Cadmium Increases GLUT1 Substrate Binding Affinity *in Vitro* While Reducing Its Cytochalasin B Binding Affinity†

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ABSTRACT: Cadmium stimulates glucose transport in fibroblasts, apparently by increasing the intrinsic activity of GLUT1 [Harrison, S. A., Buxton, J. M., Clancy, B. M., & Czech, M. P. (1991) *J. Biol. Chem. 266,* 19438−19449]. In the present study, we examined whether cadmium affects the binding *in vitro* of purified GLUT1 to glucose and cytochalasin B. Cadmium inhibited cytochalasin B binding to GLUT1 competitively by reducing its binding affinity with an apparent inhibition constant of approximately 0.2 mM. However, D-glucose displaced cytochalasin B bound to GLUT1 as effectively in the presence of cadmium as in its absence, and detailed analysis of this displacement revealed that cadmium in fact increases the substrate binding affinity significantly. These findings suggest that cadmium induces a specific conformational change in GLUT1 that interferes with cytochalasin B binding but enhances substrate binding. This is the first clear demonstration in which the substrate and cytochalasin B binding activities of GLUT1 are differentially affected, which may offer insight into the workings of the glucose transporter.

A family of specific membrane proteins known as facilitative glucose transporters catalyzes the uptake and release of glucose and other metabolizable sugars by animal cells (Carruthers, 1990). Six distinct isoforms (GLUT1-5 and 7) have been identified in this family (Pessin & Bell, 1992). Although 40−65% identical in amino acid sequence and showing a common transmembrane topology, these isoforms differ in their tissue-specific expression, subcellular localization, and sensitivity to hormonal and/or metabolic modulation (Bell et al., 1993). Of these isoforms, GLUT1 is particularly important with its ubiquitous expression in animal cells and possible link to oncogenicity suggested by its abundant expression upon cellular transformation (Birnbaum et al., 1987). In fact, most of the currently available biochemical information on this protein family has been obtained with this isoform (Mueckler, 1994). GLUT1 has been available as a pure and functional protein, and its structure and functional properties have been studied extensively (Bell et al., 1993; Lachaal et al., 1996).

The function of these transporters in cells is known to be tightly regulated in response to hormonal and metabolic signals (Pessin & Bell, 1992). An example of the modulation of intrinsic activity of this protein family include that of GLUT1 in clone 9 cells (Shi et al., 1995) and in pigeon erythrocytes (Diamond & Carruthers, 1993) where hypoxia or inhibition of oxidative phosphorylation stimulates glucose uptake severalfold without increasing the plasma membrane GLUT1 content, the only isoform expressed in these cells. In muscle cells and adipocytes, insulin stimulates glucose transport by inducing subcellular redistribution of GLUT4, the major isoform in these cells, and to a lesser extent GLUT1, from an intracellular pool to the cell surface (Cushman & Wardzala,1980; Suzuki & Kono, 1980). The increased D-glucose transport by insulin in adipocytes, however, significantly exceeds the increase in GLUT4 in the plasma membrane due to redistribution (Baly & Horuk, 1987; Joost et al., 1988), suggesting that insulin also increases the intrinsic activity of GLUT4 and/or GLUT1. Exact mechanisms underlying the modulation of glucose transporter catalytic activity, however, are currently unknown.

Cadmium (Cd) is a group IIb metal cation that affects the functionality of some proteins by interacting readily with exposed thiols (Ezaki, 1989; Perez-Garcia et al., 1996). Group IIb metal cations, including Cd, were shown to stimulate glucose transport in rat adipocytes (Ezaki, 1989). Subsequently, Harrison et a1. (1991) have shown that incubation of 3T3-L1 adipocytes with Cd results in a large (7−11-fold) stimulation of 3-*O*-methyl-D-glucose (3-OMG) flux with little or no increase in the plasma membrane GLUT4 and GLUT1 levels, and concluded that cadmium increases the intrinsic activity of glucose transporters in 3T3-Ll adipocytes. This conclusion, however, contradicts the observation by Ezaki (1989), with isolated rat adipocytes, that cadmium stimulates D-glucose transport more than 10-fold, but this stimulation is accompanied by an equally large increase in plasma membrane GLUT1/4 immunoreactivity, indicating that the stimulation is due to transporter recruitment. This apparent contradiction strongly suggests that the biochemical mechanisms by which cadmium stimulates GLUT function differ in different cell types and may involve interaction with cell-specific proteins. This consideration prompted us to examine, in the study reported here, whether Cd(II) can modulate GLUT1 function directly *in vitro*.

