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Liposomes fuse with sperm cells and induce activation by delivery of impermeant agents

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Abstract

Sperm cell activation is a critical step in fertilization. To directly investigate the cell signaling events leading to sperm activation it is necessary to deliver membrane impermeant agents into the cytoplasm. In this study, the use of liposomes as possible agent-loading vectors was examined using (1) the octadecylrhodamine B (R18) and NBD phosphatidylethanolamine (NBD DHPE)/rhodamine phosphatidylethanolamine (rhod DHPE) fusion assays in bulk samples, (2) membrane transfer of fluorescence from liposome membranes labeled with R18 and rhodamine-tagged phosphatidylethanolamine (TRITC DHPE), and (3) lumenal transfer of impermeant calcium ions from liposomes to sperm cells, a process that stimulated sperm cell activation. Intermediate-sized unilamellar liposomes (98.17 ± 15.34 nm) were prepared by the detergent-removal technique using sodium cholate as the detergent and a phosphatidylcholine/phosphatidylethanolamine/cholesterol (2:1:1 mole ratio) lipid composition. In the R18 fusion assays, self-quenching increased logarithmically with increasing concentrations of R18 in the liposome membranes; addition of unlabeled sperm to R18-labeled liposomes lead to a rapid release of self-quenching. In the NBD DHPE/rhod DHPE resonance energy transfer (RET) fusion assay, RET was rapidly reduced under similar conditions. In addition, individual sperm became fluorescent when TRITC DHPE-labeled liposomes were incubated with unlabeled sperm cells. Incubation of sperm cells with empty liposomes did not significantly affect sperm cell activation and did not alter cell morphology. However, incubation with Ca (10 mM)-loaded liposomes resulted in a time-dependent increase in sperm cell activation (7.5-fold over controls after 15 min). We conclude that liposomes can be used for direct loading of membrane-impermeant agents into sea squirt sperm cell cytoplasm, and that delivery occurs via fusion and content intermixing.

Keywords: Liposome; Membrane fusion; R18; Liposome carrier; Passive targeting; Sperm activation

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1. Introduction

The delivery of impermeant substances into the cytoplasm of cells is a major problem for cell biologists. Three primary approaches prevail: permeabilization, microinjection, and liposomal delivery. Permeabilization, which allows entry of materials found in the external medium, has been attained by electroporation [1,2] and by pore-forming proteins or detergents [3,4]. Microinjection, which allows delivery of specific materials directly into the cytoplasm, can be used only with large cells [5]. Liposomal delivery involves fusion of the liposomes with the plasma membrane. This allows introduction of lipophilic materials from the liposome's phospholipid bilayer into the plasma membrane or hydrophilic materials from the liposome's lumen into the cytoplasm [6–8]. The relative success of each technique depends on the material being delivered and the sensitivity of the cell to perturbation by the process, and, most importantly, whether or for how long the researcher expects the cells to function following the perturbation. Because sperm cells are designed for efficient delivery of genomic material, they are relatively small and have minimal cytoplasmic volume. Perhaps because of this latter situation, they are very sensitive to membrane perturbation; keeping these cells alive in order to observe cellular responses following such a perturbation is very difficult. We have developed a liposomal delivery system that allows sperm cells to remain functional for hours following delivery of substances into their cytoplasm.

Liposomes have been found useful as models for the study of membranes and as vectors for drug delivery to cells. In the latter role, they have low toxicity effects on cells when used either in vivo [9,10] or in vitro [11]. Thus, liposomes are a desirable vehicle for delivery of cytoactive agents and, in some cases, are more effective than incubation in high concentration of the free drug [12].

Since sperm cells fuse with eggs to form zygotes, they are potential targets for fusion with artificial membranes. The interaction between sperm cells and liposomes has been used to investigate the biology of reproduction. Mammalian sperm cells have been found to fuse with liposomes after undergoing the acrosome reaction [13], and have been transfected with DNA using liposomes as vectors [14,15]. Liposomal delivery of DNA to sperm has produced more viable sperm and allowed for a greater percentage of transfection in eggs fertilized by these sperm when compared to electroporation or incubation with free DNA [15]. Liposomal introduction of specific phospholipids into ram sperm can alter sperm activation and increase egg penetration [16]. Recently, by incorporation of putative binding proteins into the liposomal bilayer, liposomes have also been used to investigate the nature of sperm–egg binding [17–19]. However, in none of these studies were non-nucleic acid, hydrophilic substances loaded into the liposome lumen and delivered into sperm cytoplasm.

We are interested in studying the role of cell signaling in an early step in the fertilization process, namely sperm cell activation. Activation prepares the sperm for penetration of the egg's protective barriers, a process that ultimately leads to fusion with the egg cell membrane. The study of these events requires sperm cells in which the activation process can be observed and manipulated. Sperm activation in ascidians (sea squirts) provides such a model since it is characterized by mitochondrial translocation, a process easily observed by light microscopy and characterized by the rounding of the mitochondrion and its movement off the head and down the tail [20]. Ascidian sperm activation depends on a rise in intracellular Ca concentration that initiates cellular events leading to the actin:myosin-dependent mitochondrial translocation necessary for sperm penetration of the egg outer layers [21]. In our model, the rise in intracellular calcium is produced by both entry of external Ca and the release of Ca from internal stores, which is dependent on a cascade of membrane and cytosolic signaling events including a proposed inositol 1,4,5-trisphosphate (IP_3)-mediated response [22]. In many cases, components of the pathway involved in this signaling process can only be studied if membrane-impermeant agents are introduced into the cytosol.

