Biochimica et Biophysica Acta, 732 (1983) 327–331
Elsevier

BBA Report

BBA 70105

KINETICS OF CALCIUM PHOSPHATE-INDUCED FUSION OF HUMAN ERYTHROCYTE GHOSTS MONITORED BY MIXING OF AQUEOUS CONTENTS

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(Received March 21st, 1983)

Key words: Membrane fusion; Erythrocyte ghost; Calcium phosphate

We have adapted the terbium fusion assay (Wilschut, J. and Papahadjopoulos, D. (1979) Nature 281, 690–692), which has proven to monitor the mixing of internal contents during phospholipid vesicle fusion in a reliable manner (Hoekstra, D. (1982) Biochim. Biophys. Acta 692, 171–175), to study the fusion of erythrocyte ghosts as induced by the combined action of Ca^{2+} and phosphate. Using this assay, it became possible to reveal, for the first time, the kinetics of fusion of a biological membrane vesicle system. The rate of fusion was critically dependent on the concentration of Ca^{2+} and phosphate. Prior addition of phosphate was essential for induction of fusion. Initial fusion was largely non-leaky, but in a process secondary to the fusion event the ghosts gradually released their contents. It is suggested that the experimental approach presented in this paper, would facilitate efforts to elucidate the mechanism of fusion of biological membranes.

Membrane fusion is of fundamental importance in a variety of biological processes [1] and, to elucidate its mechanism(s), artificial phospholipid vesicles have been used extensively as a model system [2]. Although lipid vesicles may form too simple a system to clarify completely the mechanism of biological membrane fusion, the validity of employing such vesicles in the study of fusion has been clearly demonstrated [2]. In particular, these studies emphasized the importance of investigating initial fusion events in order to correlate early changes in the structural and physical properties of the bilayer lipids with the onset of membrane fusion [3–8]. The recent development of fusion assays (5, 9–12), allowing the continuous monitoring of the kinetics of vesicle fusion, has greatly facilitated the study of the initial events of this process. Therefore, the adaptation of these assays to more complex systems such as biological membranes, would appear highly relevant to studies aimed to clarify the mechanism(s) of physiological membrane fusion. Fusion of erythrocyte ghosts appears to be a suitable model for this purpose [13–6], particularly because the molecular architecture of their membranes has been extensively characterized [17,18]. In this paper we describe the kinetics of fusion of erythrocyte ghosts as induced by Ca^{2+} and phosphate. The experimental approach relies upon the possibility to encapsulate terbium (Tb) and dipicolinic acid in separate ghost populations. Calcium phosphate-mediated fusion of ghosts [14,15] will then result, upon mixing of their aqueous contents, in the formation of the fluorescent $Tb(DPA)_3^{3-}$ complex, which can be monitored continuously.

Human erythrocyte ghosts were prepared

Abbreviations: ACP, amorphous calcium phosphate $(Ca_3(PO_4)_2)^{20}$; OCP, octacalcium phosphate $(Ca_4H(PO_4)_3 \cdot 2.5\ H_2O)^{20}$; HAP, hydroxyapatite $(Ca_5(PO_4)_3OH)^{20}$; DPA, dipicolinic acid.

according to Steck and Kant [19] with 5 mM sodium phosphate (pH 8.0) as hemolyzing buffer. Packed ghosts were then resuspended in approx. 10 vol. of a solution (buffered with 10 mM Hepes, pH 7.4) containing 7.5 mM $TbCl_3$/75 mM sodium citrate or 75 mM dipicolinic acid (sodium salt)/50 mM NaCl, respectively. After 10 min at 2°C, the suspension was transferred to 37°C, 1 mM Mg^{2+} was added and the mixture incubated for 45 min to induce resealing of the ghosts. Non-entrapped material was subsequently removed by repeated washing and centrifugation (20 min, 22000 × *g*). The final pellet was suspended in 120 mM KCl/30 mM NaCl/10 mM sodium phosphate/0.1 mM EDTA (pH 7.4) (approx. 0.4–0.5 mg/ml of protein), and was kept on ice for the duration of the experiment. Under these conditions leakage of encapsulated Tb and dipicolinic acid, determined by addition of excess dipicolinic acid or Tb/citrate, respectively, to the ghost preparations in the absence of EDTA, was found to be less than 1%/h. The Tb fluorescence scale was calibrated by lysing the same amount of Tb-containing ghosts as used in the fusion assay by addition of cholate (1%, followed by sonication) in the absence of EDTA and subsequent addition of excess dipicolinic acid.

