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Binding of Cytochalasin B to Platelets

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The binding of cytochalasin B (CB) to human platelets and to isolated platelet cytosol and membranes has been analyzed with [^{3}H]CB. High- and low affinity classes of saturable binding sites were associated with intact platelets. Binding at very low concentrations of CB (i.e., high-affinity binding) was partially prevented by 100 mM D-galatose or D-glucose and to a much lesser extent by L-glucose. Binding to platelet cytosol also involved two classes of sites with affinities and capacities similar to those observed with the whole cells. None of this binding, however, was affected by 100 mM D-galactose. Saturable binding to platelet membranes occurred at sites with a uniform binding affinity. Approximately 52% of this binding was prevented by 1 M D-galactose and another 15% by cytochalasin E (CE). We hypothesize that binding in the cytosol is to monomeric (low-affinity) and polymerized (high-affinity) actin, whereas membrane binding (high-affinity only) occurs primarily at sites involved with galactose transport.

Key words: cytochalasin B, platelets, cytochalasin binding

The fungal metabolite cytochalasin B (CB) is a potent inhibitor of cellular contractile processes and hexose transport [1–4]. Platelets have been useful in studying this toxin because these cells manifest a variety of contractile functions, including shape change, aggregation, and clot retraction, all of which are suppressed by CB [5–7]. Recently we have also shown that this compound interrupts platelet transport of D-galactose [8]. These effects are presumably mediated by CB binding to filamentous acting (F-actin) and to membrane structures involved with transport [9–11]. CB binding to muscle actin and to the hexose transporter of red cells has been well documented [9,10,12]. Binding of CB to bovine and human platelets also has been described, although the exact subcellular localization of the binding has remained uncertain [13–15].

Our interest in CB binding to platelets stems from recent studies of CB inhibition of galactose transport by these cells [8]. Previously we had identified two kinetically distinct platelet transporters for D-galactose [16]. Although CB inhibited both of these, the mechanism of inhibition appeared to be different for each. The high-affinity transporter was competitively inhibited by CB (Ki ~ 1.7 μM), whereas inhibition of

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low-affinity transporter was by a mixed mechanism (Ki ~0.8 μM). We reasoned that CB might be used to identify the platelet membrane structures mediating galactose transport, as has been done to isolate the hexose transporter from red cells [12]. Therefore, we sought to confirm earlier reports that CB binding to platelets can be quantitated and that a portion of this binding is associated with the membrane [14, 15].

MATERIALS AND METHODS

CB, CE, D-galactose, L-galactose, D-glucose, L-glucose, and phenylmethylsulfonyl fluoride were purchased from the Sigma Chemical Company (St. Louis, MO). Leupeptin was obtained from Boehringer Manneheim Biochemicals (Indianapolis, IN). [4-^{3}H]cytochalasin B (574 GBq/mmol) and NCS Tissue Solubilizer were bought from the Amersham Corporation (Arlington Heights, IL). Econofluor and Aquasol were from the New England Nuclear Corporation (Boston, MA).

Unlabeled and tritiated CB were mixed in phosphate-buffered saline (PBS: 90 mM NaCl, 20 mM Na_2HPO_4), containing 10 mM Na_2EDTA, pH 7.4, to produce a stock solution of 82 μM CB (4.2×10^4 dpm/nmol). The CB concentration of subsequent dilutions was checked by measuring the radioactivity dilution.

Protein concentrations were measured with the BCA kit from Pierce Chemical Company (Rockford, IL) with bovine serum albumin used as a standard.