In this study, we investigated the use of liposomes as transport vectors for membrane-impermeant agents to sperm cells of the sea squirt, *Ascidia ceratodes*. We show that sperm cells can fuse with liposomes in a time-dependent manner and that liposomes can be used to deliver membrane-impermeant agents to these cells.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA), and were used with no further purification. Cholesterol (Chol), *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes), potassium chloride, sodium chloride, calcium chloride, magnesium phosphate, magnesium chloride, sodium carbonate and chloroform (99.9% A.C.S. HPLC grade, kept anhydrous by storage with 0.4-nm molecular sieves) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3*a*, 7*a*,12*a*-trihydroxy-5*b*-cholanic acid sodium salt (sodium cholate) was purchased from Calbiochem (La Jolla, CA, USA). Octadecylrhodamine B chloride (R18, 1.4 mM in ethanol), *N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (rhod DHPE), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD DHPE) and *N*-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (TRITC DHPE) were obtained from Molecular Probes (Eugene, OR, USA). All aqueous solutions were made in water obtained using a Barnsted Ultrapure water system (nanopure water).

2.2. Tunicate maintenance and dissection

Ascidia ceratodes were collected from Princeton Harbor, Half Moon Bay, CA, USA. The animals were maintained in natural seawater aquaria at 13°C for no more than 6 weeks with tri-weekly one-quarter volume water changes. The sperm cells, defined as dry sperm, were needle dissected directly from sperm ducts and stored on ice for immediate use.

2.3. Artificial seawater preparation

Artificial seawater (ASW) was prepared as follows: $CaCl_2$(10 mM), NaCl (423 mM), KCl (9 mM), $MgCl_2 \cdot 6H_2O$ (22.9 mM), $MgSO_4 \cdot 7H_2O$ (25.5 mM), and $NaHCO_3$ (2.02 mM) in nanopure water at pH 6.8. This abnormally low pH for seawater was used to minimize spontaneous activation of sperm cells.

2.4. Preparation of empty and lumen-loaded liposomes

Liposomes were prepared by the dialysis method [23,24] using a Mini Lipoprep unit (Sialomed) with 10-kDa-cutoff dialysis membrane (Diachema). Routinely, a lipid solution was prepared by dissolving a 2:1:1 mole ratio mixture of DSPC/DSPE/Chol (32 mg–40 μmol, 15 mg–20 μmol, and 8 mg–20 μmol, respectively) into chloroform (55 μl). NBD DHPE (0.5 mol%) and rhod DHPE or TRITC DHPE (0.25 mol%) were substituted for equivalent concentrations of DSPE when tagged liposomes were made. Dialysis was against a KCl (740 mM) saline adjusted to pH 8.0 with Hepes (50 mM); this dialysis buffer was also used to prepare the detergent solution (sodium cholate, 54.82 mM). All solutions were heated to 60°C and a mixed micellar solution was prepared by combining 950 μl of detergent solution with 50 μl of lipid solution to yield 73 μmol total lipid per ml mixed micellar solution. The mixed micellar solution was then dialyzed for 14 h against the dialysis buffer (1:1000 v/v ratio) at 60°C and produced about 1 ml of packed liposomes. Thus, the total lipid was estimated to be 73 μmol per ml in liposome form; dilutions were made from these packed liposomes and total lipid concentration of diluted liposomes was determined on this basis. Liposomes prepared in this manner contained the dialysis buffer, but hereafter are called 'empty liposomes'. Ca-loaded liposomes were prepared by dialyzing the mixed micellar solution against dialysis buffer with added $CaCl_2$ (10 mM). Liposome size was measured by dynamic laser scattering (MicroTrac Ultrafine Particle Analyzer) using the refractive index determined by a hand-held refractometer (American Optical). Sizing, unilamellar character, and intactness were verified by morphometric analysis of freeze-fracture replicas. Packed liposomes could be stored at 4°C and used for a 2-week period following their preparation.

2.5. Freeze-fracture procedure

Liposomes were cryoprotected by equilibration in

15% methanol-dialysis buffer and plunge-frozen in liquid nitrogen-cooled propane. Freeze-fracturing was performed on a Balzers Freeze Etch Apparatus BA360M at −110°C, etched slightly at −100°C for 1 min, Pt shadowed at 45° for 4 s and C-coated at 90° for 8 s at minimum vaporization current. Replicas were cleaned in distilled water for 30 min and mounted onto copper grids. Transmission electron micrographs were taken on an Hitachi H-7000.

