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BINDING OF [^{3}H]CYTOCHALASIN B AND [^{3}H]COLCHICINE TO ISOLATED LIVER PLASMA MEMBRANES

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Summary

The binding to isolated hepatocyte plasma membranes of radioactively labelled inhibitors of microfilamentous and microtubular protein function ([^{3}H]cytochalasin B and [^{3}H]colchicine, respectively) was studied as one means of assessing the degree of association of these proteins with cell surface membranes. [^{3}H]Cytochalasin B which behaved identically to the unlabelled compound with respect to binding to these membranes was prepared by reduction of cytochalasin A with NaB^3H_4. The binding was rapid, readily reversible, proportional to the amount of membrane and relatively insentive to changes of pH or ionic strength. At 10^{-6} M [^{3}H]cytochalasin B, glucose or *p*-chloromercuribenzoate, an inhibitor of glucose transport inhibited binding by about 20%; treatment of membranes with 0.6 M KI which depolymerizes F actin to G actin caused about 60% inhibition of binding. These two types of inhibition were additive indicating two separate classes of binding sites, one associated with sugar transport and one with microfilaments. Filamentous structures with the diameter of microfilaments (50 Å) were seen in electron micrographs of thin sections of the membranes. At concentrations greater than 10^{-5} M [^{3}H]cytochalasin B, binding was proportional to drug concentration, characteristic of non-specific adsorption or partitioning. Intracellular membranes of the hepatocyte also bound [^{3}H]cytochalasin B, those of the smooth endoplasmic reticulum to a greater extent than plasma membranes.

[^{3}H]Colchicine bound to plasma membranes in proportion to the amount of membrane and at a rate compatible with binding to tubulin. However, other properties of the binding including effects of temperature, drug concentration and antisera against tubulin were different from those of binding to tubulin. Hence, no evidence was obtained for association of microtubular elements with these membranes. Despite this there appeared to be an interdependence

Abbreviation: PCMB, *p*-chloromercuribenzoate.

between microtubule and microfilament inhibitors: vinblastine sulfate stimulated [^{3}H]cytochalasin B binding and cytochalasin B stimulated ^{3}H colchicine binding. [^{3}H]Colchicine also bound to intracellular membranes, especially smooth microsomes.

Introduction

The presence of filamentous proteins including microtubules and microfilaments in a wide variety of cell types has been demonstrated [1—3] and it seems likely that they are ubiquitous elements of cell structure. They participate in cellular functions involving cell form and movement of either the whole or a part of the cell [4]. In order to contribute to these functions these filamentous structures apparently require interactions with subcellular membranous organelles including the cell surface plasma membrane. Furthermore, an expanding body of data indicates that these proteins also act as important determinants of the intramembranous arrangement and in turn the function of some components of the cell surface membrane [5,6]. Much of the evidence for the membrane association of the microtubular and microfilamentous systems has been obtained using intact cells [7]. Recently, however, isolated plasma membranes from some cell types have been shown to retain elements of the microfilament system [8—11]. The present communication describes the interaction of radioactively labelled inhibitors of microfilament ([^{3}H]cytochalasin B) and microtubule ([^{3}H]colchicine) function with isolated hepatocyte plasma membranes. The findings are consistent with the binding of [^{3}H]cytochalasin B to filamentous proteins attached to the membrane in a manner stable enough to withstand the rigors of the membrane isolation procedure. The properties of the [^{3}H]colchicine binding, however, do not support the existence of a significant amount of microtubular elements in association with the isolated membranes. A preliminary report of this work has already been presented [12].

Methods

Materials. Cytochalasins A and B were purchased from Aldrich Chemical Co. NaB^3H_4 (11 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England. [^{3}H]Colchicine (ring C-*methoxyl*-^{3}H; 3 Ci/mmol) was from the same source. Colchicine, vinblastine sulfate and PCMB (*p*-chloromercuribenzoate) were from Sigma. Pre-coated thin-layer chromatography sheets (silica gel F-254) were from E. Merck, Darmstadt.

Isolation and characterization of membranes. Plasma membranes were isolated by the method of Ray [13] from male Wistar rats (150—200 g) which were starved for about 15 h prior to death. Smooth and rough microsomes were prepared from livers of similar animals according to the procedure of Rothschild [14] and the Golgi apparatus according to Sturgess et al. [15]. Each of these membrane fractions were monitored for purity by assays of appropriate marker enzymes and by electronmicroscopic analysis. The rough microsomal, Golgi and plasma membrane fractions were shown by morphometric analysis to contain 82% rough microsomes, at least 70% Golgi complexes and

88% plasma membranes, respectively [16]. Specific activities and recoveries of marker enzymes were in line with those determined by the original authors and are presented in detail elsewhere by us. [16,17]. The various membrane preparations at concentrations from 2 to 10 mg of protein per ml were frozen in liquid N_2 and stored at −80° C. These treatments did not alter the specific [^{3}H]cytochalasin B or [^{3}H]colchicine binding activity.

