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Rapid report

Membrane fusion mediated by ricin and viscumin

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Abstract

The ribosome inactivating plant proteins (RIPs) ricin and viscumin but not *Ricinus communis* agglutinin are able induce vesicle–vesicle fusion. A model is suggested in which the toxicity of the RIPs is partially determined by their fusogenicity. Herein, fusion is hypothesized to allow the RIPs to leak across endocytic vesicles to approve their access to cytoplasmic ribosomes. © 1998 Elsevier Science B.V.

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The lectins ricin and viscumin from *Ricinus communis* and *Viscum album* are known to inactivate ribosomes. These proteins, which are among the most potently toxic compounds [1] can be selectively targeted to specific cell types, such as cancer cells, by linking them to antibodies [2]. Their binding to cell surface receptors is followed by endocytosis [3]. It has been shown that the subsequent delivery into the cytosol across the membrane of an intracellular compartment not only requires ATP [4] and membrane proteins [5] but also depends on the retrograde transport from the Golgi complex to the endoplasmic reticulum (ER) [6]. The A-chain of these heterodimeric proteins is catalytically active whereas the B-chain binds oligosaccharide moieties. The concentrations required for 50% inhibition of cell-free protein synthesis in rabbit reticulocyte assay were estimated to be 1.5 [7], 5.5 and 12 ng/ml [8] for the A-chains of ricin, *R. communis* agglutinin (RCA) and viscumin, respectively. RCA is 2000-fold less toxic than ricin when injected intraperitoneally into mice [9] whereas viscumin is as effective as ricin [10]. The difference in cytotoxicity observed with different lectins does not simply reflect the difference in biological activity of the respective A-chains [7]. Rather, it appears to be due to differences in the protein–lipid interactions which in turn are crucial for the delivery of the A-chains into the cytosol of target cells [7].

Along with the A-chain of ricin other lectins, namely concavalin A [11] and annexin V [12] were reported to induce membrane fusion. In turn, the fusion event is known as an expedient means of accomplishing the passage into the cytoplasm [13]. Therefore, we have undertaken a comparative analy-

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sis of the fusion capabilities of ricin, viscumin and RCA in a model system where small unilamellar vesicles were fused with planar bilayer membranes (BLM).

Ricin and RCA were purified from *R. communis* seeds as described earlier [14]. Viscumin was isolated from *V. album* [15]. Different lectin isoforms were separated on a FPLC chromatograph (Pharmacia, Sweden) using Mono S HR column (5×5) with a linear NaCl gradient (0–500 mM) in 15 mM citric buffer, pH 4.2.

Protein-free liposomes containing nystatin were prepared as described by Woodbury and Miller [16]. Around 2 mg of a lipid mixture were dissolved in 0.2 ml chloroform to which 10 μl of nystatin stock solution (50 μg) had been added. The lipids used were: 45 mol% phosphatidyl-ethanolamine (PE); 9 mol% phosphatidyl-choline (PC); 18 mol% phosphatidyl-serine (PS) (all from Avanti Polar Lipids, Alabaster, Alabama); 18 mol% ergosterol and 10 mol% monosialoganglioside (GM1; both Sigma, Dreisenhofen, Germany). After evaporating 0.5 ml of a buffer solution (150 mM NaCl, 10 mM HEPES and 10 mM MES, pH 7.5) were given to the dry lipid film. The suspension was sonicated until its turbidity became minimal.

BLM were formed by a conventional method [17] in a hole, 0.6 mm in diameter, of a diaphragm dividing a Teflon-chamber. The membrane forming solutions contained 14 mg bacterial PE and 6 mg diphytanoyl-PC (Avanti Polar Lipids) per milliliter solvent. The latter was a mixture of *n*-decane, chloroform and methanol (7:2:1 by volume, all Merck, Darmstadt, Germany). The membrane was sur-

