.55
100	7.13	8.18	6.99
90	1.15	2.04	1.60
80	4.60	3.71	4.11
70	3.91	2.75	5.71
60	3.37	1.47	3.23
50	5.14	3.83	4.11
40	19.86	8.89	3.56
30	10.00	15.28	12.15

The micrograms of protein remaining in the pellet before (*control*) and after two regimens of CB treatment. Total reduction in the actin region is 10.9 [*CB* (10^{-6})] and 16.3 [*CB* (10^{-5})]. The reduction in total protein in the pellet is mainly attributable to the protein in this region. The reduction in the 140-kdalton range is unexplained (see text). The increase in the 30-kdalton region may be attributable to the effect of CB on other proteins (i.e., 50, 60, 70, 80, etc.).

network of microfilaments. Some of the filaments were attached to the canalicular membranes (Fig. 1 *a*). The RR-positive surface coat was retained on the outer surface of canalicular membranes. The CB-treated BC fractions at both dosage levels were similar morphologically. The pericanalicular microfilaments were dissociated from the canalicular membranes (Fig. 1 *b*). The RR-positive surface coat was also removed from the canalicular membrane surface. Moreover, networks of filaments or filament segments were found in the negatively stained preparations of the supernates obtained from the CB-treated BCM fractions (Fig. 1 *c*), whereas no filamentous structures were detected in the supernate from the DMSO-treated BCM controls.

Typical results of analysis of SDS PAGE are shown in Fig. 1 *d*. It illustrates that many polypeptides are released from the BCM membrane fraction by the CB treatment and are recoverable in the supernate (compare slots *3* and *5*). The wide band is in the actin region.

Data on the polypeptides released are shown in Tables I–III. The molecular weight range is shown on the left; it should be noted that a number of polypeptides correspond to the percentages of the total polypeptides present at each molecular weight indicated. No corrections were made for possible variations in the intensities of staining of individual polypeptides by Coomassie Brilliant Blue. These studies have shown that CB has a specific effect on the polypeptides released from the BCM. In the control incubations, 24% of the membrane protein was released into the supernate, which is in close agreement with the value of 20% released by incubation in $NaHCO_3$, $NaHCO_3/CaCl_2$, or K_2HPO_4/KH_2PO_4 (37). Moreover, the polypeptides released, in general, appeared similar to the polypeptides remaining in the pellet after incubation. Incubation in CB released 35–42% of the BCM protein. The tabulated results show the release of a number of polypeptides. The largest component was in the 40-kdalton range; from the gel it can be seen that this is due mainly to a polypeptide of 42–44 kdaltons. This polypeptide corresponded to the actin as shown in the standard (Fig. 1 *d*) and as shown in reports by others (8, 37). A second change of significant but lesser magnitude is noted in the 140-kdalton region. Whether this protein is a subcomponent of myosin or represents an actin binding protein or another protein perhaps unrelated to actin is unknown.

DISCUSSION

Although microfilaments commonly lie in close proximity to the plasma membranes of nonmuscle cells, they are not generally considered an integral part of the plasma membrane (26, 28, 30, 32). It has been proposed that microfilaments (and microtubules) may be linked in some way to integral membrane

TABLE III

Comparison of Polypeptides in the Supernates Only

Molecular mass	Control	CB (10^{-6}) treatment	CB (10^{-5}) treatment
kdaltons			
170	0.77	0.41	0.42
160	0.70	0.12	0.09
150	2.91	1.66	1.03
140	0.77	4.84	4.88
120	1.82	0.76	0.81
110	1.14	0.04	0.04
100	2.51	—	—
90	0.22	—	—
80	1.65	0.23	—
70	1.40	0.04	—
60	1.11	0.04	—
50	1.77	0.80	—
40	5.50	21.44	29.69
30	2.03	4.05	4.36

Total micrograms of protein released in supernate. There is increase in actin band by 15.94 μg in *CB* (10^{-6}) and 24.19 in *CB* (10^{-5}). However, not all that increase is attributable to release of actin because actin was reduced in pellet by 10.97 μg in *CB* (10^{-6}) and 16.3 in *CB* (10^{-5}), which leaves a 4- to 8-μg increase not accounted for by release of actin; it could result from the action of CB on other proteins. The change in the 140-kdalton range is unexplained (see text).

