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Change in intravesicular volume of liposomes by freeze-thaw treatment as studied by the ESR stopped-flow technique

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The intravesicular volume of phospholipid vesicles was measured by a spin-label method coupled with the ESR stopped-flow technique. The internal volume was defined as the volume from which a spin-broadening agent, tris(oxalato)chromate ion, but not a spin label, TEMPONE, was excluded. The internal volume of sonicated phospholipid vesicles was about 0.4 μl/mg lipid. Freeze-thaw treatment increased this value 10–50-fold. However, repeated freeze-thaw treatment did not increase the value further. On the other hand, biomembrane vesicles (sarcoplasmic reticulum membrane vesicles) showed no change in internal volume with freeze-thawing. The permeability of the phospholipid vesicle membranes measured as the rate of transmembrane diffusion of the spin-broadening agent into the vesicles was very low even after freeze-thawing. These findings clearly demonstrated that freeze-thaw treatment induced fusion of hundreds of small vesicles to form giant liposomes, the membrane of which was impermeable to the complex ions. Optimal conditions for the formation of giant liposomes were investigated with reference to the composition of the suspension medium and the method of freezing and thawing. Within the range of 0.143–2 M KCl, a concentration of 0.143 M produced the largest increase in internal volume for asolectin vesicles, whereas 2 M was optimal for egg-yolk phosphatidylcholine vesicles. Freezing in either liquid nitrogen or solid CO_2/ethanol and thawing in cold air was the best procedure for increasing the internal volume by freeze-thaw treatment.

Introduction

We have reported in the previous paper [1] that freezing and then thawing a suspension of phospholipid vesicles induced the formation of giant vesicles up to tens of micrometers in diameter depending on the type of phospholipids and the composition of the suspension medium used. Among various kinds of phospholipids, L-α-phosphatidylcholine (PC), a mixture of L-α-phosphatidylethanolamine (PE) and L-α-phosphatidyl-L-serine (PS), or asolectin (AL, soybean phospholipid) resulted in the formation of giant liposomes. Employing these phospholipids for membrane reconstitution, the freeze-thaw technique is successful in the formation of giant proteoliposomes at appropriate protein/lipid ratios. Giant proteoliposomes prepared by this procedure have already been used by several authors in electrophysiological studies of ion channels in biomembranes usually inaccessible to a patch-pipette electrode [2–8].

Since in all of these studies, the formation of giant vesicles was observed by light microscopy and since only the excised-patch recording technique was applied, some of the important properties of the vesicles prepared by the freeze-thaw technique are still not clear. For example, it has yet to be determined how permeable the membrane of these vesicles is to ions or what the average increase in intravesicular volume is as a result of the freeze-thaw treatment.

There have been several reports concerning measurement of the intravesicular volume of frozen-thawed phospholipid vesicles [9,10]. Pick used isotope-labeled compounds to measure the trapping volume of the vesicles [9]. The compounds trapped inside the vesicles were separated by column chromatography and the trapping volume was calculated from the radioactivity of the vesicle fraction. Oku and MacDonald used a

Abbreviations: ESR, electron spin resonance; Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; TEMPONE, 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PS, L-α-phosphatidyl-L-serine; AL, asolectin; (oxalato)chromate, potassium tris(oxalato)chromate(III) trihydrate; SR, sarcoplasmic reticulum.

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fluorescence method, in which fluorescence from an untrapped fluorophore, calcein, was quenched with external $CoCl_2$ and the trapped calcein was measured without separation [10]. Although these two studies closely examined the conditions for increasing the internal volume of the vesicles on freeze-thaw treatment, some of their results conflicted. For example, high ionic strength had an inhibitory effect on the increase of trapping volume according to Pick [9], whereas it had a stimulatory effect according to Oku and MacDonald [10].

In the present study, we investigated quantitatively the effect of freeze-thaw treatment on the internal volume of liposomes under various conditions. For this purpose, we used the ESR stopped-flow technique in combination with a spin label, TEMPONE, and a paramagnetic broadening agent. The method was found to be rapid and sensitive, and allowed us to measure the internal volume without separating the vesicles. In addition, the membrane permeability of the liposomes before and after the freeze-thaw treatment was investigated using the same method.