Determination of acetylcholinesterase activity [19] in the sealed ghost membranes in the absence and presence of Triton X-100 revealed that 90–95% of the erythrocyte ghost membranes were oriented right-side-out.

Fig. 1 shows the fluorescence tracings that were obtained when different Ca^{2+} concentrations were added to a 1 : 1 mixture of Tb- and dipicolinic acid-containing ghosts in the presence of 10 mM phosphate. At Ca^{2+} concentrations less than 1.4 mM, significant fusion could not be detected, while maximal fusion, both with respect to the initial rate (approx. 20%/min) as well as the extent of Tb fluorescence increase (approx. 40%), was observed at 1.75 mM.

A further increase in the Ca^{2+} concentration led to a considerable decrease in the initial fusion rate. Most surprisingly, under those conditions a second fusion event became apparent after the initial event has levelled off (Fig. 1). The 'initial' rate of this second fusion event appeared to be very similar to the rate observed at 1.75 mM Ca^{2+}, although the maximal fluorescence reached in this case was substantially lower. When Ca^{2+} (1.75 mM) was added to ghost-containing media of various phosphate concentrations, the rate as well as the extent of fusion increased linearly with increasing phosphate concentration, showing a threshold at 7 mM phosphate and levelling off at phosphate concentrations of at least 10 mM (not shown).

Fusion did not take place when ghosts were added to a medium that contained Ca^{2+} and phosphate at concentrations that otherwise induced optimal fusion (1.75 mM and 10 mM, respectively,

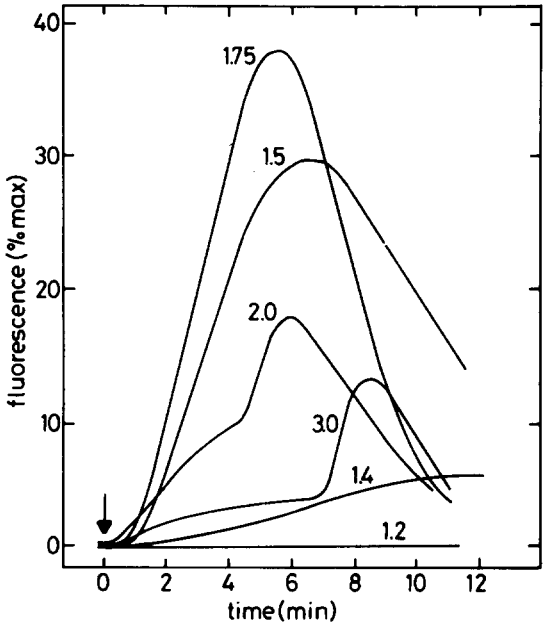

Fig. 1. Dependence of calcium phosphate-induced fusion of erythrocyte ghosts on the Ca^{2+} concentration. Equal amounts of Tb- and dipicolinic acid-containing ghosts were mixed in a medium of 120 mM KCl/30 mM NaCl/0.1 mM EDTA and containing 10 mM sodium phosphate (pH 7.4) at 30°C, to give a final total concentration of approx. 40–50 μg/ml protein. Final incubation volume was 2 ml. Fusion was initiated by injection of Ca^{2+} (arrow) to the final mM concentration as indicated. Continuous monitoring of Tb-dipicolinic acid fluorescence (λ_{ex} = 276 nm; λ_{em} = 545 nm) was carried out with a Perkin Elmer MPF 43 spectrophotofluorometer, using a cut off filter (> 520 nm) between sample and monochromator. The sample chamber was equipped with a magnetic stirrer, and the temperature was controlled with a thermostatically controlled circulating water bath. It should be noted that the presence of EDTA (0.1 mM) and Ca^{2+} in the external medium would effectively prevent Tb-dipicolinic acid formation if release of ghost contents into the external medium were to occur when monitoring fusion.