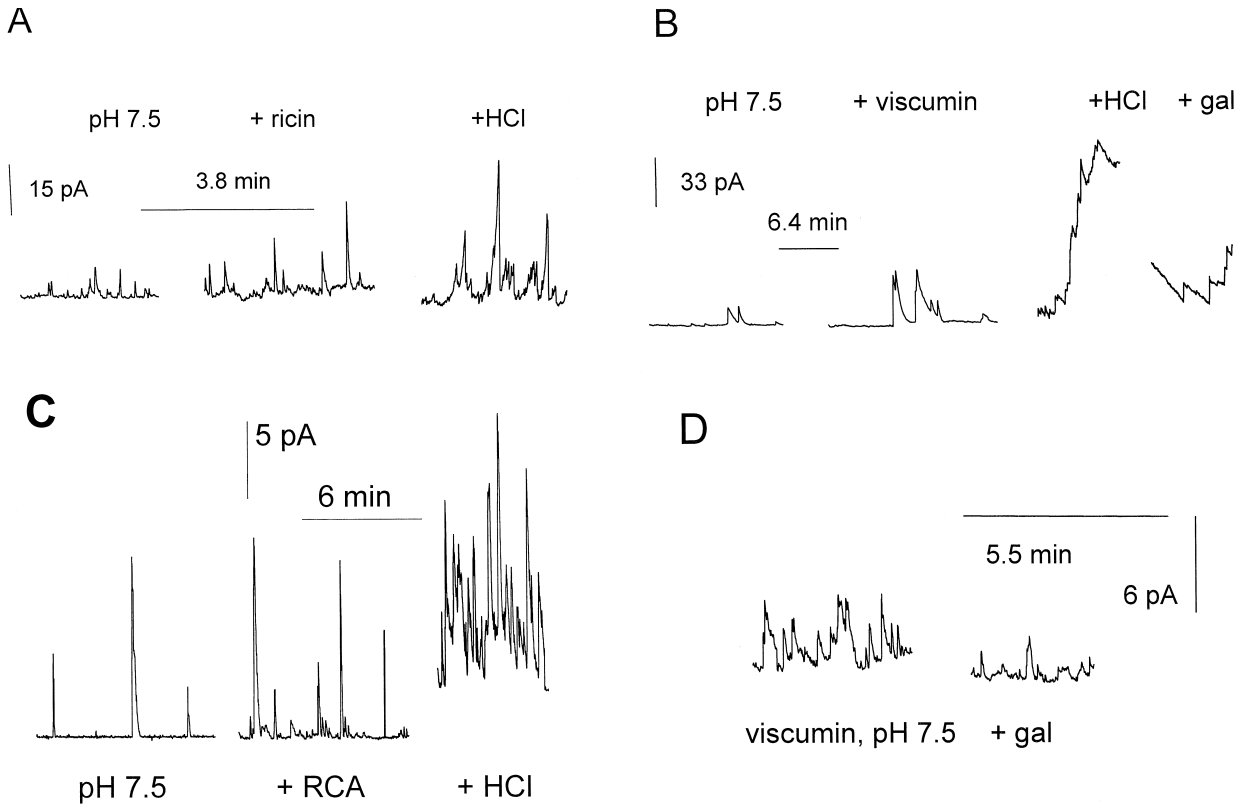

Fig. 1. The effect of ricin (A), viscumin (B), and *R. communis* agglutinin (C) on the liposome fusion with a planar lipid membrane at pH 7.5 and after a subsequent acidification to pH 5.2. Spikes in the current across ergosterol and GM1 free bilayers appeared after fusion with GM1 and nystatin containing liposomes. Both the vesicles and the proteins were added to the hypertonic side of the membrane. The solution was buffered with 10 mM HEPES and 10 mM MES. (A) A 750 to 150 mM NaCl transmembrane gradient was established. Lipid and ricin concentrations were 53 and 54 μg/ml, respectively. (B) NaCl gradient was 650/350 mM. Lipid and viscumin concentrations were 54 and 27 μg/ml. (C) NaCl gradient was 600/150 mM. Lipid and RCA concentrations: 80 and 26 μg/ml. (D) Inhibitory effect of galactose on the liposome fusion. NaCl gradient was 550/150 mM. Lipid and viscumin concentrations: 135 and 27 μg/ml, respectively.

rounded by the buffer solution used for liposome preparation. The solution was agitated by magnetic bars.