Materials and methods

Chemicals

Egg-yolk L-α-phosphatidylcholine (egg PC, Coatsome C-10) was obtained from Nichiyu Liposome Co. (Tokyo, Japan). According to the analytical data sheet provided by the manufacturer, the purity of the egg PC was high. It was shown that the egg PC contained 98.5% PC, 0.7% lyso-PC, and 0.8% sphingomyelin, while PE, neutral lipids, and free fatty acids were undetectable. Egg-yolk L-α-phosphatidylethanolamine (PE, type III), L-α-phosphatidyl-L-serine (PS) from bovine brain, and asolectin (soybean phospholipid, type II-S) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The asolectin was partially purified by washing with acetone [11]. The spin label reagent, 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl (TEMPONE), was obtained from Eastman (Rochester, NY, U.S.A.). The paramagnetic broadening reagent, potassium tris(oxalato)chromate (III) trihydrate ((oxalato)chromate), was synthesized according to Bailar and Jones [12].

Membrane preparation

Phospholipid vesicles were prepared by a sonication method. Briefly, a phospholipid suspension in a buffer solution was sonicated for an appropriate period of time with a bath-type sonicator (model NS50-05U, Nihon Seiki, Tokyo, Japan), under a nitrogen atmosphere. The freeze-thawing treatment was performed as follows unless otherwise stated: The sonicated membrane suspension in a test tube (17.5 mm × 130 mm, Advantec, Japan) was immersed in liquid nitrogen and kept for about 1 min until frozen completely, after which the frozen suspension was thawed slowly in a cold room (4° C). Sarcoplasmic reticulum membrane vesicles were prepared from rabbit skeletal muscle as described previously [13].

Stopped-flow ESR apparatus

An X-band ESR spectrometer (model PE-1X, JEOL, Tokyo, Japan), equipped with a 100 kHz field modulator and a cylindrical TE_{011} mode cavity, was used. The constitution and performance of the stopped-flow apparatus were described in the previous paper [14]. Briefly, the flow of sample solution was controlled with a stop-valve located downstream from the observation cell. When the valve was open, solutions in two 15-ml sample reservoirs were delivered by nitrogen gas pressure into a Diflon mixing chamber (a modification of model O3, Otsuka Electronics, Hirakata, Japan) coupled with an observation cell (thick-walled quartz capillary, 1 mm inner diameter). The open-valve period was set so that 100 μl of solution was delivered each time. The flow rate was set at 3.3 ml/s.

ESR measurement

Two isotonic solutions, A and B, were placed in the sample reservoirs of the stopped-flow apparatus. Although there were some modifications in some experiments, a typical composition of the solutions was as follows. Solution A contained membrane vesicles (20 mg lipid/ml), 1 mM TEMPONE, 143 mM KCl, and 10 mM Mops-KOH (pH 7.0), while solution B contained 100 mM (oxalato)chromate and 10 mM Mops-KOH (pH 7.0). When effect of salt concentration was examined, a certain amount of KCl was further added equally to both solutions A and B. The sweep rate of the ESR magnetic field was usually 20 G/min unless otherwise stated. Sweeping was started from a field just below the position of a signal peak at the time of mixing of the two solutions so that the peak was recorded 5–10 s after mixing. For measurement of the time course of (oxalato)chromate permeation, the magnetic field was kept at the peak of the TEMPONE signal and the peak height was monitored continuously. All the ESR measurements were performed at room temperature (24 ± 1° C). The osmotic pressure of the solutions was measured with an osmometer (Type M, Knauer, F.R.G.).