Electronmicroscopy. Freshly prepared membranes were suspenden in cold (2° C) 2.7% glutaraldehyde in 0.07 M NaH_2PO_4, pH 7.4, and pelleted by centrifugation. After fixation the membranes were rinsed in the same buffer, postfixed with 1% OsO_4 in 0.03 M veronal buffer, pH 7.4, blocked stained with 1% uranyl acetate in 25% ethanol, dehydrated in graded ethanol solutions and embedded in Spurr epoxy resin. Ultrathin sections were cut on a Porter Blum Mt-2 ultramicrotome and viewed in a Philips EM 201 electron microscope at 60 kV.

Preparation of [^{3}H]cytochalasin B. [^{3}H]Cytochalasin B was formed from cytochalasin A by reduction with NaB^3H_4 according to Lin et al. [18]. 1.25 mg of cytochalasin A was dissolved in 1 ml of isopropanol. To this was added 0.5 ml of isopropanol containing 0.15 mg NaB^3H_4 (100 mCi). After stirring for 15 min at room temperature, 25 µl of 1 M HCl was added to stop the reaction. The solution was concentrated to a volume of approx. 25 µl using a N_2 stream and spotted onto a thin-layer chromatography sheet which had been prerun in chloroform/ethyl acetate (1 : 1, v/v). Authentic cytochalasins A and B were spotted on the same sheet which was developed for 3 h at room temperature in the same solvent system in which it had been prerun. The product of the reduction of cytochalasin A was resolved into three ultraviolet-absorbing spots: two having the same R_F values as cytochalasin A and cytochalasin B and the third migrating slightly less rapidly than cytochalasin B and presumed to be dihydrocytochalasin B [18,19]. The silica at the spot having the same R_F as cytochalasin B was scraped from the sheet and eluted with 1 ml of absolute ethanol. Aliquots of the eluate were used for determination of ^{3}H radioactivity and absorbance at 200 nm. The latter value was used to calculate the amount of cytochalasin B present by interpolation from a straight line relating A_{200nm} and various amounts of authentic cytochalasin B [18]. From these determinations a specific activity of 4.1 Ci/mmol was calculated for the [^{3}H]cytochalasin B formed.

Binding of [^{3}H]cytochalasin B to membranes. Membranes were suspended in a total volume of 0.2 ml of 0.05 M Tris · HCl, pH 7.4, containing an appropriate amount of [^{3}H]cytochalasin B and incubated at 22° C. At the end of the incubation period the suspension was centrifuged at 10 000 × g for 5 min in a Beckman 152 microfuge. The supernatant was immediately removed by aspiration and a measured aliquot retained for determination of radioactivity. The tip of the microfuge tube containing the pellet was excised and the pellet dissolved in Protosol (New England Nuclear) prior to liquid scintillation counting. Each assay was performed in duplicate or triplicate and the amounts of [^{3}H]-cytochalasin B in the supernatants and pellets used to calculate the total recovery of the labelled drug for each incubation. Data were rejected when these recoveries were outside the 95—105% range.

Binding of [^{3}H]colchicine. The assay was carried out in essentially the same manner as with [^{3}H]cytochalasin B except that the stability of the complex

formed permitted rinsing of the pellet after centrifugation. Membranes were incubated in 0.2 ml of 0.05 M Tris · HCl, pH 7.4, containing 1 mM $MgCl_2$. To terminate the reaction, 0.2 ml of the same buffer (ice-cold) was added and centrifugation carried out immediately (10 000 × *g*, 5 min). Aliquots of the supernatant were retained for ^3H counting. The pellets were rinsed three times with 0.4 ml of the same ice-cold buffer with centrifugation between each rinse. The pellets were dissolved in Protosol and ^3H determined by liquid scintillation counting. Replicate assays and determinations of recoveries were performed and utilized in the same way as in the [^3H]cytochalasin B binding.

Protein determination. Protein was determined by the fluorimetric procedure of Böhlen et al. [20] using bovine serum albumin (Fraction V, Sigma) as standard.

Results

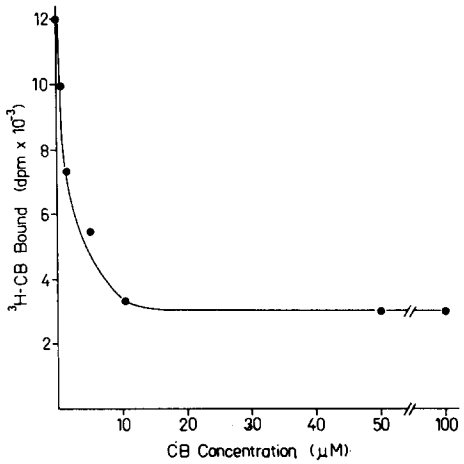
Binding of [^3H]cytochalasin B to isolated plasma membranes

Dependence of binding on amount of membrane. The binding of [^3H]cytochalasin B (10^{-6} M; 0.8 μCi) increased linearly with increasing amounts of plasma membrane from 15 to 300 μg. All the data reported below are from experiments performed using amounts of membrane within this range.

