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Enhanced Internalization of Ricin in Nigericin-Pretreated Chinese Hamster Ovary Cells

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Biochemical and electron microscopic autoradiographic studies with [^{125}I]ricin have revealed that nigericin-pretreated Chinese hamster ovary cells are more efficient than untreated cells in the internalization of the toxin into the cells. These results suggest that the enhanced rate of internalization of ricin in nigericin-pretreated cells may account for the enhancement of cytotoxicity of ricin in Chinese hamster ovary cells by nigericin.

In an accompanying paper, we have shown that the cytotoxicities of ricin and *Pseudomonas aeruginosa* exotoxin A are enhanced by the pretreatment of Chinese hamster ovary (CHO) cells with the monovalent ionophores, nigericin and monensin (9). These two toxins differ in their surface receptors as well as in their intracellular biochemical targets (1, 3, 5, 8). Enhancement of the cytotoxicities of ricin and *Pseudomonas* toxin, but not that of diphtheria toxin, in nigericin-pretreated cells suggests the existence of a common step in the intoxication process of CHO cells by ricin and *Pseudomonas* toxin which is not shared by the intoxication process of diphtheria toxin. This common step must reside between the initial bindings of these two toxins to their respective surface receptors and the intracellular enzymatic activities manifested by their A subunit or domain (9).

In the present paper, we present evidence that the enhanced cytotoxicity of ricin in nigericin-treated cells is due to an increased internalization of ricin into the CHO cells.

MATERIALS AND METHODS

Chemicals and radiochemicals. Galactose, lactoperoxidase, pronase, bovine serum albumin, and proline were purchased from Sigma Chemical Co. Nigericin was obtained from Eli Lilly & Co. Ricin was supplied by the Miles Laboratories, Inc. Na^{125}I (carrier free; specific activity, 4 mCi/nmol) was purchased from Amersham Corp. Powdered α-MEM and α-MEM suspension culture media were purchased from Flow Laboratories. Fetal calf serum was obtained from Microbiological Associates.

Methods. A CHO cell line auxotrophic for proline (*pro*$^-$) was used for this study, and the method of culture was as described in an accompanying paper (9).

The methods for the iodination of ricin and for the measurement of binding and internalization of iodinated ricin into CHO cells were described in an accompanying paper (10). [^{125}I]ricin was at least 80% as toxic as the unlabeled toxin based on plating efficiency assay.

Electron microscopic studies. Samples were fixed in a suspension of 2% glutaraldehyde in tissue culture medium, centrifuged to obtain a pellet, and left overnight at 4°C. Samples were postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.34) for 1 h at room temperature, dehydrated through a series of ethanol and propylene oxide and embedded in Epon 812 epoxy resin. Thin sections were cut on a Sorvall MT 2-B microtome, mounted on 300 mesh Formvar-coated grids, and stained with uranyl acetate and Reynolds lead citrate. Grids were carbon coated in a Denton evaporator and applied to microscope slides with double-stick tape. The slides were then coated with a thin layer (purple interference at region of grids) of Ilford L-4 emulsion and stored with desiccant in light-tight containers at 4°C. At specified times grids from each sample were developed in Kodak Microdal X developer and fixed in 24% sodium thiosulfate for 4 min. Grids were examined and photographed with a Hitachi 11-E electron microscope. At least 25 cells from each sample were photographed, and grains were scored for cellular location by using a half-distance of 100 nm (11).

RESULTS

Effect of nigericin on the binding of [^{125}I]ricin to CHO cells. The presence of nigericin during the binding assay did not affect the time course or the extent of binding of [^{125}I]ricin to CHO cells at either 0 or 37°C. The kinetics (time course and concentration dependence) of binding of [^{125}I]ricin in the presence of nigericin was essentially the same as that in its absence (data not shown). On the other hand, there was a slight increase in the binding of [^{125}I]ricin at 0°C and a greater increase at 37°C, compared with 0°C, to CHO cells which had been pretreated with 10 nM nigericin for 72 h at 37°C before the binding assay (data not shown).

The increase in the total cell-bound [^{125}I]ricin in nigericin-pretreated cells at 37°C suggests the interesting possibility that nigericin pretreatment resulted in a more efficient uptake of ricin into the CHO cells.

