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TEMPERATURE- AND DOSE-DEPENDENT INTERNALIZATION OF CONCANAVALIN A IN MONOLAYER CULTURE

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SUMMARY: When rat hepatoma cells (R117-21B) were incubated for 20 h at 37°C with ^{125}I-labeled concanavalin A at low concentrations (0.5-10 μg/ml), only 20-30 % of the cell-associated radioactivity was released by α-methyl-D-mannoside, but at high concentrations (50-500 μg/ml), 60-80 % of the cell-associated radioactivity was released. At 4°C, the cell-associated radioactivity decreased with the increase in concentration of concanavalin A, and more than 80 % of the cell-associated radioactivity was released by α-methyl-D-mannoside. These results suggest that the amount of cell-associated concanavalin A is related to the physicochemical state of the plasma membrane, which can be altered by the incubation temperature or by the concentration of concanavalin A, the transitional concentration being 5-10 μg/ml.

It is well known that concanavalin A (Con A) binds to sugar moieties contained not only in proteins, but also on the cell surface (1-5), and that Con A mimics the effect of insulin on the regulation of certain enzymes in cultured cells (6-9). In our study (10,11), Con A elicited insulin-like effect on the induction of tyrosine aminotransferase activity in rat hepatoma cells (R117-21B and R-Y121B). These findings suggest that Con A and insulin may share the same binding sites. In fact, the pretreatment of cells with Con A decreased the binding of insulin (12-14), although in certain cases the latter addition of wheat germ agglutinin or Con A increased the binding of insulin. In addition, the inhibitory effect of Con A on insulin binding was observed with solubilized insulin receptors (15). Recently, however, we showed that the effects of Con A depended on the order of addition of Con A and insulin to the incubation medium in which cells were cultured (16).

In the present study, the binding of Con A to R117-21B cells cultured in a monolayer has been investigated in detail, and it has been

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shown that there is temperature- and dose-dependent accumulation of Con A on the cell surface after the long-term incubation of cells at high Con A concentrations.

MATERIALS AND METHODS

Chemicals. Con A and actinomycin D were purchased from Calbiochem (San Diego and La Jolla, Calif., U.S.A.). Cytochalasin B was from Sigma Chem. Co. (St. Louis, Mo., U.S.A.). Colchicine was from E. Merck (Darmstads, Germany). Cycloheximide was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Alpha-methyl-D-mannoside, potassium cyanide were from Nakarai Chemicals, Ltd. (Kyoto, Japan). Con A labeled with ^{125}I (~40 μCi/μg) was purchased from New England Nuclear (Boston, Mass., U.S.A.).

Cell culture. A line of rat hepatoma cells (R117-21B) (17) was established from Reuber rat hepatoma cells (H4-II-E) (18), and used for the previous studies on the induction of tyrosine aminotransferase activity (19), and on the metabolism of the thyroid hormones (20). Cell cultivation procedures were described in detail in these previous papers. The cells were maintained in glass culture flasks in a modified Eagle's minimum essential medium containing 0.5 % fetal bovine serum, and for experiments, cells were inoculated in 35-mm plastic culture dishes (Corning, N.Y., U.S.A.).

Binding assay of Con A. The method used in the binding assay of Con A to cells cultured in a monolayer was basically the same as the previously reported method used for the binding assay of insulin (19). After cells reached confluence, they were incubated with labeled Con A in 1 ml of the incubation medium containing 0.5 % bovine serum albumin instead of fetal bovine serum, and then washed twice with ice-cold phosphate-buffered saline, pH 7.4, without Ca^{2+} and Mg^{2+}.

RESULTS

When R117-21B cells cultured in monolayer were incubated with ^{125}I-labeled Con A at both 4°C and 37°C, the amount of cell-associated Con A increased with time and did not reach a plateau after 1 h of incubation, but did at 12 h. Even at very high Con A concentration (1 mg/ml) the cell-associated radioactivity was approximately 10 % of the total radioactivity in the dish after 24 h of incubation at 37°C (data not shown). This result shows that Con A binding at 1 mg/ml was not saturated, although the amount of total cellular protein per dish was less than 1 mg.

