Biochimie 92 (2010) 1886–1894

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Differential involvement of rat sperm choline glycerophospholipids and sphingomyelin in capacitation and the acrosomal reaction

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ARTICLE INFO

Article history:
Received 28 May 2010
Accepted 28 August 2010
Available online 17 September 2010

Keywords:
Acrosome reaction
Capacitation
Ceramides
Cholesterol
Glycerophospholipids
Sphingomyelins

ABSTRACT

Rat spermatozoa main lipid classes and their fatty acids were studied to assess their possible changes in capacitation and the acrosomal reaction (AR), induced *in vitro*. Capacitation-associated protein tyrosine phosphorylation, and the efflux of 30% of the total cholesterol from gametes to the medium, took place concomitantly with the release of a similar percentage, *i.e.*, a larger amount, of the total phospholipid, mostly after hydrolysis of the major choline glycerophospholipids (CGP). Main medium lipid metabolites after capacitation were lyso-CGP and polyenoic fatty acids typical of CGP (22:4n-9, 22:5n-6), as free fatty acids (FFA). The AR, induced by a calcium ionophore, resulted in further phospholipid loss, but the produced metabolites remained in the gametes. CGP decrease in AR accounted for some additional FFA and lyso-CGP, but mostly for (22:5n-6-rich) diglycerides. Hydrolysis of sphingomyelins (SM) to ceramides also occurred, mostly affecting species with very long chain polyenoic fatty acids. Quantitatively, CGP and SM were the lipid classes decreasing the most after capacitation and AR, respectively. The massive cholesterol and phospholipid loss from the gametes during capacitation is thus associated with protein phosphorylation, a function that has been located to the sperm tail. The lipid metabolites produced during AR, by accumulating in the gamete heads, could be implicated in sperm–oocyte interactions.

1. Introduction

Spermatozoa undergo a significant process of maturation in the epididymis to attain their final size and shape, acquire progressive motility, and develop their signaling pathways. Competence to fertilize a metaphase II-arrested oocyte is only acquired after another maturational process known as capacitation, which *in vivo* requires that spermatozoa spend some time in the female reproductive tract [1,2]. The finding that spermatozoa can be capacitated *in vitro* in media that contain the ions and macromolecules that resemble those that are normally produced by uterine and oviductal epithelial cells was the first of a long series of relevant discoveries in the field of mammalian fertilization [3].

Sperm capacitation involves a series of biochemical changes that include an increase in metabolism and energy expenditure, loss of cholesterol from the plasma membrane, increased membrane fluidity and polarization, increased intracellular Ca^{2+} and bicarbonate concentration, augmented intracellular pH, increased levels of reactive oxygen metabolites, and stimulation of second messenger-activated cascades [4,5]. Bicarbonate/CO_2, Ca^{2+} ions, and bovine serum albumin (BSA) are required for this process [6]. Incubation for a few hours in capacitation-inducing media leads to activation of a unique form of soluble adenylyl cyclase (sAC), cAMP production, protein kinase A (PKA) activation, and phosphorylation of several proteins in their tyrosine residues [7,8]. It is accepted that BSA facilitates capacitation by promoting the efflux of sterols (mainly cholesterol) from the sperm plasma membrane [9,10].

Abbreviations: AR, acrosomal reaction; AR cells, acrosome-reacted cells; Cer, ceramide; CGP and EGP, choline and ethanolamine GPL, respectively; DRG, diradylglycerols (total diglycerides, not distinguishing into diacyl-, alk-1-enyl, 2-acyl-, and 1-alkyl, 2-acyl-glycerol subclasses); FFA, free fatty acids; GPL, glycerophospholipids; Lyso-CGP and lyso-GPE, lyso-derivatives of CGP or EGP, respectively; SM, sphingomyelin; VLCPUFA, 2-OH VLCPUFA, very long chain polyunsaturated or polyenoic fatty acids, normal (or non-hydroxylated) and 2-hydroxylated, respectively.

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doi:10.1016/j.biochi.2010.08.015

Incubation of mouse sperm in a medium that contains BSA [11], or beta-cyclodextrins [12], enables concomitantly the release of cholesterol from the sperm membrane to the medium, protein tyrosine phosphorylation, and capacitation.