c.f. Fig. 1). Furthermore, simultaneous injection of Ca^{2+} and phosphate induced fusion at a rate of only 9% of that obtained under optimal conditions, while the maximal level of fluorescence attained under these conditions was only 10%. However, when the ghosts were preincubated first with 10 mM phosphate for 10 s, prior to the addition of 1.75 mM Ca^{2+}, the rate of fusion was approx. 85% of its optimal value. Finally, Ca^{2+} alone, up to 15 mM in the absence of phosphate or addition of Ca^{2+} prior to phosphate did not induce fusion of ghosts. Therefore, the addition of phosphate prior to the addition of Ca^{2+}, appeared to be an absolute necessity for calcium phosphate-induced fusion of ghosts. The conditions under which fusion was observed are in qualitative agreement with those presented by Zakai et al. [14]. Thus, optimal fusion was observed when ghosts were preincubated in 10 mM phosphate prior to the addition of 1.75 mM Ca^{2+}. It appears, however, that calcium phosphate-induced fusion of ghosts under these conditions is faster than previously described (less than 5 min in this study, whereas Zakai et al. [14] reported 15–20 min), a result presumably due to the inability to recognize early fusion events by phase-contrast microscopy, i.e. to distinguish local cell-cell fusion [16] from aggregation of ghosts.

The increase in $Tb(DPA)_3^{3-}$ fluorescence appeared to be of a transient nature, presumably reflecting the leakage of the complex from fused ghosts. We therefore monitored, in parallel experiments, the kinetics of leakage by encapsulating the $Tb(DPA)_3^{3-}$ complex in ghosts. Subsequently, fusion was induced similarly as in the fusion assay, and the release of the complex into the medium was followed as a quenching of the Tb fluorescence due to rapid dissociation of the $Tb(DPA)_3^{3-}$ complex by EDTA and Ca^{2+}. As indicated in Fig. 2, in the presence of 1.75 mM Ca^{2+} significant leakage was observed after approx. 4 min. By that time the fluorescence level obtained in the corresponding fusion assay had almost reached its maximum before starting to decline, suggesting that both phenomena were closely related. After approx. 12 min the residual relative fluorescence in the fusion assay was virtually negligible. Consistently, at that time the leakage of contents was essentially complete.

Although the later stages of fusion (after 4 min)

