Quantitative measurement of membrane fusions induced by calcium and polyethylene glycol using the porin function

Eisaku Yoshihara and Taiji Nakae

Institute of Medical Sciences and School of Medicine, Tokai University, Isehara 259-11, Japan

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1. INTRODUCTION

Fusion of biological membranes is a fundamental process for a number of essential cellular activities and fusion with liposome membranes is of great interest from the standpoint of their potential use as a vehicle for drug delivery, or gene transfer, etc. A number of workers reported the methods that have been used to detect membrane fusions (review [1]); however, most of these methods have limitations which prohibit their general application.

We have developed a quantitative assay system that can be used to monitor the extent of vesicle fusion induced by a variety of fusogens, e.g. calcium and polyethylene glycol (PEG). The principle of the present assay method is to fuse vesicles containing porin (porins are the pore-forming membrane proteins from the outer membrane of *E. coli*, [2,3]) with the K^+-loaded vesicles lacking porin. The unfused vesicles lacking porin (porinless-vesicles) were quantified by determination of the extent of fluorescence quenching of a cyanine dye, 3,3′-dipropylthiodicarbocyanide iodide [diS-C_3-(5)] upon dilution of the mixture with Na^+-containing medium in the presence of valinomycin [4]. The porin containing vesicles (porin-vesicles) do not accumulate diS-C_3-(5) due to the instantaneous equilibrium of K^+ and Na^+ across the membranes via porin pores.

2. MATERIALS AND METHODS

Porin was extracted from *E. coli* B as in [3]. Phospholipids were purchased from SRL. DiS-C_3-(5), PEG-4000 and valinomycin were purchased from Nippon Kanko Sikiso, Nakarai and Sigma, respectively. The porin-vesicles and porinless-vesicles were reconstituted as in [5].

3. RESULTS AND DISCUSSION

3.1. *Standard assay procedure*

Equal amounts of the porin- and the porinless-vesicles [0.16 μmol phosphatidylserine (PS) and 0.04 μmol phosphatidylcholine (PC) in 10 μl of a solution containing 100 mM K-gluconate and 10 mM Tris–HCl, pH 8.0] were mixed and the fusion reaction was started by adding 10 μl of 30 mM $CaCl_2$. The reaction was stopped by adding 970 μl of the above buffer after 3 min at 28°C. The porinless-vesicles in the reaction mixture were quantified as follows by the fluorescence quenching of diS-C_3-(5) as in [4], since the porin-vesicles do not participate. A portion of the above reaction mixture (20 μl) was diluted with 2.5 ml of a solution of 100 mM Na-gluconate, 3.3×10^{-9} mol diS-C_3-(5) and 10 mM Tris–HCl (pH 8.0) and, after 1 min, 1.2×10^{-10} mol valinomycin in 2 μl ethanol was added. Fluorescence intensity was recorded at 670 nm (fig.1). A rapid decrease of

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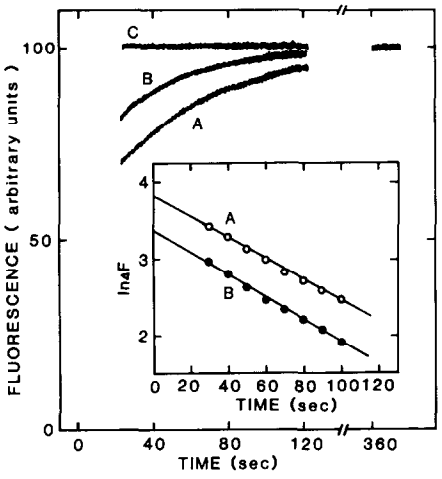