The ability to undergo the acrosome reaction (AR) is the most significant change in spermatozoa after capacitation [13]. *In vivo*, the AR occurs once spermatozoa have penetrated the layer of *cumulus* cells that surround the egg and are exposed to the zona pellucida (ZP) [14]. By activating rapid molecular signaling events in sperm cells, cumulus cell-derived progesterone primes capacitated spermatozoa to undergo the AR on the ZP surface [15]. In AR, multiple fusion events take place on the sperm head by creating points of contact between the inner leaflet of the plasma membrane and the outer leaflet of the membrane covering the acrosome [16]. The AR is strictly dependent on Ca^{2+} influx, which is controlled by a variety of calcium ion channel types [17], and entails the calcium-dependent interaction of the same proteins that are involved in most membrane exocytotic mechanisms [18].

Except for cholesterol, the information on how other endogenous lipids of mammalian spermatozoa are affected by capacitation and AR is quite limited. Rat spermatozoa show peculiarities in their PL fatty acids that may facilitate assessment of how such lipids are affected during sperm functions. For example, the major sperm choline glycerophospholipids (CGP) are rich in two polyunsaturated fatty acid (PUFA), 22:5n-6 and 22:4n-9 [19], and the major sphingo-phospholipid, sphingomyelin (SM) contains high proportions of very long chain polyunsaturated fatty acids (VLCPUFA), this including "normal" or non-hydroxylated and 2-hydroxylated VLCPUFA [20,21]. The aim of this study was to examine quantitatively and qualitatively the main lipids of rat spermatozoa, with a focus on their fatty acids, in order to investigate their involvement in capacitation and AR. We show here that each of these processes has different lipid "protagonists". The main effect of capacitation is that, along with cholesterol, sperm cells loose a significant amount of their GPL, mostly CGP. The main effect of AR is that the gametes gain an important proportion of endogenously produced diglycerides and ceramides.

2. Materials and methods

2.1. Spermatozoa

Male 3–4-month-old Wistar rats, maintained and manipulated in accordance to the rules on the care and use of laboratory animals issued by the National Institutes of Health, USA, were employed to obtain the caudal region of epididymes. These were minced in PBS and incubated at 37 °C for 10 min in an orbital shaker to facilitate the swim-out of sperm cells into the medium. The supernatant sperm suspensions were centrifuged at 700 × *g* for 15 min at room temperature. The gametes were washed by careful re-suspension in PBS and collected by centrifugation.

2.2. Incubations and study of spermatozoa

Sperm cells were suspended and incubated in the medium developed by Biggers, Whitten and Whittingham [22]. For sperm capacitation, this medium included 25 mM $NaHCO_3$, 1.7 mM $CaCl_2$, and 4 mg/ml fatty acid-free bovine serum albumin. The same medium, with no additions except 25 mM NaCl to maintain osmolarity, was used as control. Both media were buffered to pH 7.4 with 25 mM HEPES. Sperm suspensions were diluted to attain a concentration of 5×10^6 spermatozoa per ml of medium. Incubations were performed under a humidified atmosphere of 5% CO_2/95% O_2, at 37 °C for 5 h. To induce the acrosomal reaction, spermatozoa incubated as described above were exposed to the calcium ionophore A23187, added in a small volume of a DMSO solution (final concentration: 10 μM), and incubation proceeded for an additional period of 30 min. Three completely independent experiments (preparations, incubations) were conducted.

2.2.1. Protein tyrosine phosphorylation

To determine the protein kinase A-dependent phosphorylation at tyrosine residues induced by capacitation, the procedure described for mice [7,8] was applied to rat spermatozoa. Briefly, after incubations, aliquots of rat sperm suspensions were washed, treated with 2-mercaptoethanol and subjected to SDS-PAGE (12.5% polyacrylamide). Proteins were transferred to membranes using an electrophoretic blotting system and incubated with peroxidase-labeled anti-phosphotyrosine PY-20 for immunodetection of tyrosine-phosphorylated proteins. After washing the membranes, a biotinylated antiperoxidase antibody from sheep was added and the reactive bands were detected by chemiluminiscence.

2.2.2. Sperm membrane cholesterol

Paraformaldehyde-fixed control and capacitated spermatozoa were incubated for 30 min at 4 °C in the dark in a freshly prepared solution of filipin (Sigma) (0.7 mM in PBS, from a DMSO solution). At the end of this incubation, the gametes were washed (twice) in PBS and centrifuged (10 min at 700 × *g*). Sperm suspensions were mounted between silylated slide and coverslips (sealed with nail polish) and observed in a fluorescence microscope (Nikon Optiphot-2, Tokyo, Japan) equipped with a Hg lamp and a UV filter block UV-2A (330–380 nm).

