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Interaction of calcium and cholesterol sulphate induces membrane destabilization and fusion: implications for the acrosome reaction

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Cholesterol sulphate is a potent stabilizer of membrane bilayer structure in both dielaidoylphosphatidylethanolamine and egg phosphatidylethanolamine model membranes, however, the addition of calcium abolishes this bilayer stabilization. Calcium also induces fusion and leakage of egg phosphatidylethanolamine large unilamellar vesicles containing cholesterol sulphate, but has no effect on fusion or leakage of egg phosphatidylcholine large unilamellar vesicles containing cholesterol sulphate. With egg phosphatidylethanolamine liposomes, the initial rate, and extent of fusion, at constant calcium concentration, vary inversely with the mol percentage of cholesterol sulphate present in the vesicle membrane. The interaction of calcium and cholesterol sulphate, which causes membrane destabilization and fusion in phosphatidylethanolamine containing model systems, may play a role in the acrosome reaction in human sperm.

Introduction

Phospholipids in biological membranes normally exist in a bilayer arrangement, however, for the fusion of two membranes to occur, this bilayer structure must be transiently perturbed. Certain lipids, such as PC, in purified form, organize into stable bilayers. Other lipids undergo transitions from bilayer to non-bilayer phases such as the inverted hexagonal phase (H_{II}) [1]. Some examples include unsaturated diacyl phosphatidylethanolamines [2], phosphatidylethanolamine plasmalogens [3], and some acidic phospholipids such as cardiolipin in the presence of calcium [4]. It has been proposed that non-bilayer forming lipids may be involved in membrane processes such as fusion [5–7]. This is not to say that inverted hexagonal phase structures, per se, need to form in biological membranes in order for fusion to occur. Structures related to the H_{II} phase, perhaps intermediates in the L_α to H_{II} phase transition, however, have been proposed as fusion intermediates [5,6,8]. The formation of the H_{II} phase offers a measurable parameter, which allows the stability and fusogenic potential of model membranes to be predicted with some degree of accuracy. We have previously shown a correlation between the effect of a membrane additive on the L_α to H_{II} phase transition temperature and its effect on membrane fusion [9].

Cholesterol sulphate (CS) is a component of human erythrocyte, sperm, myelin and keratinocyte plasma membranes [10–13]. In erythrocytes, CS can act as a membrane stabilizer and inhibit hypotonic hemolysis [14]. CS inhibits the fusion of Sendai virus to both human erythrocyte and liposome membranes (unpublished observation). CS also reduces the fertilization efficiency of rabbit spermatozoa [15]. In human spermatozoa, CS is localized in the plasma membrane, mainly in the acrosome region, and can account for as much as 20% of the sperm head surface area [11]. Roberts et al. have proposed that CS acts as a membrane stabilizer in human sperm during maturation and epididymal transit [11]. Sterol sulphatases in the female reproductive tract would then be responsible for the removal of the sulphate moiety from CS rendering the sperm membrane more fusogenic as part of the capacitation process [16].

There is an absolute requirement for Ca^{2+} in the acrosome reaction of mammalian spermatozoa, al-

Abbreviations: DEPE, L-α-dielaidoylphosphatidylethanolamine; egg PE, egg phosphatidylethyanolamine; egg PC, egg phosphatidylcholine; DSC, differential scanning calorimetry; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; CS, 5-cholesten-3β-ol-sodium sulphate; L_β, gel phase; L_α, liquid crystalline phase; H_{II}, inverted hexagonal phase; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulphonic acid; ANTS, 1-aminonaphthalene-3,6,8-trisulphonic acid; DPX, *N*-*N*′-*p*-xylylenebis(pyridinium bromide); CHEMS, cholesterol hemisuccinate.

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though the exact function of Ca^{2+} in this process is poorly understood [17]. We have found that Ca^{2+} abolishes the bilayer stabilizing effect of CS on egg PE MLVs and that Ca^{2+} induces the rapid fusion of egg PE/CS LUVs but not egg PC/CS LUVs. We propose that one of the roles of calcium in the acrosome reaction may involve its interaction with CS in the sperm membrane which changes the effect of CS on lipid polymorphism, and could promote membrane fusion.

