EFFECTS OF CYTOSKELETAL PERTURBANT DRUGS ON ECTO 5′-NUCLEOTIDASE, A CONCANAVALIN A RECEPTOR

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ABSTRACT

Differences in cell morphology, concanavalin A-induced receptor redistributions, and the cooperativity of the inhibition of 5′-nucleotidase (AMPase) by concanavalin A (Con A) have been investigated in ascites sublines of the 13762 rat mammary adenocarcinoma cells treated with microfilament- and microtubule-perturbing drugs. By scanning electron microscopy MAT-C1 cells exhibit a highly irregular surface, covered with microvilli extending as branched structures from the cell body. MAT-A, MAT-B, and MAT-B1 cells have a more normal appearance, with unbranched microvilli, ruffles, ridges, and blebs associated closely with the cell body. MAT-C cells have an intermediate morphology. Treatment of MAT-A, MAT-B, or MAT-B1 cells with Con A causes rapid redistribution of Con A receptors. Both cytochalasins and colchicine cause alternations in the receptor redistributions. Receptors on MAT-C1 cells are highly resistant to redistribution, even in the presence of cytoskeletal perturbant drugs.

The cooperativity of the inhibition of AMPase by Con A was investigated in MAT-A and MAT-C1 cells. Untreated cells exhibit no cooperativity. If either subline is treated with colchicine, cytochalasin B or D, or dibucaine, cooperativity is observed. Lumicolchicine has no effect. Theophylline or dibutyryl cyclic AMP prevents the effects of either colchicine or cytochalasin. The concentration required for half-maximal induction of cooperativity is 0.3–0.4 μM for both colchicine and cytochalasin D, which is in the appropriate range for specific microtubule and microfilament disruptions. The effectiveness of the cytochalasins ($E > D > B$) is consistent with their known effects on microfilaments. No direct correlation was observed between the induction of cooperativity and drug-induced changes in Con A receptor redistributions or cell morphology. The morphology of MAT-A cells is grossly altered by cytochalasins or dibucaine and somewhat less by colchicine. MAT-C1 cells exhibit more minor alterations in morphology as a result of these drug treatments. The results of this study indicate that the inhibition of AMPase, which is a Con A receptor, is a different process from the redistribution of the bulk of the Con A receptors, possibly reflecting short range membrane interactions rather than global effects on the cell.

KEY WORDS cytoskeleton · 5′-nucleotidase
Con A receptors · morphology · cell surface

The cell surface has been implicated in numerous biological phenomena (11). In spite of the obvious importance of the cell surface, the methods for evaluating surface changes are still rather unsophisticated. Much attention has been given in recent years to the use of lectins as tools in these studies (1). Although this approach has had considerable success, it suffers from two difficulties. (*a*) The reactions involved are often complex, involving multiple receptors with possibly different properties. In many cases, the observed effect is the end result of multiple, sequential processes, making interpretation difficult. (*b*) Quantitation of lectin reactions is often difficult. We have sought to eliminate some of these problems by studying a single concanavalin A (Con A) receptor, the enzyme 5′-nucleotidase (AMPase). Con A specifically inhibits AMPase in intact cells (7, 9, 29), in isolated membranes (4, 5, 7, 14, 24, 25), or in purified form (9, 28). Since the reaction occurs at the cell surface, it can be studied without prior disruption of the cells (7, 9, 29). The enzyme reaction is simple; kinetically, there appears to be a single enzyme form in the intact cells we have studied (7). The Con A inhibition of the enzyme is rapid and appears to involve a direct interaction between the enzyme and Con A (9, 28).

In previous studies, we have shown that the inhibition exhibits cooperativity (5) which is dependent on conditions that alter the "state" of the membrane in isolated membranes (7). In intact cells or cell surface envelopes isolated from Zn^{++}-stabilized cells the inhibition is noncooperative (7). If the envelopes are extracted with glycine-EDTA-mercaptoethanol, a procedure which dissociates and solubilizes attached cytoskeletal elements containing actin, α-actinin, and actin binding protein (20, 21), the inhibition becomes cooperative. Likewise, release of the enzyme from envelopes by detergent causes the inhibition reaction to become cooperative (7). These results provide circumstantial biochemical evidence for an effect of membrane-cytoskeleton interactions on the behavior of the ecto-enzyme. We postulated that this membrane-cytoskeleton interaction imposes restrictions on the membrane enzyme which are reflected in the inhibition reaction (7). If this hypothesis is correct, one would expect that treatment of the intact cells with drugs which disrupt cytoskeletal elements should also cause a change in the cooperativity of the inhibition.

To test this hypothesis, we have examined the effects of various cytoskeletal perturbants on sublines of the 13762 rat mammary adenocarcinoma (8) that differ in morphology and in Con A-induced redistribution of receptors. The cooperativity of the Con A inhibition of AMPase is sensitive to perturbations by a variety of agents under conditions which indicate specific alterations of cell microtubules and microfilaments. There is no correlation between the abilities to induce cooperativity and to cause redistribution of Con A receptors, as measured with fluorescent Con A. Thus the change in cooperativity appears to reflect a different facet of cell surface behavior than the movement of the bulk of the Con A receptors and may be useful in analyzing subtle cell surface changes induced by physiological alterations of the cell.

A preliminary report of a portion of this work has been presented (6).

MATERIALS AND METHODS

Materials

Cytochalasins were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisc. Ionophore A23187 was a gift of Dr. Robert Hosley of Lilly Research Laboratories, Indianapolis, Ind. Con A, colchicine, enzyme substrates, and buffers were from Sigma Chemical Co., St. Louis, Mo. All other chemicals were reagent grade. The 13762 mammary ascites adenocarcinomas (Mason Research Institute Tumor Bank, Worcester, Mass.), were maintained in Fischer 344 strain female rats (60–90 d old) by intraperitoneal injection of 0.2–0.3 ml of ascites fluid from rats 6–10 d post-injection (7). The cells were removed from the peritoneal cavity by aspiration and washed three times in ice cold *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES)-buffered saline with centrifugation at 210 *g*·min before use.

Lumicolchicine was prepared by UV irradiation of a dilute ethanolic solution of colchicine and characterized by its spectrum, as reported previously (19).

5′-Nucleotidase Assay

Enzyme activity was determined by a modification of the coupled spectrophotometric assay of Ipata (12), measuring the production of inosine from adenosine at 265 nm in a 3-ml Vol containing 50 mM Tris (pH 8.0), 1 mM $MgCl_2$, 125 mM sodium chloride, and 4–5 U adenosine deaminase. Samples were incubated 10 min at 37°C before assaying at 37°C with 0.1 mM AMP (7).