2.2.3. Acrosomal status

Small portions taken from gamete suspensions were spread onto slides, stained for 2 min at room temperature with Coomassie Blue G-250 (prepared in 50% methanol containing 10% glacial acetic acid), washed to remove dye excess, and examined under a Zeiss bright field microscope (Germany) [23]. The percentage of acrosome-reacted spermatozoa was determined using isothiocyano-fluoresceinated *Pisum sativum* agglutinin (FICT-PSA) [24]. After lectin binding, the spermatozoa were examined by fluorescence microscopy as described above. A total of 100 spermatozoa were scored after each treatment.

2.3. Lipid and fatty acid analysis

Lipid extracts, prepared from cells and media [25], were taken to dryness under N_2, and resuspended in chloroform:methanol. Aliquots were taken for total lipid phosphorus and cholesterol measurement. Phosphorus was determined after digestion with perchloric acid [26]. Cholesterol was determined using a standard enzymatic/colorimetric procedure.

Lipids were resolved into classes by analytical or preparative TLC and identified with the aid of commercial standards. Lipid extracts were spotted on TLC plates (500 μm-thick silica gel G) to separate the neutral lipids (ceramides, cholesterol, diacylglycerols, free fatty acids), by running chloroform/methanol/ammonia (90:10:0.2, by vol) up to the middle, and then hexane/ether (80:20, by vol) up to the top, of the plates. The total polar lipids at the origin of these plates were recovered, aliquots were taken for analysis and the remainder was used for separation of representative classes (glycerophospholipids, sphingomyelin, seminolipid), using two-dimension TLC [26].

After TLC, lipids were eluted from the silica support with chloroform/methanol/acetic acid/water [27], followed by centrifugation (three elutions), partition (1 M ammonia for most lipids, water in the case of FFA and PA) and drying under N_2. The eluted and dried

SM and Cer were routinely exposed to mild alkali to remove potential lipid contaminants with ester-bound fatty acids [28], and separated again by TLC.

Dried lipid class samples were dissolved in (N_2-saturated) anhydrous methanol containing 0.5 N H_2SO_4 under N_2 to obtain fatty acid methyl ester (FAME) derivatives. Internal standards for quantitative analysis were added at this step, and all samples under N_2 were warmed at 45 °C overnight. Before GC, FAME were routinely purified by TLC using silica gel G plates that had been previously cleaned with methanol:ethyl ether (75:25, v/v) and dried. Ordinary (non-hydroxy) FAME were separated with hexane:ether (95:5, by vol), eluted, dried, and analyzed directly. The 2-hydroxy (2-OH) FAME from SM and Cer were separated using hexane/ether (80:20, v/v), eluted, dried, and converted into O-TMS derivatives before GC analysis [21]. After GC, the 2-OH FA of SM and Cer were added to the rest of the FA to obtain the total amounts of fatty acids in both lipids. The instrumentation and conditions employed in this study for the GC analysis of fatty acids were the same as previously described [21].

The amounts of total or individual lipid classes in the figures are represented by the amounts of their fatty acids, directly as they were obtained, with no further corrections or calculations. For comparison with the corresponding controls, the two-tailed Student's *t*-test was employed for statistical analysis.

3. Results

3.1. Capacitation and acrosomal reaction in rat spermatozoa

Rat spermatozoa incubated for 5 h in the medium that supports capacitation showed a significant increase in the intensity of tyrosine phosphorylation of proteins (Fig. 1A). Only a few of the proteins that become phosphorylated in capacitation-supporting media have been identified to date, the first one being a member of the HSP-90 family of proteins, in the 84–86 kDa range [29]. The intensely stimulated phosphorylation observed in the 30–120 kDa range, with a pattern resembling that described for mice and other mammals [7,30], was taken as evidence that the gametes used in this study were capacitated.

Also as in other species, rat spermatozoa incubated in capacitating conditions showed a fainter intensity of filipin fluorescence in comparison with the corresponding controls (Fig. 1B). This loss of fluorescence, associated with the depletion of cholesterol induced by capacitation, was another widely accepted criterion of capacitation that was met by rat spermatozoa. The vanished fluorescence could be observed from the whole surface of the gametes (Fig. 1B), including the small head and the large tail, allowing the inference that the tail was the main source of most of the cholesterol lost during capacitation.