2.6. Liposome–sperm cell fusion assay by membrane mixing

2.6.1. R18 self-quenching assay

Fusion of R18-labeled liposomes with sperm cells was measured by monitoring the relief of R18-fluorescence quenching [25,26]. Liposomes (73 μmol total lipid/ml liposomes) were labeled with R18 (1.4 mM ethanolic stock) by incubating liposome samples in different concentrations of R18 (0.6–5.0 μM) to give 0.5–3.5 mol% membrane-associated R18. The solution was incubated with agitation at room temperature for 1 h, settled and resuspended repeatedly to remove excess R18, diluted into ASW (1:500; approximately 146 nmol total lipid per ml of liposome suspension), and a 2-ml aliquot placed into plastic cuvettes with continuous mixing in an Hitachi F-2000 fluorescence spectrophotometer to measure fluorescence (560 and 590 nm, excitation and emission, respectively). After measuring baseline fluorescence of liposomes alone for several minutes, dry sperm (2 μl) or ASW (2 μl) were added. Fusion occurred rapidly and the increased intensity of fluorescence was stable over several more minutes. Maximum fluorescence (F_∞) was the average fluorescence measured after the addition of Triton X-100 (0.5% v/v). Incubation of R18-labeled liposomes also resulted in the gain of fluorescence by individual sperm cells.

2.6.2. NBD DHPE/rhod DHPE resonance energy transfer assay

Fusion of NBD DHPE- and rhod DHPE-labeled liposomes with sperm cells was monitoring by measuring the extent of resonance energy transfer (RET) [27]. Briefly, NBD DHPE- and rhod DHPE-labeled liposomes were excited at 465 nm to excite the NBD and fluorescence intensity at 534 nm (NBD peak emission wavelength) and 582 nm (rhodamine peak emission wavelength) were monitored over time in an Hitachi F-2000 fluorescence spectrophotometer using the manufacturer's Intracellular Cation Measurement software package. Liposomes alone were monitored for several minutes to establish baseline emissions before sperm cells were added. Emission scans were also performed before and after the addition of sperm cells to labeled liposome preparations.

Liposomes used in sperm fusion experiments contained NBD DHPE (0.25 mol%) rhod DHPE (0.5 mol%); the remainder of the DHPE was unlabeled. This mixture produced the highest F_{582}/F_{534}, thus optimizing the opportunity to observe loss of RET (data not shown).

2.6.3. Transfer of membrane fluorescence assay

As an additional test of fusion, TRITC DHPE-labeled liposomes were incubated with normal sperm cells. TRITC DHPE-labeled liposomes were made by adding TRITC DHPE (1 mg) along with unlabeled DSPE (14 mg) during liposome manufacture. The TRITC DHPE-labeled liposomes were incubated with unlabeled sperm cells in ASW for 15 min at room temperature with continuous mixing. Sperm cells were attached to glass coverslips for 7 min, washed with ASW and fixed in 1% formaldehyde. Sperm cells were then excited by epi-illumination in the standard rhodamine wavelengths (for both R18- and TRITC DHPE-labeled liposome exposed sperm cells) and photographed with an Olympus BMAX 60 equipped with a PM30 photometer.

2.7. Sperm activation assay

Unlike echinoderm and mammalian sperm, ascidian sperm cells do not undergo an exocytic process (the acrosome reaction) in preparation for penetration of egg vestments [28]. Instead, the defining characteristic is mitochondrial translocation [20]. In unactivated sperm cells, the mitochondrion lies along side the nucleus in the head of the sperm; upon activation, the mitochondrion rounds up, moves to the base of the head, migrates off the head onto the tail, and translocates down the tail (Fig. 1). This morphological change was used to assay sperm activation. Sperm cells, taken directly from the sperm duct, were diluted at a concentration of 1 μl/ml into: (1) pH 6.8 ASW (the sperm control), (2)

1:500 dilution of empty liposomes in pH 6.8 ASW (the liposome control), (3) 1:500 dilution of Ca-loaded liposomes in pH 6.8 ASW (the lumen-loaded experimental) or (4) 1:500 dilution of membrane-labeled liposomes in pH 6.8 ASW (the membrane-labeled experimental) and gently agitated during incubation. Samples (200 μl) were taken at 0, 1, 5, 10, and 15 min of incubation. Incubation continued while sperm cells were allowed to attach to coverglasses for an additional 5 min. They were then washed with pH 6.8 ASW and fixed in 1% formaldehyde in ASW (15 min). Coverglasses were mounted in 50% glycerol in ASW and sealed with fingernail polish. Cells were viewed with a 40× phase objective on an Olympus BH2 light microscope and percentage of sperm activation was determined by counting 300 sperm cells per experiment in randomly chosen fields. Experiments were performed in triplicate with sperm cells dissected from three different animals. Activated sperm cells were defined as those with mitochondria rounded and at the base of the head or on the tail. Positive controls were determined using egg extracts made by incubation of whole eggs (fresh or frozen) with pH 2.0 seawater for 1 h and adjusted to pH 6.8 before incubation with sperm.

In all sperm activation assays, viability of sperm was determined by sperm motility either prior to fixation or in parallel experiments. For experiments involving liposome fusion and transfer of membrane labels, live sperm were examined in the fluorescence microscope to verify that labeled cells were motile. The images presented below were taken from samples fixed in formaldehyde to eliminate poor focus due to cellular motility; however, they are representative of observations made of live, motile sperm.

3. Results and discussion

Ascidian sperm cell heads are very small compared to other sperm cells [29], those of *Ascidia ceratodes* measure approximately 1.5×4.5 μm, and therefore are difficult to study using techniques such as microinjection. Until now, only membrane-permeable agents and techniques entailing cell membrane permeabilization have been used to investigate the cell signaling events indirectly. In light of recent use of liposomes as delivery vehicles to various cells, we have developed a methodology to prepare liposomes and use them to load sea squirt sperm cells with membrane-impermeant agents. This application will allow more direct investigation of intracellular signaling events.

