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A cell free system reveals that capacitation is a prerequisite for membrane fusion during the acrosome reaction

Ben Spungin, Tova Levinshal, Sara Rubinstein and Haim Breitbart

Department of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel

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Plasma and outer acrosomal membranes were extracted from bovine spermatozoa and used in an in vitro fusion assay. Fusion was revealed by monitoring the merging of lipids using the chlorophyll *a*–*N,N'*-dioctadecyloxacarbocyanine-*p*-toluene sulfonate (DCY) method [(1984) Biochim. Biophys. Acta 769, 531–542]. The requirement for capacitation, as well as the effects of pH, calcium and spermine, on membrane fusion in our cell-free system were similar to those observed in vivo on the acrosomal reaction. This demonstrates for the first time that capacitation and alterations in intracellular pH and calcium concentration, which must precede the acrosomal reaction, are required for the membrane fusion event.

Sperm; Acrosomal reaction; Exocytosis; Membrane fusion; Secretion; Fertilization

1. INTRODUCTION

During mammalian fertilization, the spermatozoan penetrates the cumulus oophorous of the ovum, and then binds to the zona pellucida with its plasma membrane intact. Zona binding induces the sperm cell to undergo the acrosomal reaction, which involves multiple fusions between the outer acrosomal membrane and the overlying plasma membrane. This vesiculation releases the acrosomal contents, including a variety of hydrolytic enzymes, and is required if fertilization is to proceed further [2]. The acrosomal reaction occurs only in spermatozoa that have undergone a process of maturation known as capacitation, which, in vivo, occurs after exposure of the cells to the female reproductive tract.

The acrosome arises from the Golgi apparatus during spermatogenesis [4], and the acrosomal reaction is considered to be an exocytotic event, although the topology of fusion is different from that in other cases of secretion. While the acrosomal reaction is of the 'PF-PF' type (plasmatic membrane faces fuse) [5], the fusion is not focal, but involves instead the formation of plasma and outer acrosomal membrane hybrid vesicles. This membrane shedding is thus an unusual feature of acrosomal exocytosis.

Although the mechanisms involved in signal generation have been well characterized, much less is known about the membrane fusion event itself [42]. Considerable progress has been made over the past few years in the identification of cellular components required for intracellular membrane fusion. This has been made possible by the development of cell-free systems in which membrane fusion occurs among reconstituted, extracted components [6–14]. Cell-free systems are important because they allow the membrane fusion event to be isolated from other stages of exocytosis, such as membrane trafficking. Early fusion events in the endocytotic pathway [8,10,12,15,16], and vesicular transport between the endoplasmic reticulum and the Golgi system [9,17–19], as well as within the Golgi stack [20–22], have been reconstituted. Cell-free models of exocytosis have proven more difficult to obtain because plasma membrane vesicles are often isolated without the requisite orientation (cytoplasmic surfaces exposed). Nadine et al. [6] observed fusion of isolated pancreatic zymogen granules with plasma membranes extracted from the same cells. While the involvement of a GTP-binding protein in the regulation of fusion in their system was implicated, calcium (known to activate exocytosis in intact pancreatic acinar cells) did not activate fusion. It thus appears that some regulatory components of in vivo fusion were not recovered intact.

In this report we describe a cell-free system in which sperm plasma and outer acrosomal membranes fuse. We used the chlorophyll *a*–*N,N'*-dioctadecyloxacarbocyanine-*p*-toluene sulfonate (DCY) method of Gibson and Strauss [1] to monitor fusion. In this method, separate populations of membranes are labeled with either chlorophyll *a* or DCY and fusion is revealed by the ability of the probes to diffuse from one membrane species to the other. Other techniques which reveal fusion by the mixing of vesicle contents [23,24] were not practical in our system because the isolated outer acro-

Correspondence address: B. Spungin, Department of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel. Fax: (972) (3) 535 1824.

somal membranes do not close to form sealed vesicles enclosing an internal volume.

The requirement of capacitation for the acrosomal reaction is retained in membrane fusion in our cell-free system. Furthermore, the effects of pH, calcium and spermine mimic those observed in vivo on the acrosomal reaction. This suggests that the regulatory components of the system were obtained essentially intact. We conclude that capacitation and physiological alterations in intracellular pH and calcium concentration which precede the acrosomal reaction in vivo are required for the membrane fusion event of the acrosomal reaction.