The vesicles were added to the *cis* side of the BLM. Their reliable and non-selective fusion to the planar membrane was achieved due to an increase of the *cis* salt concentration to 450–650 mM [16]. The fusion of every single vesicle with the BLM was detected as a sudden jump in the transmembrane current induced by the simultaneous insertion of many nystatin channels from the ergosterol containing liposome [16]. The increase in conductance was transient (first trace in Fig. 1, panel A), i.e., it decayed back to baseline before the next fusion event because ergosterol, required for the integrity of the nystatin pore, diffuses away into the sterol-free planar bilayer after liposome fusion. The current across the BLM was monitored via a picoamperemeter (model 428, Keithley Instruments, Cleveland, OH) connected to a *x–t*-recorder. Both the stirring rate and the osmotic gradient were varied to achieve nearly the same fusion rate in all experiments. For every single membrane the average spike amplitude and the average time interval between two neighbouring spikes were measured before (U_1 and t_1) and after (U_2 and t_2) protein addition to the *cis* side of the BLM. The experimental results achieved with different membranes were compared in terms of the ratios R_A and R_f:

$$R_A = U_2/U_1,\ R_f = t_1/t_2 \quad (1)$$

The comparatively low variation of these parameters allowed an interpretation of the results. Furthermore, each experiment (from 20 to 40 spikes) was carried out at least six times to get statistically significant results.

After the addition of ricin to the *cis* side of the membrane (Fig. 1, panel A) the spike amplitude increased two-fold ($R_A = 2.2 \pm 0.4$) whereas the frequency did not change. The agglutinin induced opposite effects. It increased the fusion rate ($R_f = 2.0 \pm 0.5$) but not the amplitude of the fusion spikes (Fig. 1, panel C). Only viscumin was able to affect both parameters ($R_f = 1.8 \pm 0.4$, $R_A = 2.2 \pm 0.4$). Most pronounced was the effect of viscumin at acidic pH (Fig. 1, panel B). The fusion rate observed ($R_f = 8.2 \pm 1.1$) was at least four times the fusion rate induced by acidification alone or by a pH drop in the presence of ricin (Fig. 1, panel A) or RCA (Fig. 1, panel B). In the absence of the lectins, the modest pH sensitivity of the liposome fusion rate ($R_f = 1.8 \pm 0.6$) may be contributed to the high amount of ethanolamines present in the model membranes [13]. Galactose competitive to the monosialoganglioside (GM1) receptor molecules incorporated into the liposomes inhibited the fusion modulating effect of viscumin both at pH 7.5 (Fig. 1, panel D) and at pH 5.2 (Fig. 1, panel B).

Each fusion spike (Fig. 1) represents the fusion of a single liposome carrying a package of preformed nystatin channels [16]. There are two plausible explanations for the two-fold increase of the fusion spikes observed after the addition of both ricin and viscumin. The first is that lectin binding to GM1 containing liposomes resulted in a slower dissolution of the nystatin–ergosterol complexes which led to larger fusion spikes. This seems not to be the case because a prolonged decay time per picoampere has not been observed for the fusion spikes after the addition of the ribosome inactivating plant proteins (RIPs). The second explanation is that RIP-induced vesicle–vesicle fusion resulted in larger liposomes, which in turn gave rise to larger fusion pulses. Because the BLM conductance is raised in a single step, it is excluded that aggregates of vesicles are responsible for the effect. To support this assumption experimentally, two populations of liposomes were prepared by extrusion through 100 nm and 400 nm pore filters [18] using the small-volume apparatus LiposoFast (Avestin, Ottawa, Canada). Upon fusion with the planar bilayer the average fusion spike for the smaller vesicles was 2.0 ± 0.5 pA whereas the large vesicles elicited spikes of 6.6 ± 2.1 pA (Fig. 2). Consequently, the enlarged amplitude of the fusion spike observed after the addition of viscumin and ricin (Fig. 1) reflects the fusion the BLM with large vesicles that are originated by vesicle–vesicle fusion.