Effect of nigericin on the internalization of [^{125}I]ricin into CHO cells. To distinguish surface-bound ricin from the internalized molecules, we have measured internalization of [^{125}I]ricin by the procedure detailed in an accompanying report (10). Internalization of [^{125}I]ricin into nigericin-treated cells is enhanced as compared with that in the untreated cells (Fig. 1, 2, and 3).

Internalization of ricin is temperature, time, and concentration dependent (10). Like the binding of [^{125}I]ricin in CHO cells, internalization was concentration dependent and required a very high concentration of ricin in order to saturate this receptor-mediated process. Over the entire range of ricin concentrations tested, nigericin-pretreated cells were more efficient in the uptake of [^{125}I]ricin than were the control cells, especially at high external concentrations of ricin (Fig. 1b). Internalization of [^{125}I]ricin in nigericin-treated cells was detectable at a lower temperature than in the untreated cells (Fig. 2). At 37°C, nigericin-pretreated cells internalized

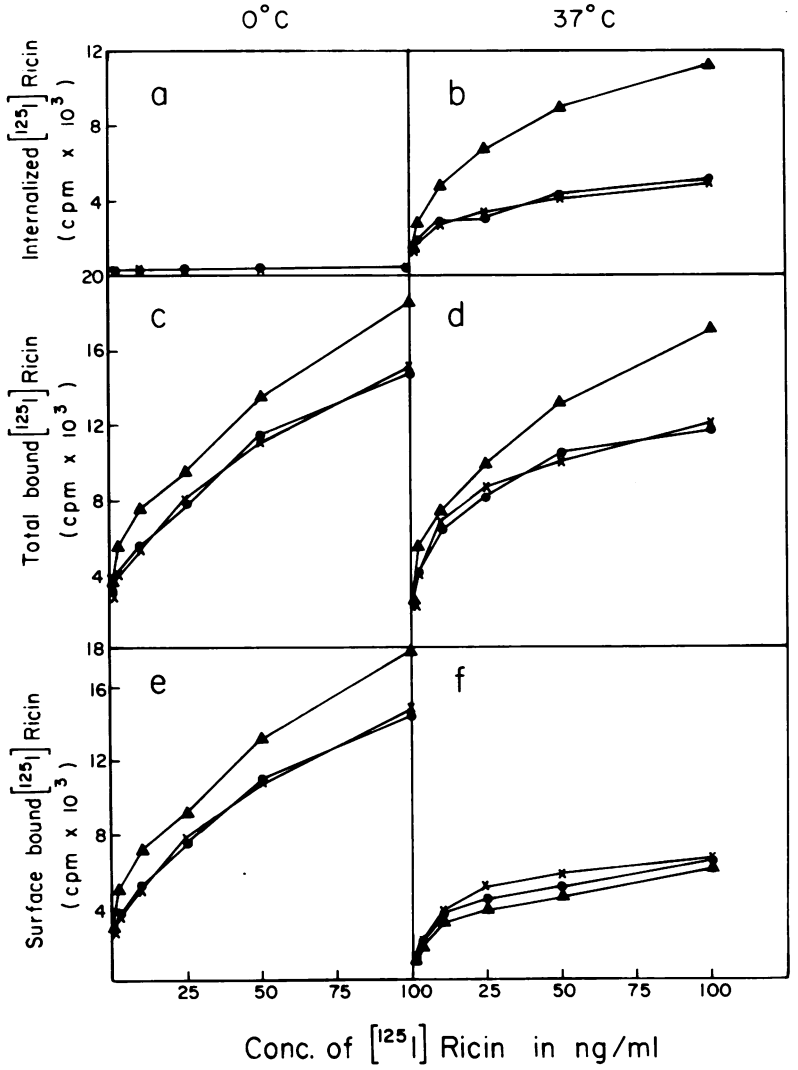

FIG. 1. *Concentration dependence of the binding and internalization of [^{125}I]ricin in control and nigericin-pretreated cells grown at 0°C (a, c, and e) or 37°C (b, d, and f). Symbols: ●, control cells; ×, control cells with 10 nM nigericin present during the binding assay; ▲, cells pretreated with 10 nM nigericin for 72 h at 37°C before the binding assay.*