Results and Discussion

Sonicated phospholipid vesicles (20 mg lipid/ml) were prepared from egg PC, AL or a mixture of PE and PS with a weight ratio of 7 : 3 in a buffer solution containing 143 mM KCl and 6.45 mM Mops-KOH (pH 7.0). The intravesicular volume of the sonicated vesicles was measured using the ESR stopped-flow apparatus constructed by us [14]. A vesicle suspension was admixed, using the stopped-flow apparatus, with an equal

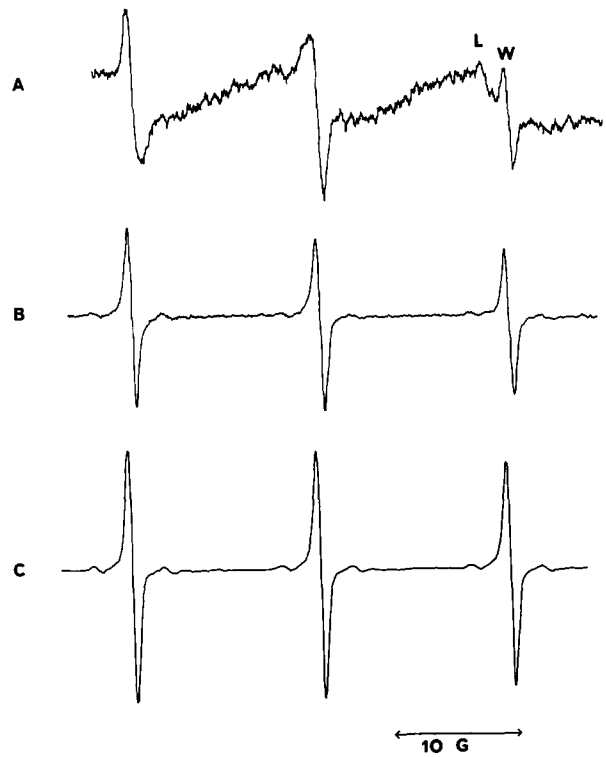

Fig. 1. ESR spectra after mixing using a stopped-flow apparatus. All the solutions contained 1 mM TEMPONE, 72 mM KCl and 8.3 mM Mops-KOH (pH 7.0), in addition to sonicated asolectin vesicles (20 mg lipid/ml) (A and C) or frozen-thawed asolectin vesicles (20 mg lipid/ml) (B), and 50 mM $K_3Cr(C_2O_4)_3$ (A and B). The amplifier gain is 1600 (A), 200 (B), and 20 (C).

volume of an isotonic solution of 0.1 M (oxalato)chromate and 10 mM Mops-KOH (pH 7.0). An example of the ESR spectra of the resulting mixtures containing sonicated AL vesicles is shown in Fig. 1A. The spectral line designated W originates from the spin labels in the aqueous phase inside the vesicles, while the line designated L originates from spin labels in the lipid phase [15]. Comparison of the amplitude of spectral line W with that of the corresponding spectral line for a sample suspension from which the paramagnetic line-broadening reagent was omitted allowed the intravesicular volumes to be estimated. Fig. 1C shows a spectrum of the AL vesicle suspension which did not contain (oxalato)chromate, but was otherwise the same as the sample used to obtain Fig. 1A. The intravesicular volume (V_{in} in μl/mg lipid) of vesicles in the sample for Fig. 1A or 1C can be calculated according to the following equation,

$$V_{in} = 1000 c^{-1} (h_{AW}/G_A)/(h_C/G_C)$$

where h_{AW} represents the peak height of signal W in Fig. 1A recorded with amplifier gain G_A, h_C represents the height of the high-field peak in Fig. 1C recorded with amplifier gain G_C, and c represents the concentration of phospholipid vesicles (mg/ml).

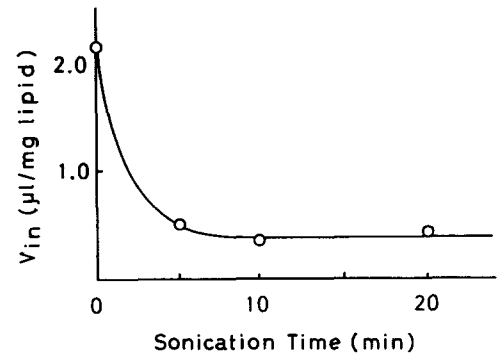

Fig. 2. Effect of sonication time on the internal volume. Asolectin multilamellar liposomes (40 mg lipid/ml) in 0.143 M KCl and 10 mM Mops-KOH (pH 7.0) were sonicated with a bath-type sonicator under a nitrogen atmosphere. Intravesicular volume (V_{in}) was measured by the ESR stopped-flow technique as described in the text.