Electron Microscopy

Cells (10^7/ml) for scanning electron microscopy were added dropwise, with shaking, to 10 Vol of 2% glutaraldehyde in 0.1 M sucrose, 0.1 M cacodylate, pH 7.4, at 37°C and incubated for 120 min. The cells were then placed on serum-coated coverslips prepared by incubating sterile coverslips in 10% calf serum MEM

(Eagle's minimal essential medium) for at least 2 h. The cells were allowed to settle onto the coverslips and were left undisturbed at 4°C overnight. They were then washed twice in cacodylate-sucrose buffer and treated with 1.0% osmium tetroxide at room temperature for 15 min. The samples were dehydrated with a graded series of ethanol and dried at the critical point of CO_2 in a Polaron E3000 critical point drying apparatus (Polaron Instruments Inc., Lexington, Penn.). The coverslips were mounted on stubs and coated with 100 Å of gold with a Hummer II. Samples were viewed with a JEOL JSM-35 scanning electron microscope operated at 15 Kv, and micrographs were recorded on Polaroid type 55 negative films.

Visualization of Receptors by Fluorescence Microscopy

Redistribution of Con A receptors was investigated using fluorescein isothiocyanate-conjugated Con A (FITC-Con A). Cells were suspended at 1×10^7/ml in PBS + 15 mg/l of phenol red with 1 mg/ml glucose. An aliquot of cells (usually 0.2 ml) was placed in a 12 × 75-mm test tube to which the appropriate drug had been added. Drugs were added in PBS except for cytochalasin B, which was added in dimethyl sulfoxide (DMSO). The final concentration of DMSO was never greater than 0.1%. The cells were preincubated with the drug for an appropriate time in a gyratory shaking water (37°C) or ice water bath at ~80 rpm. FITC-Con A was added to the final concentration stated and incubated for the appropriate time. The cells were then fixed with a tenfold excess of freshly prepared 4% paraformaldehyde in PBS, pH 7.4, at the same temperature as the final incubation for 60 min. The fixed cells were washed three times in PBS, suspended in PBS, mounted on slides, sealed and viewed with a ×50 or ×100 objective on a Wild M-12 research grade microscope equipped with 200 W high pressure mercury lamp, dark field condensor, and FITC filter system. Cells were photographed on Tri-X film developed with Diafine developer, or with Kodak Kodacolor 400. To establish the distribution of Con A receptors on untreated cells, control samples were prefixed with fresh 4% paraformaldehyde in PBS before addition of FITC-Con A. Cells incubated with drugs were also fixed in 4% paraformaldehyde for 60 min at the temperature of the incubation, washed three times in PBS, resuspended to 1×10^7/ml in PBS and incubated with FITC-Con A at 100 μg/ml after the same procedure described above.

RESULTS

Cell Characterization

The 13762 rat mammary adenocarcinoma is a dimethylbenzanthrene-induced solid tumor originally isolated by Segaloff (26). This tumor was adapted for ascites growth and the ascites tumor separated into three morphologically distinct sublines, MAT-A, MAT-B, and MAT-C (2). During investigations on some of the properties of these cells, we discovered that the MAT-B and MAT-C sublines had become significantly altered by passage of the cells in vivo for ~6 mo. We have designated the variants MAT-B1 and MAT-C1 and have continued their passage. The properties which we shall describe in this report have been stable for more than one year. The variant sublines are maintained by weekly passage and are stored as frozen stocks.

The key properties in differentiating the new cell sublines were their agglutinability, 5′-nucleotidase activity, and periodate-Schiff staining. MAT-C1 cells failed to agglutinate when tested with antisera against MAT-A, but the MAT-B1 cells did agglutinate. This failure of the MAT-C1 to agglutinate is not due to the absence of the appropriate antigens, which could be demonstrated by indirect immunofluorescence, but is an intrinsic property of the cell surface, as shown by the behavior of these cells in the presence of lectins. MAT-C1 cells are strongly stained by periodate-Schiff reagent, indicating a heavy surface carbohydrate coat. In contrast, the MAT-B1 cells are readily agglutinable by MAT-A antiserum and show little staining with periodate-Schiff reagent. They can be distinguished from the other sublines by the virtual absence of 5′-nucleotidase activity (8).

Fig. 1 shows scanning electron micrographs of the five sublines. The MAT-C1 cells show extensive branched microvilli extending from the cell body (Fig. 1 *E*). When the cells are viewed straight on, the microvilli appear clustered, and the branching is not so obvious. The branches are observed more readily at the periphery, viewed from an angle (Fig. 1 *F*). The MAT-B1 cells also have numerous microvilli (Fig. 1 *C*), but they are unbranched, often curved and do not show the clustered appearance. MAT-B1 cells more often exhibit ruffles, ridges, and blebs at the cell surface than do MAT-C1. The MAT-A (Fig. 1 *A*) and MAT-B (Fig. 1 *B*) sublines are similar to MAT-B1 and cannot be readily distinguished on the basis of morphology. The MAT-C cells (Fig. 1 *D*), from which MAT-C1 were derived, are intermediate in morphological characteristics, exhibiting numerous microvilli extending straight from the cell body but few ruffles, ridges, or blebs. Most of the MAT-C cells have no branched microvilli, although a small population does exhibit branching, suggesting that the MAT-C1 cells are a variant subpopulation selected during passage in vivo.

Con A Receptor Redistributions on the Different Ascites Sublines and Cultured Line

In examining the Con A-induced redistribution using FITC-Con A, we have defined two types of