Competition by unlabelled cytochalasin B. Fig. 1 shows the effect of increasing concentrations of native cytochalasin B on the binding of 10^{-6} M [^3H]cytochalasin B to plasma membranes. A 10^{-6} M concentration of the unlabelled compound reduces the amount of bound radioactivity by about 50% of the difference between the amount bound with no unlabelled drug and that at high concentrations ($>10^{-5}$ M). This provides evidence that the [^3H]cytochalasin B and unlabelled cytochalasin B behave identically with respect to their interaction with these membranes. The amount of [^3H]cytochalasin B bound which is not effectively competed for at drug concentrations $\geqslant 10^{-5}$ M is considered "non-specific binding" (approx. 25% of the total). In subsequent experiments the proportion of the total binding persisting in the presence of 10^{-4} cytochalasin B is subtracted from the total binding in its absence to obtain "specific binding".

Rate of binding. Fig. 2 shows the time course of binding of [^3H]cytochalasin B (10^{-6} M) to plasma membranes. The association is rapid with 65% of maximal binding occurring within 2 min (the earliest time tested) and 75% within 10 min. The reaction is near completion by 30 min with only a slightly higher amount bound at 2 h. When membranes which had bound [^3H]cytochalasin B for 2 h were separated from the drug-containing solution, dissociation occurred at a rate only slightly less than that of the "on" reaction. Hence the binding is a very readily reversible reaction.

Effect of pH and ionic strength. The curve relating the extent of binding to the pH of the incubation medium presented in Fig. 3A is relatively featureless although there is somewhat more binding at pH 5.5 than over the rest of the range. Similarly, the binding is fairly independent of ionic strength. Fig. 3B shows that the amount of [^3H]cytochalasin B bound increases by only about 30% between 0 and 1.2 M NaCl. These observations make it unlikely that



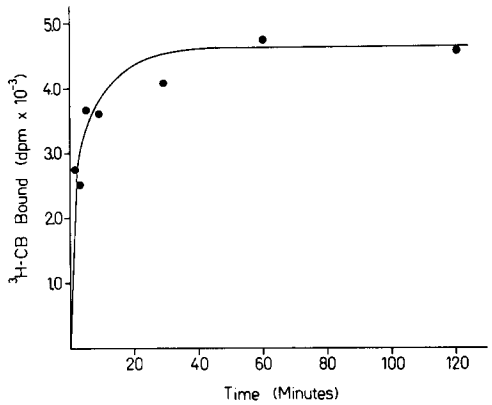


Fig. 1. Effect of increasing cytochalasin B concentrations. 292.5 μg amounts of plasma membrane protein were incubated in the presence of 1 μM [^{3}H]cytochalasin B (0.8 μCi) at the unlabelled cytochalasin B concentrations indicated.

Fig. 2. Time course of [^{3}H]cytochalasin B binding to plasma membranes. 87.5 μg of membrane protein were incubated with 1 μM [^{3}H]cytochalasin B (0.8 μCi) for the times indicated.

charged groups are important in the binding of cytochalasin B to these membranes.

Effect of glucose and PCMB. The transport of monosaccharides across the plasma membrane of a number of cell types is inhibited by cytochalasin B [21—25]. In some instances kinetic evidence indicates that the inhibitor binds to the sugar carrier [20—22]. In fact, in the human erythrocyte plasma membrane, the sugar carrier is the highest affinity cytochalasin B binder [23,24]. In

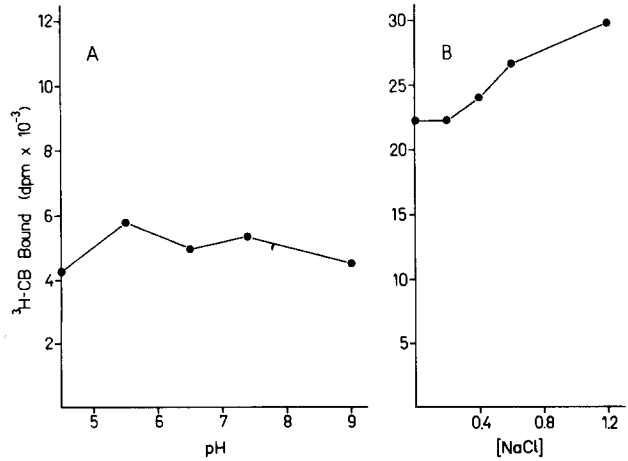


Fig. 3A. Influence of pH on the binding of [^{3}H]cytochalasin B to plasma membranes (97.5 μg protein). (B) Influence of salt concentration on [^{3}H]cytochalasin B binding to plasma membranes (250 μg protein). In both experiments 1 μM [^{3}H]cytochalasin (0.8 μCi) was present

TABLE I

INFLUENCE OF D-GLUCOSE, PCMB AND KI ON [^{3}H]CYTOCHALASIN B BINDING TO PLASMA MEMBRANES

Binding of 1 μM (0.8 μCi) [^{3}H]cytochalasin B to 125 μg of membrane protein. Concentrations of additions were: D-glucose, 0.5 M; PCMB, 1 mM; KI, 0.6 M.