MATERIALS AND METHODS

Materials. Cadmium sulfate and cytochalasin B were obtained from Sigma, while [^{3}H]cytochalasin B was purchased from Amersham. Outdated human whole blood was supplied by the American Red Cross, Buffalo, NY.

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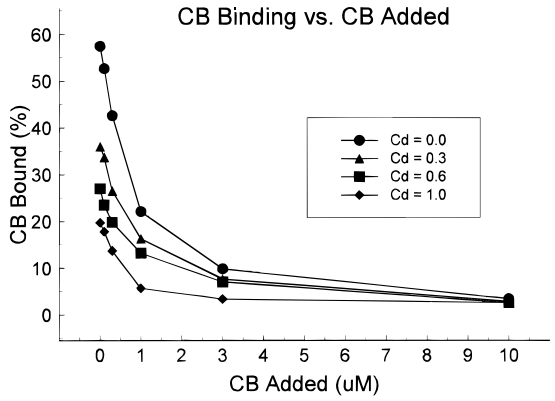

FIGURE 1: Cytochalasin B bound as a function of CB added to a fixed amount of transporter preparation., with cadmium concentration as a parameter. Cadmium concentrations (millimolar) are indicated on the figure.

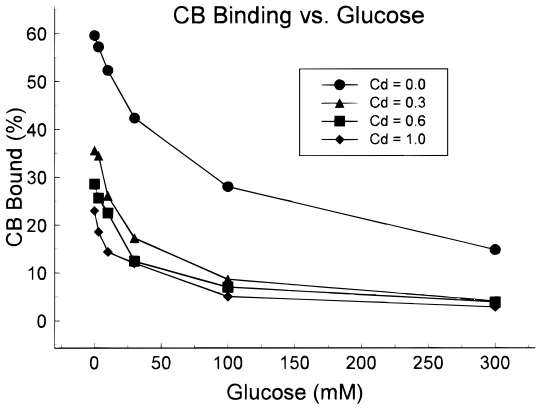

FIGURE 2: Fraction of a fixed amount (0.1 μM) of cytochalasin B which is bound as a function of glucose concentration. Cadmium concentration (0−1.0 mM) is a parameter, with values as shown on the figure.

Purification and Reconstitution of GLUT1 Protein. The preparation used here is essentially identical to those used previously (Chin et al., 1992). The GLUT1 in this preparation is 75 −80% functional in terms of cytochalasin B binding activity (with a specific binding activity of 14.5−15 nmol/mg of protein).

Cytochalasin B Binding Assay. Equilibrium binding of cytochalasin B to control and Cd(II)-treated GLUT1 (30−60 min) were measured in the absence and presence of an increasing concentration (up to 500 mM) of glucose as described (Jacobs & Jung, 1985) using six different cytochalasin B concentrations (10^{-8}−10^{-5} M) and a tracer amount of [^{3}H]cytochalasin B. Radioactivities were measured in an LKB 1209 Rackbeta liquid scintillation counter.

RESULTS

Cytochalasin B (CB), a well-known competitive inhibitor of GLUT1 (Jung & Rampal, 1977) and other members of the facilitative glucose transporter family (Carruthers, 1990), inhibits by directly binding to the transporter. This binding is readily displaced by D-glucose but not by L-glucose, with a simple one-to-one stoichiometry; it thus provides a useful assay for GLUT1 substrate binding activity (Pinkofsky et al., 1978; Sogin & Hinkle, 1980). We have undertaken equilibrium CB binding experiments in order to estimate the effect which cadmium association with the transporter protein exerts upon the apparent binding affinities of the transporter for both cytochalasin B and glucose. Data was collected in two series of experiments: in the first, the fraction of cytochalasin B (CB) bound was assayed as a function of CB added, with cadmium concentration ($CdSO_4$) as a parameter. In the second series, the fraction bound of a fixed quantity of added CB (0.1 μM) was measured as a function of glucose concentration, again with cadmium concentration as a parameter. In both instances, the extent of CB binding, using labeled CB, was determined from the distribution of radioactive label between the supernatant solution and the spun-down pellet of membrane fragments assumed to contain the transporter protein. In neither set of experiments was the binding of glucose or cadmium measured directly. In a control experiment, sulfate (Na_2SO_4) in the absence of cadmium was shown to have no effect upon CB binding.

