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THE USE OF AQUEOUS SPACE MARKERS TO DETERMINE THE MECHANISM OF INTERACTION BETWEEN PHOSPHOLIPID VESICLES AND CELLS

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Summary

A method has recently been introduced that quantitates the extent of phospholipid vesicle-cell interactions by following the amount of a vesicle-entrapped water-soluble fluorescent probe, carboxyfluorescein (CF) that becomes cell associated (Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) Science 195, 489—492). We have characterized some of the properties of this probe in sonicated phospholipid vesicles. The CF undergoes a pH-dependent quenching as previously reported and both a pH- and temperature-dependent efflux from vesicles. Decreasing the pH from 7.4 to 5.0 results in almost a 100-fold increase in CF efflux from the vesicles. The simultaneous measurement of cell-associated tritiated lipid and CF fluorescence reveals a discrepancy between the two markers with the tritiated phospholipid becoming associated to a 5—10-fold greater extent than the CF. In the presence of cells the leakage of CF from vesicles increases from 1.5- to 10-fold depending on the vesicle composition. This data suggests that interpretations of cell-vesicle interactions followed by the CF technique or other aqueous space markers should be done with caution. However, in experiments where the leakage of CF from vesicles can be controlled, the technique can provide useful information.

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Abbreviations: CF, carboxyfluorescein; DPPC, dipalmitoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine. The terms vesicle, phospholipid vesicle, and liposome are used interchangeably in the paper.

Introduction

Recently a technique to explore cell-liposome interactions using a self-quenched concentration of a water-soluble fluorescent probe entrapped in vesicles was described by Weinstein et al. [1]. This technique has been used to study the interaction of neutral phospholipid vesicles with a number of cell types [1—3]. The resulting fluorescent microscopic image of these cells is a combination of a diffuse intracellular fluorescence indicative of probe localization in the cytosol and areas of more intense fluorescence indicating localization of the probe in discrete regions of the cell [1,2]. Such results have been interpreted as evidence for substantial levels of cell-vesicle fusion or transfer of vesicle contents to the cell [2]. However, when the fluorescent probe is a lipid analog embedded in the bilayer of the vesicle, punctate non-uniform areas of fluorescence appear on the cell surface following incubation of the cells with the labelled vesicles [4]. Measurement of the lateral diffusion of these lipid analog probes on the cell surface by the Fluorescence Receovery After Photobleaching technique reveals that they do not show typical lipid lateral diffusion values [4] but are immobile. If a substantial level of vesicle-cell fusion had occurred, one would expect to observe a more uniform distribution of the fluorescent phospholipid in the plasma membrane and a lateral diffusion coefficient characteristic of a lipid molecule diffusing in the bilayer [5,6]. Both the image and photobleaching results suggest that the majority of the vesicles associated with the cells are adsorbed on the cell surface [4]. This apparent discrepancy between the mechanism of cell-vesicle interactions determined by these two different techniques prompted us to quantitate cellular uptake of vesicles containing encapsulated CF and tritiated phospholipids in the vesicle bilayer and to investigate some of the properties of vesicle-entrapped CF. The results reported here suggest that the use of CF-containing vesicles to delineate the pathways of cell-vesicle interactions must be approached with caution. However, this technique can be utilized in a simpler model system to study calcium-induced vesicle-vesicle fusion [7].

Methods

Materials. Palmitic acid (puriss), stearic acid (puriss), were obtained from Fluka, A.G., Switzerland. Phosphatidylcholine was extracted from egg yolk as described [8]. Dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidylcholine (DSPC) were synthesized by the method of Robles and Van der Berg [9] and contained greater than 99% of the appropriate fatty acid. Phosphatidylserine was extracted from bovine brain as previously described [8]; [^{3}H]DPPC was prepared as previously described [8]. All phospholipids including [^{3}H]DPPC were purified on silicic acid and found to be pure on thin-layer chromatography. Carboxyfluorescein was obtained from Eastman Kodak, recrystallized from ethanol-water by the method of Blumenthal et al. [3], and the stock solution was prepared by titrating the appropriate amount of CF with 1 M KOH until the pH was 7.4. The phosphate-buffered saline contained 137 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4.