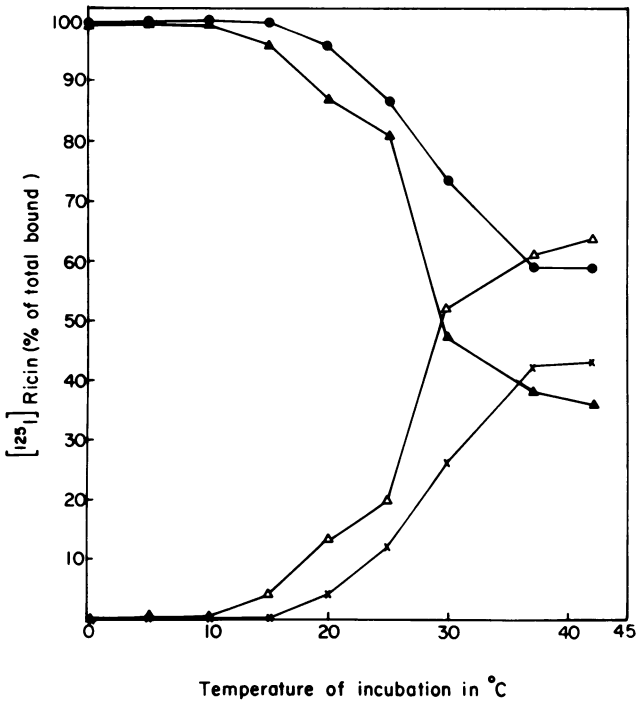

FIG. 2. *Temperature dependence of [^{125}I]ricin internalization in control* (×, ●) *and nigericin-pretreated* (△, ▲) *cells. The data are expressed to indicate ricin internalized* (×, △) *or remaining at the cell surface* (●, ▲) *as the percentage of total cell-associated ricin at the given temperature.*

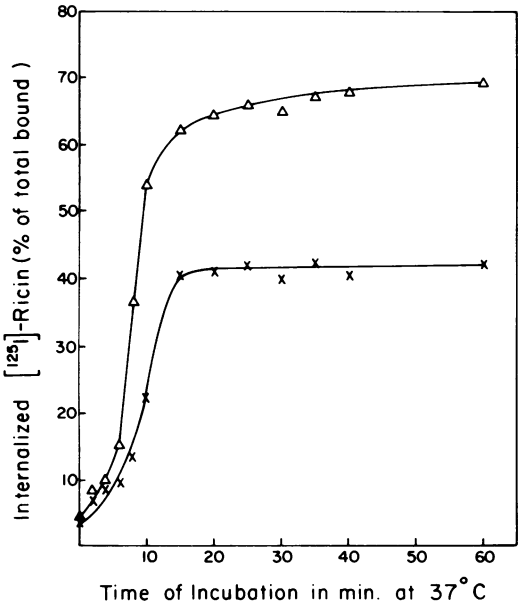

FIG. 3. *Time course of internalization of [^{125}I]ricin in control* (×) *and nigericin-pretreated* (△) *cells.*

50% more [^{125}I]ricin than did the control cells (Fig. 3). Arrhenius plots indicated a clear-cut difference between binding and internalization of [^{125}I]ricin with respect to temperature dependence (Fig. 4). The enhancement of internalization of [^{125}I]ricin in nigericin-treated cells is reflected in an apparent reduction in the activation energy for the internalization process. No sharp transition temperature can be discerned for both control and nigericin-treated cells.

Scatchard analysis of binding of [^{125}I]ricin revealed a minimum of two classes of binding sites with different affinities (Fig. 5a). Similar differences in affinities were seen when internalization was measured (Fig. 5b). These results suggest that binding to receptors of both high and low affinities may lead to the internalization of this toxin. Pretreatment of CHO cells with nigericin did not affect the number or the affinities of these binding sites. On the other hand, the numbers of binding sites of both high and low affinities which are used for internalization appeared to be increased in nigericin-pretreated cells (Fig. 5). Similar results were obtained with the *ts*-1-1 mutant line of CHO cell which was defective in the binding of [^{125}I]ricin (Fig. 6). These results are summarized in Table 1. The pH optimum of internalization of [^{125}I]ricin was not altered in the nigericin-pretreated cells (data not shown).