In order to estimate the amount of nonspecific binding of Con A to cells cultured in a monolayer, R117-21B cells were incubated with Con A under various conditions. The incubation period with Con A was a 30 min or 60 min to reduce the amount of Con A internalized by cells. When Con A and α-methyl-D-mannoside were incubated together with cells, Con A binding decreased with increase in mannoside concentration and was less than 2 % of the total Con A at 10 mM α-methyl-D-mannoside (Table 1). The effect of

Table 1. Effects of α-methyl-D-mannoside on Con A binding to cells

Condition	Cell-associated radioactivity (cpm/dish)	Percent of total	Percent of control
Con A + mannoside (30 min)			
0 (mM)	5,460 ± 90	13.4	100
1	2,700 ± 300	6.6	49
10	760 ± 30	1.9	14
50	480 ± 40	1.2	9
100	470 ± 10	1.2	9
Mannoside (30 min), then Con A (30 min)			
0 (mM)	4,370 ± 70	10.7	100
1	2,300 ± 400	5.7	53
10	630 ± 30	1.5	14
50	370 ± 10	0.9	8
100	350 ± 10	0.9	8
Con A (30 min), then mannoside (30 min)			
0 (mM)	7,600 ± 100	18.7	100
1	5,890 ± 10	14.5	78
10	4,200 ± 100	10.3	55
50	2,910 ± 10	7.2	38
100	2,900 ± 30	7.1	38

Cells (3.4×10^6 cells/dish) were incubated with Con A and α-methyl-D-mannoside at 25°C. The incubation medium consisted of 0.8 ml of culture medium containing 0.5 % bovine serum albumin, 0.1 ml of ^{125}I-labeled Con A (40,700 cpm, 1 μg) and 0.1 ml of α-methyl-D-mannoside at various concentrations. The final volume of the incubation medium was 1 ml. The value given is the mean ± S.D. for the values of three culture dishes.

α-methyl-D-mannoside on Con A binding to cells reached a plateau at 50 mM. Similar results were obtained from the experiments in which α-methyl-D-mannoside was incubated with cells before Con A addition. On the other hand, when cells were first incubated with Con A, a large portion of cell-associated radioactivity was not released by 100 mM α-methyl-D-mannoside, although the incubation time of Con A with cells was 60 min. In our previous study, after 1 h of incubation of cells at 25°C, approximately 30 % of cell-associated Con A was not released by 50 mM α-methyl-D-mannoside (10).

Con A binding in the presence of 50 mM α-methyl-D-mannoside added before Con A might represent nonspecific binding of Con A to cells. However, since this value was small (∿1 % of the total radioactivity in the dish) compared with that in the absence of the mannoside, and since we did not know the degree of distribution of this value to the released and unreleased radioactivities from the cells by the mannoside, original values were shown throughout the present study.

When the binding of Con A to cells was carried out at various initial concentrations of Con A at 4°C, the ratio of cell-associated Con A to total Con A in the dish decreased with the increase in the concentration of Con A (Fig. 1, left panels). When the cells were incubated with Con A at 37°C, the ratio of cell-associated Con A to total Con A in the dish rose with concentration at low Con A concentrations (<1-5 μg/ml), but fell at high (>1-5 μg/ml). This phenomenon was observed at 37°C, but not at 4°C as shown in Fig. 1.