Materials and Methods

Egg phosphatidylethanolamine (egg PE), egg phosphatidylcholine (egg PC) and dielaidoylphosphatidylethanolamine (DEPE) were obtained from Avanti Polar Lipids (Birmingham, AL). *N*-[Tris(hydroxymethyl)-methyl]-2-aminoethanosulphonic acid (Tes) from Calbiochem (La Jolla, CA). 1-Aminonaphthalene-3,6,8-trisulphonic acid (ANTS) and *N-N'-p*-xylylenebis(pyridinium bromide) (DPX) were purchased from Molecular Probes (Junction City, OR). Cholesterol sulphate (CS) and cholesterol hemisuccinate (CHEMS) were obtained from Steraloids, (Wilton, NH). NaCl was from BDH Chemicals (Toronto, ON). $MgCl_2$, $CaCl_2$ and EDTA were obtained from Fisher Scientific (Fairlawn, NJ).

ANTS / DPX fusion assay

Lipid mixtures were dried down from chloroform/methanol (2 : 1, v/v) solutions with a stream of nitrogen to form thin films inside Pyrex tubes. The films were then placed in a vacuum evaporator equipped with a cold trap for at least 2 h to remove any residual solvent. The lipid films were then hydrated in either 25 mM ANTS, 89 mM NaCl, 10 mM Tes (pH 7.4) or 90 mM DPX, 47 mM NaCl, 10 mM Tes (pH 7.4) as previously described [18]. Large unilamellar vesicles were prepared by first freeze-thawing the lipid suspensions five times, then extruding them through 0.1 μm polycarbonate membranes ten times using an Extruder (Lipex Biomembranes, Vancouver, BC). Vesicles containing ANTS or DPX were separated from non-encapsulated material on a Sephadex G-75 gel filtration column eluted with 150 mM NaCl, 10 mM Tes, 0.1 mM EDTA (pH 7.4). The vesicles were kept on ice until use within the same day as preparation. Routinely, equal concentrations of both ANTS and DPX containing vesicles (40 nmol of lipid phosphorus each), were suspended in 150 mM NaCl, 10 mM Tes, 0.1 mM EDTA (pH 7.4), final volume 2 ml. ANTS fluorescence was monitored with a Perkin-Elmer MPF-44 Spectrofluorometer using 360 nm excitation and 530 nm emission wavelength. Fluorescence emission was monitored as a function of time relative to the initial fluorescence which was taken to be 0%, and baseline (shutters closed) which was taken as 100% fusion.

Leakage assay

Leakage of vesicle contents was monitored using LUVs containing 12.5 mM ANTS, 45 mM DPX, 68 mM NaCl, 10 mM Tes (pH 7.4) as described above. Calcium was added to the vesicles (80 nmol lipid phosphorus in 2 ml final volume) and the increase in ANTS fluorescence was followed using an excitation wavelength of 360 nm and emission wavelength of 530 nm. Increase in ANTS fluorescence reflects leakage of ANTS and DPX from vesicles [18]. 100% leakage was taken as the fluorescence obtained after the addition of 0.1 % Triton X-100.

Differential scanning calorimetry

Phospholipids (Avanti Polar Lipids) were dissolved in chloroform/methanol (2 : 1, v/v). CS (Steraloids) was dissolved in methanol. Aliquots of phospholipid and sterol solution were mixed to obtain the desired concentrations. The solvent was evaporated with a stream of dry nitrogen, depositing the lipids as a film on the walls of a glass test-tube. Samples were placed in a vacuum evaporator equipped with a liquid nitrogen trap for 3 h to remove any residual solvent. The dried lipid film was resuspended in 20 mM Pipes, 150 mM NaCl (pH 7.4) with or without Ca^{2+}, by vigourous vortexing at about 45 °C. Lipid suspension and buffer were equilibrated in an ice bath for one hour, then degassed under vacuum and loaded into the sample and reference cells, respectively, of an MC-2 high-sensitivity scanning calorimeter (Microcal Co.). A heating scan rate of 45 °C/h was employed. Transition temperatures and enthalpies were calculated by fitting the observed transitions to a single Van't Hoff component using the DA2 software package from Microcal.