Preparation of lipid vesicles. Unilamellar vesicles were made as described pre-

viously [8] by sonication of multilamellar vesicles. Lipid concentration was determined by phosphate analysis [8]. The transition temperature of the (DPPC/DSPC, 1 : 1) sonicated vesicles has previously been reported at 43°C [10]. The CF-loaded vesicles were separated from non-encapsulated CF on a 1 X 25 cm Sephadex G-75 (fine) column. The vesicles were immediately placed on ice and the fluorescence in the absence and presence of Triton X-100 was determined. To obtain a value for the fluorescence per vesicle it is important, particularly for the higher concentrations of CF, to immediately measure the fluorescence of an aliquot since even a slight amount of leakage from the encapsulated quenched material can significantly increase the fluorescent signal.

Fluorimetric measurements of CF vesicles. Carboxyfluorescein fluorescence was measured in an Aminco-Bowman spectrofluorimeter equipped with an averaging digital photometer and a jacketed cuvette holder in which the temperature could be adjusted by circulating water [11]. The excitation wavelength was 490 nm, emission wavelength was 550 nm with a Corning 3-68 sharp cut-off filter inserted between the sample compartment and photomultiplier. A 1 mm slit was positioned on the excitation side of the cuvette, a 1 mm slit on the emission side, and a 2 mm slit before the photomultiplier. The fluorescence was followed by recording the signal on a strip chart recorder (Omniscribe). Efflux of CF from vesicles containing 200 mM CF was followed by placing 3 ml of the vesicle suspension in the appropriate buffer solution in a magnetically stirred cuvette and recording the increase in fluorescence as the CF leaked out of the vesicles. Under these conditions, continuous exposure of the dye to the excitation beam did not result in a significant amount of bleaching of the CF and the rate of increase of fluorescence is a direct indicator of CF leakage from the vesicles. The total fluorescence of the sample was determined by adding 0.1 ml 10% Triton X-100 to the cuvette to release all of the CF. The buffer solutions used to determine the effect of pH on efflux and relative fluorescence were composed of citric acid/sodium phosphate in 100 mM NaCl. The pH adjusted to within ±0.01 pH unit. The temperature of the samples were measured both before and after the efflux measurements with a digital thermocouple temperature probe (Bailey Instruments, Saddlebrook, NJ) to 0.1°C. For the measurements made at the lower temperature regions, the increase was followed for at least 15 min. The rate of CF release was calculated as the fraction of the total CF in the vesicles released per h. For measurement of cell-associated CF, the cells were incubated and washed according to the protocol of Table I and the total CF fluorescence in the presence of 0.1% Triton X-100 was measured in a 5 mm cuvette containing 1 ml of suspended cells. The blank consisted of cell similarly treated in the absence of CF.

To determine the amount of CF released in the presence of cells, the cells were separated from the vesicles by centrifugation (8 min, 200 X g) and the CF fluorescence was measured in the supernatant at zero time and at 60 min, in the absence and presence of Triton X-100. The percentage release per h was then calculated as the 60 min fluorescence (F_{60}) corrected for the zero time fluorescence (F_0) divided by the total Triton X-100 fluorescence (F_{total}) times 100.

Preparation of cells. L1210 cells were cultured as previously described [12]

in RPMI 1640 with 10% fetal calf serum. Prior to use the cells were washed three times in RPMI 1640 without serum and resuspended at a density of $1 \cdot 10^7$ cells/ml. Human lymphocytes were obtained by phlebotomy from a healthy subject on the day of the experiment and separated by the Ficoll-hypaque technique [13], washed three times in Eagles balanced salt solution and finally resuspended in RPMI 1640.

All incubations of cells with vesicles were carried out in the absence of serum at 37°C. The exact conditions of the incubation are given in Table I. [^{3}H]-DPPC associated with the cells was determined by liquid scintillation counting of the cell-vesicle complex solubilized in 1% Triton X-100 in 10 ml ACS (Amersham, Arlington Heigths, IL).