Studies of binding and internalization of [^{125}I]ricin in control and nigericin-pre-

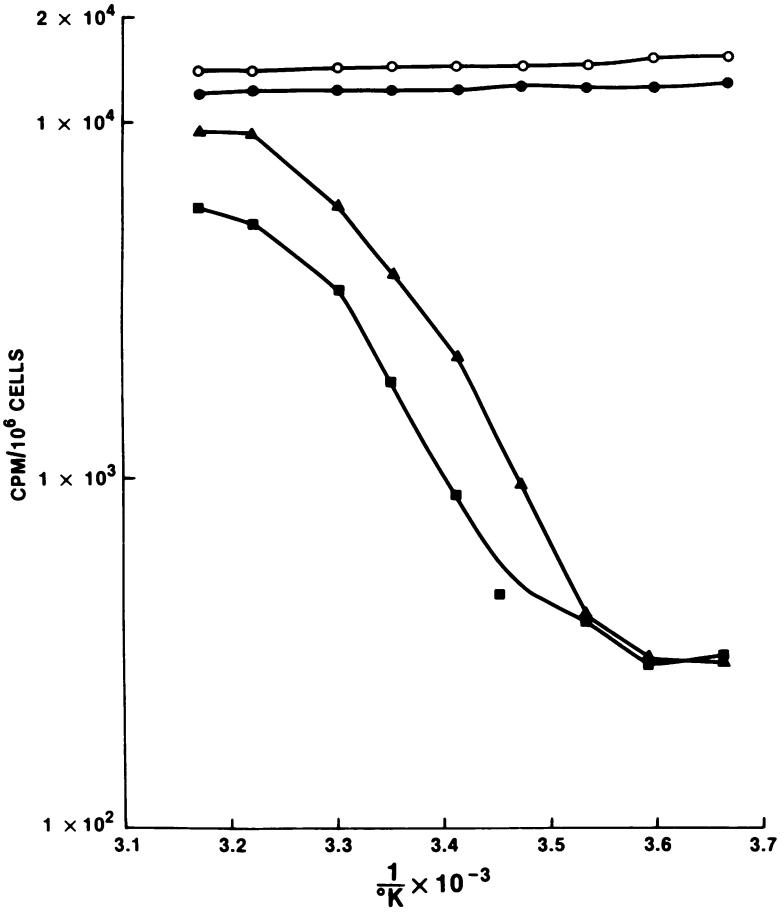

FIG. 4. *Effect of temperature on the binding and internalization of [^{125}I]ricin in control and nigericin-treated CHO cells. Symbols: ●, binding of [^{125}I]ricin in control cells; ○, binding of [^{125}I]ricin in nigericin-pretreated cells; ■, internalization of [^{125}I]ricin in control cells; and ▲, internalization of [^{125}I]ricin in nigericin-pretreated cells. The logarithm of counts per minute (CPM) bound or internalized per 10^6 cells (ordinate) is plotted against the inverse of temperature ($1/°K \times 10^{-3}$, abscissa).*

treated cells by thin-section electron microscopy and autoradiography. To confirm that nigericin-pretreated cells are more efficient in the uptake of [^{125}I]ricin into the cells, we employed thin-section electron microscopy and autoradiography to distinguish internalized ricin from cell surface-bound molecules. At 0°C, nearly all of the grains remained at the cell surface (≤2 half-distances of the grains) in both control and nigericin-treated cells (Fig. 7 and 8). At 37°C, grains corresponding to the internalized [^{125}I]ricin were found inside the cells (>2 half-distances of the grains away from the cell boundaries), whereas some grains remained on the cell surface. In nigericin-treated cells, more grains were found, especially those inside the cells. A statistical analysis of the total number of grains and their distribution is shown in Table 2. Under identical conditions, nigericin-treated cells internalized about 50% more ricin than the control cells. The correlation between the biochemical data and the results obtained with autoradiography is excellent.

DISCUSSION

In an accompanying paper, we have shown that nigericin and monensin selectively enhance the cytotoxicities of ricin, abrin, and *Pseudomonas* toxin in CHO cells, but do not enhance the cytotoxicity of diphtheria toxin (9). We have also shown that the increased cytotoxicity of ricin in nigericin-pretreated cells is mediated by the same surface receptors as those used in the untreated cells. Inasmuch as the biochemical target for ricin differs from that for *Pseudomonas* toxin and the cytotoxicity of diphtheria toxin is not altered in the nigericin-pretreated cells, these results strongly suggest that nigericin

pretreatment has affected a rate-limiting step in the intoxication process common for ricin, abrin, and *Pseudomonas* toxin. The step presumably lies between the binding to the surface receptors and their final enzymatic inactivation of protein synthesis in the cytosol.