Platelet Binding Studies

Platelets were separated from the fresh blood of normal donors using a discontinuous gradient of arabinogalactan (10% over 20%), as previously described [17]. The cells were suspended in PBS–EDTA, pH7.4, and quantitated with a Coulter S-Plus (Coulter Electronics, Inc., Hialeah, FL). Platelets were collected from aliquots of the suspension ($0.8–1.5 \times 10^9$ cells/ml) by centrifugation (15,600*g* × 4 min) and resuspended in the same buffer containing specified concentrations of CB (6 nM–80 μM) and in some cases hexose. After 5 min at room temperature four 0.1-ml aliquots of the CB-platelet suspension were filtered through separate 0.4-μm polycarbonate filters (Nuclepore Corporation, Pleasanton, CA). The filters were dissolved in 0.5 ml NCS Tissue Solubilizer at 50°C. After neutralization with 25 μl glacial acetic acid, the solution was mixed with 5 ml Econofluor for liquid scintillation counting. Filter radioactivity derived from buffer-phase [^{3}H]CB was measured by filtering 0.1-ml aliquots of supernatant prepared by centrifugation of the cell suspension. This value was subtracted from the radioactivity of the filters used for the aliquots of whole suspension. The difference represents cell-associated (bound) [^{3}H]CB.

Membrane and Cytosol Separation Analysis

Platelets were collected from normal donors by apheresis in acid-citrate-dextrose (USP Formula A). EDTA was added to a concentration of 5 mM. The cells were centrifuged at 1,000*g* for 3 min to sediment any remaining leukocytes and red cells. The platelets were separated from the plasma by centrifugation through a discontinuous gradient (10% over 20%) of arabinogalactan, as previously described [17]. The isolated platelets were washed once with PBS–EDTA, pH 6.6, and resuspended in the same buffer containing 1 mM leupeptin and 1 mM phenylmethysulfonyl fluoride. In this they were frozen (−70°C) and thawed four times and were sonicated

(20 kHz, 10 s × 6) after each thawing. The platelet lysate was layered over 30% sucrose and centrifuged at 100,000*g* for 1 hr at 25°C. The upper fraction (cytosol) was removed and dialyzed for 24 h against PBS–EDTA, pH 7.4, at 4°C. The interface layer (membranes) was washed once by dilution in PBS–EDTA, pH 6.6, and repeat centrifugation (100,000*g* × 1 h) over 30% sucrose. The interface layer then was dialyzed for 24 h against PBS–EDTA, pH 7.4, at 4°C.

The cytosol and membrane fractions were assayed for lactate dehydrogenase activity (LH kit, Sigma Diagnostics, St. Louis, MO) to determine the degree of membrane contamination with cytosolic protein [18]. In addition, the isolated cytosol and membranes, as well as whole platelets, were reduced with 1% 2-mercaptoethanol and dissolved in 1% sodium dodecylsulfate (SDS) by heating at 100°C for 5 min. The samples were then electrophoresed through a linear gradient of 4% to 20% polyacrylamide with the Laemmli system of buffers (SDS–PAGE) [19]. The slabs were stained with Coomassie blue. The degree of actin polymerization in the cytosol was estimated by measuring the ability of the cytosol to inhibit deoxyribonuclease in the presence and absence of guanidine hydrochloride [20].

Equilibrium Dialysis

Samples of cytosol or membrane (0.2 ml) in PBS–EDTA, pH 7.4, were placed on one side of a dialysis chamber (Fisher Scientific, Springfield, NJ; M_r cut-off, 6,000), and equal volumes of specified concentrations of [^{3}H]CB in the same buffer were placed on the opposite side. In some studies 200 mM D-galactose was included with the [^{3}H] CB solution (to give a final concentration at equilibrium of 100 mM D-galactose). The chambers were rocked for 24–48 h at room temperature before 0.1-ml samples were removed from both sides for liquid scintillation counting in 10 ml Aquasol. Preliminary studies showed that equilibrium was reached by 24 h. The concentration of CB on the buffer side (unbound CB) was subtracted from the concentration of CB on the cytosol or membrane side (unbound plus bound CB) to give the concentration of CB bound to the material under study.

Competitive Binding With D-Galactose and Cytochalasin E

Two-tenths-milliliter aliquots of isolated membrane (0.9 mg protein/ml) were mixed with 0.05 ml of PBS containing [^{3}H]CB to give a final CB concentration of 9.5 nM. In parallel experiments the mixtures included 10 μM CB, 10 μM CE, 1 M D-galactose, or the combination of 1 M D-galactose and 10 μM CE. Unbound [^{3}H]CB was separated from bound by centrifugation (1,000*g* × 15 min) through ultrafiltration membranes (Centrifree; Amicon, Danvers, MA). [^{3}H]CB in the filtrate was quantitated by liquid scintillation spectrometry as described above and subtracted from total [^{3}H]CB to give the bound fraction.

