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Steroid-induced perturbations of membranes and its relevance to sperm acrosome reaction

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The interaction of progesterone, 17-α-hydroxyprogesterone, testosterone and estradiol with membrane vesicles prepared from phosphatidylserine (PS), from the total lipids of human and hamster spermatozoa, from the lipids of hamster spermatozoal plasma and acrosomal membrane and with the native membranes of hamster spermatozoa have been investigated by 90° light scattering and fluorescence spectroscopy. The results indicate that progesterone decreases the fluidity of membranes, aggregates membrane vesicles, induces fusion of membrane vesicles and also renders them permeable to hydrophilic molecules like carboxyfluorescein. But, testosterone and estradiol at the same concentration had very little effect on membrane fluidity, membrane aggregation, fusion and leakage. The above membrane perturbing activities of the steroids is discussed in light of the recent findings that progesterone induces acrosome reaction in human and hamster spermatozoa [11,18].

Introduction

The mammalian sperm acrosome reaction, an exocytotic event, involves the fusion of the outer acrosomal membrane with the overlying plasma membrane resulting in the formation of membrane vesicles and in the simultaneous release of the acrosomal contents [1,2]. The acrosomal enzymes released during the acrosome reaction aid the spermatozoa in penetrating the various investments of the oocyte. Hence, it is obvious that the acrosome reaction is one of the central events preceding fertilization.

Molecules such as serum albumin, glucosaminoglycans, prostaglandins, biogenic amines, proteinases and zona pellucida components have been suggested as physiological initiators [3–6] of the acrosome reaction. In mouse, ZP3, one of the three glycoproteins associated with the zona pellucida of the mouse oocytes is the physiological initiator of the sperm acrosome reaction [3,4]. The human follicular fluid also has the capacity to induce the acrosome reaction in human spermatozoa and the activity was attributed to a 50000 mol. wt. fraction of the follicular fluid [7–10]. A subsequent study identified progesterone and 17-α-hydroxyprogesterone in the fluid of preovulatory human ovarian follicles as the active factors involved in the induction of acrosome reaction [11].

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The spermatozoa, during its sojourn in the male reproductive tract and its residence in the female reproductive tract, is exposed to a variety of steroids including testosterone, estradiol and progesterone. Since these steroids are all hydrophobic, one would expect them to bind to spermatozoa and elicit effects. But this does not seem the case, since, though progesterone and 17-α-hydroxyprogesterone could induce the acrosome reaction and the influx of Ca^{2+} into sperm, the other steroids do not [12]. The exact reason(s) as to why only certain steroids interact with spermatozoa and induce physiological effects such as the acrosome reaction are not known. The binding of progesterone to human sperm has been known for a long time [13,14], and this is probably mediated by a receptor in the plasma membrane [12]. With respect to steroid-induced effects on spermatozoa, it would not be too far fetched to predict that only those molecules which are capable of binding to sperm membranes and capable of inducing a number of membrane perturbations (such as changes in the membrane fluidity, induce aggregation of membranes, leaks in membranes, and fusion of membranes) would be capable of inducing the acrosome reaction in spermatozoa. Earlier studies have indicated that membrane fusion is generally accompanied by a number of membrane perturbations For instance, the Ca^{2+}-mediated fusion of PS vesicles is preceded by interaction of Ca^{2+} with the vesicles, aggregation of the vesicles, fusion of the vesicles and leakage and collapse of the bilayer [15–17]. In this report, we have studied the effect of four different

steroids (progesterone, 17-α-hydroxyprogesterone, testosterone and estradiol) on membrane vesicles prepared from synthetic lipids, from the total lipids of human and hamster spermatozoa, from the lipids of hamster spermatozoal plasma ad acrosomal membrane and on membrane vesicles of hamster spermatozoa in an effort to understand how these steroids interact with membranes and perturb them so as to achieve fusion. The results are discussed keeping in mind that progesterone and 17-α-hydroxyprogesterone induce acrosome reaction in human ad hamster spermatozoa [11,18].

Materials and Methods

Materials

Hepes, sucrose, Tris, BAEE, AMP, pyruvate, NADH, hyaluronic acid, pyrene, terbium chloride, 8-anilino-1-naphthalenesulfonate (ANS) and dipicolinic acid sodium salt were obtained from Sigma, St. Louis, MO, USA. Carboxyfluoresein, octadecyl rhodamine B chloride, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoylphosphatidylethanolamine (*N*-NBD-PE) and *N*-lissamine rhodamine B phosphatidylethanolamine (*N*-RH-PE) were from Molecular Probes, OR, USA. All other reagents were of analytical grade.

Collection of spermatozoa

Human ejaculates were obtained by masturbation from healthy donors (age 20–40 years) and allowed to liquefy at 37°C for about 45 min. Only those semen samples which showed very good motility (> 75% motile cells) and appeared morphologically homogeneous under the phase contrast microscope were used. Following liquefaction, the semen samples were suspended in 30 ml of Hepes buffer (5 mM Hepes; 150 mM NaCl (pH 7.4)) and washed by centrifugation at $1000 \times g$ for 10 min at room temperature. The pellet was recovered and washed two more times with 30 ml of Hepes buffer as above. The washed pellet was suspended in a minimal volume of Hepes buffer and layered on 20 ml of 1.3 M sucrose containing 0.9% NaCl and centrifuged for 30 min at $6000 \times g$ at 5°C. The pellet thus obtained was free of seminal plasma and was further washed twice at $1000 \times g$ for 10 min. This washed pellet was used immediately for the preparation of membranes or stored at −20°C for future use.