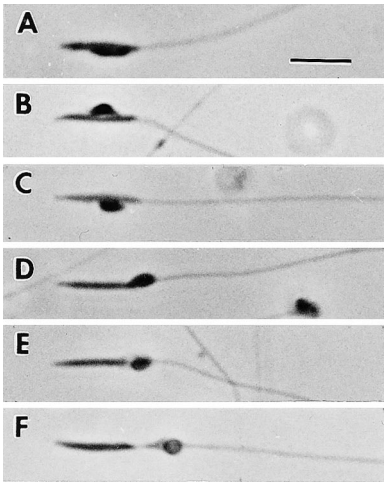

Fig. 1. Phase contrast light micrographs of ascidian sperm cells. (A) Sperm cells treated with pH 6.8 ASW remain unactivated. Sperm cells treated with high pH (9.4) ASW or other activating agent are activated and may be seen in various stages of mitochondrial translocation. In the initial stages of activation, the mitochondrion rounds (B) and moves to the base of the head (C). In the later stages, it transitions off the head (D), onto the tail (E) and translocates down the tail (F). Bar = 10 μm.

3.1. Liposome preparation

To limit alteration of the membrane lipid composition and hence to minimize potential interference with in vivo transmembrane cell signaling events, the phospholipid composition of the liposomes was chosen to match the phospholipid composition of ascidian sperm cells as closely as possible. Published analysis of whole, dry ascidian (*Ciona intestinalis*) sperm cells revealed that DSPC, DSPE and Chol were present in a ratio of 2.5:1:1.2, respectively [30], a ratio very close to the 2:1:1 used in the manufacture of liposomes for this study. Even though it was present in the sperm, phosphatidylserine (PS) was left out of our liposome preparation mixture for three reasons. First, we wanted to avoid the inhibitory effect PS has on lipid intermixing between membranes [31]. Second, since seawater contains a relatively high level of Ca (10 mM), we wanted to avoid Ca-induced aggregation of PS-containing

vesicles [31]. And, third, since the phospholipid distribution in the bilayers during liposome preparation could not be controlled, we could not restrict PS to the inner leaflet as is common in most plasma membranes [32]. DSPE and Chol were used not only to accommodate the natural composition of ascidian sperm cell membranes but also due to their physical properties and effects. DSPE has a weak surface hydration [33], spontaneously promotes the formation of a nonlamellar inverted hexagonal phase [34], and thus favors destabilization and fusion of bilayer membranes [35]. Chol was incorporated in order to enhance liposome stability and to avoid phase transition effects caused by phospholipids [36].

3.2. Liposome formation

It was desirable to prepare the liposomes using a fast procedure that reproducibly yielded intermediate-sized unilamellar vesicles (IUV) of a constant diameter that could encapsulate macromolecules. The dialysis method is a simple technique that is reported to form vesicles of a relatively homogeneous size distribution [37]. In this method, a mixed micellar solution is prepared by mixing phospholipids in an organic solvent with another lipid-like chemical (e.g., bile salts) at the midtransition temperature and above the critical micellar concentration (CMC) [38,39]. Specifically, we used sodium cholate (well above its CMC of 9–15 mM) in the detergent-removal technique since it is known to facilitate the production of unilamellar vesicles of a uniform intermediate size [24]. To form the liposome vesicles, the mixed micellar solution was dialyzed for continuous and controlled removal of the detergent [40,41]. The dialysate was chosen in light of previous studies suggesting minimal effects of this solution on detergent-permeabilized ascidian sperm cell viability as determined by motility (Koch, unpublished data). The pH of the dialysate was adjusted to 8.0 to prevent the formation of large unilamellar vesicles and to eliminate significant effects of variations in cholesterol content on vesicular size [37].

IUVs, prepared by dialysis from DSPC/DSPE/Chol (2:1:1 molar ratio), were 98.17 ± 15.34 nm in diameter (mean ± S.D.) as determined by dynamic light scattering. Two groups of large structures were detected with diameters approximately 1.3 and 5.0 μm. Freeze-fracture electron micrographs verified that the vesicles were unilamellar and intact (Fig. 2A). Morphometric analysis of the freeze-fracture images showed that the primary group of liposome profiles centered around 100 nm (Fig. 2B). These profiles were produced by fracture at various levels of the spherical liposomes, thus the most abundant profile approximates the diameter of the most abundant objects. These data are consistent with the light

