FUSION OF PHOSPHOLIPID VESICLES CONTAINING A TRYPSIN-SENSITIVE FLUOROGENIC SUBSTRATE AND TRYPSIN

A new method to study membrane fusion activity in a model system

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Received 6 August 1979

1. Introduction

It is well established that fusion of membranes is involved in several biological processes [1–4]. Numerous attempts have been made to elucidate the molecular mechanisms underlying membrane fusion, and various model membrane systems have been applied for this purpose [5–11].

A number of those studies rely on transfer of spin-labelled lipids between vesicles [12] or on changes in NMR spectra [13–15] as criteria for fusion, leaving considerable uncertainty concerning the question whether the change in membrane composition observed is entirely due to fusion or partly to molecular exchange. The ambiguity of such observations calls for cautious interpretation of experimental data. Despite these complications substantial evidence has been obtained during recent years for the occurrence of fusion as a result of vesicle–vesicle interaction [12,16,17]. Thus far, however, scanty attempts have been made to demonstrate the coalescence of aqueous intravesicular compartments as a result of fusion events. The methods published so far to demonstrate fusion on the basis of this criterion have shown more or less serious shortcomings. Even recent work [18,19] involving the use of the luciferase/ATP system leads to ambiguous results as indicated by the conflicting conclusions reached in [18] and [19]. The need for complicated corrections in this luminescence system makes quantitation and kinetic measurements of the fusion process difficult.

We developed fluorogenic peptide substrates in which a fluorogenic group and a quenching group are attached to the same molecule such that the molecule is non-fluorescent. Separation of quencher and fluorophore as a result of proteolytic cleavage results in a fluorescence increase proportional to the rate of hydrolysis. Such substrates can be designed according to special requirements and provide the means for specific and very sensitive quantitative enzyme assays [20]. In this report we present a simple method to monitor the coalescence of intravesicular compartments based on the proteolytic digestion of a fluorogenic trypsin-sensitive substrate by trypsin.

The presence of trypsin inhibitor in the medium entirely precludes extra-vesicular proteolysis and thus interference by leakage phenomena.

2. Experimental

Egg yolk PC was obtained from Sigma. PS was isolated from bovine brain extract (Sigma) by the method in [21] and purified by preparative thin-layer chromatography on silica gel. The lipids were routinely checked for impurities and repurified if necessary. Unilamellar phospholipid vesicles were prepared from a mixture of PC and PS (molar ratio 1:1) by sonication with a Branson B15 probe sonifier, at a final lipid

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; Tris, Tris (hydroxymethyl) aminomethane; ABz, *o*-aminobenzoyl; Phe(NO_2), *p*-nitro-L-phenylalanyl; Cbz, benzyloxycarbonyl; Boc, *t*-butyloxycarbonyl

concentration of 10 mM in 50 mM NaCl/50 mM Tris·HCl (pH 7.4)/0.1 mM EDTA containing either substrate or trypsin (see below). Sonication was performed at room temperature under an atmosphere of N_2, followed by centrifugation (Eppendorf) to remove titanium shed by the probe.

The fluorogenic substrate Abz–Gly–Arg–Phe-(NO_2)–NH_2 was prepared by stepwise synthesis using the *N*-hydroxysuccinimide ester coupling method. The amide Boc–Phe(NO_2)–NH_2 was prepared from ammonia and Boc–Phe(NO_2). After removal of the Boc group with trifluoroacetic acid, the amide peptide was coupled with Boc–Arg(NO_2). Then Cbz–ABz–Gly was added [22]. The resulting Cbz–ABz–Gly–Arg(NO_2)–Phe(NO_2)–NH_2 was purified by column chromatography on silica. Analysis calculated for $C_{32}H_{36}N_{10}O_{10}$ (mol. wt 721.6): *N*, 19.4; found *N*, 19.7. Protecting groups were finally removed by treatment with HF, the tripeptide amide. HF moved as a single spot on thin-layer silica plates. R_F in *n*-butanol–acetic acid–water (4:1:1, by vol.) = 0.6. Analysis calculated for $C_{24}H_{38}N_9O_6F_7$ (681.6): *N*, 18.49; found *N*, 18.29. Using this substrate with trypsin as the enzyme a K_m was found of 7.7×10^{-5} M measured at the optimal pH of 8.3 in 0.2 M Tris·HCl. Authentic ABz–Gly was used for calibration of fluorescence readings.