2. MATERIALS AND METHODS

Frozen bovine sperm pellets were obtained from The Artificial Insemination Service, Hafetz Haim, Israel, and maintained in the laboratory in liquid nitrogen. Chlorophyll *a* was obtained from Sigma and used as a 1.1×10^{-4} M ethanolic stock solution. DCY was obtained from Eastman Kodak and used as a 4×10^{-5} M ethanolic stock solution. Spermine, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 3-[*N*-morpholino]propanesulfonic acid (MOPS), benzimidine and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma. All other reagents used were of analytical grade.

In vitro capacitation was accomplished by the method of Parrish et al. [25]. Sperm pellets were thawed and washed several times in 155 mM NaCl, 10 mM histidine, pH 7.4, and finally suspended in glucose-free TALP (100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.29 mM KH_2PO_4, 21.6 mM lactic acid, 1.5 mM $MgCl_2$, 1 mM pyruvate, 3 mg/ml bovine serum albumin, 10 mM HEPES, pH 7.4), containing 10 μg/ml heparin. The cells were incubated in this medium for 4 h at 37°C with shaking. In control samples (uncapacitated sperm), heparin was omitted from the incubation medium.

2.1. *Preparation of sperm membranes*

Cells were suspended in 10 mM histidine, 0.5 mM EDTA, 1 mM PMSF, 1 mM benzimidine, pH 7.4, brought to 4°C and their plasma membranes removed by Ultraturrax [26]. The membranes were collected by differential centrifugation and suspended in HBS (150 mM NaCl, 10 mM HEPES, pH 7.0). Outer acrosomal membranes were then removed from the same cells by a modification of the procedure of Zahler and Doak [27,28]. The cells were centrifuged twice through 1.3 M sucrose in HBS and incubated for 30 min in 0.2 M sucrose, 10 mM HEPES, and 5 mM EDTA, at 37°C with shaking, to loosen the outer acrosomal membrane. The cells were then brought to 4°C and Ultraturraxed to remove the outer acrosomal membranes. The membranes were then collected by differential centrifugation and suspended in HBS. The plasma and outer acrosomal membranes were further purified on a 1.3 M/1.75 M sucrose step gradient made in HBS. The plasma membranes collect at the HBS/1.3 M interface, and the outer acrosomal membranes at the 1.31/1.75 M interface. The purified membranes were collected, washed once and resuspended in HBS.

2.2. *Fluorescent labeling of membranes*

Four populations of fluorescently labeled membranes were prepared: chlorophyll-labeled plasma membranes, DCY-labeled outer acrosomal membranes, and doubly labeled plasma or outer acrosomal membranes.

2.2.1. Chlorophyll-labeled plasma membranes

Plasma membranes containing 400 μg protein (determined by the method of Lowry [29]) were mixed with 32 μl chlorophyll *a* stock, brought to 4 ml with cytosolic medium (110 mM KCl, 5 mM NaCl, 10 mM MOPS, pH 6.8), and sonicated for 15 min. (Attempts to introduce the probes by shaking were not successful, and sonication for only 5 min resulted in very weak signals.)

2.2.2. DCY-labeled outer acrosomal membranes

Outer acrosomal membranes containing 400 μg protein were mixed with 40 μl DCY stock, brought to 4 ml with cytosolic medium, and sonicated for 15 min.

2.2.3. Doubly labeled membranes

Plasma or outer acrosomal membranes containing 400 μg protein were mixed with 16 μl chlorophyll *a* stock and 20 μl DCY stock, brought to 4 ml with cytosolic medium, and sonicated for 15 min.

2.3. *Fluorescence measurements*

These were carried out on a Shimadzu RF 5000 spectrofluorophotometer. Equal volumes of separately or doubly labeled plasma and outer acrosomal membranes were mixed in the reaction cuvette, and energy transfer was measured by exciting the donor (DCY) at 486 nm, and observing and sensitized fluorescence in the acceptor (chlorophyll *a*) at 676 nm. Since energy transfer between the two probes is efficient only when their dipoles are parallel, and is quenched in an aqueous environment [30], it will only occur when the two amphipathic probes are embedded in the same membrane. Therefore, fluorescence emission from the receptor is not observed after aggregation without fusion, and this was verified directly on our system (Fig. 3).

The reactions were carried out at room temperature with constant stirring of the mixture. Typically, the fluorescence was monitored for 1 min to obtain an initial fluorescence level. A fusogenic agent was then added to the cuvette, and the fluorescence monitored for another 10 min. The extent of fusion was calculated by comparing the time (t) dependence of the measured fluorescence intensity of the separately (SL(t)) and doubly (DL(t)) labeled runs. The initial emission intensity of the separately labeled run, SL(0), is due to the self fluorescence of the reaction components. The difference in the initial fluorescence of the two runs, i.e. DL(0)–SL(0), is due to energy transfer in the doubly labeled membranes, and is the maximum increase (100%) which could occur upon fusion of the separately labeled membranes. Changes in DL(t), i.e. DL(t)–DL(0), are observed due to altered turbidity of the reaction mixture, dilution effects, and incorporation or release of labels, and these should affect SL(t) in an identical fashion. In order to eliminate these artifacts from our measurements, we take as the extent of fusion:

$$\frac{SL(t)-SL(0)-(DL(t)-DL(0))}{DL(0)-SL(0)}$$

The extent of fusion is thus expressed as the fraction of maximum fluorescence due solely to energy transfer in the separately labeled run.