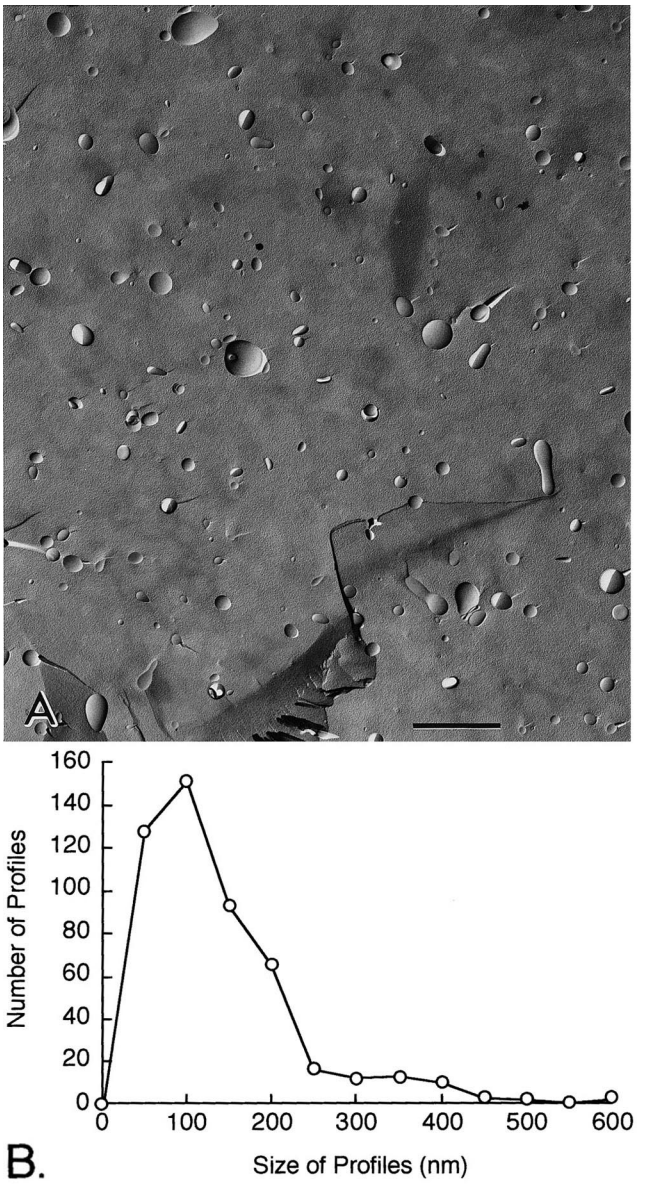

Fig. 2. (A) Electron micrograph of freeze-fracture replica of packed liposomes. Bar = 1 μm. (B) Histogram of liposome profile diameters found in freeze-fracture replicas ($n = 504$; bin size = 50 nm).

scattering data. The largest profiles seen in the electron micrographs (Fig. 2A), represented by the rightward trailing edge of the profile-size distribution (Fig. 2B), are so few in number that they represent an insignificant number of opportunities for fusion with sperm; indeed, primarily IUVs were observed by light microscopy to interact with sperm cells. The large structures seen in dynamic light scattering analysis were not seen in freeze-fracture replicas and probably represented aggregates of IUVs produced during preparation of liposomes for that analysis. However, aggregates were occasionally seen to be in contact with sperm cells during fusion experiments (see below).

3.3. Sperm cell–liposome fusion

Several studies have reported that cells can take up liposomes by endocytosis [42–45] and by phagocytosis [46], and that fusion and release of lumenal contents occurs in the endosomal compartment. This type of uptake is very unlikely in ascidian sperm cells since they are designed solely for genome delivery and the appropriate intracellular compartments do not exist [29]. In this study, interaction between liposomes and sperm cells depended only on passive targeting; i.e., no cell-specific proteins have been incorporated into the liposome bilayer – fusion depends on electrostatic attraction between polar head groups and interactions between phospholipids of the closely apposed bilayers. Therefore, it was important to demonstrate that this unguided fusion had occurred. We did this by demonstrating the mixing of membranes in three different types of experiments, by revealing the introduction of lumenal contents in one experiment, and by using the combination in the third set of experiments.

3.4. Sperm cell–liposome fusion as measured by the R18 self-quenching assay

To assess fusion activity between sperm cells and liposomes, the R18-fusion assay was used. This method is based on monitoring the increase in R18 fluorescence which occurs upon dilution of the indicator following fusion of labeled and non-labeled membranes and which results from the relief of self-quenching. Self-quenching relief is inversely proportional to the surface density of the fluorophore and hence a decrease in R18 surface density is accompanied by an increase in fluorescence [26].

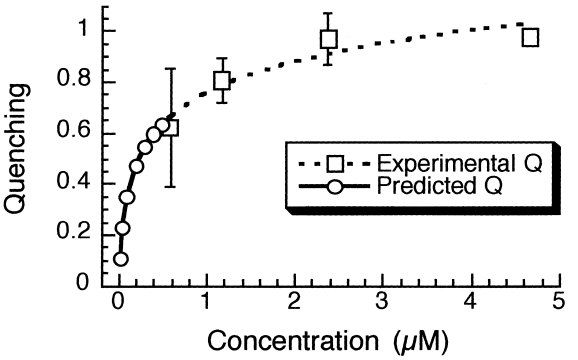

Fig. 3. Self-quenching of R18 fluorescence increases with increasing R18 membrane association. Open squares represent the R18 fluorescence following ethanolic injection of R18 at different concentrations (to convert to molarity, v μM = 1.37 * w mol%). These points can be fit by a logarithmic equation ($y = 0.8093 + 0.40669 \log x$; $R = 0.9486$; dashed line). Open squares and solid line represent predictions made by extrapolations based on this relationship. Note that quench equals zero when R18 concentration equals zero.

Empty liposomes were labeled with R18 using the ethanol-injection method [25,26]. In this method, R18 spontaneously incorporates into phospholipid membranes at concentrations high enough to result in self-quenching. Quenching (Q) was calculated according to $Q = 1 - F/F_\infty$ where F is the average fluorescence recorded from R18-loaded liposomes prior to sperm or ASW addition, and F_∞ is the maximum fluorescence as defined in Section 2 [25]. Incorporation of R18 into liposome membranes yielded a Q value of 0.88 ± 0.03 (mean ± S.D., $n = 4$ batches), in close agreement with other reports of quenching of 0.9 or greater [25].