Results

We have found CS to be potent in raising the L_α to H_{II} phase transition temperature of egg PE model membranes using differential scanning calorimetry (Fig. 1). 10 mol% CS raises the L_α to H_{II} phase transition temperature of egg PE from 38 to 51 °C. CS also broadens the L_α to H_{II} phase transition of egg PE but has negligible effect on the enthalpy of the transition which remains at 250 ± 30 cal/mol. The addition of Ca^{2+} broadens the L_α to H_{II} phase transition of pure egg PE by promoting a high temperature shoulder on the transition, but Ca^{2+} has little effect on the temperature or the enthalpy of this transition (Fig. 1). Addition of Ca^{2+} to egg PE/10 mol% CS MLVs lowers the L_α to H_{II} phase transition temperature (temperature of maximum excess heat capacity) from 51 to 42 °C and again causes a high temperature shoulder to be formed (Fig. 1). The L_α to H_{II} phase transition of synthetic PE, such as DEPE, is sharper than that of egg PE, and has a greater transition enthalpy. Thus the effects of additives

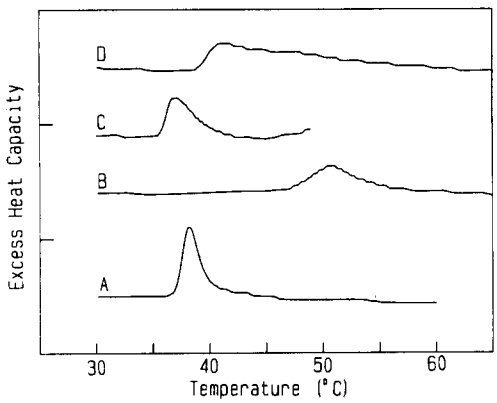

Fig. 1. Representative DSC scans of (A) egg PE, (B) egg PE/10 mol% CS, (C) egg PE with 10 mM Ca^{2+} and (D) egg PE/10 mol% CS with 10 mM Ca^{2+}. Lipid concentration was 10 mg/ml PE in 20 mM Pipes, 150 mM NaCl (pH 7.4), with or without Ca^{2+}. A scan rate of 45 C°/h was used. The interval between calibration marks on vertical axis represents 200 cal/C° per mol PE.

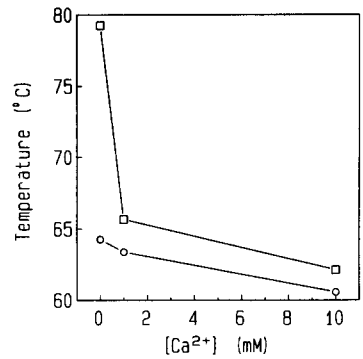

Fig. 2. Dependence of the L_α to H_{II} phase transition of DEPE (○) and DEPE/10 mol% CS (□) on Ca^{2+} concentration. Buffer and scan rate were the same as for Fig. 1.

on the L_α to H_{II} phase transition can be determined with greater accuracy. CS can dramatically raise the L_α to H_{II} phase transition temperature of DEPE. 10 mol% CS raises the L_α to H_{II} phase transition temperature of DEPE from 64 to 79°C. In a plot of temperature of the transition vs. mol fraction of CS a slope of $+173 \pm 8$ C°/mol fraction is obtained (data not shown). A large positive slope is indicative of a good bilayer stabilizing molecule in PE model membranes.

In the presence of 1 mM Ca^{2+} the L_α to H_{II} phase transition temperature of DEPE with 10 mol% CS drops 13°C, from 79°C to 66°C (Fig. 2). In contrast, the addition of 1 mM Ca^{2+} to pure DEPE has little effect on the temperature (Fig. 2). In all cases the enthalpy of the L_α to H_{II} phase transition remains unaltered.

The addition of CS to DEPE broadens the L_β to L_α phase transition and lowers the temperature at which it occurs (Fig. 3). When 100 mM calcium is added to pure DEPE MLVs the temperature of the L_β to L_α phase transition is raised from 36.7 to 39.1°C (Fig. 3). When calcium is added to DEPE MLVs containing 10 mol% CS, the temperature of the L_β to L_α phase transition is raised compared to that of the same sample in the absence of Ca^{2+}, but the endotherm remains broadened and the enthalpy of the transition is unchanged (Fig. 3).