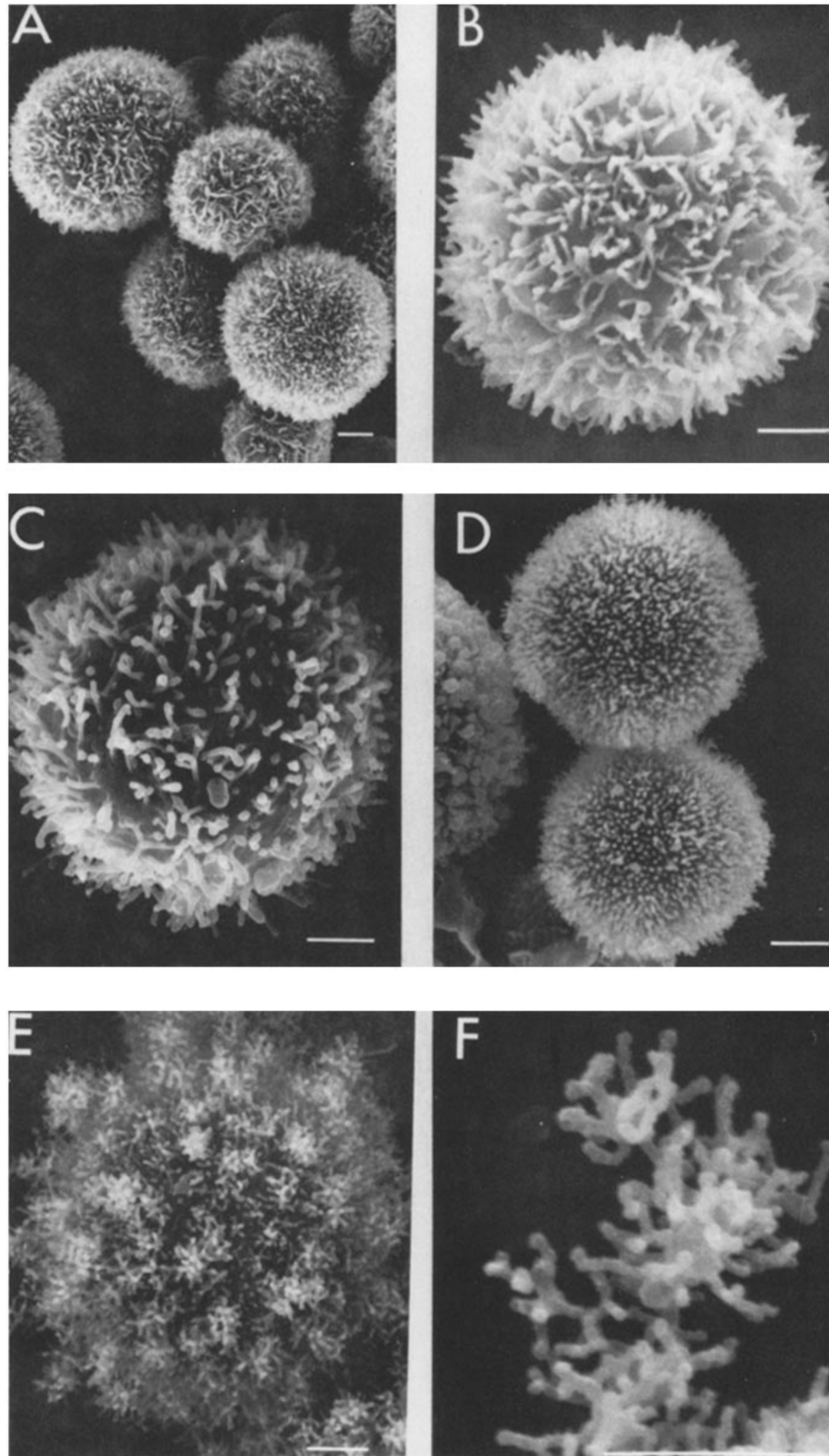

FIGURE 1 Scanning electron micrographs of 13762 mammary ascites adenocarcinoma cells. Cells removed from the animals were fixed onto serum-coated coverslips using 2% glutaraldehyde and prepared for scanning electron microscopy. (*A*) MAT-A, × 2,500; (*B*) MAT-B, × 4,800; (*C*) MAT-B1, × 4,600; (*D*) MAT-C, × 2,500; (*E*) MAT-C1, × 4,500; and (*F*) MAT-C1, × 16,300. Bars, 2 μm. The cells shown are representative of the morphology of >90% of nondividing cells observed for MAT-B, MAT-B1, MAT-C, and MAT-C1 and >80% for MAT-A.

receptor movement. Long-range redistribution (LRR) refers to the collection of all visible fluorescent-labeled receptors into a single region of the surface of the cell, essentially identical to "capping." Intermediate-range redistribution (IRR) is used to describe the formation of collections of fluorescent material at multiple points on the cell periphery. There appear to be two distinct types of IRR. In the first case, observed most frequently during treatments with cytochalasin B, the fluorescence is collected in multiple protuberances on the cell periphery. In the second case, the fluorescence is collected at multiple points on the cell periphery that do not protrude and that appear similar to "patches" described in other systems. Both are sensitive to azide. Although they often appear differerent by microscopy and may arise via different mechanisms, there is considerable subjectivity in trying to differentiate between IRR types. Therefore we have chosen to enumerate them as one category and indicate in the text the predominant form. No redistribution (NR) is scored for a complete ring of fluorescence.

The concentration dependence of the Con A-induced receptor redistribution is shown in Fig. 2 for the five ascites sublines and the cultured line. Three of the ascites sublines (A, B, and B1) and a subline adapted to cell culture show high degrees of intermediate-range redistribution at the lowest Con A concentration used. The amount of long-range redistribution increases with increasing Con A concentration. In the other two ascites sublines (MAT-C and MAT-C1) no redistribution is observed at the lowest Con A concentrations. As the Con A concentration is increased, the MAT-C subline shows increased receptor redistribution, ranging up to 50% IRR and 20% LRR at 100 μg Con A/ml under the conditions used. The MAT-C1 fails to undergo significant redistribution at any Con A concentration tested.

Effects of Cytoskeletal Perturbants on Con A Receptor Redistribution

When the sublines which are able to undergo Con A-induced redistribution are treated with cytoskeletal perturbants, the redistribution patterns are changed, as shown in Table I for MAT-B1 cells. Cytochalasin B causes the receptors to be collected into multiple protuberances at the cell surface. Colchicine causes substantial shedding of the receptors, leaving most of the cells with a uniform fluorescence which suggests that no redistribution has occurred. The kinetics of the redistribution process in colchicine (data not shown) suggest that the receptors collect at the cell surface into "patches" which are shed rather than collecting into a cap. All of the redistribution phenomena are inhibited by azide, suggesting an energy dependence. All of the redistributions required the presence of Con A.

MAT-C1 cells do not undergo redistribution of their Con A receptors under any of the conditions tested using the drugs indicated in Table I. Only

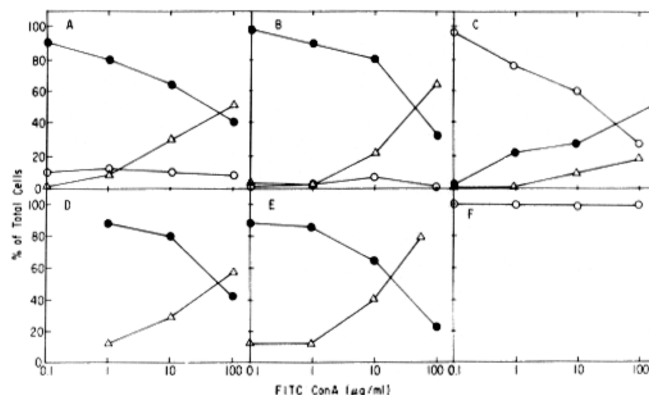

FIGURE 2 The effect of FITC-Con A concentration on the redistribution of lectin receptors of the 13762 MAT sublines. Cells were suspended at 1×10^7/ml in PBS and incubated with 0.01-100 μg/ml FITC-Con A at 37°C for 60 min. Cells were fixed with 10 Vol of 4% paraformaldehyde at 37°C, washed three times with PBS and 100 cells scored as follows: no redistribution (NR) ○——○, intermediate range redistribution (IRR) ●——●, or long range redistribution (LRR) △——△. (*A*) MAT-A, (*B*) MAT-B, (*C*) MAT-C, (*D*) MAT-B adapted to cell culture, (*E*) MAT-B1, and (*F*) MAT-C1.

a ring-type fluorescence is observed, although some "clustering" of microvilli is observed under strenuous treatment conditions (e.g., colchicine plus cytochalasin). However, no movement of the receptors on the microvilli is discernible under these conditions by fluorescence microscopy. Thus, the Con A receptors on the microvilli appear to be immobile within the limits of resolution of the fluorescence microscope.