For entrapment, the substrate was dissolved in a small volume of 1 M HCl. Subsequently 50 mM NaCl/50 mM Tris·HCl/0.1 mM EDTA was added and adjusted to pH 7.4 by addition of 1 M NaOH. Final concentration of substrate was 4.8 mg/ml. The lipid suspension formed upon adding the substrate solution to the dried lipid film was then sonicated as described above.

Entrapped substrate was freed from non-encapsulated substrate by elution with the same buffer on Sephadex G-100.

Trypsin (from bovine pancreas, Boehringer) was entrapped similarly by addition of an enzyme-containing solution (5 mg/ml in 50 mM Tris/50 mM NaCl (pH 7.4)/0.1 mM EDTA) to the dried lipid film. Encapsulated trypsin was separated from non-entrapped trypsin by chromatography on Sepharose CL-6B. Soy-bean trypsin inhibitor was purchased from Merck. Fluorescence measurements were determined with a Perkin-Elmer MPF 43 fluorescence spectrophotometer with excitation and emission wavelengths of 360 nm (slidwidth 8 nm) and 410 nm (slidwidth 10 nm), respectively. Lipid phosphate was assayed according to [23].

3. Results and discussion

The fluorogenic substrate ABz–Gly–Arg–Phe-(NO_2)–NH_2 was designed for the assay of trypsin and other trypsin-like enzymes. The main features of the peptide are:

(i) The presence of an arginine residue in the center of the molecule to satisfy the specificity requirements of the enzyme;

(ii) The placement of the *o*-aminobenzoyl fluorophore and the *p*-nitro–L-phenylalanyl quenching residue on each side of the cleavable Arg–Phe-(NO_2) peptide bond;

(iii) The substituted carboxyl group in the form of amide which precludes the formation of charge which, when situated next to the cleavable peptide bond, is not well tolerated by trypsin;

(iv) Substituted terminal groups which prevent the hydrolysis by exopeptidases.

As shown in fig.1, the fluorescence of the intact molecule is very low. The hydrolysis by trypsin results

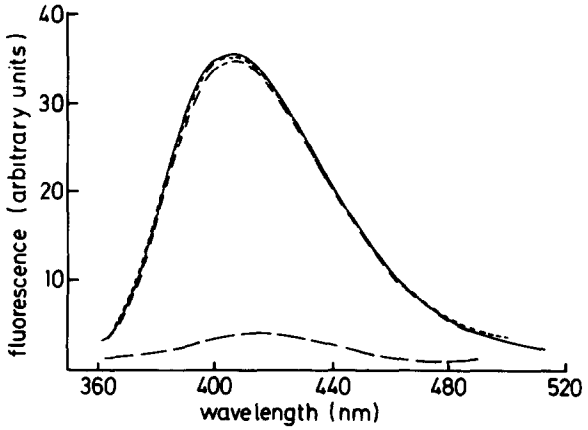

Fig.1. Characteristics of the fluorescence emission spectra of ABz–Gly–Arg–Phe(NO_2)–NH_2·HF (0.03 mM) in buffer (50 mM NaCl/50 mM Tris·HCl (pH 7.4), 0.1 mM EDTA), (— —); after addition of trypsin (100 μg), (———); in presence of 1% Triton X-100 (— — —); and in presence of 2.5 μmol phospholipid vesicles (– – – – –), after trypsin digestion. Excitation wavelength was 360 nm.

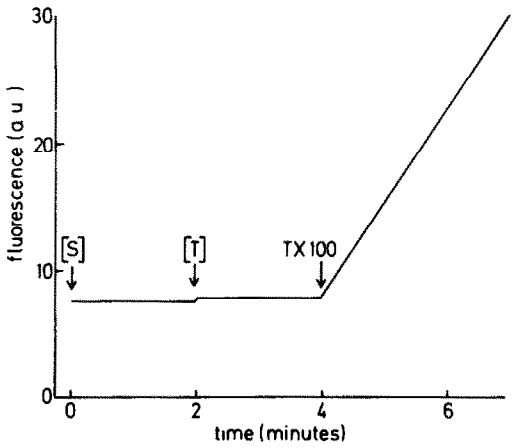

Fig.2. Interaction of substrate [S] and trypsin [T] containing vesicles in absence and presence of Triton X-100 (TX100). Substrate-vesicles (1.1 μmol) were added to the incubation buffer followed by 1.2 μmol trypsin vesicles in 2 ml final vol. Degradation of substrate, resulting in fluorescence increase, is only observed after addition of Triton X-100 (1% final conc.).

in an ~9-fold increase in fluorescence due to the separation of the quenching Phe(NO_2) group from the highly fluorescent *o*-aminobenzoylglycine. The emission spectrum was not influenced by the presence of phospholipid vesicles or of Triton X-100.