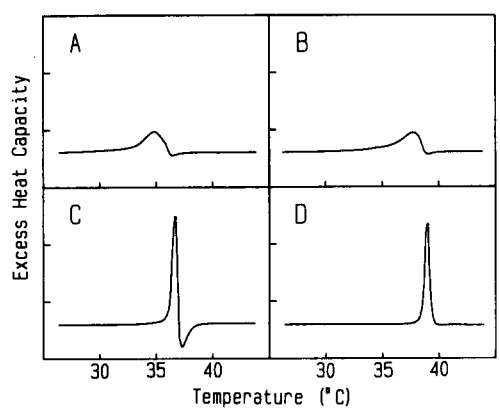

Fig. 3. DSC scans of the L_β to L_α phase transition of DEPE MLVs and the effects of CS and calcium. (A) DEPE with 10 mol% CS, (B) DEPE with 10 mol% CS and 100 mM Ca^{2+}, (C) DEPE, (D) DEPE with 100 mM Ca^{2+}. Lipid concentration was 1 mg/ml. Buffer and scan rate were the same as in Fig. 1. The interval between calibration marks on the vertical axis represents 5 kcal/C° per mol PE.

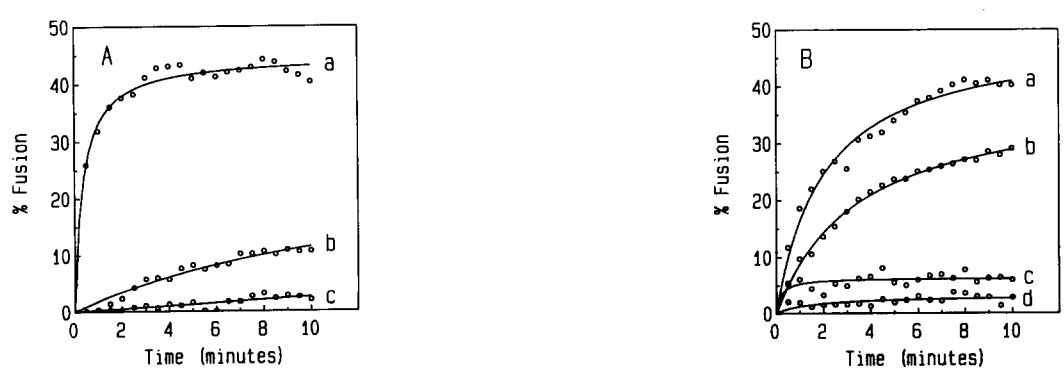

Fig. 4. (A) Dependence of fusion of egg PE/10 mol% CS LUVs on Ca^{2+} concentration, Ca^{2+} as follows: (a) 14.5 mM, (b) 1.2 mM, (c) 0.1 mM. Vesicles were suspended in 150 mM NaCl, 10 mM Tes, 0.1 mM EDTA (pH 7.4, 37°C), final volume 2 ml. Calcium was added from a concentrated stock solution in Tes buffer. (B) Dependence of fusion on mol percentage of CS in egg PE vesicles; (a) 10 mol% CS, (b) 15 mol% CS, (c) 20 mol% CS, (d) 25 mol% CS. Calcium concentration was 2.4 mM.

Fig. 5. (A) Dependence of leakage of egg PE/10 mol% CS LUVs on Ca^{2+} concentration. Calcium concentrations were: (a) 14.5 mM, (b) 4.9 mM, (c) 1.2 mM. Buffer and temperature same as in Fig. 4. (B) Dependence of leakage on mol percentage of CS in egg PE vesicles. CS mol percents were 25 mol% (○), 20 mol% (●), 15 mol% (□) and 10 mol% (■). Calcium concentration was 2.4 mM, temperature 37 °C.

The addition of Ca^{2+} to egg PE/10 mol% CS LUVs induces rapid fusion, as measured by the ANTS/DPX fluorescence quenching assay (Fig. 4). Both the rate and extent of fusion of egg PE/CS LUVs depends on calcium concentration. The results for egg PE LUVs containing 10 mol% CS are shown as an example (Fig. 4A). As the concentration of calcium is increased, the rate and extent of fusion also increases. At higher calcium concentrations rapid fusion followed by leakage of the fusion products occurs. The sensitivity of fusion to calcium concentration is particularly evident at low calcium concentrations. Calcium also induces leakage of egg PE/CS LUVs (Fig. 5A). As with fusion, the rate and extent of leakage is dependent on the concentration of calcium.