Effects of Cytoskeletal Perturbants on the Cooperativity of the Inhibition of AMPase by Con A

Previous studies on the inhibition of AMPase by Con A have shown no cooperativity (Hill coefficient, 1) in intact 13762 MAT-A rat mammary ascites adenocarcinoma cells and their Zn^{++}-stabilized cell surface envelopes, which retain attached cytoskeletal elements (7). Pronounced cooperativity (Hill coefficient, 2) was observed for deoxycholate-solubilized envelopes and EDTA-extracted envelopes, which have become fragmented and have lost substantial amounts of their cytoskeletal elements.

Since these results suggest an involvement of the cellular cytoskeleton in the behavior of the AMPase, we have investigated the effects of specific cytoskeletal perturbants on the cooperativity of its inhibition in intact cells. If either MAT-A or MAT-C1 cells are treated with an appropriate concentration of colchicine, which disrupts cellular microtubules (10), cooperativity is induced (Fig. 3 and Table II). Lumicolchicine, a structural analog of colchicine which does not alter microtubule polymerization (19, 33), is not effective in inducing cooperativity (Fig. 4 and Table II). The concentration dependence of the colchicine effect on the cooperativity parameter (Hill coefficient) is shown in Fig. 5 and exhibits a half-maximum of 0.4 μM colchicine. The low concentration necessary for the effect implicates microtubule disruption rather

TABLE I

Effect of Cytoskeletal Perturbant Drugs on Redistribution of MAT-B1 Con A Receptors

Treatment	NR	IRR	LRR
		% cells	
Cell (prefixed) + Con A	100	0	0
Cell + Con A	0	32	68
Cell + 10 mM azide + Con A	100	0	0
Cell + 1 μg/ml cytochalasin B + Con A	20	80	0
Cell + 1 μg/ml colchicine + Con A	90	10	0
Cell + 1 μg/ml cytochalasin B + 1 μg/ml colchicine + Con A	10	90	0
Cell + 10 μg/ml dibucaine	28	16	57

All incubations contained 10^7 cells/ml in PBS and 1.0 mg/ml glucose. FITC-Con A concentration was 100 μg/ml. Drug and Con A incubations were performed sequentially for 60 min each. Control cells fixed after drug treatment but before Con A treatment showed 100% NR.

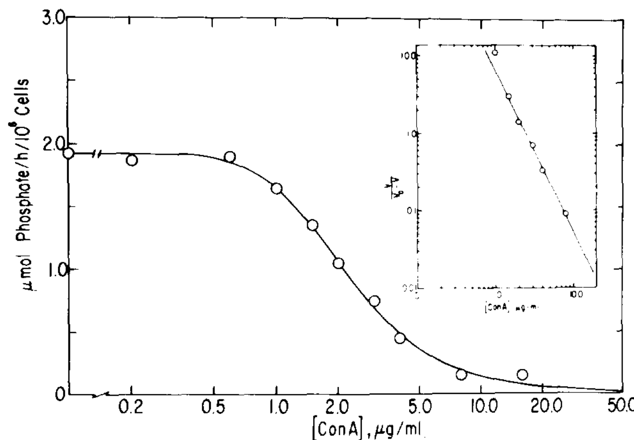

FIGURE 3 Effect of colchicine on the Con A inhibition of the 5'-nucleotidase of 13762 MAT-C1 cells. Cells, prepared as described in Materials and Methods, were suspended in assay buffer (50 mM Tris, 125 mM NaCl, 1 mM $MgCl_2$, pH 8.0) at 2×10^5 cells/assay and incubated for 30 min in a shaking water bath at 37°C with 0.01 mM colchicine. Subsequently, an additional incubation of 10 min at 37°C with Con A was carried out before assaying at 37°C. The inset shows the Hill plot (n = 2.1).

than some nonspecific perturbation as the cause of the change in cooperativity.

Cytochalasins B and D are effective perturbants of cell morphology as a result of their ability to alter microfilament structures (18, 32). Cytochalasin D is apparently more specific, since it is not as effective as cytochalasin B in inhibiting transport (30). The effects of both cytochalasins were tested on MAT-A and MAT-C1 cells, and cooperativity was observed in each case (Table II). Dimethylsulfoxide, in which the cytochalasins were dissolved for addition to the suspended cells, has no effect at the concentration used for the cell incubations. The concentration dependence for the cytochalasin D effect on the cooperativity is shown in Fig. 6. Again, the low concentration (half-maximal, 0.3 μM) required suggests a specific microfilament disruption rather than a nonspecific membrane perturbation as the cause of the cooperativity change. A further indication of the specificity was obtained by comparing the effects of cytochalasins B, D, and E at the concentration observed for the half-maximal effect for cytochalasin D (Table III). The order of effectiveness (E > D > B) is exactly that found for binding to the spectrin-actin cytoskeletal complex of the erythrocyte membrane (15) and for promoting morphological changes of 3T3 fibroblasts (16).

TABLE II

Effects of Cytoskeletal Perturbants on Hill Coefficients of Con A Inhibition of Nucleotidase in MAT-A and MAT-C1 Cells

Perturbant	Concentration	Cell type	Hill coefficient
	mM		
None	—	A	0.87 ± 0.06 (3)
None	—	C1	0.85 ± 0.08 (4)
Cytochalasin B	0.02	A	2.1 ± 0.1 (2)
Cytochalasin B	0.02	C1	1.9 ± 0.4 (4)
Cytochalasin D	0.02	A	2.1
Cytochalasin D	0.02	C1	2.0
Colchicine	0.1	A	1.9 ± 0.05 (3)
Colchicine	0.1	C1	2.05 ± 0.08 (2)
Colchicine	0.01	A	2.05
Colchicine	0.01	C1	2.1
Dibucaine	0.2	C1	2.05 ± 0.14 (2)
Dimethyl sulfoxide	28 (0.2%)	C1	0.98
Lumicolchicine	0.1	C1	0.93

Numbers in parentheses denote numbers of determinations. Experiments were performed as indicated for Fig. 3.