The mature cauda epididymal spermatozoa from adult hamsters was obtained as follows: the hamsters were briefly anaesthetised with ether, killed by cervical dislocation and the cauda region of the epididymis was dissected free of blood and suspended in Hepes buffer. Epididymis from four to five animals were minced to a fine paste, suspended in 30 ml of Hepes buffer and filtered through a fine nylon mesh. The suspension of spermatozoa in the filterate was washed thrice with 30 ml of Hepes buffer by centrifugation at $2000 \times g$ for 10 min and then layered on 20 ml of 1.3 M sucrose containing 0.9% NaCl and centrifuged (as described for human sperm) so as to obtain a sperm pellet free of other fluids. The pellet was washed as above and either used immediately for the preparation of membranes or stored at −20°C for further use.

Preparation of spermatozoal membranes

The plasma and outer acrosomal membrane fractions of the cauda epididymal spermatozoa of hamster were isolated as described earlier [19,20] following essentially the method described by Zahler and Doak [21] for bull spermatozoa. Protein was determined by the method of Lowry et al. [22] and inorganic phosphate by the method of Chen et al. [23]. The activities of the enzymes Na^+/K^+-ATPase [24], Ca^{2+}-ATPase [25], Mg^{2+}-ATPase [26], alkaline phosphatase [27], acid phosphatase [28], 5'-nucleotidase [29], acrosin [30], hyaluronidase [31], succinate dehydrogenase (SDH) [32] and lactate dehydrogenase (LDH) [33] were assayed using standard methods.

Preparation of lipids and liposomes

Lipids were isolated from spermatozoa and spermatozoal plasma and acrosomal membranes by the method of Folch et al. [34]. PS was purified from bovine brain [35]. Lipid concentrations were estimated by the method of Stewart [36]. The above lipids were used for the preparation of liposomes (small unilamellar vesicles) by sonication (Branson B-50 sonifer) of an aqueous dispersion of the lipids in Hepes buffer to clarity.

Pyrene excimer fluorescence

All fluorescence measurements were recorded on a Hitachi 650-10S fluorescence spectrophotometer with 4 nm excitation and emission bandpass. Pyrene was incorporated into sperm plasma membrane (SPM) and sperm acrosomal membrane (SAM) by rapidly mixing a stock solution of 2 mM in methanol with the membrane (100 μg/ml Hepes buffer) such that the final concentration of methanol did not exceed 1%. Pyrene was excited at 333 nm and the emission spectra were recorded from 360 to 530 nm.

90° light scattering

Aggregation of liposomes was monitored by 90° light scattering in the fluorescence spectrophotometer by setting both the excitation and emission monochromators at 400 nm.

Fusion of liposomes

The ability of liposomes derived from various sources to fuse under different experimental conditions was studied using four methods: the Tb^{3+}-dipicolinate

method [17], the octadecyl rhodamine B chloride (R-18) method [37], the resonance energy transfer (RET) fusion method [38] and by gel filtration.

In the Tb^{3+}-dipicolinate method, two populations of small unilamellar vesicles (SUV) were prepared separately such that one population contained 150 mM $TbCl_3$ and 150 mM sodium citrate and the other contained 150 mM dipicolinic acid. The way this was done [39] was to disperse the lipids in 5 mM Hepes (without NaCl, pH 7.4) containing the above constituents and then sonicated to clarity to obtain the SUVs. The vesicles were then separated from the non-encapsulated material by gel filtration on a Sephadex G-75 column using 5 mM Hepes (pH 7.4) containing 100 mM NaCl and 1 mM EDTA as the elution buffer. Liposomes from the two different populations were mixed in 5 mM Hepes (pH 7.4) containing EDTA (0.1 mM) and Ca^{2+} (> 1 mM) as described [39] and the steroids were added from a stock solution (5 mg/ml) in methanol. Measurements were made at 491 nm (λ_{ex} = 276 nm) [39]. The maximum fluorescence of Tb^{3+} was determined as described [17].

In the R-18 assay [37], vesicles in Hepes buffer were incubated at room temperature for 30 min with an aliquot of a stock solution (2 nmol) of R-18. After incubation, the R-18 labelled vesicles were mixed with the unlabelled vesicles such that the proportion of labelled : unlabelled vesicles was 1 : 4, respectively. The initial fluorescence due to the labelled vesicles was taken as zero and the fluorescence intensity after addition of Triton X-100 was taken as 100% (infinite dilution). Fusion of the vesicles was monitored as an increase in fluorescence emission intensity at 580 nm when excited at 560 nm. The emission and excitation slit widths were 5 nm.

In the RET method the total lipids of hamster spermatozoa were used to prepare vesicles containing 0.8 mol% each of the fluorescent donor lipid (*N*-NBD-PE) and the acceptor (*N*-RH-PE) [38,40]. These labelled vesicles were then mixed with unlabelled vesicles at a ratio of 1 : 4 and fusion was initiated by the addition of the steroid The extent of fusion between the vesicles was measured by following the changes in fluorescence intensity at 530 nm following excitation at 450 nm. The fluorescence of the vesicles at 530 nm before the addition of the fusogenic agent was taken as zero level fusion and the intensity attained after the addition of the Triton X-100 as 100%.