The results of these experiments are shown in Figures 1 and 2, respectively. The binding data is also presented in Tables 1 and 2, together with the corresponding apparent affinity coefficient for CB binding.

Table 1: CB Binding vs CB in the Presence of Cadmium

CB (μM)	Cd = 0.0 mM		Cd = 0.3 mM		Cd = 0.6 mM		CD = 1.0 mM	
	% bnd	K_C'	% bnd	K_C'	% bnd	K_C'	% bnd	K_C'
0.0	57.5	0.21	36.0	0.50	27.0	0.76	19.7	1.1
0.1	52.7	0.20	33.7	0.49	23.5	0.84	17.8	1.2
0.3	42.6	0.21	26.5	0.56	19.8	0.89	13.7	1.5
1.0	22.1	0.21	16.3	0.60	13.2	0.97	5.7	3.7
3.0	9.8	−0.13	7.7	0.61	7.0	0.93	3.4	5.1
10.0	3.4	−0.18	2.9	−0.20	2.6	0.96	2.6	0.96

Table 2: CB Binding vs Glucose in the Presence of Cadmium

Glu (mM)	Cd = 0.0 mM		Cd = 0.3 mM		Cd = 0.6 mM		CD = 1.0 mM	
	% bnd	K_C'	% bnd	K_C'	% bnd	K_C'	% bnd	K_C'
0	59.6	0.20	35.6	0.59	28.6	0.83	23.0	1.13
3	57.2	0.23	34.5	0.62	25.6	0.97	18.6	1.49
10	52.3	0.28	26.1	0.95	22.5	1.16	14.4	2.1
30	42.3	0.43	17.3	1.64	12.4	2.46	12.0	2.6
100	28.0	0.85	8.7	3.7	7.0	4.7	5.0	6.7
300	14.8	1.99	4.1	8.3	3.9	8.8	2.8	12.2

As a basis for the interpretation of these experimental results, a simple multiple association model of the following form was assumed:

$$\begin{aligned} \mathbf{T} + \mathbf{C} &\overset{K_C}{\longleftrightarrow} \mathbf{TC} \\ \mathbf{T} + \mathbf{G} &\overset{K_G}{\longleftrightarrow} \mathbf{TG} \\ \mathbf{T} + \mathbf{X} &\overset{K_X}{\longleftrightarrow} \mathbf{TX} \\ \mathrm{TX} + \mathrm{C} &\overset{K_{XC}}{\longleftrightarrow} \mathrm{TXC} \\ \mathrm{TX} + \mathrm{G} &\overset{K_{XG}}{\longleftrightarrow} \mathrm{TXG} \end{aligned} \tag{1}$$

in which T symbolizes transporter protein; C, cytochalasin B; X, cadmium; and G, glucose. Each binding reaction is characterized by the dissociation constant shown. On the basis of previous results (Pinkofsky et al., 1978), it is assumed that the association of CB and D-glucose is strictly competitive; thus the complexes TCG and TCGX are precluded.

Two changes of notation are introduced to simplify subsequent development. First, the concentrations of CB,

cadmium, and D-glucose are normalized with respect to the corresponding dissociation constant. Thus, for example, the equilibrium concentration of the complex TC is given by

$$[TC] = [T][C]/K_C = [T]\,\underline{C} \qquad (2)$$

where $\underline{C} \equiv [C]/K_C$. The underlined notation, $\underline{C}$ signifies the normalized CB concentration variable, with a similar notation for the normalized concentrations of cadmium and D-glucose. Second, rather than the explicit dissociation constants for the double complexes TXC and TXG, we utilize the parameters α_G and α_G, defined as follows:

$$\alpha_C \equiv K_C/K_{XC} \qquad \alpha_G \equiv K_G/K_{XG}$$

With these notations, and dropping the brackets on the symbols for the other concentrations, then we have the equilibrium relationships

$$TC = T\,\underline{C} \qquad TXC = T\,\underline{X}\,\underline{C}\,\alpha_C$$

$$TX = T\,\underline{X} \qquad TXG = T\,\underline{X}\,\underline{G}\,\alpha_G \qquad (3)$$

$$TG = T\,\underline{G}$$

In addition, the total number of binding sites, T_T, is given by the sum of all complex forms, and the free sites. Then we have

$$T_T = T(1 + \underline{C} + \underline{G} + \underline{X} + \underline{X}\,\underline{C}\,\alpha_C + \underline{X}\,\underline{G}\,\alpha_G) \qquad (4)$$

The amount of cytochalasin B bound to the transporter, C_B, can be expressed as

$$C_B = TC + TXC = (1 + \alpha_C)\,T\,C/K_C \qquad (5)$$

With the help of eq 4, the free transporter site concentration, T, can be replaced, yielding the relation

$$C_B = \frac{C\,(1 + \alpha_C\,\underline{X})T_T}{K_C[1 + \underline{X} + \underline{C}\,(1 + \alpha_C\,\underline{X}) + \underline{G}\,(1 + \alpha_G\,\underline{X})]} \qquad (6)$$

which can be rearranged into the form

$$C\,(T_T/C_B - 1) = K_C\,\frac{1 + \underline{X} + \underline{G}\,(1 + \alpha_G\,\underline{X})}{1 + \underline{X}} \equiv K_C' \qquad (7)$$

K_C' is the apparant CB dissociation "constant" which, as eq 7 indicates, is a function of glucose and cadmium concentrations. In the absence of both glucose and cadmium, the apparent constant K_C' converges, as it should, to K_C. Examination of the way in which K_C' varies with the glucose and cadmium concentrations thus provides a means of evaluating α_C and α_G, the primary objective of this study.

CB binding data, in the absence of glucose and cadmium (Table 1), is plotted in standard Scatchard form in order to estimate K_C. This plot is shown in Figure 3. In addition, the *x*-axis intercept of the line fitted to the data provides an estimation of the total transporter binding capacity, T_T, which is needed to evaluate eq 7. Since the total transporter present may vary significantly between experimental series, T_T must be estimated independently in each set of experiments.

As the plot of Figure 3 demonstrates, the binding data conform quite well to the linear Scatchard plot, with the exception of those corresponding to the two highest CB concentrations. These points are excluded; their deviation

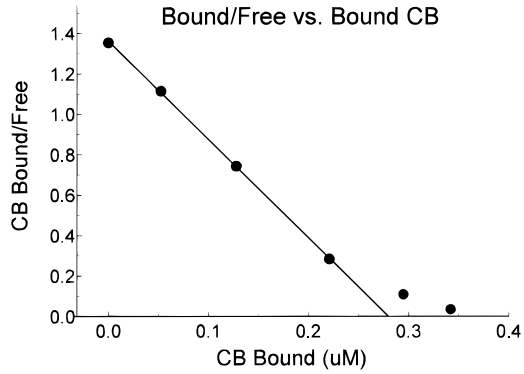

FIGURE 3: Scatchard plot showing CB binding as a function of free CB, in the absence of both cadmium and glucose. A linear curve-fit to the four lowest concentration points yields an estimate of the CB dissociation constant, K_C, of 0.206 ± 0.002 mM. The two higher concentration points were discarded as corrupted by nonspecific CB binding. Extrapolation of the fitted line to the axis gives a value of 0.28 μM for the total transporter binding sites; this value is used subsequently in the first experimental series to allow calculation of the apparent K_C' for each experimental point.