Not only are fusion and leakage of egg PE/CS LUVs dependent on calcium concentration, but they are also dependent on the mol% of CS in the vesicle membrane. As the mol% of CS is increased, the sensitivity to calcium-induced fusion and leakage is reduced (Fig. 4B and Fig. 5B). Thus at higher mol% of CS, more calcium is required to induce fusion and leakage.

Calcium has no effect on the fusion or leakage of egg PC/10 mol% CS LUVs (Fig. 6). Identical concentrations of calcium induce rapid and extensive fusion and leakage of egg PE/10 mol% CS LUVs (Fig. 6).

Discussion

CS significantly raises the L_α to H_{II} phase transition temperature of both egg PE and DEPE (Figs. 1 and 2), while cholesterol has very little effect on the temperature of the L_α to H_{II} phase transition of PEs [19,20]. The difference between CS and cholesterol may be due, in part, to the different polar headgroups of these two molecules. Cholesterol has a poorly hydrated and relatively small polar hydroxyl group. In comparison, CS possesses a charged and hydrated polar sulphate group. The sulphate group of CS could disrupt hydrogen bonding interactions between PE headgroups. Disruption of hydrogen bonding interactions between cholesterol and fatty acids in the stratum corneum by CS has been proposed [21]. The sterol portion of the molecule provides sufficient hydrophobicity for spontaneous parti-

Fig. 6. Calcium induced fusion (A) and leakage (B) of egg PE/10 mol% CS LUVs (○) and egg PC/10 mol% CS LUVs (●). Assay conditions were the same as in Fig. 4. Calcium concentration was 19.2 mM.

tioning of CS into the membrane but because of its constrained ring structure, does not produce much hydrocarbon splay. The difference in effects on the L_α to H_{II} phase transition temperature can also be explained in terms of intrinsic radius of curvature [22]. CS increases the intrinsic radius of curvature of a PE bilayer to a greater extent than does cholesterol because of its larger charged and more hydrated headgroup, and thereby stabilizes the bilayer phase.

The addition of calcium to PE MLVs containing CS abolishes the bilayer stabilizing effect of CS (Figs. 1 and 2). This could be due to lateral phase separation of the CS within the membrane, or possibility due to neutralization of the negative charge of the sulphate moiety, diminishing headgroup repulsion and reducing surface hydration. The addition of calcium to PE MLVs alone has little effect on the L_α to H_{II} phase transition temperature (Fig. 3). Thus the calcium effect most likely involves interaction with the CS in the membrane.

The addition of CS to DEPE broadens the L_β to L_α phase transition and lowers the temperature at which it occurs (Fig. 3). This is similar to the effect of cholesterol on the L_β to L_α phase transition of DEPE [19,20], and the ability of CS to affect the L_β to L_α phase transition of dipalmitoylphosphatidylcholine MLVs has previously been reported [23]. Calcium raises the L_β to L_α phase transition temperature but does not broaden the transition or affect the enthalpy to any great extent. The addition of calcium to DEPE MLVs containing CS raises the L_β to L_α phase transition temperature, as with pure DEPE, but the endotherm remains broadened and the enthalpy not appreciably changed. If calcium were inducing lateral phase separation of CS, an increase in the cooperativity and enthalpy of the L_β to L_α phase transition of DEPE would be expected when calcium was present. This is not observed.

Stabilization of PE membranes by CHEMS, another negatively charged cholesterol ester has been previously reported [24]. We have found that CHEMS raises the L_α to H_{II} phase transition temperature of DEPE, and has a slope of 171 ± 10 C°/mol fraction CHEMS (data not shown). This slope is similar to that of CS in DEPE which is 173 ± 7 C°/mol fraction. Although these two compounds have similar bilayer stabilizing potencies, the effect of pH and Ca^{2+} are quite different. When the CHEMS was protonated, it changed from a bilayer stabilizer (increased the L_α to H_{II} phase transition temperature) to a potent bilayer destabilizer (lowered the L_α to H_{II} phase transition temperature) [24]. In contrast we find that the bilayer stabilizing effect of CS is relatively pH insensitive over the pH range 4.5 to 7.5 [25]. This may be due to the pK of the sulphate group, which requires a lower pH to become protonated.