Addition	[^{3}H] Cytochalasin B binding (dpm)
None	5311
D-Glucose	4394
PCMB	4581
D-Glucose + PCMB	4371
KI	1202
D-Glucose + KI	112
PCMB + KI	29
D-Glucose + PCMB + KI	301

order to determine whether or not some portion of the binding to the hepatocyte plasma membrane was to the sugar transport system, the effects of glucose and an inhibitor of glucose transport (PCMB) on [^{3}H]cytochalasin B binding was tested. As shown in Table I, either a high concentration of glucose (0.5 m) or 1 mM PCMB caused approximately a 20% reduction of binding. L-Glucose was without effect while phlorizin (1 mM), another inhibitor of glucose transport, also caused nearly a 20% decrease in binding. Hence, it appears likely that this proportion of the binding which we observe at 10^{-6} M [^{3}H]cytochalasin B is to the glucose transport system. The combination of glucose and PCMB caused no further inhibition over that caused by either agent alone.

Effect of 0.6 M KI. Actin filaments (F actin) are rapidly depolymerized (to G actin) by 0.6 M KI [9]. Since these filaments may be the site of action of cytochalasin B responsible for most if not all effects of the drug except inhibition of sugar transport, the influence of pretreatment of the isolated plasma membranes with 0.6 M KI was tested. The result was a 60% reduction in binding (Table I). If cytochalasin B were binding to two separate classes of sites in the hepatocyte plasma membrane, the sugar carrier and filamentous protein, then inhibition of the first should be additive with inhibition of the second. Table I also shows that this is in fact the case: the exposure to glucose and/or PCMB together with 0.6 M KI resulted in a degree of inhibition equal to the sum of the two separately. In this situation there is little residual binding of [^{3}H]cytochalasin B to the membranes indicating the absence of other classes of binding sites with sufficient affinity to complex cytochalasin B at 10^{-6} M.

Electron microscopic detection of microfilaments associated with the isolated plasma membranes. Fig. 4 shows the presence of filamentous structures in a thin section of the isolated plasma membranes. These filaments which are approx. 50 Å in diameter exhibit no ordered arrangement with respect to each other or the membranes. They appear to be mainly in contact with the larger sheets or ribbons of membrane rather than the smaller vesicles. The points of contact of the filaments with membrane are not clearly defined, although their proximity after the vigorous membrane purification procedure attests to a strong association. We have not been able to demonstrate that these filamen-