Results

Cell-associated CF and tritiated phospholipid radioactivity were simultaneously determined with both L1210 cells and human lymphocytes (Table I). Regardless of thecomposition of the lipid vesicles, the tritiated phospholipid became associated to a much greater degree (at least 5-fold) than the entrapped CF. Since tritiated lipid has previously been shown to give reasonable estimates of the increase in cell-associated lipid when measured by chemical analysis [14] or by certain water-soluble entrapped components [15], the discrepancy between the lipid and CF associated with the cells can be attributed to the leakage of CF from vesicles following contact with the cells. In fact, experimentally significant increased leakage of CF into the incubation medium from DPPC/DSPC and phosphatidylserine/phosphatidylcholine vesicles incubated with cells is also observed (Table II). This is particularly noticeable when vesicles (DPPC/DSPC) were incubated below their transition temperature with cells. Since the DPPC/DSPC vesicles have a broad transition range centered at 43°C but starting around 30°C, the increase in CF leakage may be a function of the anomalous increase in permeability and ability of proteins to insert into the bilayer that occurs in the vicinity of the transition temperature (see for

TABLE I

UPTAKE OF VESICLE LIPID [^{3}H]DPPC AND VESICLE CONTENTS (CF)

$2 \cdot 10^6$ cells were incubated with 200 nmol vesicle lipid in 1 ml RPMI 1640 without serum at 37°C in a slanted position while being rotated at 12 rotations per h. Incubation time was 1 h for the L1210 cells and 0.5 h for the human lymphocytes. The vesicle contained 200 mM CF and $2.55 \cdot 10^5$ cpm [^{3}H]DPPC per nmol lipid. At the end of the incubation period the cells were washed (three times) with 10 ml ice-cold phosphate-buffered saline and centrifuged at 200 × *g* for 8 min to separate cells and associated material from vesicles (three times). The final cell pellet was suspended in 1 ml phosphate-buffered saline with 0.1% Triton X-100 and the fluorescence and radioactivity associated with the cells were determined. The values for the L1210 cells are the mean and standard error from three separate experiments, for the lymphocytes from a single experiment done in triplicate.

Vesicle composition	Cell type	Uptake (%) at 37°C	
		[^{3}H]DPPC	CF
Phosphatidylcholine	L1210	0.42 ± 0.069	0.0074 ± 0.0021
DPPC/DSPC (1 : 1)	L1210	1.5 ± 0.35	0.33 (2)
Phosphatidylserine/phosphatidylcholine (1 : 9)	L1210	0.55 ± 0.037	0.057 ± 0.017
Phosphatidylcholine	Human lymphocytes	0.143 ± 0.028	0.0044 ± 0.0016

TABLE II

RELEASE OF VESICLE-ENTRAPPED CF INTO MEDIUM FOLLOWING INCUBATION WITH L1210 CELLS

200 nmol vesicle lipid containing 200 mM CF were incubated in 1 ml RPMI 1640 without serum at 37°C for 1 h in the presence or absence of $2 \cdot 10^6$ L1210 cells. At the end of this period 10 ml ice-cold phosphate-buffered saline were added and the cells separated from the vesicles by centrifugation at 200 × *g* for 8 min (three times). 1 ml of the supernatant was analyzed for CF fluorescence in the absence and then in the presence of 0.1% Triton X-100. The values in the presence of cells are the mean and standard error for three separate experiments, in the absence of cells the mean from duplicate measurements, that agree to within 10% in a single experiment.

Vesicle composition	Percent CF release per h at 37°C	
	Cells present	Cells absent
Phosphatidylcholine	9.5 ± 2.4	6.0
DPPC/DSPC (1 : 1)	36.4 ± 8.4	3.4
Phosphatidylserine/phosphatidylcholine (1 : 9)	12.3 ± 3.0	4.0

example, ref. 16, and references therein). One consistent observation is that vesicles below their transition temperature (e.g. DPPC/DSPC) become leakier when in the presence of cells (Table II) but paradoxically yielded a CF fluorescence value more in line with that obtained with the tritiated lipid (Table I). This phenomenon has also been observed with five different cell lines in 15 separate experiments [28]. Although we can speculate that this is related to the multiple binding sites on the cell for vesicles below their transition temperature [20] one class of sites resulting in a high rate of leakage, another in a low rate of leakage, we have no evidence that this is the case. In any event the effect of the lipid physical state on CF leakage would complicate comparisons between vesicles of different lipid composition. Medium that had been incubated with cells for 60 min, separated from the cells by centrifugation, and then incubated with CF-loaded phosphatidylcholine vesicles did not cause an increase in CF leakage. This suggests that the presence of cells is necessary for the increase in vesicle permeability to occur.