3. RESULTS

Fig. 1 shows the effects of 200 μM calcium on membrane fusion. Fusion was only observed in the presence of calcium at pH 7.4. The rate of fusion, determined from the initial slope after the addition of calcium, was 14% maximum/minute (% max/min). Similar results were obtained with 20 μM spermine (Fig. 2).

The measured fusion rates at pH 7.4 are summarized in Table I. At this pH, the ability of these two substances to induce fusion is over an order of magnitude greater with membranes from capacitated, as opposed to uncapacitated, cells. In either case, Ca^{2+} and spermine are equipotent fusogenic agents that do not act synergistically.

We wished to ascertain that the measured fluorescence intensity in our experiments is due to membrane

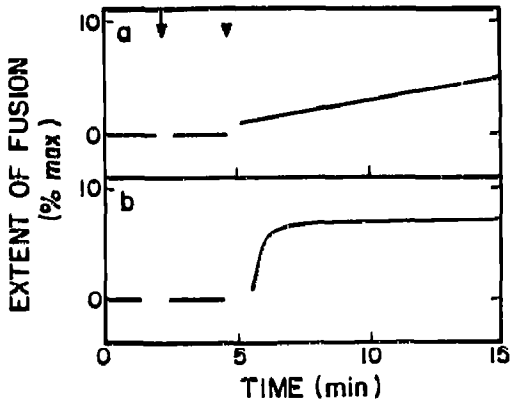

Fig. 1. The effects of 200 μM Ca^{2+} on the fusion of uncapacitated (a) and capacitated (b) plasma and outer acrosomal membranes. The membranes were combined and the pH was then raised from 6.8 to 7.4 by the addition of 1 M Tris-base, pH 10 (arrow). 200 μM Ca^{2+} was then added (arrowhead). The initial slope after the addition of calcium is the fusion rate.

fusion and not aggregation. This was done by repeating the experiment of Fig. 1b using doubly labeled plasma membranes and unlabeled outer acrosomal membranes. Membrane aggregation would not affect the initial fluorescence level, while membrane fusion would be revealed by a decrease in fluorescence due to reduced energy transfer between the probes following their dilution in the membranes. (The efficiency of energy transfer decreases with the sixth power of the distance between the probes.) As can be seen in Fig. 3, when the membranes were brought to pH 7.4 and Ca^{2+} added, a 6% decrease in fluorescence intensity was observed. This is about the maximal increase observed in our experiments (Figs. 1 and 2), and we conclude that aggregation contributes very little, if at all, to the measured fluorescence intensity in our experiments.

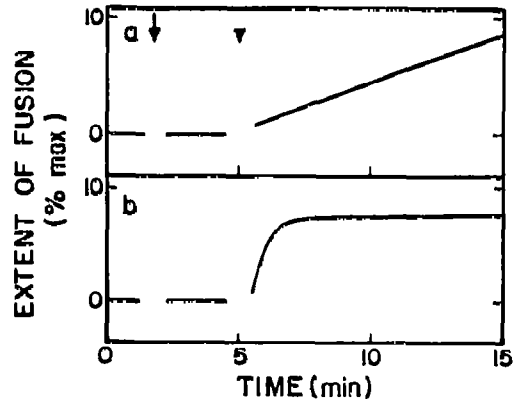

Fig. 2. The effects of 20 μM spermine on the fusion of uncapacitated (a) and capacitated (b) plasma and outer acrosomal membranes. The membranes were combined and the pH was then raised from 6.8 to 7.4 (arrow). 20 μM spermine was then added (arrowhead).

Table 1

Fusion rates of plasma and outer acrosomal membranes at pH 7.4

	Uncapacitated sperm		Capacitated sperm	
	No spermine	20 μM spermine	No spermine	20 μM spermine
No Ca^{2+}	0	0.8	0	13
200 μM Ca^{2+}	0.4	0.6	14	12

Fusion rates were calculated from the initial slope of the energy transfer measurements. Rates are given as % maximum fluorescence intensity per minute. The results are the average of at least two determinations. At pH 6.8, fusion was not observed under any of the conditions reported in the table.