Effect of Hypotonic Swelling on Cooperativity of Con A Inhibition of AMPase

Hypotonic swelling is often used in the preparation of plasma membranes. If Zn^{++} is added before homogenization, the cell is "stabilized" and cell surface envelopes can be isolated (27). If no stabilizer is added, homogenization yields vesicles

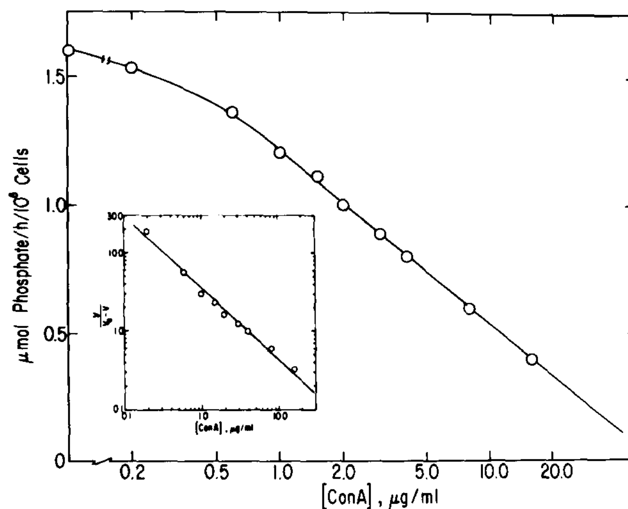

FIGURE 4 Effect of lumicolchicine on Con A inhibition of the 5′-nucleotidase of 13762 MAT-C1 cells. Cells prepared as described in Fig. 1 were incubated with 0.1 mM lumicolchicine for 30 min at 37°C. Subsequently, an additional incubation of 10 min at 37°C with Con A was carried out before assaying at 37°C. The inset shows the Hill plot (n = 0.93).

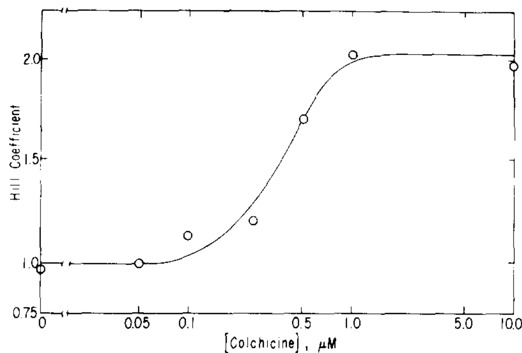

FIGURE 5 Concentration dependence of the effect of colchicine on the cooperativity of the Con A inhibition of AMPase for MAT-C1 cells.

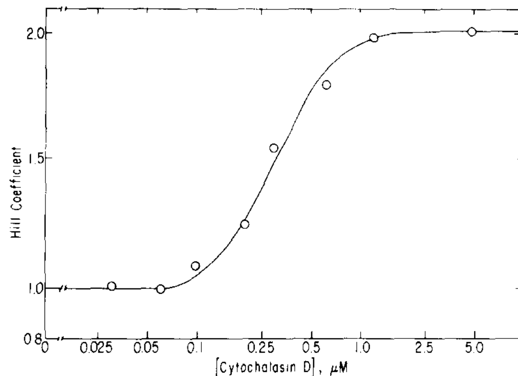

FIGURE 6 Concentration dependence of the effect of cytochalasin D on the Hill coefficient of the inhibition of AMPase by Con A for MAT-C1 cells.

and small membrane fragments (27). Electrophoretic analysis indicates that the "stabilized" envelopes have substantially greater quantities of actin and high molecular weight cytoskeletal proteins, including myosin and actin binding protein (20, 21), than do the fragments or vesicles (27). We have suggested that the effect of Zn^{++} is to stabilize cytoskeletal elements associated with the plasma membrane (21). Since membrane vesicles exhibit cooperativity (5) but envelopes do not (7), we examined the behavior of the nucleotidase under conditions used for plasma membrane preparations from the ascites cells.

When the inhibition of AMPase by Con A was examined in hypotonic solutions, it was found that the swollen cells exhibited cooperativity (Table IV). These results suggest that the disruption caused by swelling perturbs the membrane or its underlying cytoskeleton sufficiently to alter the properties of the nucleotidase. This effect on cooperativity is prevented by including Zn^{++} in the incubation mixture. No effect of Zn^{++} on the enzyme activity was noted under the conditions of treatment. Thus Zn^{++} appears to prevent the induction of cooperativity by hypotonic swelling concomitant with its stabilization of the association of cytoskeletal proteins with the plasma membrane.

Effects of Theophylline and Dibutyryl cAMP on the Cooperativity Change Induced by the Cytoskeletal Perturbants

Cytoskeletal elements have been shown to be sensitive to the effects of cyclic AMP concentration (23). Therefore we tested the ability of theophylline, which inhibits cyclic AMP phosphodiesterase and raises the cyclic AMP level in cells (31), and dibutyryl cyclic AMP to alter the induction of cooperativity of the Con A inhibition of AMPase. Theophylline or dibutyryl cyclic AMP alone has little effect on the cooperativity parameter (Table V). If cells are preincubated with 0.1 mM theophylline or dibutyryl cyclic AMP before addition of cytochalasin D or colchicine, the ability of the cytoskeletal perturbants to induce cooperativity is prevented. These results suggest that an increase in cellular cyclic AMP is able to overcome the effects of the cytoskeletal perturbants on the cooperativity of the AMPase inhibition. Previous studies have shown some effects of theophylline on the redistribution of Con A receptors that could be attributed to cytoskeletal alterations (3). Our results provide further support for the idea that the cell surface interactions with the cytoskeleton are responsive to changes in cellular cyclic AMP.

Effects of Increased Intracellular Calcium on Cooperativity

Cell surface properties, including receptor redistributions, which are known to be sensitive to intracellular calcium, can be altered by treatment of cells with calcium in the presence of ionophores. If MAT-A or MAT-C1 cells are treated with the

TABLE III

Effects of the Various Cytochalasins on the Hill Coefficient for Inhibition of MAT-C1 AMPase by Con A

0.3 μM Cytochalasin	Hill coefficient
B	0.84
D	1.58
E	1.90

TABLE IV

Effect of Hypotonic Swelling on the Cooperativity of the Inhibition of AMPase by Con A

Treatment	Cell type	Hill coefficient
Hypotonic assay medium*	MAT-C1	2.40 ± 0.02 (2)
Hypotonic assay medium	MAT-A	1.92 ± 0.05 (2)
Zn^{2+}-treated cells in hypotonic assay medium	MAT-C1	1.10 ± 0.07 (2)
Zn^{2+}-treated cells in hypotonic assay medium	MAT-A	1.29 ± 0.01 (2)

* The assay medium contained the same components used with the drug treatments except that NaCl and the drug were omitted. The incubations were the same as for Fig. 3. The cells were stabilized in 1 mM $ZnCl_2$ for 15 min (21), washed, and then incubated in the same hypotonic buffer as in the swelling experiment.

ionophore A23187 and Ca^{++}, cooperativity for the inhibition is observed without a significant change in the activity of the uninhibited enzyme (Fig. 7 and Table VI). Treatment with ionophore alone, Ca^{++} alone or ionophore plus EGTA does not cause induction of cooperativity (Table VI). These results imply that uptake of external calcium causes a change in the cell surface which is reflected in the behavior of the AMPase. The ionophore is known to release Ca^{++} from intracellular storage compartments (10). However, it appears that this release is not sufficient to trigger the cooperativity change.

The concentration dependence of the Ca^{++} effect is shown in Fig. 8. Induction of cooperativity occurs between 10 and 50 μM. Since the number of cells in the incubation mixture is small, their contribution to the volume and total Ca^{++} concentration is negligible. If true equilibration across the cellular membranes is achieved, the intracellular concentration should be very close to that added externally.