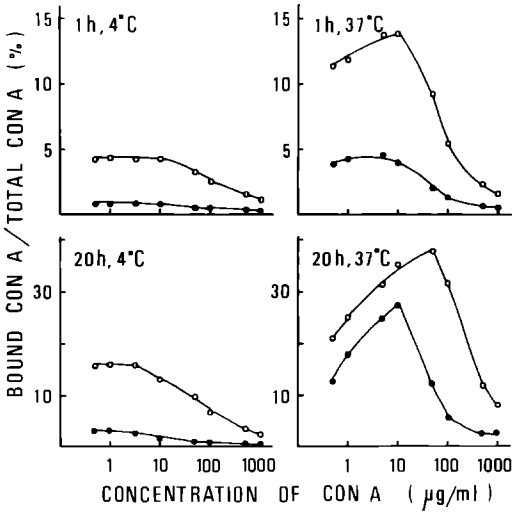

Fig. 1. The binding of ^{125}I-labeled Con A to Rl17-21B cells as a function of initial Con A concentration. The cells were incubated with ^{125}I-labeled Con A at various concentrations for 1 h at 4°C or 37°C. (○—○), Con A binding was measured without α-methyl-D-mannoside; (●—●), after the treatment of the cells with Con A, the incubation medium was replaced with fresh culture medium containing α-methyl-D-mannoside (50 mM), and then the cells were incubated for 30 min at 37°C. The release of Con A from the cells was carried out at 37°C in every case, since it is extremely slow at 4°C (10). The total radioactivity of labeled Con A in the dish was 55,400 cpm. The number of cells was 3.3 x 10^6 cells per dish. The value given is the mean for the values of two culture dishes.

When the cells were incubated with Con A at 37°C for 20 h at various concentrations of Con A, the highest cell-associated radioactivity (40 % of the total) was found at an initial concentration of 50 μg/ml (Fig. 1, lowe right panel). A large portion of the cell-associated radioactivity, obtained at 0.5-10 μg/ml Con A, was not released by α-methyl-D-mannoside, nor was it released by a large excess of unlabeled Con A. After α-methyl-D-mannoside treatment, the highest cell-associated radioactivity remaining was found at an initial concentration of 10 μg/ml Con A.

The data shown in Fig. 2 are expressed as the ratio of the radioactivity released by α-methyl-D-mannoside to total cell-associated radioactivity; the radioactivity released from the cells might represent Con A on the cell surface, and the unreleased radioactivity might represent internalized Con A. After the incubation of cells with Con A for 1 h or 20 h at 4°C, 80 % of the total cell-associated radioactivity was released from the cells by α-methyl-D-mannoside (Fig. 2). The incubation of cells with Con A at 37°C, however, decreased the amount of radioactivity released by the mannoside, and this temperature effect was greater after 20 h of incubation (Fig. 2, right panel). At 1-10 μg/ml of Con A at 37°C, more than 75 % of the total cell-associated radioactivity still remained in the cells

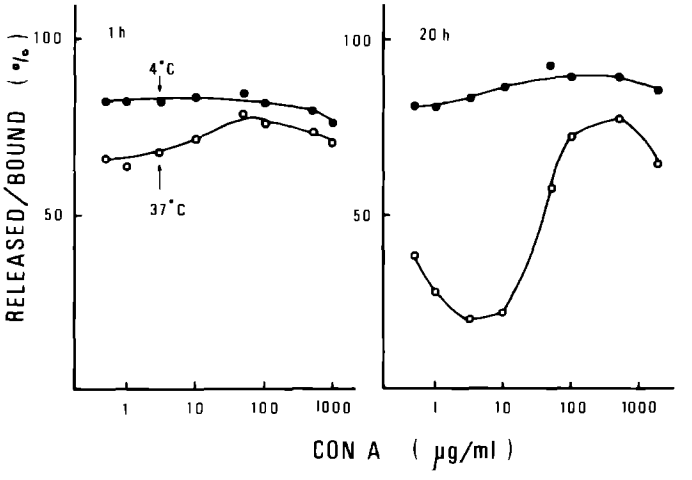

Fig. 2. The ratio of Con A released from the cells by α-methyl-D-mannoside to total cell-associated radioactivity. Other information is described in the legends to Fig. 1.

Table 2. Con A binding to cells and its internalization under various conditions.

