A Fluorescence Assay to Monitor Vesicle Fusion and Lysis*

(Received for publication, August 3, 1982)
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An assay based on the fluorescent compound 2',7'-[[bis(carboxymethyl)amino]methyl] fluorescein (calcine) has been developed to investigate vesicle fusion and lysis. The assay involves encapsulating the nonfluorescent Co²⁺ complex of calcine in one set of vesicles and EDTA in a second set. If fusion occurs, EDTA chelates Co²⁺, releasing calcine which may be assayed by means of its intense fluorescence. Leakage of either component of the reaction can be directly quantitated by titrating the external medium with the other component. This assay was used to investigate the effects of calcium ion on small phosphatidylserine vesicles, a system reported to undergo fusion. It revealed immediate and extensive leakage of the encapsulated contents; membrane fusion must, therefore, be simultaneous with, or subsequent to, loss of vesicle integrity. This assay is convenient and flexible; it should be useful in a variety of investigations of membrane-bounded compartments.

Although membrane fusion is a central theme of many biological processes, the mechanisms involved remain unclear. Attempts to model the in vivo fusion event with artificial lipid vesicles have been complicated by the lack of unambiguous assays to demonstrate and quantify vesicle fusion. Assays based upon mixing of spin-labeled lipids (1, 2), changes in NMR spectra (3, 4), and fluorescence energy transfer (5–8) do not distinguish lipid transfer or exchange from fusion. To avoid this ambiguity, other assays have been employed which monitor mixing of aqueous compartments. These typically involve encapsulating half of a two-component reaction in each of two sets of vesicles. If the appropriate vesicles fuse, the reaction can proceed and a detectable spectral change results. This approach has been the basis of assays involving firefly luciferase and ATP (9, 10), trypsin and a fluorogenic substrate (11), the interaction of arsenazo-Ca²⁺ chelates with EDTA to produce an absorbance shift (12), and terbium ion and dipicolinic acid which form a fluorescent complex (13, 14). While these may prove informative in some systems, none is entirely adequate. If fusion is transient, assays utilizing enzymatic reactions may be too slow to permit kinetic analysis, and lysis of vesicles containing large molecules as markers may be underestimated due to the geometric constraints of diffusion through a lipid barrier. Although some assay reactions can be inhibited in the external phase to avoid background due to leakage (11, 13, 14), few permit direct quantitation of lysis during the course of an experiment. In addition, other assays (9, 10, 12) require manipulations and corrections following each experiment which make quantitation and kinetic measurements difficult. The terbium-dipicolinic acid assay is largely free of these constraints but excitation of terbium requires UV light which makes it unsuitable for microscope studies.

With these considerations in mind, we have developed a new assay to monitor vesicle fusion and/or lysis based on the fluorescent compound 2',7'-[[bis(carboxymethyl)amino]methyl] fluorescein (calcine). Calcine is stable, highly fluorescent, readily available, and inexpensive. The spectral and fluorescent properties of this compound are well known (15) and are advantageous for a variety of lipid vesicle applications (16). In this study, we have combined Co²⁺ with calcine to form a chelate which does not fluoresce in the neutral pH range. However, in the presence of EDTA, a stronger Co²⁺ chelator, the intensely fluorescent free dye is released and can be readily detected at very low concentrations. Therefore, by encapsulating the quenched complex calcine-Co²⁺ in one set of vesicles and EDTA in a second set, intervesicle mixing can be monitored by fluorimetry. Furthermore, leakage of calcine and calcine-Co²⁺ can be quantitated by titrating the external medium with Co²⁺ and EDTA, respectively.