Morphological Changes Induced by Cytoskeletal Perturbants

Since the cytoskeletal perturbants are known to cause changes in cell morphology, we have examined treated cells by scanning electron microscopy. We have not attempted to interpret subtle changes in cell surface morphology, but have concentrated on changes which are readily and widely observed throughout the cell population. Only cells which are typical of the majority of a population are depicted or described. The MAT-A surface has microvilli that adhere closely to the cell body and some ruffled structures are present (Fig. 1*A*). Cells that have been washed and inoculated under conditions used for the nucleotidase assay with (Fig. 9*B*) or without Con A (Fig. 9*A*) are not greatly changed. Cytochalasin D (Fig. 9*C*) and dibucaine (Fig. 9*E*) cause pronounced and somewhat different effects on the MAT-A cells. Cytochalasin D-treated cells have a profusion of long, tangled microvilli along with small blebs. Dibucaine-treated cell bodies are distorted, showing prominent protuberances, and long, relatively straight microvilli are observed. Inclusion of Con A with these perturbants does not appear to cause any further effects (data not shown). Incubation with colchicine (Fig. 9*D*) causes less pronounced effects on morphology than cytochalasin D or dibucaine, although there is some indication of increased shedding of cell surface material in the presence of colchicine. Pretreatment of the cells with theophylline does not appear to prevent the morphological alterations caused by cytochalasin D.

The morphology of MAT-C1 cells is far less susceptible to treatments with cytoskeletal perturbants than MAT-A cells (data not shown), a result which is consistent with the observations on receptor mobilities. Only dibucaine causes pronounced

TABLE V

Effect of Theophylline on Hill Coefficient of MAT-C1 Cells Incubated with Colchicine or Cytochalasin B

Treatment	Hill coefficient
0.1 mM Theophylline	1.18
0.1 mM Theophylline + 0.02 mM cytochalasin B	1.03
0.1 mM Theophylline + 10 μM colchicine	1.00
0.1 mM Dibutyryl cAMP	1.26
0.1 mM Dibutyryl cAMP + 10 μM colchicine	1.10
0.1 mM Dibutyryl cAMP + 0.02 mM cytochalasin D	0.92

MAT-C1 cells were incubated in assay buffer containing 0.1 mM theophylline or dibutyryl cyclic AMP for 15 min at 37°C followed by an additional 30 min at 37°C with or without cytochalasin B or colchicine. Subsequently, Con A was added to the tubes which were incubated another 10 min at 37°C before assaying at the same temperature.

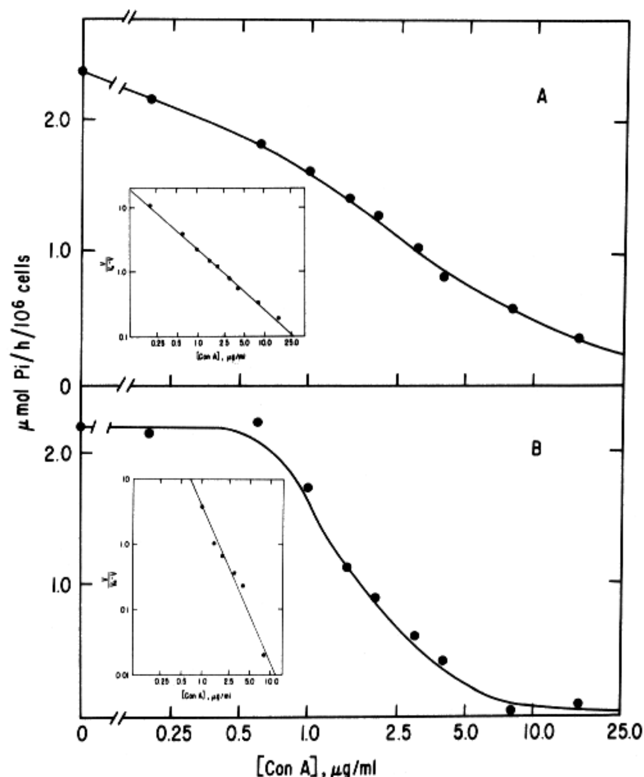

FIGURE 7 Con A inhibition of 5′-nucleotidase of 13762 MAT-C1 cells with or without Ca^{++}. (*A*) MAT-C1 cells were incubated in assay medium containing 1.5 μM ionophore A23187 with no added Ca^{++} for 30 min at 37°C in a shaking water bath. Subsequently, an additional incubation of 10 min at 37°C with Con A was carried out before assaying at 37°C. The assay was initiated by the addition of AMP (final concentration 0.1 mM). The inset shows the Hill plot (n – 0.92). (*B*) MAT-C1 cells were incubated in assay medium containing 1.5 μM ionophore A23187 and 2 mM $CaCl_2$ for 30 min at 37°C. Con A was added and an additional incubation of 10 min at 37°C was performed before assaying at 37°C. The inset shows the Hill plot (n = 2.4).

morphological changes. Cytochalasins and colchicine cause reductions in the fraction of microvilli which are branched. All of these effects appear to be partially reversed by Con A.

There is no direct relationship between changes in cooperativity and morphology upon perturbant treatment, as indicated by the following results. (*a*) The effects of colchicine, cytochalasin D and dibucaine on cooperativity are the same, even though their effects on morphology are very different. The failure of colchicine to perturb morphology substantially does not indicate that the drug is not acting on the microtubules of these cells. Metabolic labeling with glycoprotein precursors indicates that colchicine significantly enhances the rate of shedding of cell surface components in all of the sublines examined. The failure of colchicine to alter gross morphology in a spherical cell is not really unexpected, since the effects of microtubules on morphology are most evident in cells which exhibit asymmetry. (*b*) Theophylline, which prevents cooperativity changes induced by cytochalasin D, is unable to prevent the gross morphological changes caused by this agent. (*c*) MAT-A and MAT-C1 cells, which differ substantially in their morphological responses to perturbants, exhibit the same induction of cooperativity.

These findings emphasize the complexity of the behavior of components at the cell surface and of the relationship between morphology and the cell surface. Morphology is probably insensitive to many short range changes at the plasma membrane which may be important to cell functions. Such results indicate a need for parameters which

TABLE VI

Effects of Ionophore A23187 and Ca^{2+} on Hill Coefficients of Con A Inhibition of MAT-A and MAT-C1 5'-Nucleotidase

Treatment	Cell line	Hill coefficient
A23187	MAT-A	1.1
2 mM $CaCl_2$	MAT-A	1.1
A23187 + 2 mM $CaCl_2$	MAT-A	2.4
A23187 + 1 mM EGTA	MAT-A	1.1
A23187	MAT-C1	0.92
2 mM $CaCl_2$	MAT-C1	0.75
A23187 + 2 mM $CaCl_2$	MAT-C1	2.4
A23187 + 1 mM EGTA	MAT-C1	1.1

Cells were prepared, incubated with the appropriate agent, and assayed for AMPase as described in Fig. 3.

can be used to study more subtle alterations in cell surface behavior.