4. DISCUSSION

Our fusion assay monitors membrane fusion through the merging of lipid bilayers. Probes were introduced into membranes by sonication for 15 min. Attempts to introduce the probes into the membranes by shaking were not successful, and sonication for only 5 min resulted in very weak signals. The probes form stable aggregates in water, and it appears that there is a large energy barrier preventing their spontaneous uptake by the membranes. We conclude that significant exchange of probes between the membrane species without fusion does not occur. Theoretical considerations predict that no energy transfer occurs between the probes when they are located on separate membranes, and this has been confirmed in our biological system (Fig. 3), as well as in liposomes [1]. Taken together, these observations establish that our assay unambiguously reveals membrane fusion. We have not, however, unequivocally

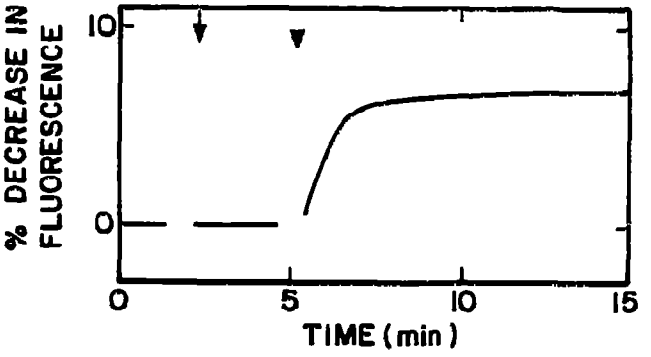

Fig. 3. The experiment of Fig. 1b was repeated using doubly labeled plasma membranes, and unlabeled acrosomal membranes. The membranes were combined and the pH was then raised from 6.8 to 7.4 (arrow). 200 μM Ca^{2+} was then added (arrowhead). Upon fusion, the membrane probes were diluted by their diffusion into the previously unlabeled membranes, thus decreasing the measured fluorescence intensity.

eliminated the possibility, raised by Ellens et al. [23], that a fusion event in our system involves only one monolayer of each membrane species. While this may be a reasonable possibility in liposomes, it does not seem likely to be the case in fusion of biological membranes where the presence of transmembrane proteins enhances membrane integrity. Indeed, the large shear forces which the membranes were subjected to during Ultraturrax treatment do not cause membrane fracturing.

The rate of fusion was taken as the initial slope of the fusion curves and as such represents the overall fusion rate which combines aggregation and fusion [37]. Work with liposomes [31–34] has shown that a combination of dehydration and structural destabilization is required for fusion. Liposomes are metastable due to their high curvature, and this enhances their fusibility. Furthermore, liposomes, being devoid of more rigid structural components (such as cytoskeletal elements, and integral membrane proteins), are more flexible than biological membranes. Our outer acrosomal membranes do not close to form sealed vesicles, indicating that these non-lipid components generate the dominant forces that determine the stable configuration of the membrane. Removal of electrostatic barriers is sufficient for fusion to occur in liposomes. Regulation of biological membrane fusion, however, must also involve mutual recognition of the fusion partners, and removal of steric barriers followed by protein modulated destabilization and bilayer-to-non-bilayer transition.

The maximum extent of fusion which we observed in our system (the plateau's in Figs. 1 and 2) is about 8%. While this is low compared to the amount of fusion which can be obtained with liposomes (about 40%) [24,31–34], it is similar to values obtained on other biological systems [12–14]. This difference may reflect the asymmetry of biological membranes and the possibility that some of the membranes have been recovered without the requisite polarity (cytoplasmic surfaces exposed). The acrosomal membranes do not close to form sealed vesicles, but rather roll into cylinders, so that, like plasma membrane vesicles, only one surface is presented for fusion. Studies on the orientation of extracted rat liver [35] and baby hamster kidney [36] cell membranes indicate that less than 30% and 15%, respectively, are obtained with cytoplasmic faces exposed. If these figures are indicative of the state of our extracted membranes, then the real extent of fusion (considering only those membranes with the requisite polarity) would be much higher than the 8% which we observe, and would be close to that obtained on liposomes.

We used the method of Parrish et al. [25] for capacitation of bovine sperm. They showed that exposure of cells to heparin for 4 h increases the fraction of fertilized oocytes in a standard in vitro assay, and this was interpreted as an increase in the fraction of capacitated sperm. After exposure to heparin, the cells probably constitute a mixed population of capacitated and uncapacitated cells, although the ratio of the two could not be determined. Similarly, the population of cells not exposed to heparin most likely contained a percentage of cells that underwent capacitation spontaneously, although their frequency in the population could also not be determined.