The level of self-quenching was dependent on the concentration of R18 present in liposomes. A logarithmic equation, which fits all the data ($R = 0.95$), predicts zero quench at zero mol% R18 (Fig. 3). This indicates that only self-quenching was occurring as has been reported by others [26,47,48].

To monitor fusion between liposomes and sperm cells, unlabeled sperm cells were added to R18-labeled empty liposomes (Fig. 4). The addition of sperm cells (at 115 s) to diluted R18-labeled liposomes (1:500) resulted in rapid relief of self-quenching. The subsequent addition of TX-100 (at 380 s)

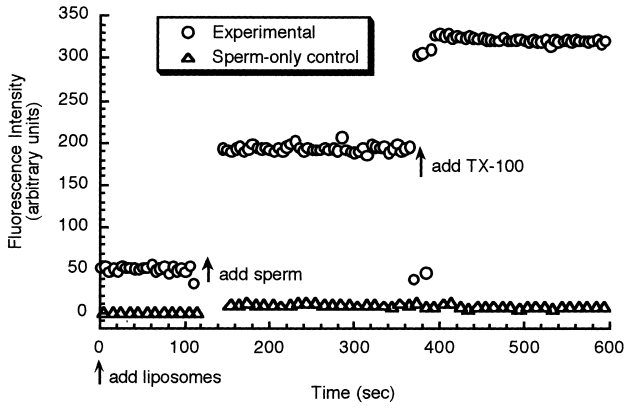

Fig. 4. Relief of self-quenching of R18 fluorescence. Fluorescence emission intensity (exc. 560 nm; em. 590 nm; open circles) collected from R18-labeled liposomes added to cuvettes at time 0 was steady until sperm cells (upward arrow at 115 s) and TX-100 (upward arrow at 380 s) were added. Following each addition, fluorescence emission intensity increased dramatically as R18 self-quenching was reduced by sperm addition and eliminated by TX-100 dissolution of liposomes (F_∞). Fluorescence emission from sperm cells in the absence of labeled liposomes (open triangles) was nearly zero. The abscissa is set at −10 fluorescence units for clarity.

yielded maximum relief of self-quenching (F_∞). In the experiment shown (one of three performed at this dilution and yielding similar results), the initial quench of 0.86 was reduced to 0.42 upon fusion with sperm cells. Similar curves were obtained using other liposome dilutions (1:50, 1:100 and 1:250; data not shown). Controls were prepared by the addition of ASW to liposome dilutions, an action that produced no change in the fluorescence intensity (data not shown). To control for labeling of sperm by non-incorporated R18, liposomes were separated from the labeling solvent by centrifugation (14 000 × g for 5 min) and the supernatant was used in a fusion assay. The addition of sperm cells to this supernatant produced a steady, low-level fluorescence similar to that recorded from sperm cells alone in ASW (as shown in Fig. 4). This result indicated that R18 was not being transferred to sperm cells through contact with the medium in which liposomes were suspended.

3.5. Sperm cell–liposome fusion as measured by the RET assay

The R18 fusion assay has been used in dozens of studies of viral fusion. Additionally, it has been compared to the resonance energy transfer (RET) assay [49], and was found to be essentially indistinguishable [26]. However, recent reports have noted the possibility that R18 can move from membrane to membrane in the absence of fusion [50]. Consequently, we have verified that fusion occurs using the NBD-to-rhodamine RET method. The mixing of dual-labeled liposomes (NBD DHPE 0.5 mol% and rhod DHPE 0.25 mol%) with sperm cells resulted in an increase in the NBD emission and a decrease in rhodamine emission (Fig. 5A,B). These results were caused by the loss of RET as NBD- and