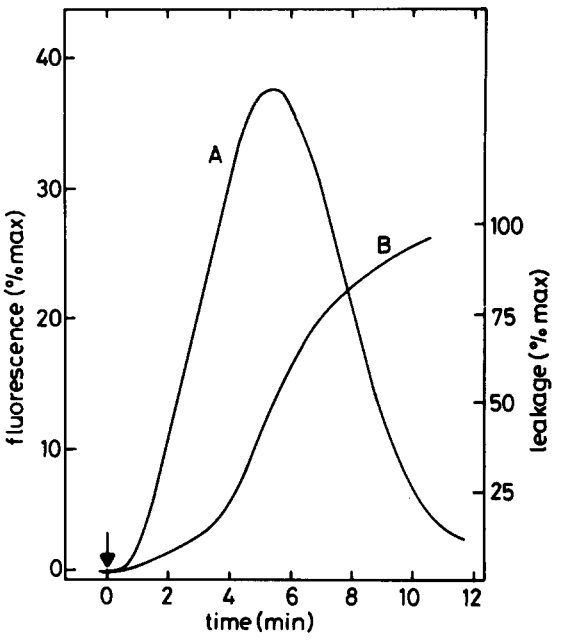

Fig. 2. Calcium phosphate induced fusion of ghosts and release of their contents. Calcium phosphate-induced fusion (1.75 mM Ca^{2+} (arrow), 10 mM phosphate) of ghosts (A) and release of contents (B) were measured in separate experiments. Leakage of calcium phosphate-induced fusion of ghosts was determined by monitoring the release of the Tb-dipicolinic acid complex into the external medium. To this end, equal volumes of the Tb and dipicolinic acid solutions were premixed, and the mixture was then encapsulated into ghosts as described. Upon leakage of the complex, the initial, high, level of fluorescence will decrease as a result of dissociation of the fluorescent Tb-dipicolinic acid complex in the medium by Ca^{2+} and EDTA [10]. Therefore, curve B represents a decrease in fluorescence intensity.

were accompanied by substantial leakage, the early events involved release of approx. 10% of their encapsulated volume per fusion event, as can be calculated from the initial rate of leakage (approx. 4%/min) and the initial rate of fusion (approx. 40%/min, i.e., twice the rate of the Tb fluorescence increase, since one round of fusion in a 1 : 1 mixture of Tb- and dipicolinic acid-containing ghosts will result in 50% of the maximal fluorescence). Finally, when using carboxyfluorescein [3,12], the kinetics of leakage of the ghost contents were essentially similar to those observed in case of leakage of the $Tb(DPA)_3^{3-}$ complex, thus excluding the possibility of substantial inward

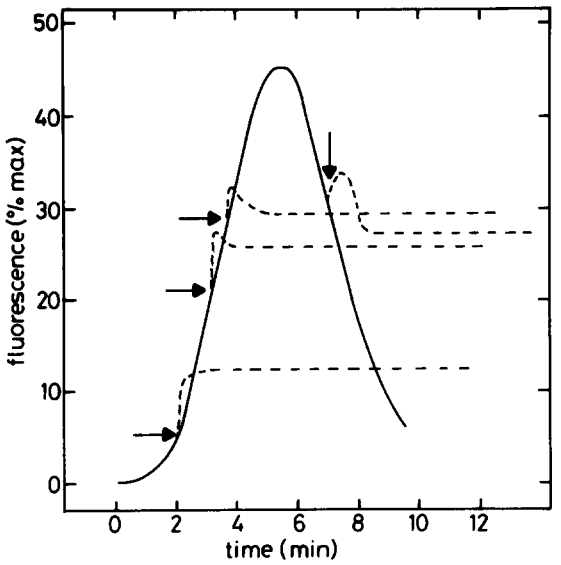

Fig. 3. Termination of calcium phosphate-induced fusion of ghosts by addition of excess EDTA. Fusion was induced by injection of Ca^{2+} to a final concentration of 1.75 mM (at zero time) under conditions as described in the legend of Fig. 1. EDTA (3.5 mM) was added at various time intervals after initiation of the fusion process (arrows). The figure is a composite of five separate experiments.

leakage of Ca^{2+} and/or EDTA from the external medium.

As shown in Fig. 3, the fusion reaction could be interrupted by addition of excess EDTA at various time intervals after initiation of the process. The net fluorescence level obtained after addition of EDTA indicated a physical separation of the fluorescent complex from the external EDTA-containing medium and thus its effective retention within the fused ghosts. Similarly, the leakage of contents as determined by the leakage assay described above (cf. Fig. 2) could be arrested by addition of EDTA.