Data Analysis

The data (unbound, bound CB) derived from the studies with whole platelets as well as with the cytosol and membrane fractions were fit with weighted least-squares regression lines by the LIGAND system of computer programs [21]. Models of one and two classes of binding sites were compared statistically [21]. Nonspecific binding was regarded as an unknown parameter and estimated simultaneously with the other parameters. The probability that the simpler, one-site model offered a better fit to the

data was expressed as a P value. Estimates of binding affinity (Kd) and capacity (R) were calculated for the statistically preferable models.

RESULTS

Membrane and Cytosol Separation

Platelet membranes were isolated virtually free of cytosolic contamination, as indicated by the very low concentration of cytosolic lactate dehydrogenase in the membrane preparation (< 80 IU/mg protein), compared with the enzyme activity in the cytosol (~19,000 IU/mg protein). In addition, the SDS–PAGE pattern of the reduced protein of the membrane fraction closely resembled the pattern published for platelet membranes [22] and was distinct from the electrophoretic pattern of the cytosolic proteins (Fig. 1). Quantitation by the deoxyribonuclease inhibition assay [20] indicated that ~50% of the cytosolic actin was in the unpolymerized state.

CB Binding Studies

Initial studies showed that [^{3}H]CB association with platelets reached a stable level within 1 min and was just as quickly reversed by diluting the CB. In addition,

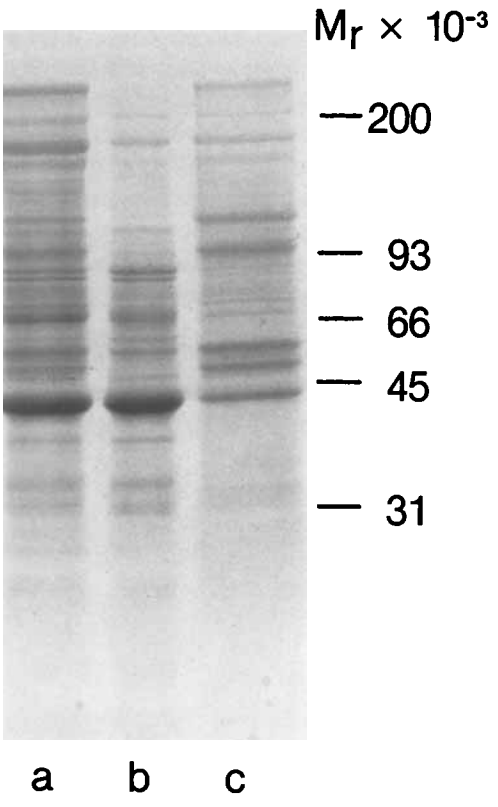

Fig. 1. SDS–PAGE (linear gradient, 4–20%) of proteins from whole platelets **(lane a)**, platelet cytosol **(lane b)**, and platelet membrane **(lane c)**. Migration distances for molecular weight standards (M_r) are shown.

100 mM D-galactose or D-glucose prevented ~40% of the CB binding to intact cells, whereas 100 mM L-glucose reduced binding by only ~8% (Table I). Since the effect of 500 mM D-galactose was no more pronounced than that of 100 mM D-galactose (data not shown), it was assumed that the lower concentration produced a maximal effect.

Scatchard plots of the data (bound/free vs. bound CB) from the experiments with whole platelets are shown in Figure 2. The measurements made in the absence of added hexose fit a model of two classes of binding sites better than a one-site model ($P = 0.002$). In the presence of 100 mM D-galactose, the two-class model was less certain but still favored ($P = 0.17$). The binding parameters for the two-class model are shown in Table II, where the binding capacities (R) have been expressed as micromoles per gram of cell protein for purposes of comparison with the membrane and cytosol data. By our measurements, 1 g of platelet protein represents $\sim 4 \times 10^{11}$ cells. Recalculating the binding capacities in terms of CB molecules bound per cell gives the following: $R_1 \sim 2.5 \times 10^5$ CB/cell in the absence of galactose and $\sim 1.8 \times 10^5$ CB/cell in the presence of 100 mM D-galactose; $R_2 \sim 3.3 \times 10^6$ CB/cell without galactose and $\sim 1.7 \times 10^6$ with galactose.