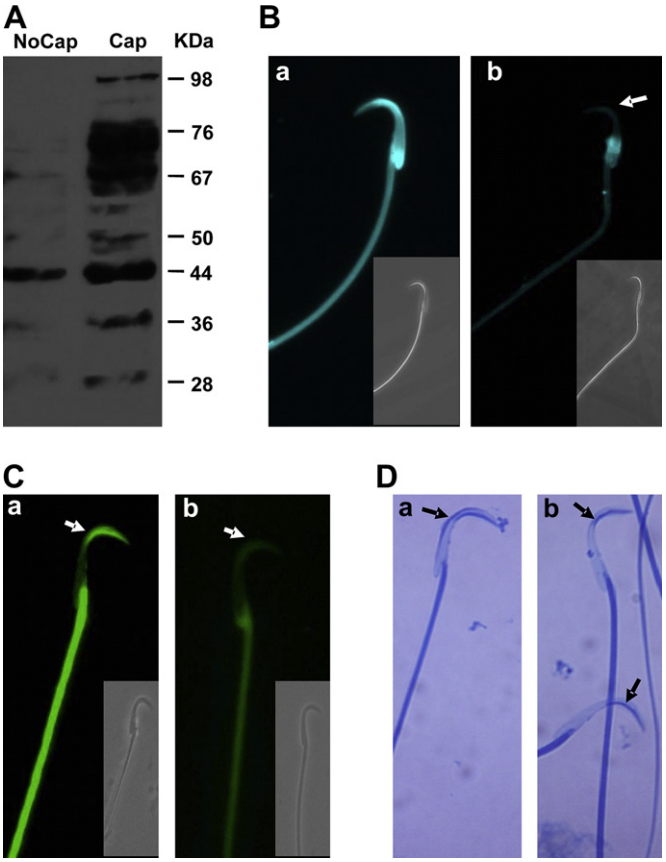

Fig. 1. Hallmarks of capacitation and acrosome reaction. *Upper panels*: Increased protein tyrosine phosphorylation and concomitant decreased cholesterol content in rat spermatozoa incubated in a capacitation-inducing medium. A: Western blot showing the tyrosine-phosphorylated proteins produced after incubation in control (No cap) and capacitating (Cap) conditions, probed as indicated in Materials and Methods. Molecular mass standards (kDa) are shown on the right. B: Filipin labeling of a spermatozoon incubated in control (a) and capacitating (b) conditions. Note the intense fluorescence on the entire surface in (a) in contrast to the faint labeling in (b), indicating cholesterol loss. *Lower panels*: occurrence of the acrosomal reaction (AR), as confirmed by fluorescent FITC-Lectin binding assay (C) and Coomassie Blue staining (D). The photographs depict a rat spermatozoon before (a), and after (b), the AR. Acrosomal caps are indicated by the arrows. At the corners, phase-contrast micrographs of the same cells.

The percentage of acrosome-reacted spermatozoa was determined using an isothiocyano-fluoresceinated agglutinin (Fig. 1C) and Coomassie Blue G-250 (Fig. 1D). Losses of green fluorescence and of the blue dye from the sperm caps, respectively, were indications that spermatozoa had undergone the acrosome reaction. The two markers used showed that almost one third of the cells had lost their acrosomal caps during the 5 h incubation required to induce capacitation (27% and 35% of the cells in control and capacitating conditions, respectively). After the additional 30 min incubation in the presence of A23187 as an evoker of AR, 62% of the gametes had completed their acrosomal exocytosis.

3.2. Main lipid changes associated to capacitation and AR

A quantitatively important efflux of cholesterol from rat spermatozoa to the BSA-containing medium was associated with capacitation (Fig. 2). As much as 31% of the total sperm sterol was lost from the cells (Fig. 3). Equivalent amounts of cholesterol to those lost from cells were recovered in the medium.

A significant loss of phospholipids also occurred during capacitation, in relative terms similar to that of cholesterol (27% of the initial amounts) (Fig. 3). Because on a mole basis rat spermatozoa contain three times more phospholipids than cholesterol, the depletion of phospholipids was quantitatively much larger than that of cholesterol. Most of the lipid phosphorus lost from the gametes was recovered in lipid form in the incubation medium (Fig. 3). Most of this lipid P was accounted for by highly polar phospholipids, notably lyso-CGP (Fig. 2).