Scatchard analysis of binding and internalization of [^{125}I]ricin revealed more than one independent class of sites in CHO cells which can bind and internalize ricin. Although a definitive and quantitative analysis of these multiple sites is not possible, we have made a qualitative in-

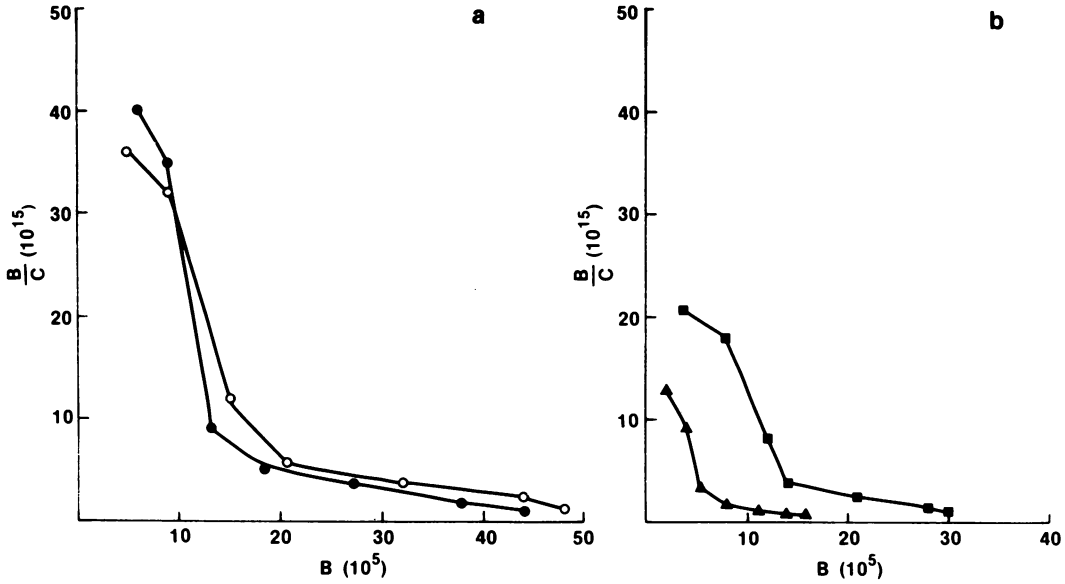

FIG. 5. *Scatchard analysis of the binding (a) and internalization (b) of [^{125}I]ricin in CHO wild-type cells as a function of ricin concentration. Symbols: ●, binding of [^{125}I]ricin to control cells; ○, binding of [^{125}I]ricin to nigericin-pretreated cells; ▲, internalization of [^{125}I]ricin to control cells; and ■, internalization of [^{125}I]ricin to nigericin-pretreated cells. The formula used was B/C = K(n − B), where B is the number of ricin molecules bound per cell, C is the number of free ricin molecules, n is the number of binding sites per cell, and K is the apparent association constant.*