In the present study, the calcine assay was used to re-examine the consequences of the interaction of calcium ions with SUV composed of PS. When Ca²⁺ is added to PS SUV, aggregates or large cochleate structures form. Upon subsequent incubation with EDTA, large vesicles are observed (17). Since this finding, a number of studies of the potential of acidic lipid vesicles to fuse in the presence of Ca²⁺ have yielded conflicting results with respect to whether fusion occurs (11, 20) and, if so, whether leakage of vesicle contents is concomitant and/or sizable (13, 14, 18, 19). Since the calcine assay is particularly appropriate for the kinetic analysis of vesicle fusion and lysis, we felt it would be an informative probe for PS SUV studies. The data reported here suggest Ca²⁺ induces immediate and extensive leakage of PS SUV.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidylserine from bovine brain was obtained from Avanti Biochemicals, Inc. 2',7'-[[bis(carboxymethyl)amino]methyl] fluorescein (calcine) was generously provided by Drs. Harvey Diehl and John Furry. A similar preparation may be obtained from the Hach Company (Loveland, CO). Stock solutions of calcine were quickly solubilized at pH 9.0 and then readjusted to pH 7.5. CF was obtained from Eastman and 4-methylumbelliferyl phosphate from Sigma. All solutions used in these studies were stored in plastic containers or carefully cleaned glassware to avoid ion contamination and possible quenching.

VESICLE PREPARATION—Vesicles were prepared by hydrating 2 mg of phosphatidylserine with 0.25 ml of 50 mM MOPS, pH 7.5, solution containing calcine at 0.8 mM plus CoCl₂ at 1.0 mM or EDTA at 20 mM. For some experiments, PS was hydrated with 50 mM CF and 0.1 mM EDTA or 10 mM 4-methylumbelliferyl phosphate. Vesicles were then vigorously vortexed for 1 min and sonicated in a water bath

* This research was supported by National Institutes of Health Research Grant GM 28404. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

This paper is dedicated to Professor David Shemin in honor of his 70th birthday.

1 The abbreviations used are: SUV, small unilamellar vesicles; MOPS, 2-(N-morpholino)propanesulfonic acid; PS, phosphatidylserine; CF, carboxyfluorescein.

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sonicator for 30 min under nitrogen. Untrapped solutes were removed by elution from Sephadex G-75 column with 50 mM MOPS, 100 mM NaCl, pH 7.5, first with 1 mM EDTA then without.

**Experimental Conditions**—In a standard experiment, calcein-Co$^{2+}$- and EDTA-containing vesicles were mixed in a 1:1 ratio in 50 mM MOPS, 100 mM NaCl, pH 7.5. The total lipid concentration was 50 μM, 25 μM of each type of vesicle, as determined by phosphate analysis (Fiske and SubbaRow modification (21)). CaCl$_2$ from a concentrated stock solution was added to a final concentration of 1.5 mM. In some experiments, either EDTA at 10 mM or CoCl$_2$ at 0.4 mM chelated with 0.4 mM citrate were present in the reaction mixture to assess the leakage of encapsulated components. The maximum fluorescence possible from a typical reaction was determined in the presence of 1.5 mM CaCl$_2$. The release of 4-methylumbelliferyl phosphate was assayed with alkaline phosphatase (Calbiochem-Behring). CF fluorescence was calibrated by addition of 0.1% Triton X-100. All reactions were with constant stirring at 28–32°C.

**Spectrofluorometric Measurements**—A Farrand spectrofluorometer was used. For calcein and CF, the excitation and emission monochromators were set at 490 and 520 nm, respectively; 488 and 520 nm interference filters were inserted and slits were removed to yield maximum intensity. Under these conditions, unloaded vesicles in the presence of Ca$^{2+}$ gave no significant signal due to light scattering. The fluorescence of 4-methylumbelliferyl phosphate was generated from its phosphate by the enzyme alkaline phosphatase. The release of 4-methylumbelliferyl phosphate was monitored by phase contrast and fluorescence.

**Microscopy**—For these studies, 2 mg of PS was hydrated with 0.25 ml of 25 mM MOPS, pH 7.5, vortexed, and sonicated as described above. The vesicles at a concentration of 50 μM were vortexed in 50 mM MOPS, pH 7.5, with 2.5 mM calcein and 8 mM calcium for 30 s and then treated with 15 mM EDTA. Citrate-Co$^{2+}$ (each at 50 mM) was then added to the reaction mixture. For the control, buffer replaced calcium. Vesicle suspensions were then observed by phase contrast and fluorescence.