The binding data for the studies with isolated membranes fit a one-class model significantly better than a two-class model ($P = 0.88$ without galactose; $P = 0.84$

TABLE I. Effect of 100 mM Hexose on the Binding of 5.7 nM Cytochalasin B to Intact Platelets

Hexose	Bound CB[a] (nmol/10^{12} cells)	P[b]
None	8.3 ± 0.12 (N = 12)	
D-galactose	4.8 ± 0.10 (N = 4)	<0.001
D-glucose	5.3 ± 0.10 (N = 4)	<0.001
L-glucose	7.6 ± 0.40 (N = 4)	0.018

[a]Mean ± 1 standard deviation; N = number of studies.
[b]P-values for the differences in mean binding in the presence and absence of added hexose were calculated by a t-test for the analysis of independent samples with unequal variances [23].

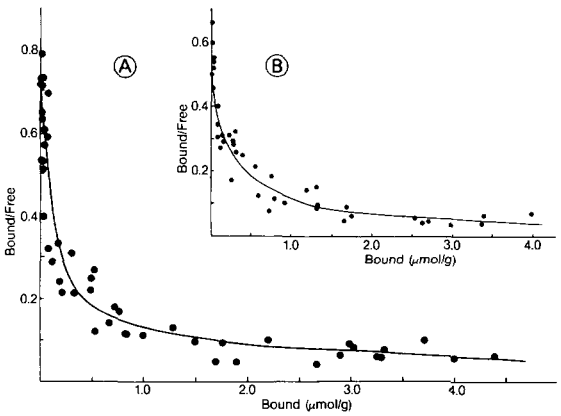

Fig. 2. Scatchard plots of CB binding to whole platelets in the absence of added hexose **(A)** and in the presence of 100 mM D-galactose **(B)**. The data are expressed for 1 mg cell protein per ml. The points have been fitted with weighted least-squares regression lines describing two classes of binding sites [21]. Binding parameters based on these curves are shown in Table II.

TABLE II. Parameters for CB Binding to Intact Platelets and Fractionated Membranes and Cytosol*

Parameter	Cells	+ Galactose	Membranes	+ Galactose	Cystol
Kd_1 (μM)	0.3	0.4	0.11	0.11	0.10
	(0.16–0.59)	(0.08–1.8)	(0.06–0.18)	(0.06–0.20)	(0.03–0.35)
R_1 (μmol/g)	0.17	0.12	0.13	0.05	0.3
	(0.10–0.30)	(0.02–0.80)	(0.10–0.15)	(0.3–0.07)	(0.015–0.07)
Kd_2 (μM)	21	7.0			20
	(8.5–50)	(2.0–24)			(2.0–154)
R_2 (μmol/g)	2.2	1.1			1.1
	(1.2–4.3)	(0.7–1.6)			(0.11–11)

*Values given are geometric means; parentheses enclose mean ±1 standard error [21].

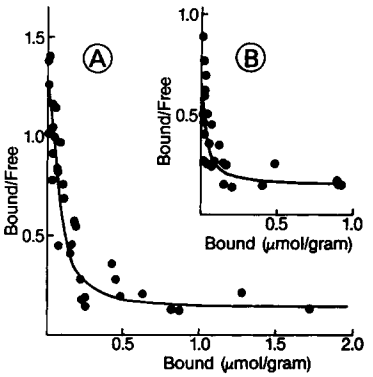

Fig. 3. Scatchard plots of CB binding to isolated platelet membranes in the absence of added hexose **(A)** and in the presence of 100 mM D-galactose **(B)**. The data are expressed for 1 mg membrane protein per ml. The points have been fitted with weighted least-squares regression lines describing single classes of binding sites [21]. Binding parameters based on these curves are shown in Table II.