Fusion of vesicles was also monitored by gel filtration chromatography of fluorescent labelled vesicles on a Sepharose 4B (1 × 60 cm) column. Vesicles were prepared from the total lipids of hamster and then labelled with the fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS) by incubating the lipids with the probe for 15 min [41]. ANS-labelled vesicles when excited at 370 nm exhibited an emission peak at 510 nm. ANS-labelled vesicles prior to and after treatment with the fusogenic agent were chromatographed on the above column and 2 ml fractions were collected and monitored for fluorescent lipid at λ emission equivalent to 510 nm following excitation at 370 nm.

Leakage of vesicles

Leakage of vesicle contents following perturbation with extraneous agents was followed by monitoring the release of carboxyfluorescein (CF) [42,43] which was entrapped at self-quenching concentrations in the vesicles. The entrapment was done as described by Nagaraj et al. [39]. Essentially the lipids were dispersed in 5 mM Hepes (without NaCl, pH 7.4) containing 100 mM CF, sonicated to clarity and chromatographed on a Sephadex G-75 column using 5 mM Hepes (pH 7.4) containing 100 mM NaCl as the elution buffer. Gel filtration separated the CF-labelled vesicles from the non-entrapped CF. Release of CF was monitored as an increase in the fluorescence emission intensity at 540 nm following excitation at 493 nm. The initial fluorescence due to the labelled vesicles was taken to be zero and complete release of CF was obtained by addition of Triton X-100 to a final concentration of 0.5%.

Results

Characterization of spermatozoal membranes

Subcellular fractionation of cauda epididymal spermatozoa from hamster yielded two membrane fractions, of which, one banded above the 1.3 M sucrose and the other at the 1.3 M and 1.75 M sucrose interface. Membranes in the 1.3 M sucrose fraction exhibited relatively high enrichments of the enzymes Na^+/K^+-ATPase, Mg^{2+}-ATPase, 5′-nucleotidase, alkaline phosphatase and Ca^{2+}-ATPase (data not shown). These enzymes are considered to be general markers of plasma membranes [44] and hence the fraction was designated as enriched sperm plasma membrane (SPM). Membranes which banded at the interface as compared to SPM exhibited low activity for the enzymes Na^+/K^+-ATPase, Mg^{2+}-ATPase, 5′-nucleotidase, and alkaline phosphtase and totally lacked Ca^{2+}-ATPase, a marker enzyme for spermatozoal plasma membranes [19,20]. But, it was associated with high acrosin (absent in SPM) and hyaluronidase activity which are marker enzymes for acrosomal membranes (data not shown). Hence the membrane fraction which bands at the 1.3 M and 1.75 M sucrose interface was designated as enriched spermatozoal acrosomal membrane (SAM). Both SPM and SAM exhibited acid phosphatase activity but were free of the mitochondrial marker enzyme SDH and the cytoplasmic marker enzyme LDH. The observations on the characterisation of hamster SPM and SAM confirm earlier studies

where in the distribution of Ca^{2+}-ATPase, Na^+/K^+-ATPase, Mg^{2+}-ATPase, 5'-nucleotidase, alkaline phosphatase, acetylcholinesterase, acrosin, hyaluronidase, α-fucosidase, β-*N*-acetylglucosaminidase, arylsulfatase, acid phosphatase, α-mannosidase, SDH and LDH was studied on subcellular fractions of the cauda epididymal spermatozoa from hamster [45]. The activities of these enzymes showed similar enrichments irrespective of whether the membranes were prepared from fresh sperm or frozen sperm pellets.

Interaction of steroids with hamster SPM and SAM

The fluorescent probe pyrene was used to monitor the changes in the fluidity of hamster SPM and SAM following treatment with progesterone, estradiol and testosterone. Fig. 1 shows the excimer/monomer (E/M) emission intensity ratio of pyrene incorporated into SPM and SAM in the presence and absence of the above three steroids. Pyrene excimer formation was more prominent in SPM ($E/M = 0.25$) as compared to SAM ($E/M = 0.20$). The steroids when added directly to pyrene did not increase the E/M emission intensity ratio of pyrene but when they were added to pyrene incorporated into SPM or SAM certain changes were observed. Progesterone decreased the E/M ratio of pyrene both in SPM and SAM to about 40% of the control in a concentration dependent manner whereas estradiol and testosterone had little or no effect (Figs. 1A, B). Hence, it appears that progesterone interacts and decreases the fluidity of SPM and SAM to a greater extent than the other two steroids.

Steroid-induced aggregation of vesicles

The 90° light scattering profiles of small unilamellar vesicles of PS, of vesicles prepared from the total lipids of hamster cauda epididymal spermatozoa and human spermatozoa are shown in Figs. 2A, B and C, respectively. Progesterone increased the scatter intensity of all the above vesicles in a concentration-dependent manner and appeared to have maximum effect on PS vesicles. Estradiol had no effect on PS vesicles but a small increase could be discerned at higher concentrations (> 20 μg) in hamster and human sperm lipid vesicles. Testosterone had no effect on the vesicles. Thus, progesterone interacts with all the vesicles and increases the scatter intensity to a greater extent than testosterone and estradiol. Earlier studies have shown that increase in scatter of vesicles could be correlated either with aggregation or fusion of vesicles [17,39]. Hence, the ability of steroids to induce fusion of vesicles was studied.