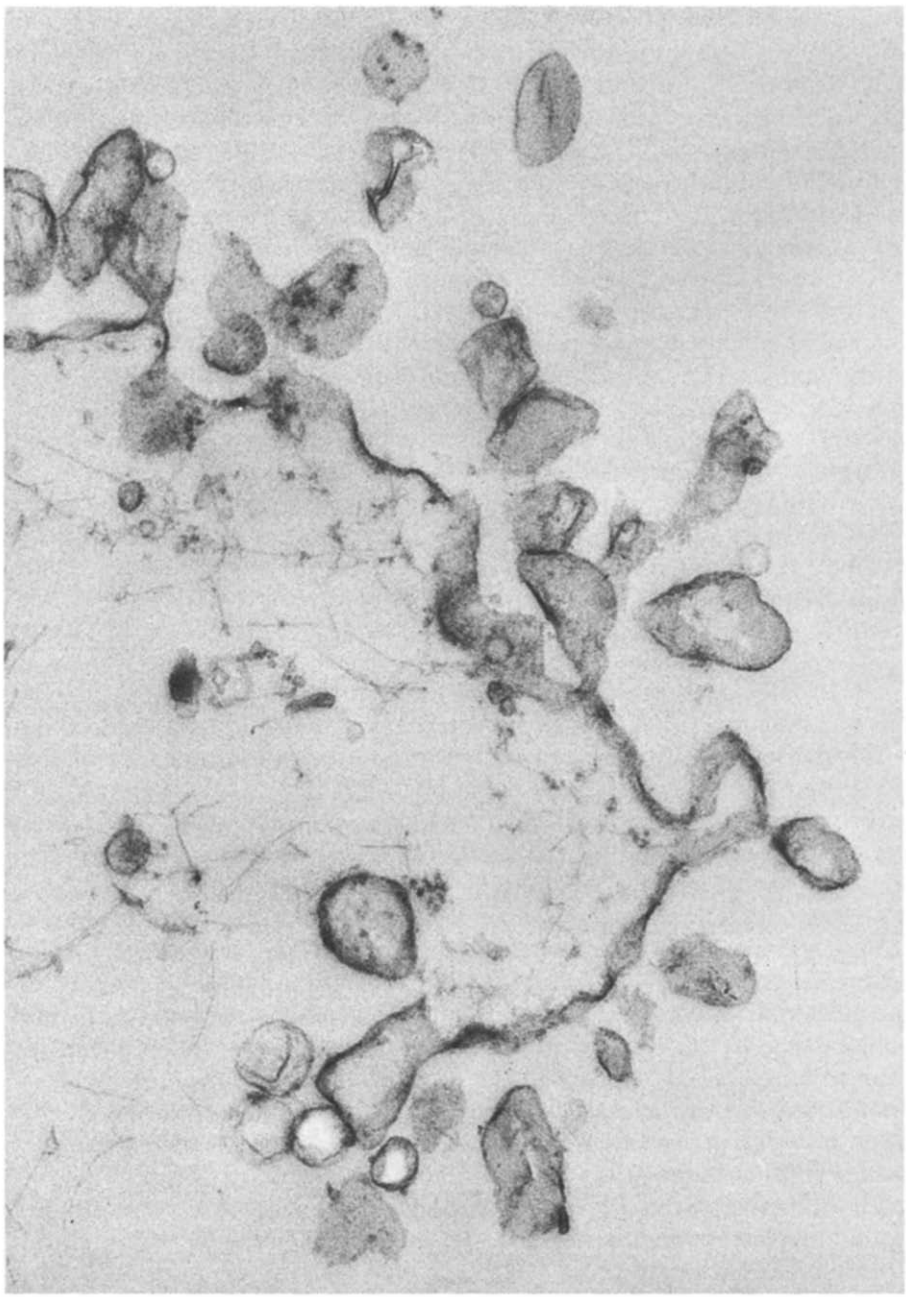


Fig. 4. Electromicrograph (magnification 35 000 ×) of a thin section through a pellet of isolated plasma membranes treated as described in Methods.

tous structures are involved in [^{3}H]cytochalasin B binding but they presently seem reasonable candidates for such a role.

Binding to intracellular membranes. Although the plasma membrane apparently is a functionally important site of cytochalasin B action on both sugar

transport and filamentous proteins, Mayhew et al. [26] have shown that microsomes from Ehrlich-Lettre ascites carcinoma cells also bind significant amounts of the drug. Therefore, the ability of different intracellular membranes of the hepatocyte to bind [^{3}H]cytochalasin B was tested. In an experiment in which plasma membranes bound 34 pmol of [^{3}H]cytochalasin B per mg of protein, Golgi membranes bound 16 pmol per mg and membranes of the rough and smooth endoplasmic reticulum 20 and 107 pmol per mg, respectively. Quantitatively, these observations differ somewhat from those of Mayhew et al. [26] in that they observed about one half as much binding to a total microsomal fraction as to a plasma membrane fraction. However, in their experiments, [^{3}H]cytochalasin B was bound to intact cells and the subcellular fractionation subsequently carried out, during which time 80% of total bound [^{3}H]cytochalasin B was lost. In addition, their microsomes were not separated into rough and smooth fractions so that the level of binding observed may have been the average of a high value to smooth membranes and a low value to rough. Considering these experimental differences together with the differences in cell types, it is perhaps surprising that our findings are as similar as they are, indicating that the plasma membrane and a microsomal fraction are major cytochalasin B binding sites. As yet we have not attempted to characterize the nature of the binding sites in the different intracellular membrane fractions.

Binding of [^{3}H]colchicine to isolated plasma membranes

The aim of these experiments was to ascertain whether or not [^{3}H]colchicine binds to the plasma membranes and, if so, to compare the properties of this binding to those of binding to isolated tubulin [1,2,27].

Dependence of binding on amount of plasma membrane. [^{3}H]Colchicine (2.5 μM; 1.25 μCi) was bound by the membranes and the amount bound was proportional to the amount of membrane present up to 400 μg of protein.

Rate of binding. Fig. 5. illustrates a time cource of binding which is not dissimilar to that exhibited by tubulin from calf brain [27,28] and extracts from cultured fibroblasts [29]. The time course is essentially unaffected by 10^{-4} M vinblastine sulfate as would be the case for binding to tubulin since its affinity for colchicine is unaltered by vinca alkaloids although they do stabilize the tubulin-colchicine complex [1]. However, the rate of binding at low temperature (22° C) was different from that of binding to tubulin in which case very little binding would occur at this temperature [27]. Only 15—20% less binding to plasma membranes occurred at 2° C than at 22° C (Fig. 5).

Effect of increasing amounts of [^{3}H]colchicine. Aliquots containing the same amount of plasma membranes (240 μg of protein) were incubated in the presence of increasing amounts of [^{3}H]colchicine and the amounts of bound radioactivity determined. Over this range of concentrations (0—25 μM) binding was directly proportional to the amount of labelled drug added and no indication of saturation of binding sites could be observed. The fact that saturation apparently did not occur even when much higher concentrations (up to 0.1 mM) of unlabelled colchicine were added indicated that at least a major portion of the binding observed must be of a non-specific nature.