with galactose) (Fig. 3). The membrane binding affinity and capacity for CB in the absence of galactose more closely approximated the values for the high-affinity class of sites observed with whole cells than the parameters of the low-affinity class (Table II). A reduction of membrane-binding capacity was observed in the presence of 100 mM D-galactose and was statistically significant ($P < 0.05$) by a t-test for the analysis of independent samples with unequal variances [23]. If the CB that is bound at a given concentration of free CB in the presence of 100 mM D-galactose is subtracted from the CB bound in the absence of galactose, the difference represents the class of binding sites eliminated by the hexose. This class has the same Kd as the class unaffected by galactose (Kd ~0.11 μM) and a capacity of ~0.08 μmol/g of membrane protein. By this analysis, therefore, ~60% of the CB binding to the membranes was prevented by hexose.

A similar result (~52%) was obtained with 1 M D-galactose and a different technique for separating bound and unbound CB (Table III). By this method 10 μM CE prevented ~15% of the CB binding, and the effects of 1 M D-galactose and 10 μM CE were roughly additive (~71% displacement).

Binding to cytosol fit a two-class model better than a one-class model ($P = 0.02$) (Fig. 4). According to the binding parameters (Kd, R), the classes correspond

TABLE III. Effect of D-Galactose and Cytochalasin E on the Saturable Binding of 9.5 nM Cytochalasin B to Isolated Platelet Membranes*

Agent	Displaced saturable CB binding (fraction)
1 M D-galactose	0.52 ± 0.006
10 μM cytochalasin E	0.15 ± 0.002
1 M D-galactose + 10 μM cytochalasin E	0.71 ± 0.014

*Mean ± 1 standard deviation, based on four measurements. Unsaturable binding, measured in the presence of 10 μM CB, has been subtracted from the totals.

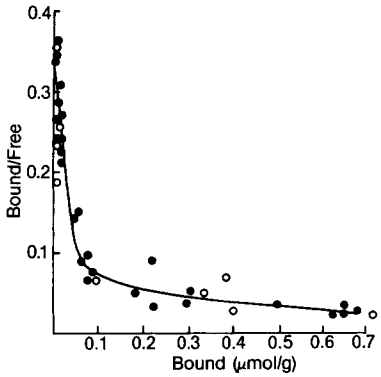

Fig. 4. Scatchard plot of CB binding to platelet cytosol. Filled circles (●) indicate measurements made in the absence of added hexose. Open circles (○) represent results in the presence of 100 mM D-galactose. The data from the studies performed without added hexose have been fitted with a weighted least-squares regression line describing two classes of binding sites [21]. Binding parameters based on this curve are shown in Table II.

to those observed with whole cells (Table III). However, binding studies performed with a wide range of CB concentrations in the presence of 100 mM D-galactose failed to demonstrate any galactose-sensitive class of sites like the ones observed with whole cells and with isolated membranes (Fig. 4).

DISCUSSION

Earlier investigators showed that bovine and human platelets bind CB in a reversible and saturable manner and that the binding can be resolved into two classes of sites on the basis of affinity (Kd ~0.1 μM and ~10 μM) [13,15]. The high-affinity class was identified in subcellular fractions assumed to contain primarily membrane fragments, whereas the low-affinity class copurified with monomeric actin [14,15]. Furthermore, a major portion of the CB binding to the membrane fraction was displaced by 500 mM D-galactose [15].