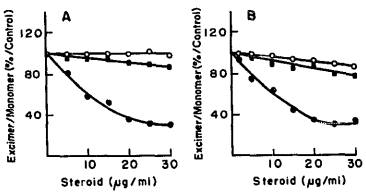

Fig. 1. Excimer/monomer intensity ratio of pyrene incorporated into plasma and acrosomal membranes of hamster spermatozoa, as a function of steroid concentration. (A) Hamster spermatozoal plasma membrane. (B) Hamster spermatozoal acrosomal membrane. Membrane protein concentration 100 μg/ml. ●, Progesterone; ○, estradiol; ■, testosterone.

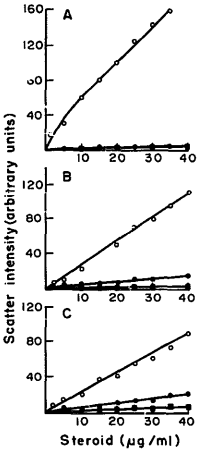

Fig. 2. 90° light scattering profiles of membrane vesicles as a function of steroid concentration. (A) PS vesicles (150 μm). (B) Vesicles prepared from the total lipids of hamster cauda epididymal spermatozoa (120 μg/ml). (C) Vesicles prepared from the total lipids of human ejaculated spermatozoa (120 μg/ml). ○, Progesterone; ●, estradiol; ■, testosterone. Increase in scatter intensity caused due to the addition of steroid alone to the buffer have been subtracted from the experimental values.

Steroid-induced fusion of vesicles

Fusion of PS vesicles by the Tb^{3+}-dipicolinate method

Fusion of vesicles could be followed by monitoring the mixing of the internal aqueous compartments of the vesicles by the Tb^{3+}-dipicolinate assay [17]. In this assay, Tb^{3+} present in the aqueous compartment of

one population of vesicles forms a fluorescent Tb^{3+} chelation complex with dipicolinic acid present in another population of vesicles. The advantage of this method is that it is very rapid. But, if leakage of vesicle contents occured prior to or during fusion, EDTA (0.1 mM) and Ca^{2+} (> 1 mM) present in the external medium would prevert the formation of the fluorescent complex and thus fusion would not be detected. When the steroids were added to hamster sperm lipid vesicles with entrapped Tb^{3+} and dipicolinic acid, only progesterone brought about a small rise in fluorescence (data not shown). Hence, this observation could either indicate that the vesicles are not susceptible to steroid induced fusion or the steroids have caused a rapid leakage of vesicle contents and hence the fusion process could not be registered.

Fusion of PS vesicles by the R-18 method

One could also use the R-18 assay method which monitors fusion of lipid bilayers of two different population of vesicles [37]. In this method, octadecyl rhodamine B chloride (R-18) is incorporated into the lipid bilayer of one population of vesicles at self-quenching concentrations and then mixed with another population of unlabelled vesicles. Upon fusion of the vesicles, the surface density of the fluorophore (R-18) decreases and there is a concomitant increase in fluorescence intensity of R-18 which could be monitored as a signal of fusion. The R-18 assay, differs from the Tb^{3+}-dipicolinic assay in that it monitors the mixing of lipid bilayers. Fig. 3 shows the time-dependent fusion of PS vesicles in the presence of steroids. Addition of progesterone brought about a rapid increase in fluorescence of PS vesicles (indicative of fusion) in a time-dependent and concentration-dependent manner. But, estradiol and testosterone had very little effect on the fusion of PS vesicles and induced only about 2% fusion in 10 min when tested at concentrations > 50 μg/ml.

Fusion of human and hamster sperm lipid vesicles by the R-18 method

The ability of steroids to induce fusion of small unilamellar vesicles prepared from the total lipids of human and hamster sperm was also monitored using the R-18 assay. As little as 2 μg of progesterone per ml was sufficient to induce fusion of hamster sperm lipid unilamellar vesicles (HASUV's) and the fusion was both time and concentration-dependent (Fig. 4A). As compared to progesterone higher concentrations of estradiol (about 40 μg/ml) (Fig. 4B) and testosterone (Fig. 4C) were required to induce fusion of HASUV's. In fact, even as much as 100 μg of testosterone per ml. brought about only 3% fusion. The human sperm lipid unilamellar vesicles (HUSUV's) also exhibited fusion in the presence of 2 μg of progesterone (Fig. 4D). But both estradiol (50 μg/ml) and testosterone (100

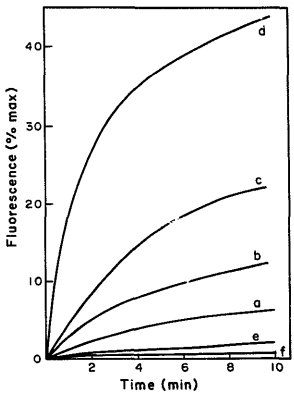

Fig. 3. Time-dependent fusion of PS vesicles by steroids monitored by R-18 assay. The R-18-labelled vesicles were mixed with the unlabelled vesicles such that the ratio of labelled/unlabelled was 1:4, respectively and the final concentration of lipid was 150 μM. The vesicles were suspended in 1 ml of Hepes buffer (pH 7.4) containing 100 mM NaCl and the experiment was initiated by the addition of steroids. The final concentration of the steroids in 1 ml buffer was as follows: a, 2 μg progesterone; b, 5 μg progesterone; c, 10 μg progesterone; d, 20 μg progesterone, e, 50 μg estradiol; f, 100 μg testosterone.