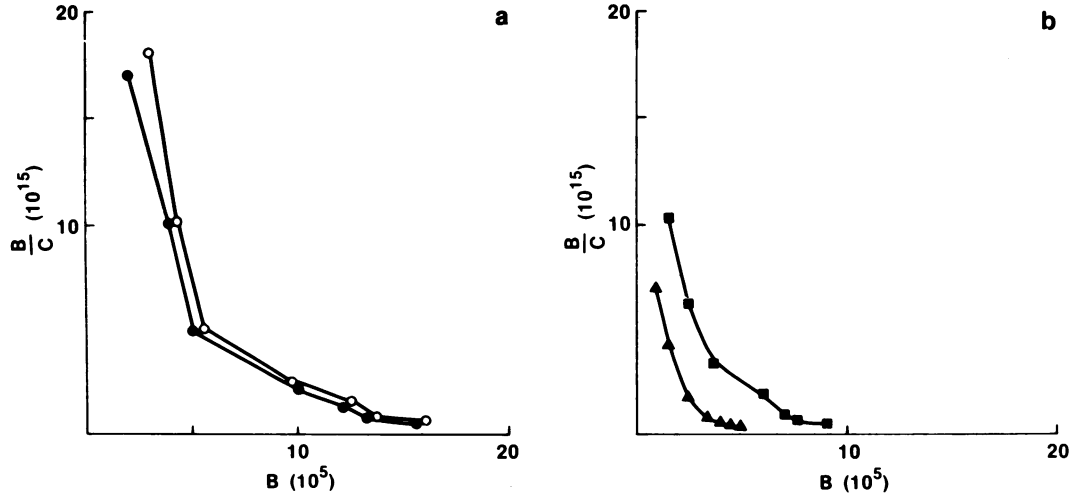

FIG. 6. *Scatchard analysis of the binding (a) and internalization (b) of [^{125}I]ricin in CHO ts-1-1 mutant cells as a function of ricin concentration. Symbols: ●, binding of [^{125}I]ricin to untreated ts-1-1 cells; ○, binding of [^{125}I]ricin to nigericin-pretreated ts-1-1 cells; ▲, internalization of [^{125}I]ricin to untreated ts-1-1 cells; and ■, internalization of [^{125}I]ricin to nigericin-pretreated ts-1-1 cells. The formula was as described in the legend to Fig. 5.*

TABLE 1. *Scatchard analysis of binding and internalization of ^{125}I in wild-type and ts-1-1 mutant CHO cells*[a]

Cell line	No. of ricin molecules bound/cell		Association constant (M^{-1})		No. of ricin molecules internalized/cell		Association constant (M^{-1})	
	n_1	n_2	K_1	K_2	n_1	n_2	K_1	K_2
CHO	3.4×10^6	1.6×10^6	1.4×10^9	3.6×10^{10}	1.3×10^6	5.1×10^5	1.2×10^9	3.4×10^{10}
CHO pretreated with nigericin	3.7×10^6	1.8×10^6	1.2×10^9	3.4×10^{10}	2.7×10^6	1.3×10^6	1.0×10^9	1.9×10^{10}
ts-1-1	1.2×10^6	4.6×10^5	2.5×10^9	4.8×10^{10}	2.4×10^5	2.4×10^5	3.5×10^9	3.8×10^{10}
ts-1-1 pretreated with nigericin	1.3×10^6	4.7×10^5	3.0×10^9	5.3×10^{10}	6.7×10^5	3.5×10^5	3.1×10^9	3.6×10^{10}

[a] The Scatchard analysis of binding and internalization of [^{125}I]ricin was carried out assuming a minimum of two independent binding sites with different affinities. The method of Klotz and Hunston (4) was used to obtain the number of sites (n_1 and n_2) and the apparent association constants (K_1 and K_2).