DISCUSSION

The control of the organization and behavior of the macromolecular components of the cell surface is not well understood (22). Our previous studies with isolated plasma membranes suggested that the behavior of the ecto-enzyme, AMPase, was influenced by the association of cytoskeletal components with the membrane (7). To test this proposal and to investigate the possibility of using the AMPase as a monitor of cell surface changes, we have examined the effects of a series of drugs known to alter cellular cytoskeletal elements on the cooperativity of the inhibition of the AMPase by Con A. Our studies clearly indicate that the cooperativity parameter is sensitive to colchicine, the cytochalasins, and dibucaine. To understand the full significance of these results it is necessary to know the mechanism of the change in cooperativity and the mechanism by which the drugs affect the nucleotidase. We have previously postulated that the cooperativity occurs as a result of the Con A-induced clustering of the enzyme molecules in the membrane (5). Our present results show no correlation between cooperativity and ability of the majority of the Con A receptors to redistribute. In the MAT-A cells the Con A receptors redistribute simply upon Con A treatment, but no cooperativity is observed without drug treatments. By contrast, in the MAT-C1 cells no redistribution of the Con A receptors is noted under any conditions examined, but drug treatments of unperturbed cells readily induce cooperativity. Thus, the two sublines are very different in terms of the redistribution of their Con A receptors, but are essentially identical in the behavior of the AMPase. This similarity of AMPase behavior between the cell types contrasts with differences in morphology and changes in morphology with drug treatments.

In interpreting these experiments, two cautions should be noted. First, the AMPase represents a small fraction of the total Con A receptors. It may exhibit behavior which is different from the more abundant Con A receptors and which would be obscured in the fluorescence assays. For example, it might be restricted in mobility in the presence of Con A in both types of cells (A or C1) unless released from restraints imposed by the cytoskeletal system. Second, the mobility required for cooperativity may be different from that necessary for movements observed by fluorescence microscopy. A shorter-range mobility or rotational effect might be involved. Obviously, the mechanism of the interaction of Con A with the AMPase will be important. Based on studies with the purified enzyme, Dornand et al. (9) have suggested that other Con A receptors might be involved in the inhibition or cooperativity, presumably through formation of heterologous complexes in which a single Con A molecule interacts with both the enzyme and another Con A receptor. It should be possible to resolve the questions about these mechanisms by studies of purified and partially purified AMPase in appropriate model membrane systems. Regardless of the exact mechanism of the enzyme-Con A interaction, it is clear that the inhibition

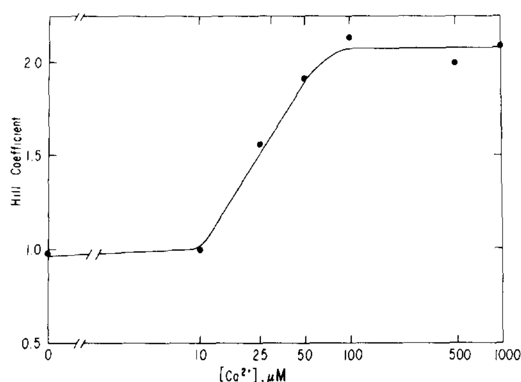

FIGURE 8 The effect of Ca^{++} on the cooperativity of Con A inhibition of 13762 MAT-C1 5'-nucleotidase. Cells were prepared, incubated, and assayed as described in Fig. 7, with increasing amounts of $CaCl_2$ added to the incubation medium containing 1.5 μM A23187.

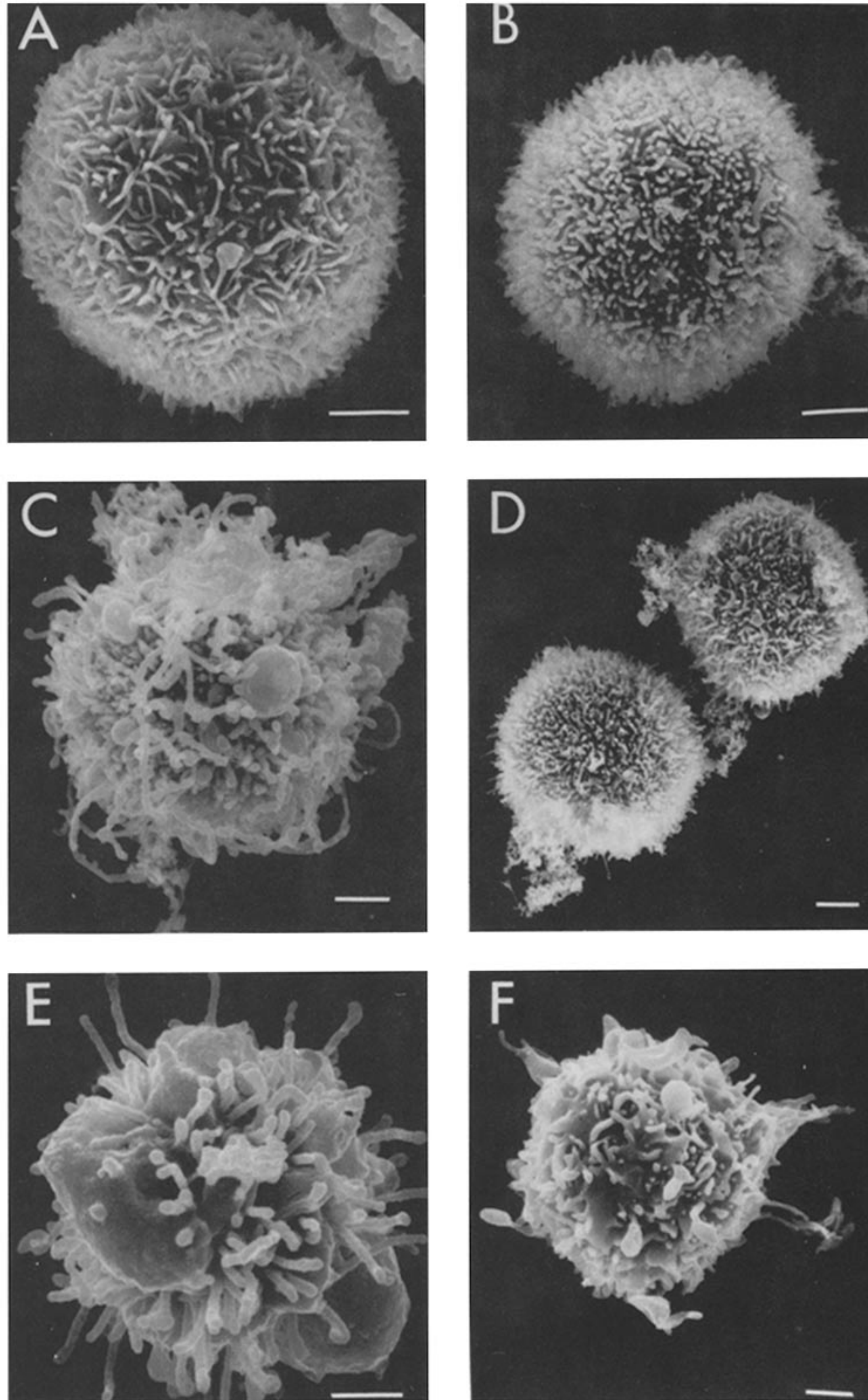

FIGURE 9 Scanning electron micrographs of MAT-A cells treated with various morphological perturbants. (*A*) No treatment except incubation in assay buffer; (*B*) Con A (3 μg/ml), × 5,000; (*C*) Cytochalasin D (20 μM), × 3,700; (*D*) colchicine (10 μM), × 2,800; (*E*) dibucaine (0.2 mM), × 5,000; and (*F*) theophylline (0.1 mM) followed by cytochalasin D (20 μM), × 3,000. Bars, 2 μm.