We have found that Ca^{2+} has a marked effect on the ability of CS to stabilize egg PE and DEPE in the bilayer phase (Figs. 1 and 2). Ca^{2+} also induced rapid fusion and leakage of egg PE/CS LUVs (Fig. 4 and Fig. 5). With CHEMS/TPE mixtures, the addition of Ca^{2+} caused the formation of aggregates, which retained their bilayer structure [24]. Thus Ca^{2+} and H^+ have different effects on the stability of PE membranes containing different negatively charged amphiphiles.

Calcium induces fusion of egg PE LUVs containing 10 mol% CS as measured by the ANTS/DPX assay for the mixing of aqueous content (Fig. 4A). Calcium also induces leakage of internal contents from these vesicles (Fig. 5A). The amount of fusion measured by the mixing of aqueous contents is quite substantial and because of the leakiness of the system, the actual extent of fusion may be even greater. Calcium could conceivably induce fusion by interacting with the negatively charged sulphate moieties and overcoming repulsive interbilayer forces. However, no fusion or leakage is observed when Ca^{2+} is added to egg PC/10 mol% CS LUVs (Fig. 6). This suggests that in addition to surmounting the electrostatic repulsion of the negative sulphate groups, Ca^{2+} also neutralizes the bilayer stabilizing capability of CS in egg PE membranes, rendering them more susceptible to fusion. Since egg PC forms stable bilayers without any additives, the addition of calcium has little effect on egg PC/10 mol% CS LUVs (Fig. 6).

We propose that the interaction of calcium with the anionic sulphate group of CS promotes fusion of PE liposomes by abolishing the bilayer stabilizing effects of CS. This does not mean that hexagonal phase structures need be present for fusion to occur. The effect of CS on the temperature of the L_α to H_{II} phase transition is a useful and relatively easy parameter to measure. This parameter allows bilayer stability to be quantitatively evaluated, and has been correlated with propensity towards membrane fusion [5,9]. However, the actual structure of any fusion intermediates is not well established. A correlation between the appearance of an isotropic ^{31}P-NMR signal (indicative of bilayer perturbation) and an increased rate of membrane fusion has been observed in model systems [8], and new structures have been observed by cryo-transmission electron microscopy [26]. The ability of a membrane to form fusogenic intermediates, appears to be correlated with its ability to form hexagonal phase structures under more extreme conditions [8].

The acrosome of mammalian spermatozoa is comprised of a large secretory granule which contains enzymes believed to be involved in penetration of the sperm cell through the zona pellucida of the oocyte [17]. Initiation of the acrosome reaction involves membrane fusion between the plasma membrane and the outer acrosomal membrane and demonstrates an absolute requirement for Ca^{2+} [17].

Langlais et al. [11] have demonstrated the presence of CS in the human spermatozoa and have calculated that it could account for up to 20% of the surface area

of the acrosomal region. They postulate that CS acts as a membrane stabilizer in the sperm membrane and during capacitation the sulphate moiety is removed by sulphatases in the female reproductive tract, thus rendering the sperm membrane more fusogenic [11]. This hypothesis is consistent with our observation of CS as a bilayer stabilizer in model systems. However, there is evidence that some CS can remain in the acrosomal membrane fragments after the acrosome reaction has occurred [27]. This implies that in some cases, not all of the CS needs to be converted to cholesterol to allow the acrosome reaction to occur. We postulate that the remaining CS can interact with Ca^{2+} and trigger the membrane destabilization necessary for initiating membrane fusion events required for the acrosome reaction. The removal of the sulphate moiety from CS would contribute to destabilization of the membrane. In addition, with less CS stabilizing the membrane, the threshold concentration of Ca^{2+} necessary to trigger membrane destabilization and fusion would be reduced.

It should be remembered, as pointed out by Langlais and Roberts [28], that there are various mechanisms involved in the capacitation process, and not all of these need to be active for successful capacitation. Thus compensatory mechanisms may exist to ensure that the acrosome reaction occurs. For example, if not all the CS molecules were removed from the spermatozoa membrane during capacitation, the remaining CS could have its bilayer stabilizing activity neutralized by Ca^{2+}, thus allowing the acrosome reaction to occur. Our results demonstrate that a combination of decreasing membrane concentration of CS and increasing concentration of Ca^{2+} can induce membrane fusion in model systems and may be involved in triggering initiation of the acrosome reaction in human sperm.

Acknowledgement

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