In the fusion assay described here vesicles were used consisting of equimolar amounts of PC and PS because a variety of methods [9,16,24,26] have demonstrated that such vesicles tend to fuse under proper experimental conditions.

When free trypsin was added to substrate-containing vesicles a very slow rate of fluorescence increase was measured. After an incubation period of 3 h at room temperature, addition of Triton X-100 showed that still >90% of the substrate was retained inside the vesicles. Similarly, trypsin vesicles did not show any enzymatic activity towards free substrate, indicating absence of trypsin at the vesicle surface.

Figure 2 demonstrates that mixing the two vesicle preparations containing the substrate and the enzyme, respectively, causes no fluorescence. Addition of Triton X-100, however, caused rapid linear development of fluorescence due to relief of intramolecular quenching, indicating proportionate degradation of the substrate. Addition of trypsin-inhibitor (50 μg/ml) prior to addition of Triton X-100 completely inhibits the fluorescence increase.

In fig.3 an experiment is shown in which substrate vesicles were incubated with trypsin vesicles in presence of trypsin-inhibitor and Ca^{2+}. It is well established that presence of Ca^{2+} is a prerequisite for fusion to occur between PS-containing vesicles [1,9,12]. Indeed, in absence of Ca^{2+}, no increase in fluorescence was observed, indicating lack of enzyme–substrate interaction and thus lack of fusion (squares). Upon addition of 5 mM Ca^{2+}, fusion occurred as indicated by the steady increase in fluorescence during the first 60 min incubation (circles). Presence of Ca^{2+} caused the formation of a slowly sedimenting white flocculate, as a result of vesicle-aggregation [15,17]. However, under the conditions used no interference from light scattering with the fluorescence measurements was observed.

The kinetics of the fluorescence development show that the process tends to level off after 60 min. By this time nearly 20% of the substrate was hydrolyzed as was indicated by addition of Triton X-100 which allows complete digestion of the substrate. Several factors may contribute to such, seemingly, limited extent of fusion:

(1) Initially, only 1/3rd of the fusion events will lead to contact between substrate-containing and enzyme-containing spaces assuming that

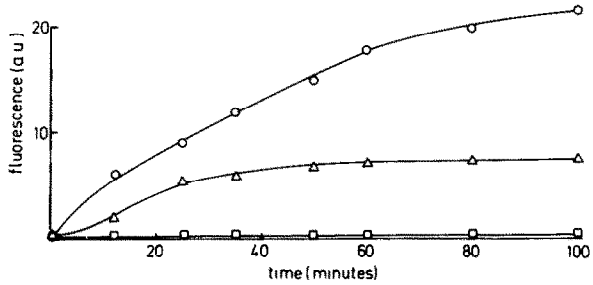

Fig.3. Fusion of 1.6 μmol substrate vesicles with 1.1 μmol trypsin vesicles and Ca^{2+}-induced leakage of vesicle contents. Incubation was carried out in 2 ml final volume 50 mM NaCl/50 mM Tris·HCl (pH 7.4)/0.1 mM EDTA at 25°C. (○——○) Substrate vesicles + trypsin vesicles in presence of 5 mM Ca^{2+} and trypsin inhibitor; (□——□) substrate vesicles + trypsin vesicles in absence of Ca^{2+} but in presence of inhibitor; (△——△) Ca^{2+}-induced leakage, i.e., fluorescence in presence of Ca^{2+} but in absence of inhibitor (corrected for a minor contribution by fluorescence arising from leakage in absence of Ca^{2+}) minus fluorescence in presence of Ca^{2+} and inhibitor.

substrate vesicles and enzyme vesicles are indistinguishable as far as their ability to fuse is concerned. If fusion would be limited to events involving only those vesicles which were initially present, at the most 1/3rd of the maximal value obtained in Triton would be attained.

(2) The trypsin concentration used was such that only 6 out of 10 vesicles contained a molecule of trypsin while 4 out of 10 were empty, assuming that the vesicles used are made up of ~4000 phospholipid molecules [9].