The fluorophore CF undergoes a pH -dependent quenching [1] so the possibility that pH might also influence the rate of CF efflux from the vesicles was examined. Fig. 1 confirms that the fluorescence from aqueous solutions of CF is pH dependent with an apparent pK_a of 6.2. In addition, CF efflux increases rapidly as the pH is lowered with the efflux reaching 7.3% per min at pH 5.0, the lowest pH value measured (Fig. 1). Thus, as the pH is lowered CF is protonated and diffuses through the phospholipid bilayer more rapidly. Measurement of the pH of the incubation medium following interaction of vesicles with cells (Table I) established that there was no change in pH of the reaction mixture during the incubation. This excludes the possible complication of medium pH changes as an explanation of the results described earlier.

In view of the leakage of CF from vesicles associated with cells and the marked pH dependence of the efflux, the influence of the internal CF concentration on fluorescence per vesicle was determined (Fig. 2). The fluorescence per vesicle goes through a maximum value near an internal CF concentration of 10 mM and then decreases to a constant value at an internal concentration of

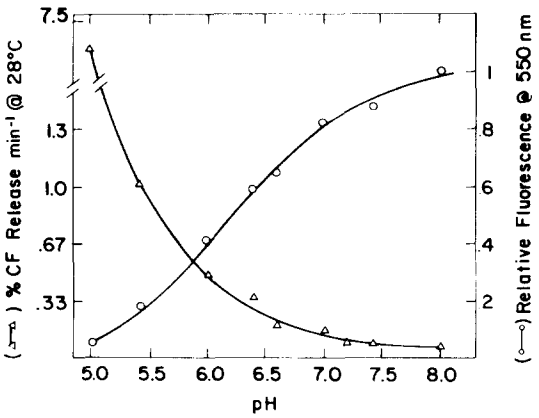

Fig. 1. Effect of pH on CF efflux from phosphatidylcholine-sonicated vesicles (△——△) and on relative CF fluorescence in aqueous solution at 550 nm (○——○) at 28°C. Values of CF efflux are the means of duplicate measurements that agreed to within 10%. Values for the relative fluorescence are the means of duplicates that agreed to within 5%. Percentage release of CF was determined as described in Methods.

100 mM. The values from 2 to 200 mM internal CF are shown in Fig. 2. This data indicates that if the 200 mM CF-containing vesicles were to lose 95% of their CF, the fluorescence from the separated vesicles would actually be greater than the original vesicle preparation.

Temperature effects on the efflux of the CF from phosphatidylcholine vesicles reveal a non-linear Arrhenius plot with a high activation energy over the

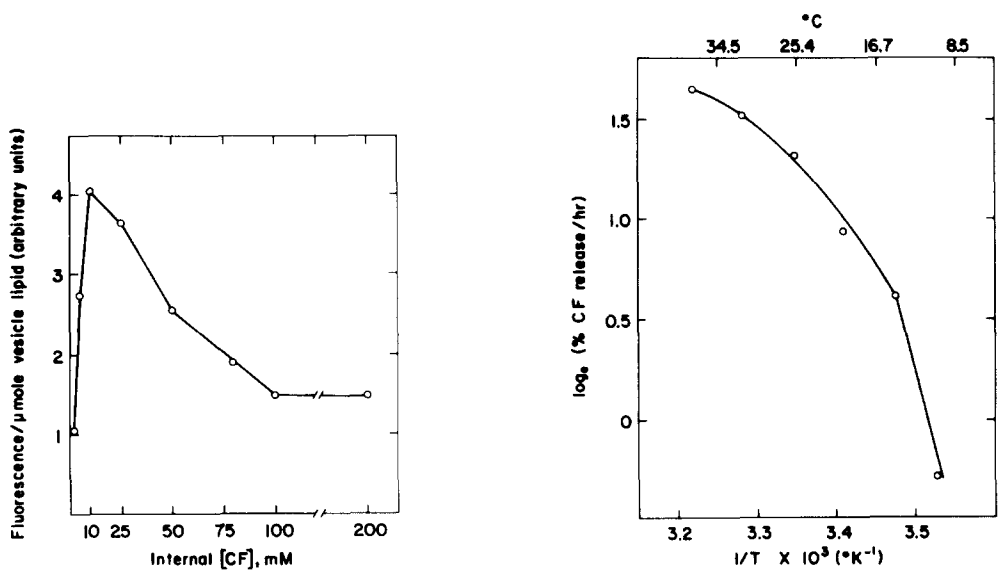

Fig. 2. Fluorescence per vesicle when loaded with a range of concentrations of CF. Values are from a single experiment in which the vesicle fluorescence was measured as soon as the vesicle-encapsulated CF was separated from unencapsulated CF.