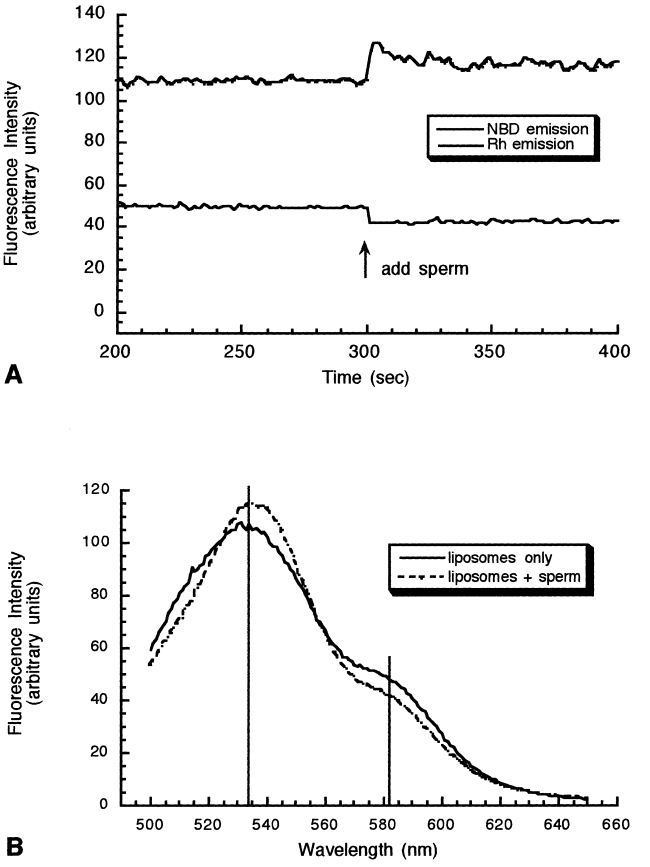

Fig. 5. Reduction of resonance energy transfer from NBD to rhodamine upon mixing of liposomes with sperm cells. (A) When NBD DHPE (0.5 mol%) and rhodamine DHPE (0.25 mol%) were mixed with sperm cells (upward arrow), the emission intensity at the rhodamine peak (582 nm; thin line) decreased and the emission intensity at the NBD peak (534 nm; thick line) increased. (B) Wavelength scans of emission intensity taken 100 s before (solid line) and 100 s after (dashed line) mixing with sperm show that these changes were focused around the peak intensities (vertical lines).

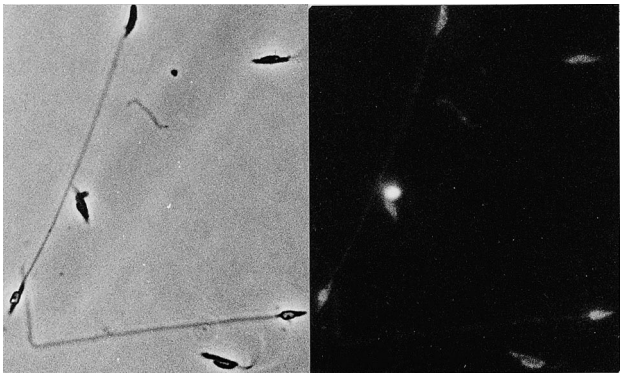

Fig. 6. Transfer of fluorescence from liposome membrane to sperm cell membrane. Sperm cells incubated with liposomes whose membranes incorporate TRITC DHPE become fluorescent themselves. Comparison of phase (left) and fluorescence (right) shows that both heads and tails are labeled. A brightly fluorescent liposome aggregate can also be seen left of center in contact with a sperm head.

rhod-labeled DHPE dispersed, an event that could only occur via liposome–sperm cell fusion and the diffusion of NBD- and rhod-DHPE away from each other as they move laterally into the surrounding sperm plasma membrane.

3.6. Sperm cell–liposome fusion as measured by transfer of fluorescence

Liposomes labeled with a fluorescent phospholipid should transfer that label to any cell with which it fuses. Thus, we prepared TRITC DHPE-labeled liposomes, mixed them with sperm cells and examined the sperm cells in a light microscope via epifluorescence. The majority of sperm cells became fluorescent (Fig. 6). Sperm cells incubated with unlabeled liposomes remained dark except for weak autofluorescence (data not shown) that illuminates only the mitochondrial region on each sperm head at a level much lower than seen in Fig. 5. Since TRITC DHPE is incorporated into the lipid bilayer during their manufacture, the only way for TRITC fluorescence to appear in sperm cells incubated with labeled liposomes is by membrane fusion and diffusion of the labeled phospholipid throughout the sperm cell plasma membrane. In nearly all cells examined, both the head and tail were fluorescent, indicating the fusion and diffusion have lead to uniform phospholipid dispersal. Heads tend to appear brighter. This may be because there is a greater surface area of membrane in the head region than the tails, thus allowing for a greater concentration of fluorescent phospholipids. On the other hand, it may be due to a selective incorporation of liposomes in the head region. However, in ascidian sperm, membrane regions structured for fusion, as found in the inner acrosomal membrane of reacted sea urchin and mammalian sperm, are exposed late during penetration, i.e., immediately prior to fusion [51,52]. Thus, in ascidian sperm, these regions would not be available for liposome fusion according to the protocol used in this study.

3.7. Lumenal delivery of impermeant agents

Liposomes of the size we have prepared are capable of entrapping macromolecules, and thus are valuable tools for possible delivery of agents to cells [53]. Although the R18, RET and membrane transfer assays confirm the interaction of the liposomes and sperm membranes, this evidence does not prove that content intermixing is occurring or that membrane-impermeant drug delivery is possible in this system. In addition, in order to use the liposomes to investigate events leading to sperm activation, it was necessary to evaluate the effect of liposome interaction on cell morphology. Our definition of sperm activation is the occurrence of mitochondrial translocation. In this process, the mitochondrion, which normally

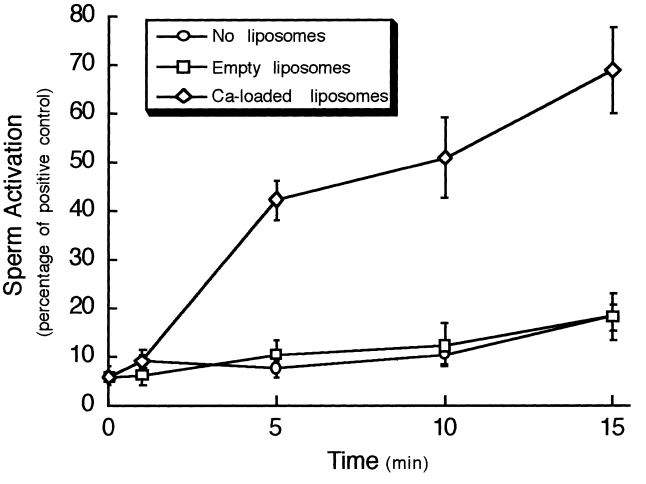

Fig. 7. Percentage of sperm activation after incubation with liposomes. Sperm were activated by Ca-loaded liposomes in a time-dependent manner (open diamonds). Empty liposomes (see Section 2 for definition; open squares) were not significantly different from sperm alone (open circles).

lies along side the nucleus in the head region, rounds up, moves to the base of the head and sometimes onto the tail [51].