**RESULTS AND DISCUSSION**

The fluorescence change accompanying the reaction of calcein with the citrate chelate of Co$^{2+}$ in the absence of vesicles is shown in Fig. 1. In the micromolar range, a 1:1 molar ratio of dye and metal ion results in about 85% quenching. Such substantial quenching indicates preferential formation of a 1:1 complex. Citrate present at equimolar ratios with Co$^{2+}$ does not produce a marked effect on the reaction of calcein and the metal ion.

Fig. 1 also shows (inset) the kinetics of calcein-Co$^{2+}$ complex formation at the concentration with which vesicles are loaded.
Assay for Vesicle Interactions

and the subsequent removal of Co\(^{2+}\) by EDTA. At 0.8 mM calcein and 1.0 mM Co\(^{2+}\), quenching is fast and little residual fluorescence remains. The release of Co\(^{2+}\) by millimolar levels of EDTA is also rapid. Under our experimental conditions (6-mm round cuvette), some quenching due to the inner filter effect is observed at 0.8 mM calcein. Therefore, in this experiment, the maximum fluorescence is underestimated. In the vesicle studies, inner filter effects are not observed because the total concentration of calcein in the reaction volume is about 50 nM.

When Ca\(^{2+}\) is added to a mixture of calcein-Co\(^{2+}\) and EDTA-containing PS SUV to a final concentration of 1.5 mM, only a small increase in fluorescence is observed (Fig. 2, solid line). The observed increase in fluorescence, and therefore free calcein, could be a consequence of EDTA-Co\(^{2+}\) formation occurring within the membrane-bounded compartment of fused vesicles. On the other hand, if the vesicle contents leak, they would become more dilute as they equilibrated with the external phase. Dilution would slow formation of the EDTA-Co\(^{2+}\) chelate but also favor dissociation of calcein-Co\(^{2+}\) and free calcein may arise in this way.

Fluorescence due to vesicle lysis can be eliminated by the presence of 0.4 mM Co\(^{2+}\) (added in 1:1 molar ratio with citrate) in the external phase. Alternatively, Co\(^{2+}\) can be added at various time points to assess the time course of calcein leakage. Fig. 2 presents the results of several such experiments. When Co\(^{2+}\) is present in the reaction medium at the time of Ca\(^{2+}\) addition, the fluorescence increase in not observed and the background fluorescence quickly diminishes (Fig. 2, ·····). The signal is stable, however, until the time of Ca\(^{2+}\) addition; it represents the residual fluorescence of the trapped calcein-Co\(^{2+}\) complex. In parallel experiments in which Co\(^{2+}\) was added after Ca\(^{2+}\), the developing fluorescence was also rapidly quenched. These data indicate that the Ca\(^{2+}\)-induced fluorescence signal is due to calcein that is accessible to Co\(^{2+}\) in the external phase. Co\(^{2+}\) in the presence of citrate (each at 0.4 mM) has no effect on PS vesicles as established by differential scanning calorimetry and microelectrophoresis.

Fig. 2 also contains results of several experiments in which 10 mM EDTA was added to the reaction medium prior to or at various time points after Ca\(^{2+}\) addition (---) to reveal the amount of calcein-Co\(^{2+}\) complex in the external phase. The first addition of EDTA indicates 17% of the calcein-Co\(^{2+}\) complex is present as untrapped marker. Subsequent