The intravesicular volume of sonicated vesicles decreased with increasing sonication period (Fig. 2). The volume, 2.22 μl/mg lipid as multilamellar vesicles at time 0, decreased with prolonged sonication and reached a plateau (about 0.4 μl/mg lipid) after sonication for 10 min. The final value was in good agreement with the calculated internal volume for small unilamellar vesicles assuming vesicle diameter as 30 nm [14]. We therefore fixed the sonication time at 10 min in subsequent experiments.

The sonicated vesicles were then subjected to freeze-thaw treatment, and formation of giant liposomes in the resulting suspension was observed by phase-contrast light microscopy as reported previously [1]. The intravesicular volume was measured as described above for sonicated vesicles. An example of the ESR spectrum obtained using frozen-thawed AL vesicles is shown in Fig. 1B. Since the volume of the membrane lipid phase compared to that of the intravesicular aqueous phase was negligible in the frozen-thawed vesicles, the highest field peak was not resolved in this case.

TABLE I

Internal volume of sonicated phospholipid vesicles before and after freeze-thaw treatment

Each value corresponds to a different preparation of sonicated vesicles. The values are averages of at least five determinations, which did not vary more than 10%. The abbreviations, −FT and +FT, represent the values before freeze-thawing and after freeze-thawing, respectively.

Lipid	V_{in} (μl/mg lipid)		Ratio (+FT/−FT)
	−FT	+FT	
Egg PC	0.35	6.52	19
Asolectin	0.21	3.43	16
	0.24	2.96	12
	0.38	5.17	14
PE/PC (7:3) [a]	0.44	23.5	54

[a] Mixture of PE and PC with weight ratio of 7:3.

The values obtained by this method for the intravesicular volume of sonicated and of frozen-thawed phospholipid vesicles are shown in Table I, which clearly indicates that the freeze-thaw treatment increased the intravesicular volume 10–50-fold. The values obtained by present method are ensemble averages. Electron microscopy revealed that sonicated vesicles were relatively homogeneous, whereas frozen-thawed vesicles were nonhomogeneous in both size and the number of lamellae (data not shown), and hence the distinct relationship between internal volume and vesicle diameter seen in unilamellar vesicles could not hold in the frozen-thawed multilamellar vesicles. Therefore, interpretation of the increase in the internal volume of the frozen-thawed vesicles cannot be straightforward. Nevertheless, the large intravesicular volume of the frozen-thawed vesicles implies that at least a dominant proportion of them were impermeable to (oxalato)chromate complex ions.

Pick reported that the freezing-thawing-sonication technique was effective for charged phospholipid vesicles but not for pure PC vesicles [9]. In contrast, in the present experiments, freeze-thaw treatment was effective even with pure PC vesicles, although we did not apply brief sonication at the last step. In the previous study, using light microscopy, we also observed giant liposomes formed from pure PC after freeze-thaw treatment [1]. The contradictory results may be due to the difference in measuring methods. Pick measured the trapping volume, which was estimated from the amount of labeled compounds trapped in the course of the freeze-thawing treatment [9], whereas we measured the internal volume directly. If the labeled compound is prevented from entering the vesicles at the freeze-thawing step for some reason, the trapping volume would be different from the internal volume.

The permeability of the liposomal membrane to the complex ion, (oxalato)chromate, was measured before and after the freeze-thaw treatment. Figure 3 shows the time course of the peak height at the highest magnetic field of the TEMPONE signal in the intravesicular aqueous phase. The peak height was constant for at least 1 min after mixing when sonicated vesicles were used (Fig. 3A). When frozen-thawed vesicles were used, on the other hand, the peak height decreased slightly just after mixing, but reached almost a plateau within 30 s (Fig. 3B, 3C). The change for AL vesicles (Fig. 3B), although within 10% of the total, was larger than that for egg PC vesicles (Fig. 3C). In general, the peak height is an indicator of the amount of TEMPONE molecules, which is a function of both the TEMPONE concentration and the internal volume of the vesicles. Since the internal volume would not change within the time scale of this experiment (a few minutes), the change in the peak height must correspond to the change in effective TEMPONE concentration, which can vary by interaction of the TEMPONE with a spin-broadening reagent, (oxalato)chromate, diffusing into the vesicle interior across the membrane. Therefore, the finding that the peak height did not change for sonicated vesicles indicates that the vesicles are highly impermeable to (oxalato)chromate ions. Since we were interested in the fast permeation phenomena, the method used here was focused on the change in short time range (within several minutes). However, in another experiment with the same principle as this method but without using stopped-flow apparatus, the amplitude of the TEMPONE signal in sonicated asolectin liposomes did not change up to 4 hours, which indicated that the sonicated asolectin vesicles were impermeable to (oxalato)chromate ions even in a longer time scale (data not shown). The peak height for the frozen-thawed vesicles decreased slightly, but the change was so small that it can generally be said that phospholipid vesicles are impermeable to the complex ion, (oxalato)chromate, even after fusion by freeze-thaw treatment. The permeability of the frozen-thawed PC vesicles was smaller than that of the frozen-thawed AL vesicles.