These considerations would decrease the maximal extent of fusion-induced fluorescence which one could expect to be exactly 20% of the maximal Triton-induced value.

The line connecting the triangles in fig.3 represents the leakage of vesicle contents into the medium during the fusion process. Considerable leakage from PS-containing vesicles as a result of Ca^{2+}/PS complex formation has been described [11,25]. In our experiments leakage does not interfere with the fusion assay because of the presence of trypsin inhibitor in the medium. Yet, the rate of leakage can easily be estimated by running the experiment in absence of inhibitor. By simply subtracting fluorescence in absence of inhibitor from that in presence of inhibitor we find the extent of Ca^{2+}-induced leakage. In absence of inhibitor ~6% of the total amount of substrate present thus appeared to be hydrolyzed in ~1 h incubation.

Similar to our observation on fusion-induced fluorescence in fig.3, the Ca^{2+}-induced leakage levels off after 60 min incubation, suggesting that the two phenomena are correlated. Considerably higher extents of leakage during Ca^{2+}-induced fusion of PS vesicles have been found [11] and a close correlation between fusion and leakage suggested. The quantitative difference between their results and ours is most likely explained by the use of different vesicle compositions. Pure PS vesicles may react much faster with Ca^{2+} to form the *trans* Ca^{2+}/PS complex as proposed [11] than vesicles containing only 50% PS. This would be in agreement with the findings [26] on the rate of phase separations in PS/PC preparations with varying molar ratios.

Under the incubation conditions as in fig.3, we observed a small increase in fluorescence when free substrate was incubated with trypsin-containing vesicles. Since trypsin inhibitor was included in the incubation medium this increase could only result from hydrolysis of substrate permeated across the membrane of the trypsin vesicles. We calculated that fluorescence thus caused contributed maximally only 3% to the total fluorescence as determined in the fusion assay. Therefore, the fluorescence increment observed upon omitting the inhibitor (but in presence of Ca^{2+}) must result from exposure of trypsin (mol. wt 24 000) during Ca^{2+}-induced fusion, indicating increasing perturbation of vesicle integrity.

To exclude the possibility of diminished trypsin activity due to inward permeation of the inhibitor across the vesicle membrane as a result of the Ca^{2+}-enhanced permeability, we fused trypsin vesicles either in presence or absence of inhibitor and isolated the aggregated vesicles. After addition of Triton X-100 to the aggregates no difference in trypsin activity was observed, indicating the absence of inhibitor in vesicles which were fused in its presence.

At present we are unable to answer the question whether the kinetics observed in fig.3 represent fusion kinetics or enzyme kinetics. Considering the very rapid rate of fluorescence increase in fig.2 caused by the same amounts of substrate and enzyme as in fig.3 but diluted over 100-fold, it would seem unlikely that the rate-limiting process in the experiment of fig.3 is the enzymatic reaction. (It should be noted that the arbitrary units in fig.2 and 3 are the same.)

We tend to conclude, therefore, that fusion of vesicles of the type we used proceeds at a relatively moderate rate. If the principal fusion event would be limited only to the fusion of vesicles of the original population with one another (primary fusion) the fusion curve in fig.3 might be interpreted as consisting of one part representing predominantly such primary fusion events ($\lesssim$1 h) and another part mainly representing fusion involving vesicles formed during the primary fusion process (>1 h).

In conclusion, the assay for vesicle–vesicle fusion as described here appears to fulfill an important criterion for a fusion assay in that it demonstrates the coalescence of the aqueous contents of separate vesicle populations with the exclusion of gross leakage or influx of medium. Besides, Ca^{2+}, which is required to induce fusion, in no way interferes with the fusion assay as such and the method allows kinetic measurements.

Substrates, such as used in this study can also be very powerful tools in assaying the interaction of phospholipid vesicles with cells. To this end intramolecularly quenched fluorogenic peptides are currently under design which would be suitable substrates for various intracellular proteolytic enzymes.

Acknowledgements

The award of a fellowship by EMBO to A.Y. is gratefully acknowledged. Part of these investigations were carried out under the auspices of the Netherlands Foundation for Medical Research (FUNGO) with financial support from the Netherlands Organization for the Advancement of Pure Research (ZWO). Miss Karin van Wijk is gratefully acknowledged for the preparation of the manuscript.

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