No channel activity could be contributed to sources other than nystatin in any of the experiments carried out with the lectins. Current fluctuations were observed only in the presence of nystatin. Furthermore, they were detectable only in the presence of a transbilayer osmotic gradient. Under the conditions of osmotic equilibrium the RIPs did not induce fusion between vesicles and the BLM.

If GM1 was not included into liposome membranes neither the fusion rate nor the amplitude of the spikes was altered by the lectins under investigation.

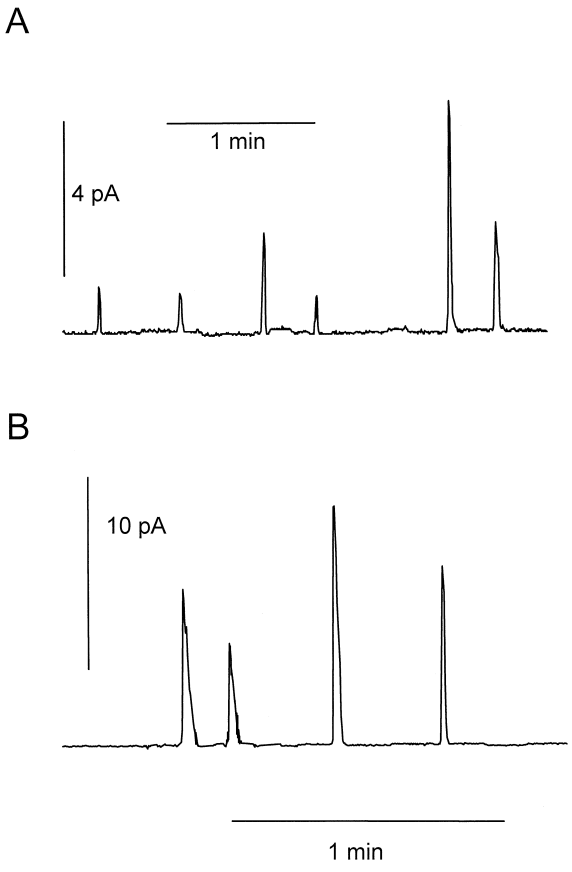

Fig. 2. Fusion of GM1 containing liposomes prepared by extrusion of multilamellar vesicles through filters of 100 nm (A) and 400 nm (B) pore diameter with an ergosterol and GM1 free planar bilayer. A NaCl gradient of 650 mM *cis* to 150 mM *trans* was established. Some 65 μg/ml liposomes were given to the *cis* side of the planar membrane.

Interestingly, under these conditions it made no difference if GM1 was incorporated into the BLM or if it were not. Addition of the glycolipid receptor to both the planar and vesicular membranes revealed in an enhanced lectin induced fusion rate (Fig. 3B) similar to the one shown in Fig. 1A. Subsequent acidification, however, was followed by multiple fusions events even in the absence of the proteins (Fig. 3A). Accordingly, the lipid composition used in the experiments shown in Fig. 1 is the only one suitable for the investigation of lectin mediated fusion. Other modifications, e.g., lowering of the PE content or an enrichment with charged PS abolished membrane fusion. From the above experiments it was concluded that in an appropriate lipid environment ricin and viscumin act as fusogens. Assuming that the lectins exhibit the same capability under in vivo conditions also, it may be suggested that fusion represents a pathway for delivering the RIPs into the cytosol. Therefore, it is required that during the fusion process the integrity of the membrane of the intracellular compartment encapsulating the RIPs is breached and leakage or lysis takes place. Then the proteins may enter the cytosol and reach their target, i.e., inhibit ribosomal protein syntheses. This hypotheses is supported by the observation that the non-fusogenic RCA is 2000-fold less toxic than the fusogens, ricin and viscumin [10], when injected intraperitoneally into mice [9].