μg/ml) were less effective in inducing fusion of HUSUV's. Thus, unlike estradiol and testosterone which do not easily induce fusion of vesicles, progesterone seems to be a more efficient inducer of membrane vesicle fusion which it does irrespective of whether the vesicles were made from synthetic lipids or lipids isolated from spermatozoa or other sources. It would also be worthwhile to check the ability of steroids to induce fusion of vesicles prepared from SPM and SAM of hamster spermatozoa.

Fusion of hamster sperm lipid vesicles by the RET method

Fusion of vesicles could also be monitored by the RET assay which also monitors the mixing of lipid bilayers. In this method fusion between hamster lipid vesicles labelled with *N*-NBD-PE and *N*-Rh-PE and non-labelled hamster lipid vesicles reduces the surface density of the fluorophores and as a consequence decreases the resonance energy transfer efficiency. Reduction in energy transfer is accompanied by an increase in *N*-NBD-PE fluorescence at 530 nm which could be monitored to determine the extent of fusion [38]. Fig. 5 shows the extent of fusion of HASUV's following addition of progesterone. The fusion was also

observed to depend on the concentration of progesterone. However, under similar conditions even 150 μg of testosterone or 50 μg of estradiol did not induce fusion.

Fusion of hamster sperm lipid vesicles monitored by gelfiltration

Gel filtration chromatography was also done to provide further evidence that the steroids induced fusion

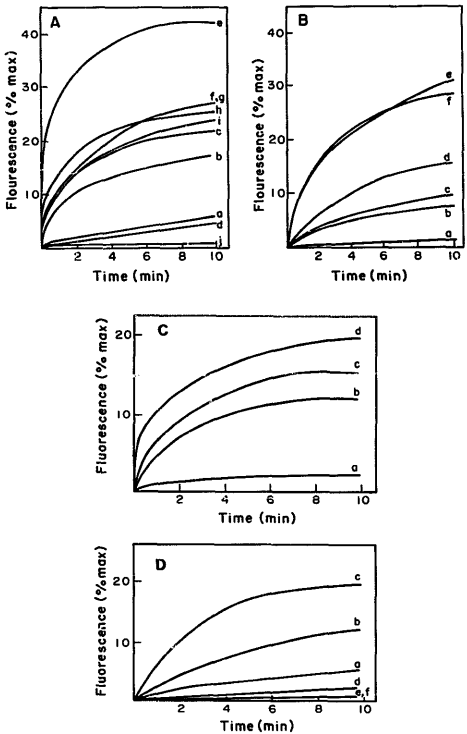

Fig. 4. Time-dependent fusion of vesicles prepared from the total lipids of hamster and human spermatozoa by steroids and divalent cations as monitored by the R-18 assay. (A) Progesterone and metal ion induced fusion of hamster sperm lipid vesicles (HASUV's). a, 2 μg progesterone; b, 10 μg progesterone; c, 15 μg progesterone; d, 4 mM Ca^{2+}; e, 15 μg progesterone plus 4 mM Ca^{2+}; f, 4 mM Mg^{2+}; g, 15 μg progesterone plus 4 mM Mg^{2+}; h, 4 mM Zn^{2+}; i, 25 μg progesterone plus 4 mM Zn^{2+}; j, control. (B) Estradiol and metal ion induced fusion of HASUV's. a, 10 μg estradiol; b, 40 μg estradiol; c, 50 μg estradiol; d, 50 μg estradiol plus 4 mM Ca^{2+}, e, 50 μg estradiol plus 4 mM Mg^{2+}; f, 50 μg estradiol plus 4 mM Zn^{2+}. (C) Testosterone and metal ion induced fusion of HASUV's. a, 100 μg testosterone; b, 100 μg testosterone plus 4 mM Ca^{2+}; c, 100 μg testosterone plus 4 mM Mg^{2+}; d, 100 μg testosterone plus 4 mM Zn^{2+} (D) Steroid induced fusion of human sperm lipid vesicles. a, 2 μg progesterone; b, 5 μg progesterone; c, 10 μg progesterone; d 50 μg estradiol; e, 100 μg testosterone; f, control. The ratio of labelled/unlabelled lipid in these experiments was 20 μg:80 μg.

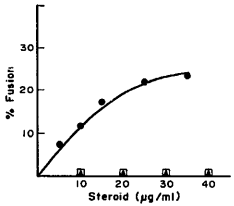

Fig. 5. Fusion of vesicles prepared from the total lipids of hamster spermatozoa by steroids as monitored by the RET method. ●, Progesterone; □, testosterone; ▲, estradiol.

of vesicles. Results indicate that vesicles chromatographed prior to addition of progesterone elute as a broad peak after the void volume (Fig. 6). But. when the vesicle mixtures were exposed to progesterone (10 μg/ml) they eluted much earlier indicating an increase in size due to fusion. However, testosterone and estradiol did not induce any such effect.