To approach these issues, we compared the effect of empty liposomes on sperm activation against that of Ca-loaded liposomes. There was no significant difference between the percentage of spontaneous activation of sperm cells in the absence of any liposomes and the percentage of activation of sperm cells incubated with empty liposomes (Fig. 7). On the other hand, incubation of sperm cells with Ca-loaded liposomes resulted in a time-dependent increase in sperm activation exhibiting a 7.5-fold increase over negative controls and reaching 75% of positive controls after 15 min. The morphology of the liposome-treated sperm cells was indistinguishable from controls in the same state of activation (data not shown). These findings not only prove that sperm fusion with empty liposomes does not affect sperm cell activity or morphology, but also prove that membrane-impermeant agents can be delivered to *Ascidia ceratodes* sperm cells using liposomes as vectors. (The argument that Ca could leak in from seawater without fusion is negated by the absence of sperm activation by fusion with empty liposomes.)

In an additional experiment that combined both membrane transfer of fluorescent molecules and lumenal contents, sperm cells were mixed with Ca-loaded, R18-labeled liposomes. Sperm cells that were activated also fluoresced in proportion to the degree of activation. The degree of activation was determined by the roundness of the mitochondrion and its location near the base of the sperm head – the rounder the mitochondrion and the nearer the base of the head, the greater the degree of activation (Fig. 1). Accordingly, sperm cells in advanced stages of activation (arrow, Fig. 8) and early stages of activation (arrowhead, Fig. 8) fluoresced at different intensities, whereas unactivated cells remained non-fluorescent (unmarked cells, Fig. 8). These results are consistent with the interpretation that the greater the number of liposomes that fused per sperm cell, the greater the delivery of Ca and R18 and the greater the degree of activation and membrane fluorescence, respectively.

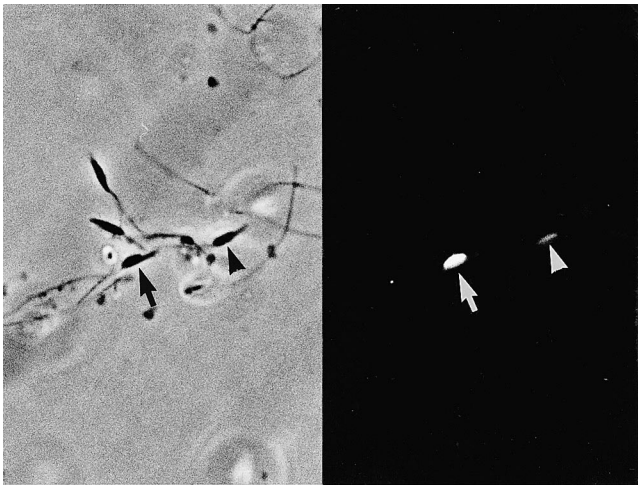

Fig. 8. Light micrograph of phase (left) and fluorescence (right) images of R18-labeled sperm. Sperm cells were incubated with liposomes having R18-labeled membranes and Ca-loaded lumens. Note that only the late-activated (arrow) or early-activated (arrowhead) sperm are fluorescent. The degree of activation was determined by the shape and location of the mitochondria.

3.8. Concluding remarks

In this paper, we have proven that liposomes can fuse with ascidian sperm cells and have described their use in delivering membrane-impermeant agents into these cells. The stimulation of Ca-dependent sperm activation following incubation with Ca-loaded liposomes was our example, but we anticipate that any hydrophilic impermeant agent can be delivered in this manner. Currently, other cell signaling molecules are being used to study several aspects of the transmembrane signaling pathway involved in coupling the initial sperm-to-egg binding event with mitochondrial translocation and subsequent penetration. Many of these agents are impermeant and their liposomal delivery would allow further elucidation of the signaling pathway. For example, this methodology has been used recently in preliminary studies to test the effects of inositol 1,4,5-trisphosphate on sperm activation [54].

Recently, several studies have reported the use of liposomes to investigate the protein interactions necessary for sperm–egg binding as well as the location of the sperm–egg membrane fusion event [17–19]. In each of these cases, fusion has occurred with membrane regions newly exposed by the acrosome reaction; i.e., the inner acrosomal membrane. Since sperm cells in our model system do not undergo an

acrosome reaction as part of the penetration process [28] and the species-specific binding event has been shown to involve protein–sugar interaction (reviewed in [51]), it will be interesting to perform similar studies using isolated sperm-surface proteins incorporated into liposomes in an attempt to distinguish between binding proteins and fusion proteins. Investigations of this type, made possible by the development of an effective liposome vector, could provide invaluable information on the process of sperm–egg binding and fusion, and significantly advance our understanding of fertilization in *Ascidia ceratodes*.

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