Earlier ricin was reported to be incapable to fuse model membranes made from fully saturated lipids with PC headgroups [19]. This failure may be originated by the absence of a lectin receptor (GM1) and the lipid dependence of the fusion event. Because unsaturation is known to promote fusion [20], the liposomes used in the present study were made from

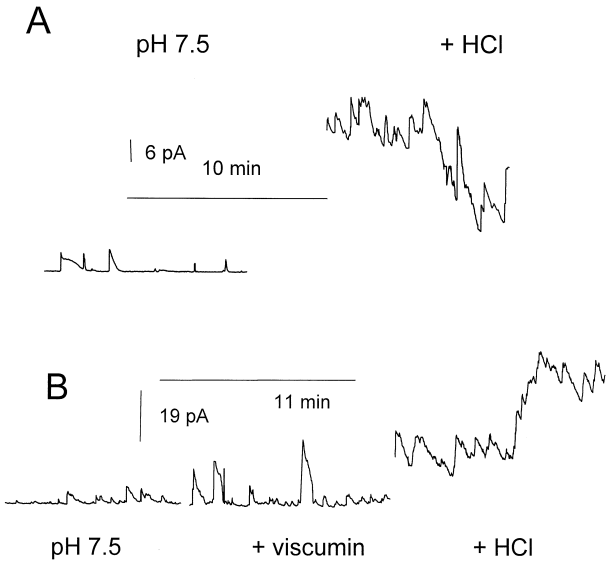

Fig. 3. The addition of GM1 to both vesicular and planar membranes caused fusion events at pH 5.2 (A) that are not distinguishable from those obtained in the presence of viscumin under the same conditions (B). A fusion stimulating effect of the protein was observed at pH 7.4 (B). A NaCl gradient of 450 mM *cis* to 150 mM *trans* was established. 27 μg/ml liposomes and 27 μg/ml viscumin were given to the *cis* side of the planar membrane.

lipids that were at least to 50% unsaturated. Fusion was further facilitated by incorporation of lipids with PE headgroups. The displacement of interfacial water by proteins is thereby eased [21]. While viscumin and ricin were shown to act as fusogens (Fig. 1) RCA induced an increase in the frequency of fusion of small vesicles with the BLM (Fig. 1) only. RCA should therefore be classified as a non-fusogenic protein capable of modulating fusion between artificial membranes after modifying intramembrane contact. This conclusion is in perfect agreement with the observation that it decreases the threshold Ca^{2+}-concentration required for fusion [22].

In general, fusion of purely lipid bilayers and biological fusion are described to be lipid dependent [20]. Alterations in the cytotoxicity of both ricin and viscumin observed after perturbations in Golgi functions [23] are accompanied by a depletion of lipids species required for fusion because the anterograde membrane transport is inhibited. Consequently, the lipid dependence of fusion provides an explanation for the inhibition of ricin cytotoxicity by brefeldin A (BFA). The latter dramatically disrupts the structure of the Golgi apparatus causing Golgi content and membrane to redistribute to the ER [24]. Commonly an alternative explanation is given. The A-chain is believed to translocate into the cytosol from the ER after having been transported from the Golgi-complex [25]. Although, recently experimental evidence in favour of retrograde lectin transport was obtained with mutant ricin, the existence of alternative pathways can not be ruled out [26]. Moreover, the intoxication of cell lines that have an aberrant Golgi complex [27,28] and the death of brefeldin A treated cells induced by immunotoxins containing ricin A-chain [29] conflict with the suggested entry of the A-chain from the ER but not with the fusion hypothesis. Here, the intracellular compartment from which the protein is delivered from depends on the capability of the protein to function as a fusogen.

Further support for the hypotheses of fusion mediated protein delivery comes from the observation that after binding to cell surface glycoproteins with signalling potency, β-galactoside-specific lectins induce changes in the level of phospholipids and inositol phosphates [30] that are known to promote fusion. Namely, diacylglycerol activation dramatically alters the kinetics of liposome fusion [31].

With respect to the role of lipids in protein mediated fusion [20] we have shown that not only the subchains of ricin and viscumin are able to aggregate [32,33] or fuse [19] lipid vesicles but the holotoxins as well. The fusogenicity of the RIPs is hypothesized allow their access to cytoplasmic ribosomes after leakage across endocytic vesicles. The differences found in their fusion capability in vitro might be relevant for a therapeutic utilization of these proteins in the treatment of cancer [34], autoimmune [35] and graft-versus-host diseases [36].

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