Condition	5 μg/ml Con A			50 μg/ml Con A		
	Released radioactivity (cpm)	Internalized radioactivity (cpm)	Total cellular radioactivity (cpm)	Released radioactivity (cpm)	Internalized radioactivity (cpm)	Total cellular radioactivity (cpm)
None	6,500 ± 300	15,400 ± 700	21,900 ± 1,000	6,800 ± 400	4,420 ± 30	11,200 ± 400
Cycloheximide (5 μg/ml)	8,200 ± 300	12,400 ± 100	20,500 ± 400	6,000 ± 200	3,690 ± 20	9.700 ± 200
Actinomycin D (5 μg/ml)	6,800 ± 200	13,800 ± 100	20,600 ± 200	6,200 ± 100	4,000 ± 200	10,200 ± 300
Cytochalasin B (5 μg/ml)	8,400 ± 400	14,100 ± 500	22,500 ± 200	7,800 ± 400	4,300 ± 400	12,100 ± 900
Colchicine (5 μg/ml)	6,300 ± 100	15,500 ± 700	21,800 ± 700	6,100 ± 300	4,100 ± 200	10,100 ± 200
KCN (10 mM)	10,200 ± 400	11,300 ± 500	21,500 ± 500	7,100 ± 300	3,500 ± 200	10,600 ± 300
Dinitophenol (1 mM)	16,500 ± 200	3,600 ± 100	20,100 ± 300	9,100 ± 100	1,400 ± 200	10,500 ± 300

After incubating the cells with Con A for 10 h at 37°C in the presence or absence of the drug, the incubation medium was replaced with fresh medium containing α-methyl-D-mannoside (50 mM) after washing the cells, and then the cells were incubated for 30 min at 37°C. The number of cells was 1.8×10^6 per dish. The total radio-activity in the dish was 56,800 cpm. The value given is the mean ± S.D. for the values of three cultured dishes.

even after α-methyl-D-mannoside treatment. On the other hand, in the cells treated with Con A at high concentrations (50-100 μg/ml), the released cell-associated radioactivity increased.

The total cell-associated radioactivity due to ^{125}I-labeled Con A bound to the cell surface and internalized was almost independent of various drugs such as cycloheximide, actinomycin D, cytochalasin B, colchicine, KCN and dinitrophenol. These results indicate that the number of Con A binding sites was almost constant under these conditions, even though the cells were incubated with Con A for 10 h at 37°C in the presence of the drug (Table 2). However, dinitrophenol and KCN clearly decreased the cell-associated radioactivity (30-70 % of the control) due to internalized Con A after 10 h of incubation at 37°C as compared with cycloheximide, actinomycin D and cytochalasin B which caused a decrease of less than 10 %.

Labeled Con A was analyzed by gel filtration chromatography using Sephadex G-100 in 0.1 % sodium dodecyl sulfate. Original Con A, which was not incubated with cells, showed two peaks due to the α subunit and β subunit (21,22), and a small iodide peak was observed (Fig. 3). After

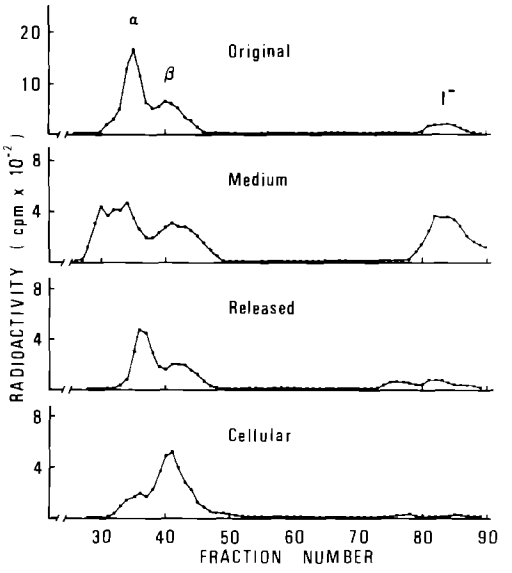

Fig. 3. Elution profiles of ^{125}I-labeled Con A after incubation with Rl17-2lB cells. The cells (2.4 x 10^6 cells per dish) were incubated with labeled Con A (10 μg/ml, 44,500 cpm) for 20 h at 37°C. Original, Con A without incubation with the cells; Medium, Con A after incubation with the cells; Released, Con A released from the cells by α-methyl-D-mannoside incubated for 30 min at 37°C; Cellular, after the treatment of the cells with α-methyl-D-mannoside, the cells were homogenized in H_2O containing 50 mM α-methyl-D-mannoside, and the radioactivity in the supernatants, after centrifugation at 100,000 g for 1 h, represents "Cellular". Gel filtration column (1 x 49 cm), with Sephadex G-100 was used. The elution velocity was 3.5 ml/h, and each fraction contained 0.5 ml. The elution buffer was phosphate buffered saline, pH 7.4, containing 0.1 % sodium dodecyl sulfate.