Fusion of hamster SPM and SAM lipid vesicles by the R-18 method

When SUV's containing R-18 were prepared from the lipids of SAM and mixed with SUV's from the lipids of SPM (which had no R-18 entrapped in them) at a ratio of (1:7) no increase in fluorescence was observed even after 30 min. But, a discernible increase in fluorescence was observed when as little as 2 μg of progesterone or 20 μg of estradiol was added to the above suspension of SUV's (Figs. 7A, B). As compared to progesterone and estradiol, even 100 μg of testosterone (Fig. 7A) did not induce fusion of these vesicles. 17-α-hydroxyprogesterone was as capable as progesterone in inducing fusion of SPM and SAM lipid vesicles (Fig. 7B).

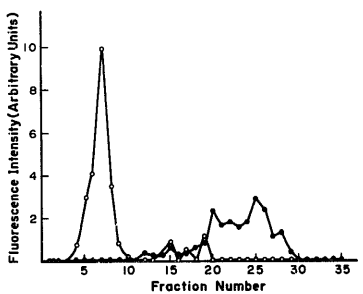

Fig. 6. Fusion of vesicles prepared from the total lipids of hamster spermatozoa by progesterone as monitored by gel filtration chromatography on a Sepharose 4B column. ●, Control vesicles; ○, vesicles treated with progesterone (10 μg/ml).

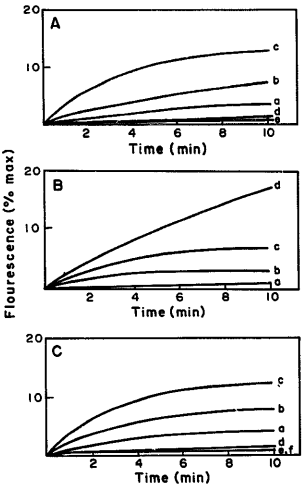

Fig. 7. Time-dependent fusion of vesicles prepared from the lipids of hamster SPM and SAM by steroids as monitored by the R-18 assay. (A) Membrane vesicles containing R-18 were prepared from hamster SAM and mixed with vesicles without R-18 prepared from hamster SPM at a ratio of 1:7 (20 μg:140 μg) (SAM/SPM). a, 2 μg progesterone; b, 5 μg progesterone; c, 10 μg progesterone; d, 100 μg testosterone; e, control. (B) Estradiol and 17-α-hydroxyprogesterone induced fusion of hamster SPM and SAM lipid vesicles. Other details are as in Fig. 5A. a, 10 μg estradiol; b, 20 μg estradiol; c, 30 μg estradiol; d, 10 μg 17-α-hydroxy-progesterone. (C) Steroid induced fusion of hamster SPM and SAM membrane vesicles by the R-18 assay. a, 5 μg progesterone; b, 10 μg progesterone; c, 15 μg progesterone; d. 25 μg estradiol;, e, 100 μg testosterone; f, control.

Fusion of hamster SPM and SAM vesicles by the R-18 method

Hamster SPM and SAM were prepared as described above and vesicles were made by sonicating the membranes in Hepes buffer to clarity. R-18 was incorporated in the SAM vesicles, mixed with SPM vesicles in a ratio of 1:4 and fusion was followed after the addition of steroids. An increase in fluorescence indicative of fusion was observed on the addition of even 5 μg of progesterone. But 25 μg of estradiol and 100 μg of testosterone had no fusogenic effect (Fig. 7C). All the

above experiments indicate that progesterone which brings about aggregation of vesicles is also an excellent fusogenic agent in the same concentration range.

The effect of Ca^{2+}, Mg^{2+} and Zn^{2+} on steroid-induced fusion of lipid vesicles by the R-18 method

The influence of divalent cations on steroid induced fusion of vesicles was also studied in the present investigation (Fig. 4). Ca^{2+}, Mg^{2+} and Zn^{2+} at a concentration of 4 mM induced fusion of hamster sperm lipid vesicles (Fig. 4A) but of the three Mg^{2+} and Zn^{2+} facilitated a greater degree of fusion than Ca^{2+} (Fig. 4A, see curves d, f and h). But, Ca^{2+} was the only cation which enhanced the steroid induced fusion of hamster sperm lipid vesicles by progesterone (Fig. 4A, see curves c–e) whereas the fusion observed in the presence of estradiol (Fig. 4B, see curves c–f) and testosterone (Fig. 4C, see curves a–d) and the metal ions was an additive effect of the steroid and the metal ions. Thus, Ca^{2+} seems to be important in the fusion event.

Release of trapped vesicle contents

The ability of the steroids to interact with vesicles and bring about leakage of the vesicle contents was studied using vesicles of PS and hamster sperm lipids with carboxyfluorescein (CF) entrapped in them at a self-quenching concentration. Release of CF due to leakage would result in enhancement of fluorescence intensity. Figs. 8A, B show the release of CF from vesicles on treatment with steroids. It is obvious that progesterone renders the vesicles considerably more leaky than testosterone and estradiol. Further, progesterone and testosterone induced CF release from vesicles was observed to be both time and concentration-dependent. The time-dependent release of CF from PS and hamster lipid vesicles indicated that progesterone induced release of CF from the above vesicles was more rapid than that of testosterone and estradiol at identical concentrations (Figs. 9A, B). It is also observed that concentrations of steroids generally required for inducing leakage of CF from the vesicles was higher than that required for aggregation or induction of fusion.