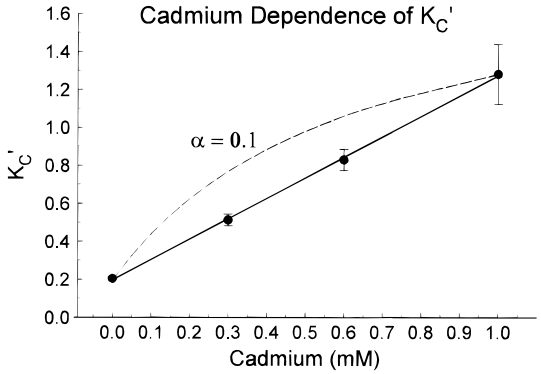

FIGURE 4: Apparent CB dissociation constant, K_C', plotted as a function of cadmium concentration, using data from the first experimental series (Table 1). As discussed in the text, the linearity of this plot indicates that α_C is zero. With $\alpha_C = 0$, from eq 8, the slope of the fitted line yields a value of 0.185 mM for the cadmium dissociation constant, K_X. Data taken from the second experimental series, Table 2, are consistent with these values within the experimental error shown.

from the line established by the remaining data most likely represents nonspecific low-affinity binding of CB to the membrane preparation. From the slope of this plot, K_C is calculated to be 0.206 ± 0.002 μM, a value consistent with that found in previous studies (Pinkofsky et al., 1978). Values of K_C calculated for individual experimental points according to eq 7 all lie within 1% of the value derived from the best-fit line. The magnitude of T_T, evaluated from the Scatchard plot intercept at the ordinate, is 0.28 μM for this experimental series.

In order to evaluate K_X and α_C, the experimental data shown in Figure 1 (series 1) were replotted, as shown in Figure 4, by computing the expression on the left-hand side of eq 7, giving the value of K_C' as a function of cadmium concentration. The plotted points are the average K_C' over the three lowest CB concentrations for each level of Cd. The data from the higher total CB experiments were excluded, based upon the problem of nonspecific binding noted above.

In the absence of glucose, eq 7 simplifies to

$$K_C' = K_C\,\frac{1 + \underline{X}}{1 + \alpha_C\,\underline{X}} \qquad (8)$$

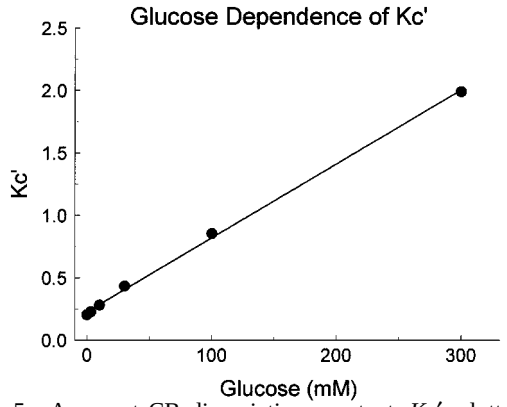

FIGURE 5: Apparent CB dissociation constant, K_C', plotted as a function of glucose added, in the absence of cadmium. From eq 9, the slope of this plot indicates a glucose dissociation constant (K_G) of 33 mM.

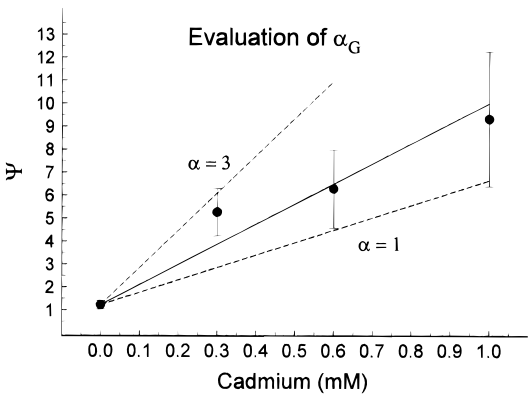

FIGURE 6: A plot of the function Ψ, as defined by eq 10, against cadmium concentration. In accord with eq 10, the slope of a linear fit to this data gives a value for α_G in the range of 1−3. The dashed lines indicate this range of slopes.

The properties of the plot shown in Figure 4 provide the basis for estimating α_C. From eq 8, for arbitrarily large cadmium concentrations, the maximum K_C' achievable is limited to K_C/α_C. From the largest value of K_C' shown in the figure, $6.5K_C$, then α_C must be less than 0.15. Furthermore, any value of α_C other than 0 or 1 will result in curvature of the plot in Figure 4. For example, the curvature introduced by an α_C value of 0.1 is illustrated by the dotted curve. On the basis of these considerations and the nearly perfect linearity of the data plotted in Figure 4, we conclude that, within the precision of the experimental data, α_C is zero. That is, the binding of CB and cadmium to the transporter protein behave competitively, and the double complex TCX does not occur. With this result in hand, the slope of the curve gives a value of 0.185 mM for K_X.