the incubation of cells with Con A at 37°C for 20 h, the radioactivity due to the last peak increased in the sample of the incubation medium, and the component eluting faster than the first peak (α subunit) also increased slightly. The elution profile of the released radioactivity was quite similar to that of the original, except that a small peak appeared before the last peak (iodide). In the cell-associated radioactivity which remained in the cells after the treatment with α-methyl-D-mannoside, the second peak (β subunit) was larger than the first. These data indicate that a large portion of the radioactivity in the cells was still Con A even after 20 h of incubation at 37°C. Consistent results were obtained from the experiments in which an increase in trichloroacetic acid-soluble radioactivity was measured after various treatments of labeled Con A with the cells at 37°C (data not shown).

DISCUSSION

Recently, Kammer and Burger (23) suggested that Con A bound to cells with three different affinities, and that the Con A unreleased from the cells by α-methyl-D-mannoside bound very strongly to cell surfaces. If there are different types of Con A binding to cells, we might expect a typical curve in Scatchard analysis as obsreved in insulin binding to cells (24). However, Scatchard plot of Con A binding to rat hepatoma cells at 25°C or 37°C was apparently linear (25), although two types of Con A binding sites on the cell surface was observed at 4°C both in rat hepatoma cells (unpublished data) and in human fibroblasts (26). Imamura et al. (27) showed by electron microscopy and with a method using ^{125}I-labeled Con A that Con A was internalized readily at 37°C. Since α-methyl-D-mannoside rapidly disrupts the binding of Con A to cells at 37°C (10), the remaining cell-associated radioactivity after mannoside treatment probably represents Con A located inside the cells (internalized Con A).

The increase in the ratio of Con A bound to cells to total Con A in the dish with the increase in Con A concentration was temperature- and dose-dependent in R117-21B cells. The former phenomenon was also observed in isolated fat cells (1) and liver plasma membranes (28,29). Internalization of insulin and epidermal growth factor in cultured fibroblasts took place at 23°C or 37°C, but not at 4°C (30). Various receptors need to move to form the cap on the cell surface, and the cap formation does not take place at low temperature (<10°C) (31,32). In the present study, the incubation of cells with Con A at 4°C did not show either cooperative binding or internalization of Con A (Figs. 1 and 2). These phenomena are due to structural changes in the plasma membranes.

The ratio of released radioactivity to total cell-associated radioactivity, varied with Con A concentrations at 37°C. If internalization or pinocytosis of Con A into cells takes place at random, the ratio of released radioactivity to total cell-associated radioactivity should be constant at various concentrations. The differences in the ratio at various

concentrations were very small in cells incubated at 4°C with Con A (Fig. 2). When cells were incubated with Con A (0.5-1000 μg/ml) at 37°C for 20 h and then treated with α-methyl-D-mannoside, the ratio of released radioactivity to total cell-associated radioactivity varied from 20 to 70 % for the different concentrations. The results indicate that the internalization of Con A was very active at low concentrations of Con A (1-10 μg/ml). When cells, treated with Con A, were scraped from plastic culture dishes with a silicone-rubber policeman, the cell membranes were broken and the cytoplasm was released (11). This Con A effect reached a plateau above 10 μg/ml. In addition, it has been shown that Con A inhibits cap formation of various receptors including those for Con A itself (33), and that this lectin inhibits phagocytosis by polymorphonuclear leucocytes (34). These results suggest that Con A restricts receptor motility on the cell surface. In addition, we showed that Con A at high concentrations (>50 μg/ml) increased insulin internalization into R-Y121B cells (16). Judging from the present and previous data, the physicochemical state of the plasma membrane might be different at low and high Con A concentrations in the incubation medium, the transitional concentration being 5-10 μg/ml.