Fig. 3. Effect of temperature on the efflux of CF from sonicated vesicles containing 200 mM CF. Conditions as stated in Methods.

lower temperature range (Fig. 3). As reported previously [1], the leakage of CF from vesicles at 0°C is very low but increases to a substantial value (0.08–0.11% per min) at 37°C.

Discussion

The mechanisms of cell-liposome interactions have been the focus of a number of investigations (for reviews see refs. 17 and 18) but no clear picture has emerged as to the contribution of different pathways to the total uptake of vesicle lipid and contents by the cells. The CF technique described by Weinstein et al. [1] appears to be a promising method for evaluating vesicle-cell interactions; however, the following points must be considered when drawing conclusions based on this method concerning cell-vesicle interactions.

First, there is a significant discrepancy between lipid and CF associated with the cell, which means that any attempt to quantitate the number of cell-associated vesicles using CF will be an underestimate [1,3]. Presumably, this discrepancy is due to leakage of CF from the vesicle interior. If the lipid label can be considered to be representative of the total number of vesicles that become cell associated, then after 30 min with the lymphocytes and 60 min with the L1210 cells, greater than 90% of the CF contents must have leaked out of the cell-associated vesicles. The experiments described here show that CF leakage is a strong function of both pH (Fig. 1) and temperature (Fig. 3). These factors complicate the use of CF as a tool to quantitate modes of cell-vesicle interactions.

Since the fluorescence per vesicle depends on the internal CF concentration, the amount of CF leakage prior to the measurement will determine the magnitude of the signal. The same number of vesicles could, therefore, result in differing fluorescent intensities depending on their internal concentration of CF. Furthermore, the site of CF leakage from the cell-vesicle complex can be a significant factor in determining what percentage of vesicles appear to interact with the cell by various pathways. For example, if the leakage of CF occurs primarly from vesicles on the cell surface, the internalized CF would then represent a larger fraction of the total cell-associated CF. On the other hand, liposomes may be internalized via endocytosis into the lysosomal apparatus. There, the low pH [19] could result in marked stimulation of CF efflux from the liposomes and the lysosomes into the cytoplasm which would appear as a diffuse intracellular fluorescence suggestive of a fusion pathway. Thus, predominant endocytosis could be mistaken for fusion. The temperature dependence of CF leakage must also be considered in interpreting results using this method. Any apparent influence of temperature on cell-vesicle interaction [20] would be complicated by temperature effects on cell-induced CF permeability. If, for example, surface adsorption of CF-containing vesicles was predominant and the vesicles were less labile to surface-induced leakage at lower temperature, adsorption would be exaggerated at 4°C compared to 37°C.

The leakage of aqueous markers may be important in other experiments as well. A number of investigators have reported on the non-equivalence of lipid and aqueous space markers in cell-vesicle interactions under certain conditions. Such discrepancies have been suggested to be due to phospholipid exchange or

leaky fusion [21,22]. Another possibility is that during the incubation of vesicles with cells, the vesicles are modified by cellular components incorporating not only cellular lipid (exchange) [21] but also protein, a notion supported by recent experimental observations [23,24]. In view of the increased vesicle permeability caused by the interaction of several proteins with vesicles [25,26] it is not surprising that vesicles that have interacted with the cell surface become leakier. Thus, experiments using markers in the aqueous space of the vesicles to follow cell-vesicle interactions should make due allowance for the possibility of significant leakage of the marker from the vesicles during incubation with cells. The inclusion of cholesterol on a 1 : 1 molar basis is one precaution that can be taken to reduce the initial permeability of vesicles to small compounds and to protect against protein-induced increases in permeability [27].

These considerations necessitate a cautious approach to the interpretation of data on cell-vesicle interactions monitored by the CF technique or other aqueous space markers. They do not pose any problem for the use of this technique to study more defined model systems such as calcium-induced vesicle fusion [7] or other model systems where the leakage problem can be more carefully controlled (Weinstein, J.N., Blumenthal, R., Ralston, E., Dragsten, P. and Leserman, L., unpublished).

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