Fig. 1. Typical tracing of the fluorescence curves. Vesicle membranes were reconstituted from 16 μmol bovine brain PS and 4 μmol egg yolk PC with or without 6.75 nmol porin trimers in 1.0 mol of a solution containing 100 mM K-gluconate and 10 mM Tris-HCl (pH 8.0) as in [5]. Sonication was prolonged for 10 min at 20°C. Part A, experiment without fusogen. The porin- and porinless-vesicles (each containing 0.2 μmol phospholipids per 10 μl of the above buffer) were mixed in a microtube (Eppendorf 3810) without fusogen. The mixture was incubated at 28°C for 3 min, diluted with 870 μl of the above buffer and 20 μl of this was mixed with 2.5 ml of 100 mM Na-gluconate, 3.3×10^{-9} mol diS-C_3-(5) and 10 mM Tris-HCl (pH 8.0). The fluorescence intensity was recorded, after addition of 1.2×10^{-10} mol valinomycin in 2 μl ethanol, at 670 nm with an excitation wavelength at 620 nm using a Hitachi 650-10 M fluorescence spectrophotometer. Part B, experiment with a fusogen. The reaction mixture, identical to that in A, was mixed with 10 μl of 30 mM $CaCl_2$ and incubated at 28°C for 3 min as above. Subsequent treatments were identical to the above. Part C, experiment with porin-vesicles only. Porin-vesicles (0.4 μmol phospholipids and 0.135 nmol porin trimers in 20 μl of the buffer) were treated as in A without fusogen. The inset shows replots of the exponential fluorescence recovery curves of A and B as a function of the logarithm of the fluorescence quenching ($\ln\Delta F$) vs time. The maximum fluorescence quenching could be obtained from the intercept of the line with the ordinate.

fluorescence (not seen in fig.1, since this occurs during the mixing time) was followed by a slow recovery due to a rapid efflux of K^+ (which generates a membrane potential, interior negative, and consequently drives the cyanine dye into the vesicles) and a slow influx of Na^+ (which causes the exit of the dye from the vesicles), respectively (fig.1). The maximum quenching value (ΔF) was obtained extrapolation of the plot of $\ln\Delta F$ vs time (fig.1, inset) to time zero, where ΔF is the extent of fluorescence quenching vs fluorescence intensity at equilibrium.

One may argue that porins in the vesicle membranes cause a porin-dye interaction, resulting in change of fluorescence emission. This seems unlikely, however, since the vesicles containing 0.37–3.7 nmol porin trimers per μmol PS showed indistinguishable fluorescence emission of the dye regardless of whether the membranes were fused or unfused (not shown).

If the quantity of the K^+-impermeable vesicles can be measured as above, the extent of fluorescence quenching in the mixture of the porin- and porinless-vesicles should be related to the proportion of the porinless-vesicles to total vesicles.

The porin- and porinless-vesicles were mixed in various ratios without fusogen and the fluorescence quenching of the mixture was determined. As shown in fig.2, the extent of fluorescence quenching was linearly related to the population of the porinless-vesicles. Treatment of the porin-vesicles (or porinless-vesicles) with 10 mM $CaCl_2$ did not affect significantly the degree of fluorescence quenching. The extent of the fusion

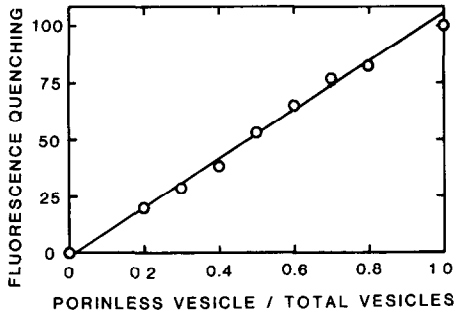

Fig. 2. Relationship between the quantity of porinless-vesicles and the extent of fluorescence quenching. Vesicle membranes were reconstituted from 1.6 μmol bovine brain PS and 0.4 μmol egg yolk PC with or without 2.4 nmol porin trimers as in the legend to fig.1. The porin-vesicles and porinless-vesicles were mixed at various ratios (total 0.013 μmol phospholipids in 6.5 μl) and the fluorescence intensity was recorded as described in the legend to fig.1. The extent of fluorescence quenching by the porinless-vesicles only was normalized to 100.

can be expressed as the fusion index (F.I.) as: F.I. (%) = $(1-\Delta F/\Delta F_o) \times 100$, where ΔF and ΔF_o are the extents of fluorescence quenching of a cyanine dye with and without a fusogen, respectively.

3.2. *Application to calcium-induced membrane fusion*

To test the validity of the present assay method we have examined the effect of calcium on the fusion of vesicles containing acidic phospholipid [6]. Equal amounts of porin- and the porinless-vesicles made of a mixture of 0.16 μmol PS and 0.04 μmol PC in 10 μl buffer were mixed with calcium and the reaction was stopped after incubation at 28°C for 3 min by diluting the reaction mixture 29-fold with the same buffer. The porinless-vesicles were quantified as above. As depicted in fig.3, the fusion reaction began at 5 mM calcium and the extent of the fusion increased gradually as calcium concentration was raised to about 20 mM. The mixture of free porins, porinless-vesicles and calcium did not increase the fluorescence intensity, suggesting negligible effect of calcium on the insertion of free porin into vesicle membrane (not shown).