Influence of pH and ionic strength. Fig. 6A shows the influence of variations in the pH of the incubation media on the binding of [^{3}H]colchicine to the

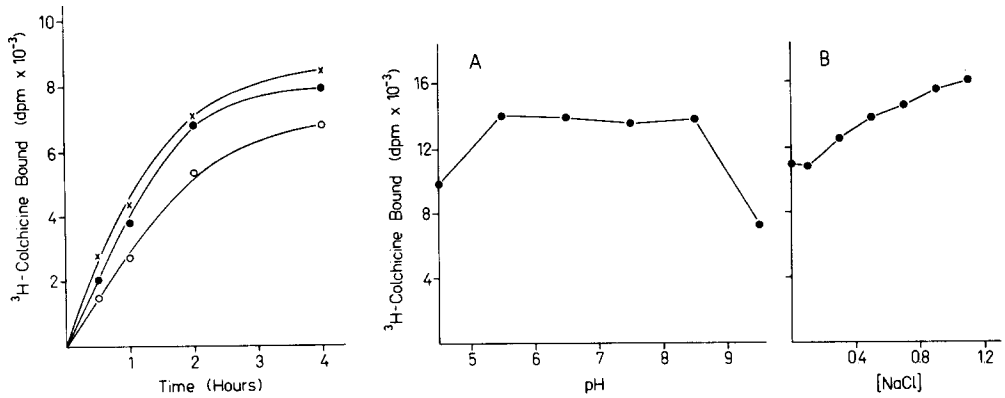


Fig. 5. Time course of [^{3}H]colchicine binding. 195 μg of plasma membrane protein incubated with 5 μM [^{3}H]colchicine (2.5 μCi) for the times indicated. •, incubation at 22°C, no additions; X, incubation at 22°C, 10^{-5} M vinblastine sulfate present; ○, incubation at 2°C, no additions.

Fig. 6.(A) Effect of pH on binding of [^{3}H]colchicine to plasma membranes. (B) Effect of salt concentration on binding. In both experiments 240 μg of membrane protein were incubated in the presence of 1.8 μM [^{3}H]colchicine (0.9 μCi).

membranes. There is little change in the level of binding between pH 5.5 and 8.5. However, at pH 4.5 and 9.5, binding was reduced by about 30 and 50% of the maximum, respectively. While these observations are consistent with the insensitivity of the binding activity of chick embryo brain tubulin [1], the rate of decay of this latter activity is greatly accelerated at pH values other than 6.7—6.8. Although decay (if it occurs) would not contribute greatly to the net level of binding observed during our time of incubation (90 min), it seems likely that some change would have been detected if the situation were as with soluble tubulin. Changes in ionic strength of incubation media also did not markedly alter binding (Fig. 6B), a gradual increase occurring with progressively higher NaCl concentrations up to 1.1 M. Binding to tubulin is also altered only slightly by ionic strength, whereas, rate of decay of binding is minimal at 0.1 M NaCl, increasing substantially below and above this concentration [1]. Hence, colchicine binding to plasma membranes resembles that to tubulin in that it does not seem to be dependent on electrostatic interactions. On the other hand, conditions of pH and ionic strength which result in increased decay of colchicine binding activity of tubulin did not influence the net amount of [^{3}H]colchicine bound by plasma membranes in 90 min.

Influence of antisera against brain tubulin. Aubin et al. [30] have recently shown that antisera against purified brain tubulin enhances the colchicine binding activity of tubulin. Although these findings were somewhat unexpected and the mechanism of the stimulation is not yet understood, we tested these antisera for possible influence on the [^{3}H]colchicine binding to plasma membranes. With two of the same antisera used by Aubin et al. [30] over a wide range of dilutions (from undiluted to 1250 times), no change in the binding activity of plasma membranes was observed. These observations also tend to indicate that the colchicine binding activity of the membranes is not due to membrane-associated tubulin.

Effect of ultraviolet irradiation of [^{3}H]colchicine on binding. Irradiation at 254 nm of [^{3}H]colchicine for 30 min before binding did not alter the level of binding although the photoderivative, lumicolchicine which is formed does not bind to tubulin [1].

Binding to intracellular membranes. In view of the indications that colchicine binding activity of hepatocyte plasma membranes does not seem to be related to the presence of microtubular elements, it was of interest to assess the relative binding activity of intracellular membranous organelles from the same cells. Membranes of the rough and smooth endoplasmic reticulum and Golgi apparatus were found to bind [^{3}H]colchicine and the extent of binding was linearly dependent on the amount of membrane present in each case over a range from 30 to 300 μg of protein. The amounts of [^{3}H]colchicine bound by 200 μg of each of these membrane fractions was as follows: plasma membranes, 2.1 pmol; Golgi membranes, 0.6 pmol; smooth microsomes, 4.1 pmol; rough microsomes, 0.8 pmol.