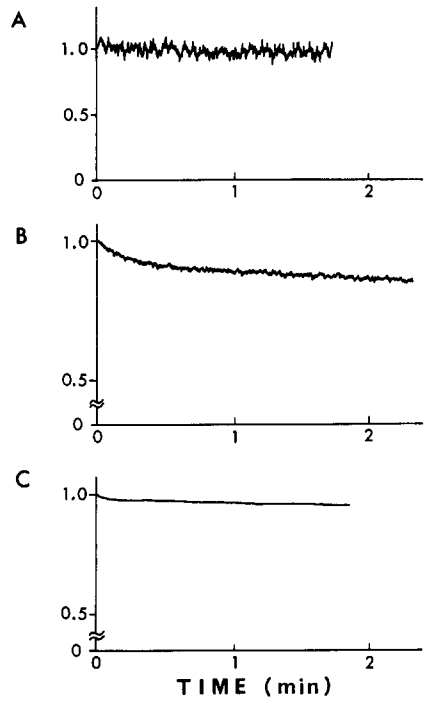

Fig. 3. Time course of the peak height of a TEMPONE signal in vesicle suspension. The vesicle suspension containing the spin label, TEMPONE, was mixed with a solution of the spin-broadening reagent, (oxalato)chromate, at time 0. The decrease in the peak height corresponds to permeation of the spin-broadening reagent into the vesicle interior. The ordinate is the relative intensity, where the value at time 0 is defined as1.0. The liposomes used were sonicated asolectin vesicles (A), frozen-thawed asolectin vesicles (B), and frozen-thawed egg PC vesicles.

The effects of KCl concentration in the suspension medium used for the freeze-thaw treatment on the change in intravesicular volume were next investigated.

TABLE II

Effect of KCl concentration on the change in internal volume caused by freeze-thaw treatment

The abbreviations, −FT and +FT, represent the values before and after freeze-thawing, respectively.

Lipid	[KCl] (M)	V_{in} (μl/mg lipid)		Ratio (+FT/−FT)
		−FT	+FT	
Asolectin	0.143	0.475	8.17	17.2
	0.5	0.479	7.67	16.0
	1.0	0.490	5.63	11.5
	2.0	0.354	1.73	4.9
Egg PC	0.143	0.598	14.0	23.4
	0.5	0.536	12.4	23.2
	1.0	0.392	16.8	42.9
	2.0	0.238	11.3	48.4