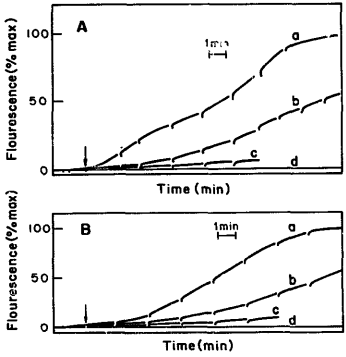

Fig. 8. Release of carboxyfluorescein (CF) from PS vesicles (150 μM) (A) and from vesicles prepared from the total lipids of hamster sperm (120 μg/ml) (B) in the presence of steroids. Arrow indicates the time point at which steroid addition was started. Breaks in the continuity of the fluorescence curves indicate successive additions of 10 μg of progesterone (a), testosterone (b), and estradiol (c). Final concentrations of the steroids was 80 μg/ml progesterone in A and B, 60 μg/ml estradiol in A and B and 100 μg and 80 μg/ml of testosterone in A and B, respectively. PS vesicles with CF entrapped served as control (d).

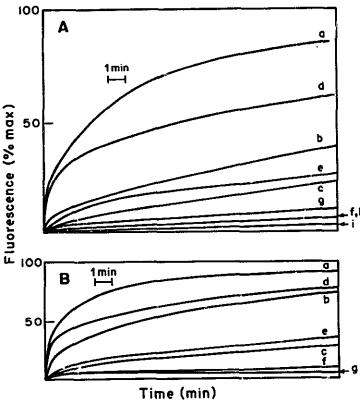

Fig. 9. Time-dependent release of CF from PS vesicles (150 μM) (A) and from vesicles prepared from the total lipids of hamster spermatozoa (120 μg/ml) (B) in the presence of steroids. (A) a, 60 μg progesterone; b, 30 μg progesterone; c, 20 μg progesterone; d, 120 μg testosterone; e, 60 μg testosterone; f, 30 μg testosterone; g, 40 μg estradiol; h, 20 μg estradiol; i, control. (B) a, 120 μg progesterone; b, 80 μg progesterone; c, 40 μg progesterone; d, 240 μg testosterone; e, 120 μg testosterone; f, 80 μg estradiol; g, control.

Discussion

In the present investigation the interaction of progesterone, estradiol and testosterone with mem-

brane vesicles was studied with a view to get an insight as to how these steroids perturb membranes and induce fusion. Such studies may reveal as to how progesterone induces the acrosome reaction an exocytotic membrane fusion event, in mammalian spermatozoa [1,2,11].

Interaction of steroids with hamster SPM and SAM which were isolated and characterised as described earlier [19,21,45,46] was followed using the fluorescent probe pyrene since steroids on interacting with membranes may alter the lipid phase fluidity of the membranes. The fluorescence emission intensity of the excimer peak of pyrene (470 nm) has been conveniently used to monitor membrane fluidity, since the formation of the excimer is related to lateral mobility of pyrene molecules in the lipid phase [47,48]. The results indicate that SPM is more fluid than SAM. Our earlier observations had also indicated that in bull spermatozoa, SPM has a more lipid phase than SAM [20]. Further, it was also observed that progesterone decreased the fluidity of both SPM and SAM to a significant extent whereas the other two steroids only had a marginal effect. Studies have also indicated that progesterone transiently decreases the plasma membrane fluidity of amphibian oocytes [49]. Changes in membrane fluidity of spermatozoa are known to precede acrosome reaction. Prior to acrosome reaction, during capacitation, rat spermatozoa incubated in the presence of BSA showed a decrease in cholesterol/phospholipid ratio, making the membranes more fluid; this change could promote fusion of SPM and SAM [50–52]. Hence, it would appear that progesterone which decreased the fluidity of SPM and SAM should inhibit the acrosome reaction. But, in fact, progesterone does not inhibit but promotes acrosome reaction. The reason for this may be that progesterone is an inducer of Ca^{2+} influx into sperm. Though it is known that Ca^{2+} is essential for the acrosome reaction, the exact mechanism is still unknown [12]. All agents that increase the fluidity of spermatozoal membranes do not necessarily induce the acrosome reaction. Seminalplasmin (SPLN), an antimicrobial protein from bull seminal plasma [53–55] binds to the surface of bovine spermatozoa [56], increases the fluidity of bovine SPM and SAM [57] but inhibits the acrosome reaction [56,58–60]. The inhibitory effect of SPLN on the acrosome reaction is attributed to the ability of SPLN to inhibit the uptake of Ca^{2+} in spermatozoa [61]. Thus, changes in the fluidity of spermatozoal membranes seem to precede acrosome reaction a membrane fusion event. But, they may not be solely responsible for the induction or inhibition of the acrosome reaction.

Progesterone which brings about distinct changes in the fluidity of membranes is also capable of aggregating membrane vesicles derived from synthetic lipids or lipids isolated from hamster and human spermatozoa. Aggregation of vesicles may precede membrane fusion [17,39,62]. In fact, the results of the present study further indicate that progesterone which most efficiently brings about aggregation of SUV's also brings about maximum fusion. But, estradiol and testosterone which aggregated the vesicles only to a very little extent (Fig. 2) either did not or marginally induced fusion of SUV's. Thus, it would appear that only those steroids which are capable of aggregating SUV's would also bring about fusion of them. Our results on membrane fusion induced by progesterone and 17-α-hydroxyprogesterone are consistent with the in vivo effects of the above steroids, i.e, the induction of membrane fusion during the acrosome reaction of human [11] and hamster (Ref. 18 and our own unpublished results) spermatozoa.