Interdependence of binding of microtubular and microfilamentous inhibitors. In interpreting the cumulative effects of microtubular inhibitors and microfilamentous inhibitors on the distribution of some plasma membrane receptors at cell surfaces and on the agglutinability of the cells, some "linkage" between membrane-associated microtubular and microfilamentous elements have been proposed [6]. However, the possible effects of one family of inhibitors on the extent and nature of reaction of the other have not been considered. Table II indicates that vinblastine, a microtubular inhibitor, significantly increases the [^{3}H]cytochalasin B binding activity of plasma membranes. Colchicine, however, did not alter [^{3}H]cytochalasin B binding. Conversely, cytochalasin B increases the [^{3}H]colchicine binding activity of the membranes. Hence, while they are not yet understood, these interactions could reflect the links between membrane-associated microtubular and microfilamentous systems which have been claimed by other workers [6]. However, in view of the lack of evidence supporting any significant association of microtubular elements with these plasma membranes, the interpretation of these effects is far from clear.

Although some of the effects on membranes of microtubule and microfilament inhibitors [6] also can be brought about by the tertiary amine local anes-

TABLE II

INTERDEPENDENCE OF MICROTUBULE AND MICROFILAMENT INHIBITORS

In Exp. 1, 400 μg of plasma membrane protein was incubated in 1 μM [^{3}H]cytochalasin B (0.8 μCi) either with no additions or with colchicine or vinblastine sulfate as indicated. In Exp. 2, 150 μg of plasma membrane protein was incubated in 2.5 μM [^{3}H]colchicine (1.25 μCi) in the presence or absence of cytochalasin B.

Addition	Exp. 1: [^{3}H]cytochalasin B binding (dpm × 10^{-3})	Exp. 2: [^{3}H]colchicine binding (dpm × 10^{-3})
None	19.0	
Colchicine (10^{-5} M)	19.1	
Vinblastine (10^{-5} M)	47.1	
None		5.8
Cytochalasin B (10^{-5} M)		8.1

thetics [31,34], tetracaine (0.1—1.0 mM) in the presence or absence of Ca^{2+} did not influence [^{3}H]cytochalasin B binding to plasma membranes. Therefore, local anesthetics may influence the arrangement of surface receptors by acting at a different stage in the control mechanism than do the microtubular and microfilamentous inhibitors.

Discussion

These investigations of the binding of [^{3}H]cytochalasin B and [^{3}H]colchicine to isolated hepatocyte plasma membranes have been carried out with the primary aim of gaining evidence for or against the association of microtubules and microfilaments with cell surface membranes. This of course is only one approach to this question and some discussion of its validity is necessary.

First, the molecular site of action of colchicine ($\leqslant 10^{-5}$ M) is now well established to be tubulin (dimer M_r = 110 000), the major subunit of microtubules [1]. [^{3}H]Colchicine binds only to the dissociated subunits and not formed microtubules. Hence, it can be used only to localize the subunits. Therefore, while our data make it unlikely that significant amounts of tubulin are associated with the plasma membrane, they do not exclude the presence of intact microtubules (electromicroscopy, however, does not reveal their presence).

The molecular site(s) of action of cytochalasin B is less well defined. It was previously claimed [35], and some investigators still support this view [36], that "sensitivity (to cytochalasins) implies the presence of some type of contractile microfilament system". However, direct binding of cytochalasin B to purified muscle proteins has not been demonstrated. This has been done in the case of the analogue cytochalasin D [37]. Forer et al. [38] did show that 1.25 mM cytochalasin B failed to interfere with the conversion of isolated G actin from skeletal muscle to F actin. Both the binding of heavy meromyosin and its ATP-induced release from actin was also unaffected. In contrast to these findings, Langsfeld et al. [9] more recently, claimed that the polymerization of actin filaments seen in association with hepatocyte plasma membranes was partially inhibited by 1 mM cytochalasin B. The apparent discrepancy between these two sets of findings could be explained in several ways.

First, very recent work from several laboratories indicate that there are proteins present in association with microfilaments of non-muscle cells which are responsible for the arrangement of these filaments into bundles [39,40]. The phenomenon, which has been termed "blebbing" [42] or "zeiosis" [43] and is induced by cytochalasins, can also be explained on the basis of disruption of the attachments of microfilaments to plasma membranes, thus permitting the membrane to distend outwards away from the microfilaments which are normally immediately subjacent to the membrane.

Despite the remaining uncertainties about all the sites of action of both cytochalasin B and colchicine, the fundamental importance of their actions on cell and membrane function makes it essential to gain an understanding of their quantitative interactions with isolated membranes as well as intact cells.

Although our main interest was in the association of microfilaments with the membranes, it was necessary to determine if cytochalasin B was bound to the sugar transport system of the hepatocyte plasma membrane as it does in eryth-

rocytes [23,24] and adipocytes [22]. The fact that glucose, a substrate of the transport system and PCMB, an inhibitor of transport, diminished ^{3}H-labelled cytochalasin B binding to the same extent indicated that cytochalasin B probably did bind to the sugar carrier as in the other cell types. The proportion of the total binding (20% at 10^{-6} M cytochalasin B) inhibited is also consistent with the affinity of the sugar carrier for the drug in other cells ($K_a \simeq 10^{-7}$ M^{-1}). The binding which was not inhibited by glucose or PCMB was totally sensitive to conditions which depolymerize actin filaments (0.6 M KI).