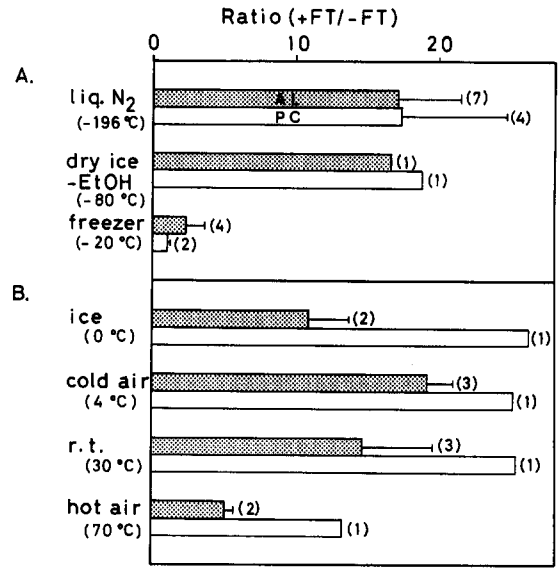

Fig. 4. Effects of the method of freeze-thawing on the change in internal volume. The averaged ratio of the internal volume after freeze-thaw treatment (+FT) to that before freeze-thaw treatment (−FT) is shown. Shaded bar and white bar represents asolectin vesicles and PC vesicles, respectively. The standard deviation and the number of data are also shown in the figure. (A) Sonicated asolectin vesicles or egg PC vesicles (20 mg lipid/ml) in 0.143 M KCl and 10 mM Mops-KOH (pH 7.0) were frozen as indicated in the figure and thawed slowly in a cold room at 4° C. (B) Sonicated asolectin vesicles or egg PC vesicles (20 mg lipid/ml) in 0.143 M KCl and 10 mM Mops-KOH (pH 7.0), were frozen in liquid nitrogen and thawed as indicated in the figure.

As shown in Table II, the effect of KCl concentration on freeze-thawing efficiency differed between AL vesicles and egg PC vesicles. The ratio of the internal volume of the AL vesicles after the freeze-thaw treatment to that before the treatment decreased as the KCl concentration increased. On the other hand, the ratio for egg PC vesicles showed an opposite tendency, although absolute values of the internal volume of the frozen-thawed vesicles were roughly constant at KCl concentrations between 0.143 M and 2.0 M. The internal volume of egg PC sonicated vesicles before freeze-thaw treatment was dependent on KCl concentration. Oku and MacDonald studied the effect of ionic strength on the formation of giant liposomes by freeze-thaw treatment using egg phospholipid, and showed that the resulting liposomes had large internal volumes when the electrolyte concentration during the freeze-thaw step was either very high (> 1 M) or very low (a few millimolar) [10]. The result obtained here is essentially consistent with, but slightly different from theirs. Although the ratio increased with the KCl concentration, the internal volume of egg PC vesicles after freeze-thaw treatment was roughly constant within the KCl concentration range of 0.143–2.0 M. We were unable to examine the condition at 0 M KCl, because the osmolarity of the two mixing solutions must be adjusted with KCl.

The effects of the method of freezing and that of thawing were also studied both for AL and PC vesicles (Fig. 4). With regard to freezing (Fig. 4A), the use of liquid nitrogen and that of solid CO_2/ethanol gave similar results for the change in internal volume by freeze-thaw treatment. The use of a freezer was not effective for increasing the internal volume. As to the method of thawing (Fig. 4B), heating with hot air produced a smaller increase in the internal volume than other methods. Thus, if the intention is to make giant vesicles by freeze-thaw treatment, freezing in either liquid nitrogen or solid CO_2/ethanol and thawing slowly in cold air may be the best choice.

Table III shows the effect of repeated freeze-thaw cycles on the internal volume of vesicles. More than one cycle of freeze-thaw treatment failed to increase the internal volume of both AL and PC vesicles, and in fact decreased it slightly. Interestingly, the internal volume of sarcoplasmic reticulum membranes from rabbit skeletal muscle did not increase as a result of single freeze-thaw treatment in several experiments (data not shown). Repeated freeze-thaw treatment did not in-

TABLE III

Effect of repeated freeze-thaw treatment on the change in internal volume

The internal volumes of liposomes (μl/mg lipid) and sarcoplasmic reticulum vesicles (μl/mg protein) were measured after repeated freeze-thaw treatments (FT). The ratios of the internal volume of frozen-thawed vesicles to those before the freeze-thaw treatment are shown in parentheses.

Number of FT	AL liposome	PC liposome	SR
0	0.39	0.66	1.16
1	5.33 (13.7)	14.8 (22.4)	1.19 (1.03)
2	3.35 (8.6)	10.8 (16.4)	1.10 (0.95)
3	3.97 (10.2)	10.6 (16.1)	1.14 (0.98)

crease the internal volume either as shown in Table III. This is the confirmation of the previous results obtained with phase-contrast microscopy [1]. Membrane proteins present at high density in the sarcoplasmic reticulum may thus block the fusion of vesicles.

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