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REFERENCES

1. Cuatrecasas, P. (1973) Biochemistry 12, 1312-1323.
2. Arndt-Jovin, D.J. and Berg, P. (1971) J. Viol. 8, 716-721.
3. Noonan, K.D. and Burger, M.M. (1973) J. Biol. Chem. 248, 4286-4292.
4. Cline, M.J. and Livingston, D.C. (1971) Nature New Biol. 232, 155-156.
5. Ozanne, B. and Sambrook, J. (1971) Nature New Biol. 232, 156-160.
6. Cuatrecasas, P. and Tell, G.P.E. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 485-489.
7. Katzen, H.M., Vicario, P.P., Munford, R.A. and Green, B.G. (1981) Biochemistry 20, 5800-5809.
8. Shechter, Y. and Sela, B.-A. (1981) Biochem. Biophys. Res. Commun. 98, 367-373.
9. Smith, J.D. and Liu, A.Y.-C. (1981) Science 214, 799-800.
10. Sorimachi, K. and Yasumura, Y. (1983) Jpn. J. Exp. Med. 53, 33-39.
11. Sorimachi, K. and Yasumura, Y. (1983) Exp. Cell Res. 149, 307-310.
12. Cuatrecasas, P. (1973) J. Biol. Chem. 248, 3528-3534.
13. Blackard, W.G., Small, E. and Ludeman, C. (1980) Metabolism 29, 691-697.

14. Capeau, J., Picard, J. and Caron, M. (1978) Cancer Res. 38, 3930-3937.
15. Hedo, J.A., Harrison, L.C. and Roth, J. (1981) Biochemistry 20, 3385-3393.
16. Sorimachi, K. and Yasumura, Y. (1984) Biochem. Biophys. Res. Commun. in press.
17. Niwa, A., Yamamoto, K. and Yasumura, Y. (1979) J. Cell. Physiol. 98, 177-184.
18. Pitot, H.C., Peraino, C., Morse, P.A., Jr. and Potter, V.R. (1964) Natl. Cancer Inst. Monogr. 13, 229-245.
19. Sorimachi, K. and Yasumura, Y. (1981) J. Biochem. 90, 1197-1204.
20. Sorimachi, K., Niwa, A. and Yasumura, Y. (1980) Biochim. Biophys. Acta 630, 469-475.
21. Wang, J.L., Cunnigham, B.A. and Edelman, G.M. (1971) Natl. Acad. Sci. U.S.A. 68, 1130-1134.
22. Abe, Y., Iwabuchi, M. and Ishii, S. (1971) Biochem. Biophys. Res. Commun. 45, 1271-1278.
23. Kammer, K. and Burger, M.M. (1983) Eur. J. Biochem. 132, 433-439.
24. De Meyts, P. (1980) Horm. Cell Regul. 4, 107-121.
25. Sorimachi, K. (1983) Cell Struct. Funct. 8, 327-338.
26. Feller, M., Richardson, C., Benke, W.D. and Gruenstein, E. (1977) Biochem. Biophys. Res. Commun. 76, 1027-1035.
27. Imamura, T., Beppu, M., Terao, T. and Osawa, T. (1981) Cell Struct. Funct. 6, 1-10.
28. Chandromouli, V., Williams, S., Marshall, S.J. and Carter, J.R., Jr. (1977) Biochim. Biophys. Acta 465, 19-33.
29. Costlow, M.E. and Gallagher, P.E. (1979) Biochim. Biophys. Acta 587, 192-201.
30. Schlessinger, J., Shechter, Y., Willingham, M.C. and Pastan, I. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2659-2663.
31. Taylor, R.B., Duffus, P.H., Raff, M.C. and de Petris, S. (1971) Nature New Biol. 233, 225-229.
32. Yahara, I. and Edelman, G.M. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 608-612.
33. Yahara, I. and Edelman, G.M. (1973) Exp. Cell Res. 81, 143-155.
34. Berlin, R.D. (1972) Nature New Biol. 235, 44-45.