Thus neither of the components of cytochalasin B binding observed have properties characteristic of partitioning in hydrophobic regions of the membrane of the type suggested by Mayhew et al. [26] in their studies of binding to intact cells. Rathke et al. [19] have also suggested a phase partitioning or adsorption since they could not demonstrate saturation of binding to cells. However, these investigators did not use [^{3}H]cytochalasin B concentrations below 10^{-5} M above which we also found the binding to be linearly dependent upon the [^{3}H]cytochalasin B concentration (Fig. 2). Hence, while both intact cells and isolated plasma membranes appear to bind cytochalasin B in proportion to the amount of drug present at concentrations greater than 10^{-5} M, this does not detract from evidence for the existence of at least two specific populations of binding sites which can be saturated at low cytochalasin B concentrations and which are apparently related to sugar transport and filamentous proteins as first proposed by Lin and Spudich [23]. It does seem reasonable to assume that the non-saturating binding at high drug concentrations may be due to non-specific adsorption or partitioning phenomena. Hauschka [44] has reported that this type of binding also occurs with chinese hampster ovary cells.

With respect to the chemical nature of the binding site(s) for cytochalasin B, the insensitivity to changes in pH and ionic strength make it unlikely that ionizable groups are directly involved. The highly reversible nature of the binding limits efforts at isolation of the actual molecular components of the membrane responsible for binding. Some method of covalently fixing the drug to its binding site(s) such as the use of a photoaffinity analogue will be required for this purpose.

The possibility of a specific role of microfilaments in association with liver plasma membranes has been raised by Phillips et al. [45] who proposed that microfilaments may be involved in events at the bile canalicular portion of this membrane during bile secretion. However, microfilaments do not seem to be exclusively localized in this subfraction of liver plasma membranes.

Filamentous structures apparently can be seen by high voltage electronmicroscopy to contact all cellular organelles except mitochondria [46]. Hence, our observations of [^{3}H]cytochalasin B binding to both smooth and rough microsomes and the Golgi apparatus as well as plasma membranes are not unexpected. The high level of binding to smooth microsomes is consistent with findings for several other cell types [25].

The biologically significant colchicine binding activity of cells is thought to be localized exclusively in the soluble fraction. This binding is to the soluble subunit tubulin which is apparently in equilibrium with formed microtubules [2]. Colchicine binding activity of particulate fractions of brain [47] and cul-

tured fibroblasts [29] have been attributed to trapping or non-specific association of tubulin with particulate material. Binding in tissue slices has also been interpreted as being to the soluble tubulin [48]. The properties of colchicine binding to liver microsomes including heat stability and dependence on drug concentration over a wide range were different from those of binding to tubulin [49]. Our findings with isolated plasma membranes were similar with respect to these characteristics, and in addition, antibodies against purified brain tubulin which enhance its colchicine binding activity [30] were without effect on binding by plasma membranes.

With respect to possible trapping of colchicine or soluble colchicine binding protein by membranous vesicles, this should happen to a far lesser extent with hepatocyte plasma membrane preparations than with microsomes since the former is only partially in the form of closed vesicles, whereas the latter is totally vesicular. However, entrapment in vesicles is apparently not the explanation of the increased binding by our smooth microsomal preparations since the rough microsomes which also consist entirely of closed vesicles (coated with ribosomes), bind even less colchicine than plasma membranes.

Although microtubules are likely involved in the movement of secretory vesicles to the hepatocyte surface for export [50,51], most if not all of the microtubules and their subunits are present in the soluble cytoplasmic fraction [52]. Hence, the way in which microtubules contribute to the movement of secretory vesicles of the Golgi to the surface plasma membrane must not depend on a permanent association with either of these membranes.

One of the most intriguing aspects of this study was the influence of a microtubular inhibitor on the binding of a microfilament inhibitor and visa versa despite the fact that colchicine binding was apparently not related to the presence of microtubular elements. Thus, cytochalasin B (10^{-5} M) caused a 30—40% increase in the binding of [^{3}H]colchicine (10^{-5} M); the microtubular stabilizing drug vinblastine sulfate (10^{-5} M) more than doubled binding of [^{3}H]cytochalasin B (10^{-6} M) whereas colchicine was without influence. The ineffectiveness of colchicine is consistent with the binding experiments which indicated the absence of unpolymerized tubulin. The effect of vinblastine may be unrelated to the presence of microtubules. Vinca alkaloids, including vinblastine, are known to exert a wide variety of effects on cells, some which are probably due to their cationic nature which permits interaction with Ca^{2+} binding sites [1]. While the mechanism of cytochalasin B action on the colchicine binding activity of the membranes is no better understood, at least the microfilaments as likely sites of cytochalasin B action are present in association with the membranes. Studies aimed at the elucidation of these mechanisms are in progress and it is hoped that it will be possible to determine whether the interdependence of binding of these microtubular and microfilamentous inhibitors is in any way related to the alleged linkages between the microtubules and microfilaments themselves [6].

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