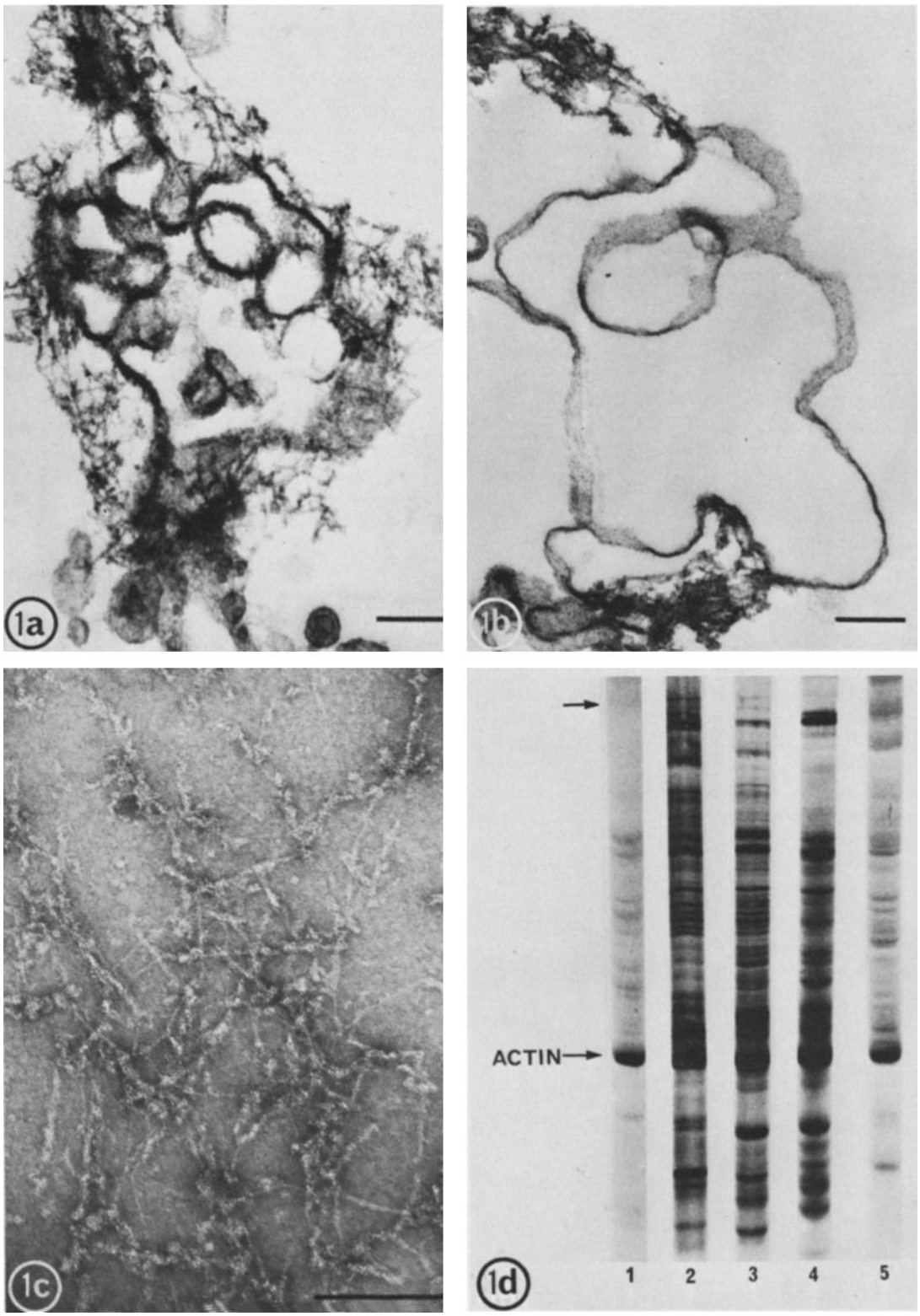

FIGURE 1 Electron microscopic appearance of bile canaliculus-enriched plasma membrane fractions from rat liver are shown in *a–c*. (*a*) A pericanalicular network of microfilaments is attached to the canalicular membrane. This is a control specimen incubated with dimethyl sulfoxide (DMSO) diluted with 3 nM phosphate-buffered saline (pH 7.4) at a final concentration of 0.2% in the absence of CB. Ruthenium red stain. Bar, 0.2 μm. × 50,000. (*b*) After incubation in CB (2.2×10^{-5} M). Note that the microfilamentous network is no longer evident. The surface coat is also removed. Compare with *a*. Ruthenium red stain. Bar, 0.2 μm. × 50,000. (*c*) Microfilamentous network in supernate from CB-treated preparation. Negative stain. 2% uranyl acetate. Ruthenium red stain. Bar, 0.2 μm. × 105,000. (*d*) SDS polyacrylamide gels of bile canaliculus-enriched plasma membrane fractions treated with CB (10^{-5} M) and controls. The actin band is marked by a labeled arrow. In all the slots, 100 μg of protein were applied to the gel, except slot *1* (actin standard) where 10 μg of protein was used. Slot *1*: an actin standard prepared from purified rabbit striated muscle. Slot *2*: BCM fraction before CB treatment (same fraction as in *a*); note the large number of polypeptides present, including a prominent actin band (~42×10^3 mol wt). Slot *3*: supernate of control BCM pellet; a number of polypeptides are released nonspecifically. Slot *4*: BCM fraction after CB treatment (same fraction as *b*). A large number of polypeptides are present but fewer than before treatment (compare with slot *2*). Slot *5*: supernate after CB treatment (same fraction as in *c*). Many polypeptides are recoverable, including a large band in the actin region. These results should be compared with the densitometric data shown in Tables I–III.