Our studies with human platelets have generally corroborated these reports. However, statistical analysis of computer-fitted models of the binding data have allowed certain observations to be made with more certainty. By such methods we have confirmed that more than one class of binding sites in whole platelets can be

distinguished on the basis of binding affinity. The dissociation constants calculated for the two-class model are in the range reported for bovine and human platelets, but the binding capacities are severalfold great [13,15]. This difference is perhaps due to a failure to reach cell saturation in the earlier studies. We also documented that physiologic hexoses (D-galactose, D-glucose) partially inhibit CB binding to intact cells but that the effect of L-glucose is much less (Table I). This effect, however, was clear only in studies that used a very low concentration of CB, which binds predominantly to the high-affinity, low-capacity class of sites. Because this class comprises $< 1\%$ of the total platelet binding capacity for CB (vide infra) its inhibition by hexose was obscured in the presence of higher concentrations of CB, which bind the low-affinity sites. Therefore, based on studies over a wide range of CB concentration, the estimated parameters for binding to cells in the presence of 100 mM D-galactose did not differ significantly from those in the absence of hexose (Table II).

CB binding to platelet cytosol resolved into two classes of sites by computer analysis of binding affinity (Table II). Neither of these classes was inhibited by 100 mM D-galactose (Fig. 4). The binding parameters for the cytosol were very similar to those for intact cells, except for R_1, which was much lower (~ 0.03 vs. ~ 0.17 μmol/g protein). This discrepancy remains unresolved because it cannot be accounted for by the small contribution of the cell membrane. It probably reflects the experimental difficulties inherent in characterizing a low-capacity class of binding sites in the presence of a class with a much greater capacity.

Actin is the probable binding protein for CB in the cytosol. Not only is actin present in higher concentration than any other protein in platelets [22], but F-actin is known to bind CB in the concentration range represented by the Kd_1 of the cytosol, whereas G-actin binds CB with a much lower affinity ($Kd > 6$ μM) [10,14]. The relatively high capacity of the low-affinity cytosolic (and cellular) binding sites is consistent with binding of one CB molecule per actin molecule, whether monomer or polymer. It has been shown that each F-actin molecule contains one CB binding site [9]. Assuming that this is true also for G-actin, we would expect that with $\sim 50\%$ of our cytosolic actin unpolymerized, the low-affinity (G-actin) binding capacity would be two- to 50-fold greater than high-affinity (F-actin) capacity, depending on the size of the polymers. The observed ~ 37-fold difference in high- and low-affinity binding capacity (Table II) therefore corresponds to an average polymer length of 37 monomers.

CB binding to isolated platelet membranes in our studies appeared to be of the high-affinity class only. Similar observations have been made before, but the purity of the membrane preparations used in those studies was not defined [14,15]. By lactate dehydrogenase measurements and by SDS-PAGE (Fig. 1), the membrane preparations in our studies were virtually free of cytosolic contamination. If one assumes that $\sim 3\%$ of the total platelet protein is membranous (which is probably an overestimate [18]), only 0.1–0.2% of the total CB binding capacity of the platelet is in the membrane.

Measuring CB binding to membranes in the presence of 1 M D-galactose and/or 10 μM CE distinguished three subclasses of saturable binding sites (Table III). The subclass from which CB is displaced by D-galactose ($\sim 50\%$ of the total, or $\sim 60\%$ if estimated from the binding data generated in the presence of 100 mM D-galactose, Table II) presumably represents the platelet hexose transporter that is competitively inhibited by CB [8]. The Ki for CB inhibition of this transporter is ~ 1.7 μM, at

which the hexose-sensitive binding site would be >90% saturated (Kd ~0.11 μM). The site to which CB binding is prevented by CE (~15% of the total) probably represents actin, some of which persisted in the membrane preparation (M_r ~43,000, Fig. 1). Since CE does not inhibit hexose transport by platelets, we would expect CE-displaceable CB binding not to involve the hexose transporters [8].

A third subclass of saturable CB binding sites in the membrane preparations (~30% of the total) was unaffected by the combination of D-galactose and CE (Table III). This subclass may involve the platelet hexose transporter that CB inhibits by a mixed-type mechanism (Ki ~0.8 μM) [8]. Another possibility, however, is that this transporter is inhibited by the unsaturable component of CB binding. This hypothesis is attractive because our kinetic data were consistent with CB inhibition of diffusion through the lipid bilayer of the platelet membrane, affecting both facilitated and unfacilitated transport [8]. Lipid solubility might produce the unsaturable binding of CB to the membranes as well as disturb diffusion-dependent processes.

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