Referring once again to eq 7, in the absence of cadmium, we have simply

$$K_C' = K_C(1 + \underline{G}) \tag{9}$$

In Figure 5, K_C' is plotted as a function of glucose concentration, using the data from the second series of experiments given in Table 2. From the slope of this line, the value of K_G is estimated to be 33 mM.

Finally, making use of these values of the dissociation constants, and setting $\alpha_C = 0$, the data can be analyzed to evaluate α_G. Equation 7 can be rearranged to

$$(K_C'/K_C - 1 - \underline{X})/\underline{G} = 1 + \alpha_G \underline{X} \equiv \Psi \tag{10}$$

in which the function on the left is defined as Ψ. Thus a plot of this function against $\underline{X}$ should be linear, with the slope of the line being proportional to α_G. This plot is shown in Figure 6. While there is considerable scatter in the data presented in this form, as indicated by the error bars, the value of α_G would clearly appear to be 1 or larger, probably lying in the range of 1−3. Upon the basis of this data, therefore, the binding of glucose to the transporter seems not to be inhibited by the presence of cadmium. Instead, cadmium association appears either to have no effect upon glucose binding or to increase the transporter's affinity for glucose by a factor between 1 and 3.

DISCUSSION

It is clear from the present study that cadmium inhibits GLUT1 cytochalasin B binding *in vitro* but probably stimulates its glucose binding activity. This is the first clear demonstration that the substrate binding activity and cytochalasin B binding activity of GLUT1 can be dissociated. Cadmium interacts avidly with free cysteine sulfhydryl in protein, and much less avidly with the lysine amino group. There are six cysteine residues in GLUT1 (Mueckler, 1994). Which of these cysteines, if any, is primarily responsible for the cadmium effects on the substrate and inhibitor binding is yet to be identified. It is relevant to note in this regard that a cysteineless GLUT1 mutant is fully functional (Wellner et al., 1995). This would indicate that none of these cysteines is required for the transporter function. This, however, would not by itself rule out the possible involvement of cysteine residue in the stimulation of GLUT1 substrate binding by Cd(II). It would be interesting to investigate whether the substrate and cytochalasin B binding of cysteineless GLUT1 are similarly affected by Cd(II). Detailed molecular structural analysis (Griffin et al., 1982) suggests that the interaction of glucose and cytochalasin B with GLUT1 may be isosteric. Results of photolabeling experiments (Holman & Rees, 1987) also suggest that glucose and cytochalasin B interact with GLUT1 at sites physically close, probably at the exoplasmic end of transmembrane helix 9 and the cytoplasmic end of the transmembrane helix 10, respectively. The cadmium-induced dissociation described here between these two parameters would provide an opportunity for selectively probing a likely role of exposed cysteine side chains in GLUT1 conformation induced by substrate and the inhibitor.

Whether cadmium causes a similar increase in GLUT1 substrate affinity in cells is not entirely clear. Harrison et al. (1991) have shown that a 3-fold increase in GLUT1 substrate affinity is associated with cadmium-induced stimulation of glucose transport in 3T3-L1 adipocytes. Our preliminary data with rat adipocytes (Lachaal et al., 1996), on the other hand, demonstrate that cadmium stimulates glucose transport in rat adipocytes up to 3.5-fold, and this stimulation is due to an increase in transport V_{max} with little change in K_m. Preliminary data (Lachaal et al., 1996; manuscript in preparation) also indicate that cadmium indeed stimulates glucose flux in purified GLUT1 vesicles and inhibits its cytochalasin B binding activity. GLUT1 transport activity in intact human erythrocytes, however, was not affected significantly by Cd(II). These findings strongly suggest that the cadmium effects on GLUT1 function are

complex and different in different cells, involving not only direct stimulation of GLUT1 intrinsic activity but also mediation by a regulatory protein or proteins that are cell-specific.

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