components and that they may control the translational mobility of certain classes of proteins (1, 5, 20). Linkage of submembrane filaments to the plasma membrane is also implicit in consideration of microfilament-mediated membrane movement. Detachment of filaments from their binding sites has been suggested as an explanation for the effects of CB on nonmuscle cells, but this has been speculative and a number of other mechanisms have also been proposed (14, 27, 33). Recent research on cytochalasins describes a number of effects, including: inhibition of actin gel formation in crude cytoplasmic extracts (9, 25, 35); a direct effect on actin, resulting in inhibition of actin filament network formation (10, 17); and reduction in the rate of actin polymerization (2, 3, 14, 15, 17). The pertinence of this information on the actions of the cytochalasins to the present study requires comment. It is possible that CB causes detachment of membrane-associated actin filaments by an as yet unknown mechanism, or the filaments may be disrupted at particular sites along their length, perhaps at the location of actin-binding proteins (10), or at points of annealing. Importantly, there is now agreement from a number of laboratories that CB inhibits actin polymerization in vitro (2, 3, 14, 15, 17) and that, in the erythrocyte membrane, at least, the polymerization site is both membrane associated and sensitive to cytochalasin B (14). The results we report with canalicular membranes suggest a membrane-associated binding site sensitive to CB. We do not know whether this site is also a polymerization site for actin. Whatever the mechanism, the functional consequences of detachment of canaliculus-associated actin filaments would be to arrest recently described bile canalicular contractions (22). It is thought that the normal function of the pericanalicular filaments is to provide a contractile force that maintains tone and hence facilitates bile flow in the bile canalicular system of the liver (21). Release of these filaments by CB would help explain the distended canalicular network, stagnation of bile, and decreased bile flow that have been shown to occur with CB treatment of rat liver in vivo (23).

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