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Ricin A-chain induces fusion of small unilamellar vesicles at neutral pH

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The interaction of ricin and its constituent polypeptides, the A- and B-chain, with small unilamellar vesicles of dipalmitoylphosphatidylcholine (DPPC) or dimyristoylphosphatidylcholine (DMPC) was investigated by means of differential scanning calorimetry measurements. The A-chain, at neutral pH, entirely shifted the endothermic peak of small unilamellar vesicles of DPPC from 37°C to 41°C at low protein/lipid ratios. The potency of either ricin or the B-chain to induce the shift of endothermic peak was much less than that of the A-chain. The A-chain was also found to cause mixing of endothermic peaks of DMPC vesicles and DPPC vesicles. These data strongly suggest that the A-chain has the ability to induce fusion of phospholipid vesicles.

Ricin; Protein-lipid interaction; Phospholipid vesicle; Fusion

1. INTRODUCTION

Ricin is a toxic lectin present in castor bean (*Ricinus communis*) seeds, which is composed of two polypeptide chains, A and B, linked by a single disulfide bridge [1]. The A-chain inhibits protein synthesis in eukaryotic cells by inactivating 60 S ribosomal subunits [2,3]. The B-chain binds divalently to galactose or *N*-acetylgalactosamine containing receptors on the cell surface and promotes the endocytosis of ricin [4].

Toxins with intracellular sites of action, like ricin and diphtheria toxin, are excellent model proteins for studying transmembrane lipid-protein interactions that occur during the internalization of physiologically active molecules.

While toxicity of ricin against susceptible cells is well characterized biochemically [5–8], the molecular mechanism of its passage into cytoplasm remains obscure. In manifesting the toxicity against cells, it is essential for either ricin or the A-chain to traverse the membrane barrier into the cytosol. However, very little information exists relating ricin structure to its ability to traverse membrane bilayer. Recently, we have reported that ricin did not interact with membranes, whereas isolated A- and B-chains effectively associated with phospholipid vesicles, and the A-chain induced a remarkable increase in membrane permeability [9].

These data suggested that the A-chain, in contrast to the B-chain and intact ricin, possesses the ability to penetrate the lipid bilayer and decrease membrane stability.

In this report, we present evidence that A-chain is able to induce, at neutral pH, fusion of small unilamellar vesicles that result probably from a strong perturbation of the lipid bilayer.

2. MATERIALS AND METHODS

2.1. *Materials*

Ricin and its constituent polypeptide chains (i.e., the A-chain and the carboxymethylated B-chain (CM-B-chain) were isolated by methods established in our laboratory [1,10,11] and were homogeneous by SDS-polyacrylamide gel electrophoresis. In the preparation of CM-B-chain, ricin was reduced with β-

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mercaptoethanol in the absence of denaturant and then carboxymethylated [1]. The obtained CM-B-chain was limitedly carboxymethylated at one cysteine residue which participated in the interchain disulfide bridge of ricin [1], and retained full binding ability to sugars.

Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) were purchased from Sigma. All reagents used were of analytical grade.

2.2. *Preparation of small unilamellar vesicles*

DPPC or DMPC (30 μmol) in 2.0 ml of a chloroform/methanol (2:1, v/v) mixture was dried under vacuum in a conical glass tube. The dried lipid was hydrated with 3 ml of 0.1 M NaCl/20 mM phosphate buffer, pH 7.4 (PBS), by repeated vortex-mixing at 50°C for 15 min. The suspension was sonicated at 50°C for 30 min using a Branson model W-185 sonifier and then centrifuged at 1500 × g for 10 min to remove titanium particles and residual multilamellar vesicles.

2.3. *Differential scanning calorimetry (DSC) measurements*

Lipid vesicles (10 μl) were mixed with 10 μl of an appropriate dilution of polypeptide in PBS, incubated at 20°C for 10 min and then sealed in aluminum pans. A reference sample was similarly prepared using 20 μl of PBS.

Measurements were carried out on a Rigaku Electric Co. DSC 8230 apparatus operating at a heating rate of 5°C/min. Differential scanning calorimetry thermograms were obtained in the temperature range from 10°C to 60°C.