The main purpose of this communication was to examine the potential application and the reliability of the Tb fusion assay in the study of fusion of biological membrane systems. We have used calcium phosphate to induce fusion of erythrocyte ghosts [14,15], a system which has been used frequently as a model for biological membrane fusion [13–16]. The potential physiological relevance of a combined action of Ca^{2+} and phosphate to induce fusion in biological systems has been suggested [4], and further details for the ghost system will be presented elsewhere (manuscript in preparation). At present it is relevant to note that calcium phosphate-induced fusion of ghosts appeared to be remarkably dependent on the Ca^{2+} concentration. Above 1.75 mM Ca^{2+}, the initial fusion rate decreased progressively (Fig. 1) with increasing Ca^{2+} concentrations, a result which was not due to increased initial leakage, as revealed by separate release experiments (not shown). In addition, at these higher Ca^{2+} concentrations a second fusion phenomenon became apparent. The nature of this second fusion event is, as yet, unclear and is currently under investigation. It is likely, however, that the remarkable Ca^{2+}-dependence of ghost fusion is related to a specific involvement of distinct calcium phosphate phases [20], initiating and/or facilitating the fusion process, as the formation of such phases appears to depend critically on the Ca^{2+}/phosphate ratio in solution [21]. The initial calcium phosphate phase that is formed upon mixing of Ca^{2+} and phosphate is the amorphous, non-crystalline, complex ACP. This ACP phase is converted to an intermediate octacalcium-phosphate complex, OCP, which, in turn, is converted to crystalline hydroxyapatite, HAP, the thermodynamically stable end product of calcium phosphate precipitations [20]. It has been suggested that fusion of phospholipid vesicles in the presence of Ca^{2+} and phosphate is stimulated when the ACP phase is converted to OCP, while membrane disruption and leakage of vesicle contents would occur upon formation of the crystalline HAP phase [4]. At relatively high Ca^{2+}/phosphate ratios, the conversion of ACP to OCP is delayed [21]. In agreement with the above suggestion that this conversion stimulates membrane fusion, the present results show a slow fusion rate and a delayed secondary fusion event at high Ca^{2+}/phosphate ratios. Additional support for this suggestion was provided by experiments showing that ATP, which also delays the conversion of ACP to OCP [22], similarly inhibited the initiation of fusion (manuscript in preparation).

Fusion was not observed (i) when Ca^{2+} was added prior to the addition of phosphate or (ii) when ghosts were added to a medium that already contained Ca^{2+} and phosphate, while simultaneous injection of Ca^{2+} and phosphate caused fusion to a very limited extent. These results indi-

cate that for effective induction of fusion calcium phosphate complex formation had to occur in or at the plane of the bilayer. It has been suggested that calcium phosphate complexes formed between adjacent erythrocytes [15] or ghosts [14] cause agglutination and eventually fusion. At present it is not clear, however, whether the formation of calcium phosphate complexes between apposing membranes per se (which may or may not be identical to those formed in bulk solution) would suffice to promote membrane fusion or that, in addition, the formation of a specific calcium-phospholipid-phosphate complex is involved in the actual fusion process. Specific calcium-phospholipid-phosphate complexes are formed only when phosphate is added prior to Ca^{2+} [23,24], thus representing conditions very similar to those which will exclusively induce the fusion of ghosts. However, since the fusion kinetics of erythrocyte ghosts can now be revealed and, moreover, the fusion process can be arrested at well-defined stages, opportunities are thus provided to examine molecular as well as morphological characteristics of the fusion product with respect to the questions raised above. In addition, the effect of exogeneously supplied compounds on calcium phosphate complex formation [22] or on the fusion reaction per se and the subsequent consequences to induce fusion of ghosts, can now conveniently be determined. It has been pointed out elsewhere [3,5,8,12] that the ability to monitor the kinetics of membrane fusion, immediately after the induction of this process, is highly relevant in order to attempt to correlate distinct membrane changes with the onset of membrane fusion, and hence, to elucidate its molecular mechanism. In this respect, the assay presented in this paper may be helpful to gain further insight in the fusion process of biological membranes, since the method can, in principle, also be adapted to study the fusion of other biological membrane vesicles, such as, for example, chromaffin granules [25].

This work was supported in part by Nato Research Grant 151.81. We thank Dr. Smit-Sibinga from the Red Cross Blood Bank Groningen-Drenthe for the generous gifts of packed red blood cells. Rinske Kuperus is gratefully acknowledged for typing the manuscript.

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