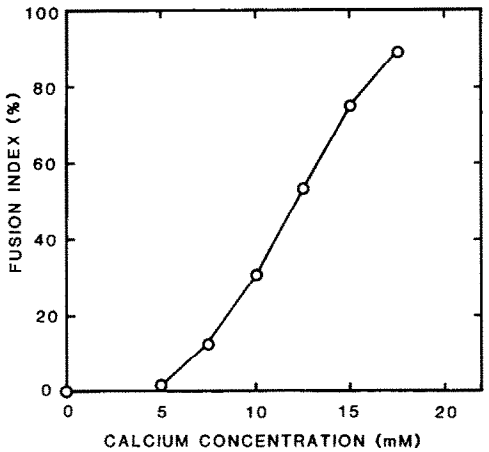

Fig. 3. Quantitative determination of calcium-induced membrane fusion. Vesicle membranes with or without porins were made as described in the legend to fig.1. Vesicles with and without porins were mixed (0.2 μmol phospholipids per 10 μl each) and the reaction was started by adding appropriate amounts of $CaCl_2$ in 10 μl of a solution containing 100 mM K-gluconate and 10 mM Tris-HCl (pH 8.0). The mixture was incubated at 28°C for 3 min and the reaction was stopped by adding 870 μl of the same buffer. Fluorescence intensity was measured as above.

3.3. *Application to PEG-induced membrane fusion*

Although high concentrations of PEG have been used for cell fusions [7], quantitative methods for PEG-induced membrane fusion seem to be unsatisfactory, since most available techniques quantitate the leakage of the vesicle contents instead of determining the true membrane fusion [8]. To test the applicability of this assay method, we have determined the effect of PEG on the fusion of PC vesicles. Both porin- and porinless-vesicles were made of PC only and fusion of these vesicles was started by adding PEG-4000. After incubation at 35°C for 10 min, the samples were diluted and the porinless-vesicles quantified as above. The plot of F.I. *vs* PEG concentration (fig.4) shows a sigmoidal shape, suggesting cooperative action of PEG molecules to evoke the fusion. The PEG-4000 concentration yielding the half-maximum F.I. was calculated to be about 28% (w/v).

In conclusion, our assay method, the quantitative determination of membrane fusion using

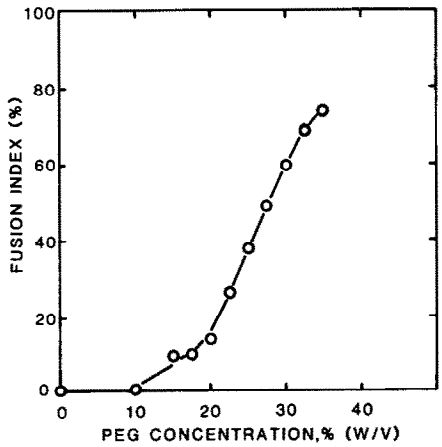

Fig. 4. Determination of PEG-induced membrane fusion. Vesicle membranes with or without 6.75 nmol porin trimers per 10 μmol PC in 1.0 ml of 100 mM K-gluconate and 10 mM Tris-HCl (pH 8.0) were prepared as in the legend to fig.1. The mixtures of 10 μl each of the vesicle suspensions (0.2 μmol PC) were combined with 40 μl of appropriate concentrations of PEG-4000 in the above buffer. These tubes were incubated at 35°C for 10 min and were diluted with 540 μl of a solution containing 100 mM Na-gluconate and 10 mM Tris-HCl (pH 8.0). Fluorescence intensity was measured as above using 20 μl of the reaction mixture.

porin, seems to be a useful technique to monitor membrane fusions irrespective of fusogens and of the leakage of vesicle contents during the fusion reaction. Since the cyanine dye could be used to monitor the presence of a membrane potential in various membranes [4,9] and the membrane potential across the liposome membranes could be generated artificially as shown here, this method has the potentiality to be used not only for studies of the mechanism(s) of membrane fusion but also for a search for fusogens.

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