process is sensitive to drug-induced alterations in membrane structure which are different from the long-range membrane effects of the type seen in bulk receptor redistributions. These more subtle alterations suggest that the interaction with or response to cytochalasin- and colchicine-sensitive structures is different for the AMPase and for the majority of the Con A receptors.

Since the AMPase cooperativity may reflect a different aspect of membrane-cytoskeleton interactions than the bulk Con A receptors, it is instructive to consider the molecular nature of these interactions. One possibility, which is consistent with current models for transmembrane interactions (22), is that the changes in cooperativity result from alterations in an interconnected microfilament-microtubule cytoskeletal network associated with the plasma membrane. The argument for a specific involvement of microtubules and microfilaments in the AMPase cooperativity change in the intact cells is based on the low concentrations of the drugs necessary for the effects, the absence of an effect of lumicolchicine, a structural analog of colchicine which does not alter microtubule polymerization, and the order of effectiveness of the different cytochalasins. In addition, we have recently observed induction of cooperativity for the Con A inhibition of AMPase in cultured fibroblasts using cytochalasins and colchicine under conditions known to cause disruption of microfilaments (18) and breakdown of microtubules (10), respectively. The treatments with cytochalasin D and colchicine cause the expected arborization and loss of cellular polarity of the fibroblasts, respectively, as viewed by light microscopy (C.A.C. Carraway, unpublished observation). Thus, it is clear that cooperativity is induced concomitantly with changes in microtubules and microfilaments, although this does not establish a cause-and-effect relationship.

The common effects of the cytochalasins and colchicine on the cooperativity also support the proposition that the agents are acting on the cytoskeletal systems rather than directly on the membrane or enzyme. A common effect on the membrane or enzyme would require that three very different agents, cytochalasin, colchicine, and dibucaine, have the same effect, while a fourth agent, lumicolchicine, which is structurally related to one of the three, has no effect at all. If the drugs are presumed to act directly on the cytoskeletal system, then their common effect stemming from two different sites of action must be explained. There would appear to be two likely possibilities. (*a*) Both microtubules and microfilaments are linked to the membrane in such a way that perturbation of either alters the properties of the AMPase. (*b*) Microtubules are linked to microfilaments, which are linked to the membrane. Perturbation of the former alters the structure of the latter to cause a change in the membrane which is reflected in the behavior of the AMPase. The latter model is more consistent with structural evidence. Polymerized actin and microfilaments have been demonstrated in association with isolated plasma membranes (13). The association of polymerized tubulin with plasma membranes of mammalian cells is more questionable. We do not find substantial amounts of tubulin polypeptide associated with plasma membrane envelopes from ascites cells, which exhibit no cooperativity, but these envelopes contain substantial quantities of actin (20, 21). Extraction of the actin and other cytoskeletal components induces cooperativity (7). These results implicate actin-containing elements as the link to the membrane involved in changes in cooperativity. This proposal is further strengthened by the observation that Zn^{++} treatment prevents induction of cooperativity by hypotonic swelling of cells, since we have previously shown that such Zn^{++} treatments decrease the dissociation of actin-associated cytoskeletal proteins from cells and plasma membranes during hypotonic swelling for membrane isolation (21).

It should be obvious from the above considerations that the evidence for the involvement of microtubules, which is based only on colchicine treatments, is less rigorous than that for involvement of actin-associated structures, which is based on both experiments with the drugs and on biochemical studies on membranes and cells. However, if the actin-associated structures are more closely associated with the membrane, as suggested above, they are the more important elements to understand in order to clarify the nature of the putative short range effects of the cytoskeleton on membrane functional activities.

What is the nature of the cytoskeleton links to the plasma membrane that influence AMPase? One possibility is a direct association of microfilaments with the AMPase at the inner surface of the plasma membrane. Mannherz and Rohr (17) have recently shown that snake venom AMPase can break the tight complex between soluble actin and DNase I and induce actin polymerization. Such results have led to the proposal that AMPase

may serve as a link by which actin filaments are connected to the plasma membrane (17). Our results on the AMPase cooperativity are consistent with such a model. However, since many cells do not express significant AMPase activity, it seems unlikely that the enzyme is the only linkage site, if indeed it does serve such a function. An alternative possibility is that disruption of the cell cytoskeleton associated with the plasma membrane in the region of the AMPase causes local changes in the membrane which are reflected in the behavior of the AMPase. Such short-range effects are consistent with cytoskeletal control over functional activities of plasma membranes without the necessity of the global changes which occur with receptor redistributions and morphological effects.

What is the potential value of the study of AMPase behavior in understanding cell surface phenomena? An important point to be emphasized is the complexity of the behavior of cell surface components and of their responses to physiological stimuli. The implication of a cytoskeleton-membrane interaction in the behavior of the AMPase provides an impetus for using it as a monitor in studies of the membrane-cytoskeleton association and the ability of that association to influence plasma membrane functions. Our results indicate that AMPase behavior is influenced by intracellular calcium and cyclic AMP concentrations. Although these effects are undoubtedly complex, investigations of the mechanisms involved in changes in the cooperativity phenomenon may provide a route to understanding some aspects of the mode(s) of action of these cellular effectors. For example, Dornand et al. (9) have shown that Con A inhibition of AMPase in intact lymphocytes shows cooperativity. We have recently found that this cooperativity can be abolished by treatment of the lymphocytes with theophylline or dibutyryl cyclic AMP (R. Doss and F. Corrado, unpublished observations), agents which reverse the effects of the cytoskeletal perturbants on cooperativity in the ascites cells. These results suggest a difference in the plasma membrane of lymphocytes, compared to ascites cells and fibroblasts, which may be related to membrane-cytoskeleton interactions. The biological significance of these plasma membrane differences is unclear but intriguing. Further investigations of the mode of association of the AMPase with other plasma membrane components and the effect of alterations of the cytoskeleton on that association are in progress to attempt to define the mechanism by which cooperativity is altered. The fact that these studies concentrate on a defined plasma membrane functional activity opens a new route to understanding putative membrane-cytoskeleton interactions.

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