**Fig. 4.** Phase and fluorescence microscopy of Ca\(^{2+}\) and EDTA-treated PS SUV in the presence of calcein. a, unloaded vesicles (present at a lipid concentration of 50 μM) treated with 8 mM Ca\(^{2+}\) in the presence of 2.5 mM calcein. Since calcein can bind up to 2 mol of Ca\(^{2+}\), the effective concentration of Ca\(^{2+}\) is about 3 mM. EDTA at 15 mM and citrate-Co\(^{2+}\) (each at 50 mM) were then added and the vesicles viewed with phase contrast. b, the same field as in a using fluorescence illumination. c, the same experiment as in a except the vesicle concentration was 500 μM. d, the same field as in c viewed with fluorescence illumination. e, vesicles treated exactly as those in a and b except buffer was added in place of Ca\(^{2+}\) and viewed with phase contrast. f, the same field as in e viewed with fluorescence illumination. The width of each frame corresponds to 27 μm.
additions of EDTA reveal the Ca\(^{2+}\)-induced release of calcein-
Co\(^{2+}\). Virtually all the delay in the fluorescence increase is due
to the EDTA reaction (compare with the first addition).
About 50% of the encapsulated complex becomes accessible
to the EDTA reaction (compare with the first addition).
In summary, the experiments depicted in Fig. 2 show that
Ca\(^{2+}\) induces the immediate release of vesicle contents. All the
fluorescence which develops when Ca\(^{2+}\) is added to PS SUV
can be attributed to the release of calcein into the external
phase. If vesicle fusion had occurred prior to lysis, one would
expect a corresponding portion of calcein fluorescence to be
inaccessible to Co\(^{2+}\) quenching. The rapid leakage of calcein-
Co\(^{2+}\) further supports the conclusion that if fusion occurs, it
is not of a nonleaky nature.
The data of Fig. 3 show Ca\(^{2+}\)-induced vesicle lysis using an
established assay (22) and verify that citrate-Co\(^{2+}\) does not alter
the response of PS vesicles to Ca\(^{2+}\). Vesicles containing
4-methylumbelliferyl phosphate were treated with 1.5 mM
Ca\(^{2+}\). Alkaline phosphatase was present externally. The rapid
increase in fluorescence is due to vesicle lysis and enzymatic
hydrolysis of the substrate to give 4-methylumbelliferone. The
data are similar when citrate-Co\(^{2+}\) (0.4 mM) is included in the
external phase. In contrast, when vesicles are loaded with
concentrated carboxyfluorescein, the fluorescence which
develops due to leakage into the external phase is much slower,
amounting to 50% in 3 min, but in good agreement with
reported results (13, 14).
Additional evidence that citrate-Co\(^{2+}\) does not significantly
interact with PS SUV is provided by the observations that up
to 10 mM, at least, the complex has no effect on vesicle light
scattering and does not induce the leakage of vesicles that are
loaded either with calcein or with carboxyfluorescein at self-
quenching concentrations.
When unloaded PS SUV in a solution of calcein are treated
first with Ca\(^{2+}\) and then with EDTA, larger vesicles result (1, 17) as shown by the phase micrograph of Fig. 4a. By compar-
ison, vesicles from the same suspension not treated with Ca\(^{2+}\)
are barely visible at this magnification (e). When the two sets of vesicles are treated with citrate-Co\(^{2+}\) to quench calcein in the
external phase and then viewed by fluorescence micros-
copy, fluorescent vesicles are apparent only in the Ca\(^{2+}\)-
treated set (b). Thus, large vesicles that have sampled the
external phase are generated by the action of EDTA on small
vesicles that have been in contact with calcium ion for no
more than 30 s. Entrapment of larger markers (i.e. ferritin)
has previously been shown to occur (23).
In conclusion, calcein forms the basis of a flexible method
to investigate changes in the bounded aqueous volume of lipid
vesicles. Whereas the study reported here demonstrates use
of the method to monitor vesicle lysis, it is equally well suited
to assay fusion in nonleaky systems or vesicle fusion and
subsequent lysis. Since calcein is excited with visible light,
fusion or lysis can be readily monitored by fluorescence mi-
coscopy. The assay has the potential disadvantage that ves-
icles are encapsulated with the Ca\(^{2+}\) chelator, EDTA. This
can be avoided, however, by encapsulating the nonfluorescent
citrate-Co\(^{2+}\) chelate and calcein in separate vesicles. Fusion
would then be revealed by fluorescence quenching. Indeed, we
have also applied this version of the assay to the problem of
PS SUV-Ca\(^{2+}\) interactions. (The results were the same as
those reported here, indicating that encapsulated EDTA does
not affect this system.) The calcein-Co\(^{2+}\) complex can also be
used to detect changes in vesicle volume because calcein-
Co\(^{2+}\) association is a function of concentration. In addition,
calcein has been used to assay the trapped volume of vesicle
preparations (24).

Acknowledgments—We thank Ruby MacDonald, Naoto Oku, and
Martin Woodle for their helpful comments. We also appreciate the
generous supply of calcein provided by Drs. Harvey Diehl and John
Furry of Iowa State University.

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