Osman et al. [11] were the first to demonstrate that progesterone and 17-α-hydroxyprogesterone from human follicular fluid were the active factors responsible for the induction of the acrosome reaction in mammalian spermatozoa. The reason for this activity of progesterone could be attributed to its ability to stimulate rapid influx of Ca^{2+} in human sperm [63]. This was recently confirmed by Blackmore et al. [12] who showed that progesterone and 17-α-hydroxyprogesterone induce acrosome reaction in human spermatozoa and hamster spermatozoa by increasing the influx of Ca^{2+} into spermatozoa. Earlier studies have also indicated that Ca^{2+} in millimolar concentrations is essential for the occurrence of the acrosome reaction [64–67] but the exact mechanism is still not clear. The present investigation also shows that the divalent cation Ca^{2+} enhances the fusion of membrane vesicles induced by progesterone. Apart, from this it was also observed that the divalent metal ions by themselves also induced fusion of the lipid vesicles. This was an anticipated observation, since it is a very well established fact that Ca^{2+} is an efficient fusogenic agent of PS vesicles [15–17]. But the observation that divalent metal ions could induce fusion of vesicles derived from the total lipids of spermatozoa could enhance the use of such vesicles from sperm as model systems to screen for factors in the female reproductive tract which induce or inhibit membrane fusion. In spermatozoa, the acrosome reaction results in the formation of hybrid membrane vesicles (due to fusion of the outer acrosomal membrane and the overlying plasma membrane) and the leakage of acrosomal contents. Thus, an acrosome inducing factor should be capable of bringing about membrane fusion and leakage at similar concentration. Based on this criteria, in the present study, only progesterone (10 μg/ml) which is capable of both these activities would qualify as an acrosome inducing factor. The results also seem to indicate that the concentration of steroids required to achieve fusion are

relatively less compared to concentrations required to achieve maximum leakage of vesicles.

The above results on the interaction of steroids with membrane vesicles clearly indicate that of the three steroids tested for membrane perturbations (such as alterations in membrane fluidity, aggregation of membranes, fusion of similar or dissimilar membranes and leakage of membranes) progesterone seems to be the most potent. In fact, the other two steroids had negligible effect on membrane fluidity and did not aggregate the membranes, and had extremely very little effect on the fusion of vesicle, and their ability to induce leakage of vesicles was also relatively less compared to progesterone. All these features make progesterone a more suitable and potential molecule for membrane perturbations and it may be these features that facilitate it in inducing the acrosome reaction in spermatozoa.

Recent studies on steroid-induced acrosome reaction and influx of Ca^{2+} [11,12,18,63] in spermatozoa and the present study have all described steroid effects at concentrations > 1 μg/ml for maximum activity. Progesterone and 17-α-hydroxyprogesterone elicited a maximum acrosome reaction in 30% of the spermatozoa at a concentration of 1 μg/ml and further increase in concentration to 20 μg/ml did not enhance the response. It is difficult to attribute any particular reason for the above observation of Osman et al. [11] but it may be worth noting that under the most optimum conditions only 40–50% of human spermatozoa undergo the acrosome reaction. In the present study on the effect of steroids on native membranes and synthetic membrane vesicles, best results were observed around 10 μg/ml of progesterone though discernible changes could be observed even at concentrations of 2 μg/ml (aggregation, fluidity and fusion of vesicles, Figs. 1–7) and at > 10 μg (leakage of vesicles, Figs. 8 and 9) of progesterone. Under in vivo conditions in human beings the concentration of progesterone in the follicular fluid of a mature follicle was about 10 μg/ml [68]. But, following ovulation the progesterone concentration in the cumulus matrix has been estimated to be only around 2 μg/ml [11]. This concentration of progesterone (2 μg/ml) was sufficient to induce the acrosome reaction in about 200 spermatozoa which reached the fertilization site in the fallopian tube out of about $280 \cdot 10^6$ spermatozoa ejaculated into the vagina [69]. Hence, the effective concentration of progesterone per spermatozoon in the fallopian tube would be around 5 ng. In the present study, the use of 10 μg of progesterone or more is therefore not high since in all the assays the membrane lipid concentration has always exceeded 100 μg, an amount equivalent to the lipid content of approx. $1 \cdot 10^7$ spermatozoa [70]. Based on this data the concentration of progesterone per spermatozoon would be about 1 pg. Further, one should also not forget that the in vitro system is devoid of other factors which facilitate the acrosome reaction [18]. It is possible that progesterone and these other factors which may be Ca^{2+} serum albumin, glucosaminoglycans prostaglandins, biogenic amines, proteins, proteinases and zona pellucida components [3–6,59] act in a coordinated fashion and increase the efficiency of steroid induced acrosome reaction in mammalian spermatozoa.

Recent studies have indicated that progesterone acts at the plasma membrane of human spermatozoa [71] by binding to non-genomic progesterone receptors on the head of the human sperm [72] and mediate calcium uptake [71–73] which was not inhibited by the progesterone receptor inhibitors RU38486 and ZK98 299. Studies directed towards characterisation of the progesterone receptor on sperm and the mechanism by which the steroid induces influx of Ca^{2+} in sperm [12] would ultimately lead to a better understanding of the molecular basis of acrosome reaction in spermatozoa.

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