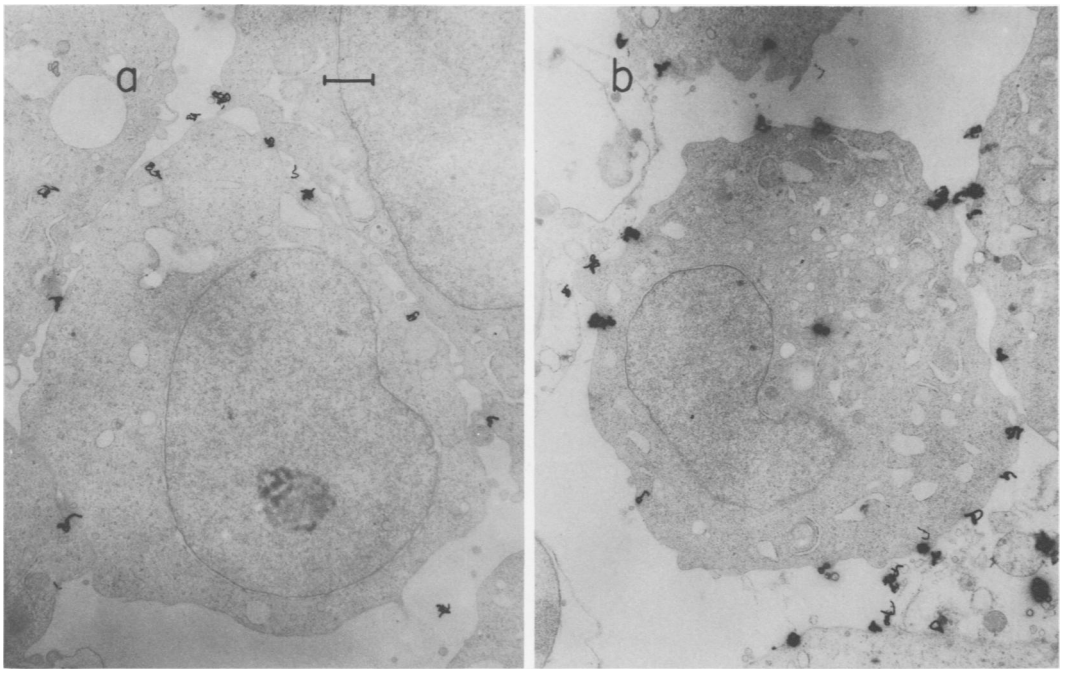

FIG. 7. *Electron microscopic autoradiograph of a thin section of control (a) and nigericin-pretreated (b) CHO cells incubated at 0°C for 20 min with [^{125}I]ricin. The positions of the grains are related to the position of [^{125}I]ricin. The experimental details are described in the text. Grains located more than 200 nm from the cell surface inside the cell represent internalized toxin. Bar, 1,000 nm.*

terpretation based on the assumption of a minimum of two classes of binding sites with different affinities; the association constants for the two classes of receptors differ by 25-fold in the wild type and by 20-fold in the *ts*-1-1 mutant. Pretreatment with nigericin does not change the affinities of both classes of receptors toward ricin. The *ts*-1-1 mutant appears to have one-third as much of both low- and high-affinity ricin receptors, and nigericin pretreatment does not alter the numbers of both classes of receptors in the wild type and in the receptor-deficient mutant. Although it may be oversimplistic to analyze the results on internalization of [^{125}I]ricin by a Scatchard plot, such analysis has provided interesting clues with regard to the relationship between binding and internalization. The apparent affinities of sites involved in the internalization of ricin are similar to those for the binding of ricin to CHO cells. These results suggest that all receptors with different affinities for ricin may participate in the internalization of ricin, and there is no need to invoke the existence of secondary intracellular sites distinct from the

surface receptors which are involved in the internalization. Furthermore, the association constants of these putative sites for internalization of ricin are not altered in nigericin-pretreated cells. On the other hand, there appears to be a two- to threefold increase in the number of sites involved in the internalization of ricin into CHO cells, even though the total binding sites of ricin remain unchanged. In the control cells, approximately 30% of the binding sites could be involved in the internalization of ricin. In nigericin-pretreated cells, 70% of total binding sites may be involved in internalization. The simplest explanation of the enhancement of cytotoxicities of ricin, abrin, and *Pseudomonas* toxin by nigericin is to postulate that receptor-bound toxins are more efficiently internalized into nigericin-pretreated CHO cells.

While the total cell-bound [^{125}I]ricin radioactivities were similar for CHO cells incubated with [^{125}I]ricin at 0 and 37°C (Fig. 1, 2c, and 2d; Table 2), the amount of [^{125}I]ricin remaining at the surface of CHO cells at 37°C was much less than that at 0°C (cf. Fig. 2e and f; Table 2). These results suggest the possibility that concomitant to the internalization of ricin, the sur-