Capacitation caused a marked enhancement in fusion rates (Table I). Since fusion of biological membranes requires the involvement of proteins, this most likely reflects membrane remodeling which occurs during capacitation. Proteins may be present in uncapacitated membranes which inhibit fusion, while fusogenic proteins may appear in the membranes during capacitation. Alteration of membrane lipids during capacitation may also occur since lipid composition affects the kinetics of liposome fusion [31,43]. Lipids with a low headgroup hydration, such as phosphatidyl ethanolamine, generally enhance membrane fusion. Secretory processes are accompanied by phospholipase C activation and the formation of diacyl glycerol, which can act in conjunction with Ca^{2+} to activate protein kinase (PKC). Breitbart et al. have recently shown that PKC is involved in the acrosomal reaction of bull sperm [38]. Activation of these enzymes may be part of a mechanism to remove steric barriers and destabilize the membrane prior to fusion. Capacitation is a prerequisite for induction of the acrosomal reaction in intact cells [3], and this requirement has been preserved in our system. The residual amount of membrane fusion which was observed with uncapacitated membranes may be due to the intrinsic fusibility of these membranes. Alternatively, it may reflect the presence of membranes from cells which underwent capacitation spontaneously.

The fusion curves for uncapacitated membranes have half-times of about 8 min in the presence of Ca^{2+} or spermine (Figs. 1a and 2a). This is similar to the half-times of fusion of pancreatic zymogen granules with the plasma membrane [6]. The kinetics of fusion of internal membranes is much slower, with half-times in the range of 20–35 min [7,8,12–14]. Fusion of internal membranes is different in other aspects, as well from fusion with the plasma membrane, in that it requires an *N*-ethylmaleimide-sensitive cytosolic factor and ATP [7–10,12,14]. These are not required for fusion of the plasma membrane with pancreatic zymogen granules [6] or the outer acrosomal membrane. The fusion curves for the capacitated membranes, on the other hand, have half-times of about 15 s. This is similar to values obtained on liposomes [24,31–34], and suggests that capacitation has altered membrane structure in such a way that barriers to fusion are substantially reduced.

Capacitation alone, however, is not sufficient for a high rate of fusion. Extra membrane factors are also involved (Table I). Of these, a pH requirement for fusion is the most stringent. Fusion was not observed under any of the other conditions tested at pH 6.8.

Bringing the pH to 7.4 can result in the onset of fusion. As in the case of fusion of liposomes [23], an elevated pH alone does not cause fusion in our system. In either system, however, physiological pH in the presence of millimolar Ca^{2+} is effective in causing membrane fusion. It has been proposed [39] that initially the acrosomal reaction is inhibited by an acidic pH which is relieved following an increase in membrane permeability to H^+ upon zona binding. There is also a Ca^{2+} requirement for the acrosomal reaction. Binding of capacitated sperm to zona pellucidae has been shown to cause a synchronous increase in membrane permeability to both H^+ and Ca^{2+} [40,41].

We found that 20 μM spermine had the same effects on membrane fusion as Ca^{2+}. Spermine at this concentration has been shown to enhance liposome fusion in a manner which is dependent on the lipid composition of the vesicles [31,43]. This indicates that spermine, a polycation, affects fusion by altering the surface electrostatic properties of the membranes. Spermine is present in seminal plasma in millimolar concentrations. Rubinstein and Breitbart have reported that at this concentration it inhibits the Ca^{2+}-induced acrosomal reaction in vitro [44], possibly by blocking phospholipase C hydrolysis of phosphinositide. We have recently re-examined the effects of spermine on the acrosomal reaction and found that, at micromolar concentrations, spermine induces the acrosomal reaction in a Ca^{2+}-independent manner (our unpublished results).

The mechanism by which Ca^{2+} or spermine induces fusion is not clear. Binding of Ca^{2+} to negatively charged lipids, causing local dehydration and charge screening, as is believed to occur during liposome fusion, is not considered to be the mechanism by which it causes fusion in biological membranes [5]. Possible roles for Ca^{2+} in membrane fusion which have been suggested include ion channel activation and other phosphorylation–dephosphorylation phenomena, activation of protein kinase C, PLA_2 and other calcium binding proteins, and effects on cytoskeletal elements. Whatever the mode of action of Ca^{2+}, H^+ and spermine on sperm membranes in whole cells, the relevant structural features of the membranes appear to have been recovered intact during our extraction procedure since our in vitro system mimics the acrosomal reaction as far as these substances are concerned. This cell-free system is thus an effective experimental tool for studying the acrosomal reaction.

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