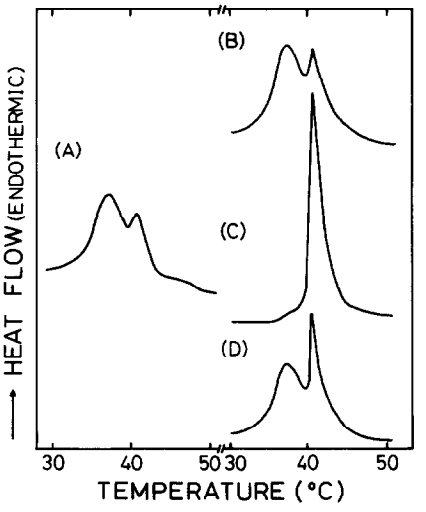

Fig.1. Effect of ricin, ricin A-chain or CM-B-chain on the DSC pattern of small unilamellar vesicles of DPPC. (A) DSC pattern of small unilamellar vesicles of DPPC in the absence of polypeptide. (B–D) DSC patterns of small unilamellar vesicles of DPPC in the presence of ricin (D), ricin A-chain (B) or CM-B-chain (C). Polypeptide (3 μM) was mixed with 7.5 mM of DPPC vesicles and incubated at 25°C for 10 min. Measurements were carried out in the temperature range from 10°C to 60°C at a heating rate of 5°C/min.

3. RESULTS AND DISCUSSION

3.1. *Membrane fusion of small unilamellar vesicles of DPPC monitored by DSC*

The DSC pattern of freshly prepared DPPC vesicles showed a main transition at 37°C associated with small unilamellar vesicles [12] and a minor transition at 41°C associated with larger vesicular structures resulting from spontaneous destabilization of the small vesicles (fig.1A).

As shown in fig.1B, this DSC pattern of DPPC vesicles was not modified by the addition of 3 μM ricin at pH 7.4. In contrast, addition of 3 μM A-chain entirely shifted the endothermic peak from 37°C to 41°C (fig.1C). 3 μM CM-B-chain, however, caused only a slight decrease of the 37°C peak and a little increase of the 41°C peak (fig.1D). This indicates that the A-chain is able to induce fusion of small unilamellar vesicles.

The area under the 37°C endothermic peak was measured and plotted as a function of the concentration of the polypeptides (fig.2). The concentration of the A-chain required to induce 50% decrease of the 37°C peak was approx. 2 μM. The A-chain/DPPC molar ratio in this case is equal to 1:3500 and this represents 1.3 molecules of A-chain/vesicle (assuming 4500 molecules of phospholipid/vesicle). Almost complete disappearance of the 37°C peak was achieved at concentrations higher than 3 μM. In contrast to this, the potency of either CM-B-chain or ricin to induce the shift of endothermic peak was much less than that of the A-chain. The evidence indicates that the induction of fusion of DPPC vesicles is due to the specific nature of the A-chain.

3.2. *Effect of the A-chain on the membrane mixing of DMPC and DPPC small unilamellar vesicles*

To confirm the potency of the A-chain to induce fusion of phospholipid vesicles, the effect of the A-chain on the membrane mixing of DMPC and DPPC small unilamellar vesicles was examined. While DPPC vesicles showed endothermic peaks at 37°C and 41°C, the DSC pattern of freshly prepared DMPC vesicles showed a main transition at 19.5°C and a minor transition at 23.5°C (fig.3A,B).

When these two vesicle preparations were mixed

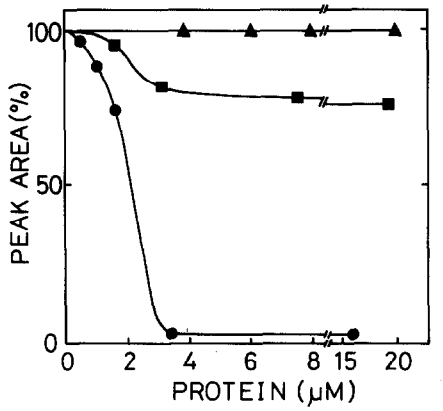

Fig.2. The decrease of the peak area of the endothermic peak at 37°C of DPPC vesicles as a function of polypeptide concentration. Different concentrations of polypeptide were mixed with 7.5 mM of DPPC vesicles and incubated at 25°C for 10 min. The area under the 37°C endothermic peak was measured and plotted as a function of polypeptide concentration. Experimental conditions were the same as in fig.1. (▲) Ricin; (●) the A-chain and (■) CM-B-chain.

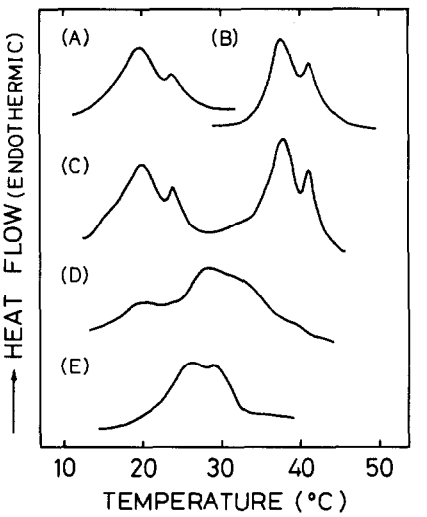

Fig.3. Effect of the A-chain on the membrane mixing of DMPC and DPPC small unilamellar vesicles. (A,B) Small unilamellar vesicles of DMPC (A) and of DPPC (B); (C) an equimolar mixture of DMPC vesicles and DPPC vesicles; (D) C + the A-chain (6 μM); (E) vesicles prepared by sonication of an equimolar mixture of DMPC and DPPC. Lipid concentration in (A) and (B) was 6 mM. Total lipid concentration in (C–E) was 12 mM. Experimental conditions were the same as in fig.1.