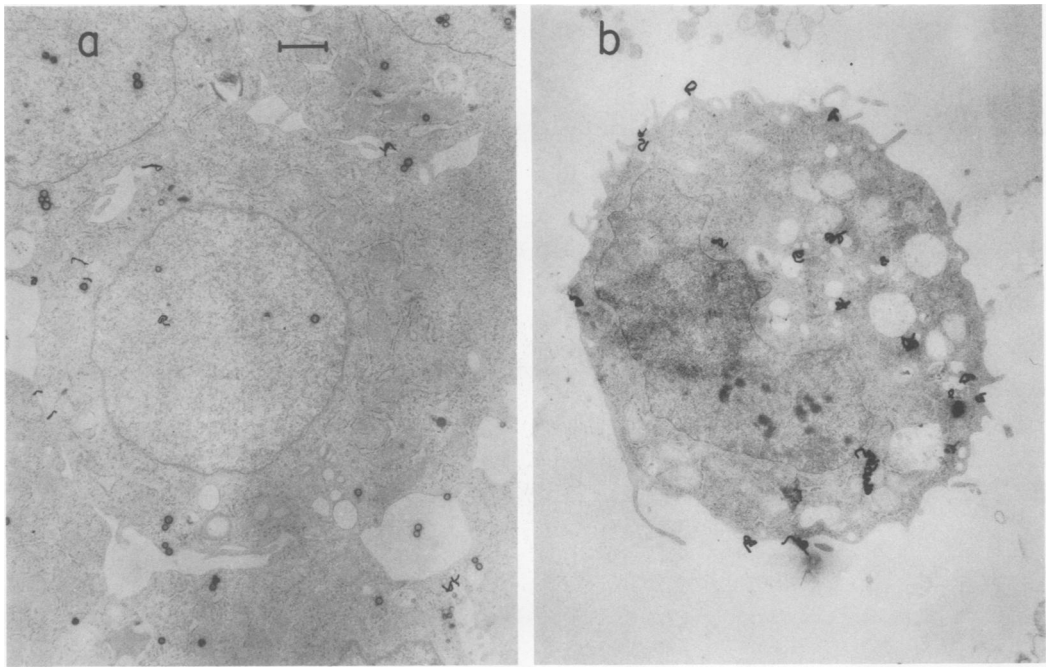

FIG. 8. *Electron microscopic autoradiograph of a thin section of control (a) and nigericin-pretreated (b) CHO cells incubated at 37°C for 20 min with [^{125}I]ricin. Other details are the same as those described in the legend to Fig. 7.*

TABLE 2. *Binding and internalization of [^{125}I]ricin in control and nigericin-pretreated cells as measured by electron microscopic autoradiography*[a]

Cells	Location of [^{125}I]ricin	Incubated at 0°C		Incubated at 37°C	
		No. of grains/cell	%	No. of grains/cell	%
Untreated	Total	13 ± 4	100	11 ± 3	100
	Inside cell	1 ± 1	7	4 ± 1	37
	At cell surface	12 ± 2	93	7 ± 2	63
Nigericin pretreated	Total	17 ± 5	100	15 ± 4	100
	Inside cell	1 ± 1	6	9 ± 2	60
	At cell surface	16 ± 3	94	6 ± 2	40

[a] The numbers of cells counted were as follows: 28 and 34 untreated cells incubated at 0 and 37°C, respectively; 35 and 40 cells pretreated with nigericin (10 nM, 72 h, 37°C) and incubated at 0 and 37°C, respectively.

face receptors for ricin are internalized. This would be consistent with previous observations on the internalization of ricin receptors in various cell lines (2, 6, 7).

The exact mechanism by which nigericin pretreatment potentiates the internalization of ricin remains unknown. The fact that this effect requires preculture of CHO cells in media containing nigericin strongly suggests that the facilitated uptake of [^{125}I]ricin into CHO cells is an indirect effect of nigericin rather than its direct effect as an ionophore. The requirement of preculture of CHO cells in nigericin-containing media suggests an alteration in the cell surface architecture and/or cell surface-cytoskeleton interaction so that the internalization of receptor-bound ricin becomes more efficient.

The presumption of a common step in the internalization of ricin and *Pseudomonas* toxin provides the rationale for the isolation of CHO mutant cell lines simultaneously resistant to both toxins by a single-step selection. Among these Ric^r Pst^r mutants might be found those defective in the internalization of ricin. This was indeed the case. We have isolated and partially characterized CHO mutants resistant to both ricin and *Pseudomonas* toxin at frequencies which suggest a single-step mutation. Two of these mutants bind normal amounts of [^{125}I]ricin at both 0 and 37°C, but have reduced internalization of [^{125}I]ricin at 37°C as compared with that of the parental strain. Both mutants retain the same sensitivity toward diphtheria toxin as in the parental strain. More interestingly, the cytotoxicities of ricin and *Pseudomonas* toxin in both mutants are not enhanced by the pretreatment of nigericin (Ray and Wu, manuscript in preparation). These results are consistent with our working hypothesis that nigericin treatment results in a facilitated internalization of both ricin and *Pseudomonas* toxin into CHO cells.

ACKNOWLEDGMENTS

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