in equimol (6 mM), each of these endothermic peaks was detected independently as shown in fig.3C. Addition of 6 μM A-chain to this mixture caused a remarkable decrease in each endothermic peak and a formation of a new component with a broad endothermic peak of 28.5°C (fig.3D). As shown in fig.3E, this newly formed endothermic peak was similar to that of vesicles prepared by sonication of an equimolar (6 mM) mixture of the two lipids. These results strongly suggest that the A-chain induces fusion of DMPC and DPPC unilamellar vesicles.

Using DPPC vesicles, we found that ricin did not interact with the vesicles, whereas isolated A- and B-chains effectively associated with them. However, only the A-chain induced a striking increase of the membrane permeability at the phase transition temperature (T_m) of DPPC vesicles [9]. We also found that the A-chain, in contrast to the B-chain and intact ricin, induced a marked increase in the turbidity of DPPC vesicles when the temperature was raised through the T_m of the vesicles (not shown). These observations indicate that perturbation of the membrane bilayer might be a prerequisite for the induction of membrane fusion.

From the quantitative analysis of hydrophobicity of ricin and its constituent polypeptides, it was revealed that the A-chain molecule has a fairly large hydrophobic region(s) when compared with the B-chain or intact ricin [13]. The specific interaction of the A-chain with the lipid bilayer of DPPC vesicles seems to occur through these hydrophobic regions on the surface of the A-chain molecule.

Membrane fusion induced by the A-chain would be the consequence of the destabilization of the lipid bilayer of phospholipid vesicles. Whether or not the phenomenon by itself is involved in the translocation process has not been elucidated. Diphtheria toxin, which requires an acidic environment for internalization [14,15], was found to induce fusion of DPPC vesicles only at acidic pH [16]. Recently, from several experimental results [5,7,14], it was proposed that the acidic environment might not be required for ricin internalization.

These observations, taken together with our present findings, suggest that the potency of the A-chain to induce fusion of phospholipid vesicles at neutral pH might be related to the transmembrane lipid-protein interaction that occurs during the internalization of ricin.

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REFERENCES

[1] Funatsu, G. and Funatsu, M. (1977) Agric. Biol. Chem. 41, 1211–1215.
[2] Sperti, S., Montanaro, L., Mattioli, A. and Stirpe, F. (1973) Biochem. J. 136, 813–815.
[3] Endo, Y., Mitsui, K., Motizuki, M. and Tsurugi, K. (1987) J. Biol. Chem. 262, 5908–5912.
[4] Olsnes, S., Refsnes, K. and Pihl, A. (1974) Nature 249, 627–631.
[5] Sandvig, K. and Olsnes, S. (1982) J. Biol. Chem. 257, 7495–7513.
[6] Wellner, R.B., Ghosh, P.C. and Wu, H.C. (1984) J. Biol. Chem. 259, 12788–12793.
[7] Ghosh, P.C., Wellner, R.B., Cragoe, E.J. and Wu, H.C. (1985) J. Cell Biol. 101, 350–357.
[8] Moya, M., Dautry-Varsat, A., Goud, B., Louvard, B. and Boquet, P. (1985) J. Cell Biol. 101, 548–559.
[9] Utsumi, T., Aizono, Y. and Funatsu, G. (1984) Biochim. Biophys. Acta 772, 202–208.
[10] Hara, K., Ishiguro, M., Funatsu, G. and Funatsu, M. (1974) Agric. Biol. Chem. 38, 65–70.
[11] Funatsu, G., Miyauchi, S., Yoshizuka, N. and Funatsu, M. (1977) Agric. Biol. Chem. 41, 1217–1223.
[12] Suurkkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1976) Biochemistry 15, 1393–1401.
[13] Yamasaki, N., Nagase, Y. and Funatsu, G. (1988) Agric. Biol. Chem. 52, 1021–1026.
[14] Sandvig, K. and Olsnes, S. (1980) J. Cell Biol. 87, 828–832.
[15] Boquet, P., Silverman, M.S., Pappenheimer, A.M., jr and Vernon, W.B. (1976) Proc. Natl. Acad. Sci. USA 73, 4449–4453.
[16] Cabiaux, V., Vandenbranden, M., Falmagne, P. and Ruysschaert, J.M. (1984) Biochim. Biophys. Acta 775, 31–36.