Capacitation resulted also in a marked reduction of the amounts of FFA in the gametes, mainly ascribable to *displacement* of FFA to the BSA-containing medium (Figs. 2 and 3). Interestingly, the sum of FFA in cells *plus* medium was lower in control than in capacitated spermatozoa (Fig. 3), indicating that during capacitation there was an important additional production of FFA from phospholipids.

After the AR, and with respect to capacitation, a small, scarcely significant amount of additional cholesterol was lost from sperm cells (Fig. 3). In the medium, the spots of cholesterol, lyso-CGP and FFA were not noticeably intensified from capacitated to acrosome-reacted cells (Fig. 2). Thus, the largest part of the efflux from cells to medium of these lipid metabolites, as that of cholesterol, took place during capacitation (Figs. 2 and 3). In the gametes in contrast, the total lipid P was further reduced, by as much as an additional −22%, from capacitation to AR. This lipid P was *not* recovered as lipid phosphorus in the medium. This is in accordance with the next section results, considering that in AR phospholipids were in part converted into phosphorus-lacking metabolites, like diglycerides, ceramides and free fatty acids.

3.3. Capacitation- and AR-associated changes in GPL and their metabolites

The data in Fig. 4 shows comparatively the amounts of representative glycerol-containing lipids and FFA in control, capacitated, and acrosome-reacted rat spermatozoa, as determined from the sum of their constituent fatty acids. Those in Fig. 5 compare, in a similar format, amounts of relevant fatty acid constituents of these lipids.

Most of the changes described for the total lipid phosphorus were ascribable to glycerophospholipids (GPL), to which choline GPL (CGP) were the main contributors (Fig. 4). The major CGP were reduced as much as nearly one fourth (−24%) between controls and capacitated, and even more (−31%) between capacitated and AR cells. In comparison, the second most abundant GPL of sperm cells, EGP, was also reduced significantly with capacitation (−28%) but was much less affected (−13%) between this condition and AR.

Consistently, CGP and EGP decreases were accompanied by differentially increased levels of lyso-CGP and lyso-EGP in cells. Lyso-CGP increased a 32% between controls and capacitated, but as much as a 79% between capacitated and acrosome-reacted cells; lyso-EGP increased 31% and an additional 18%, respectively, in each condition. Thus, although capacitation reduced similarly both GPL in relative terms, AR was much more specific in stimulating the hydrolysis of CGP in quantitative terms.

Sulfogalactosylglycerolipid (seminolipid) showed an interesting behavior: it was the less affected lipid (a non-significant −7%) after capacitation (hence its proportion with respect to the major sperm GPL increased), but was significantly decreased after incubation in AR-promoting media (−38% with respect to capacitation).

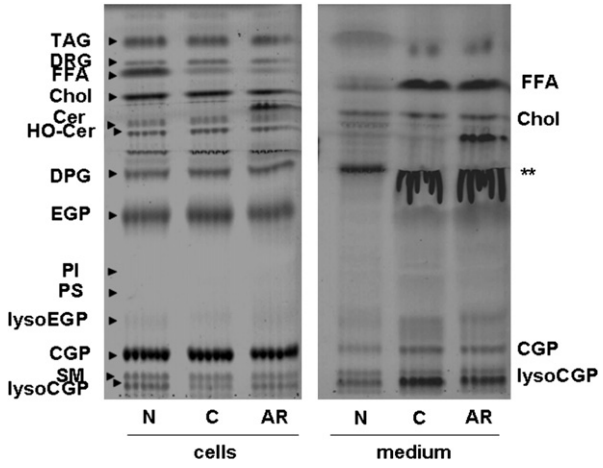

Fig. 2. Lipid profiles in spermatozoa and medium after incubation with no additions (N), in the presence of bicarbonate and albumin to induce capacitation (C), and in the presence of A23187 to induce the acrosome reaction (AR). Lipids derived from 50×10^6 sperm cells were spotted in each case, as well as the corresponding proportion of lipids from the medium. Two solvents, chloroform/methanol/acetic acid/water (50:37.5:3.5:2), run up to the middle of the plates, and hexane/ether (80:20, by vol) up to the top, were used. TAG, triacylglycerols; DRG, diradylglycerols (total diglycerides); FFA, free fatty acids; Chol, cholesterol; Cer, ceramides; HO-Cer, Cer with 2-OH fatty acids; DPG, diphosphatidylglycerol; EGP ethanolamine GPL; PI, phosphatidylinositol; PS, phosphatidylserine; CGP, choline GPL; SM, sphingomyelin; Lyso-CGP and lyso-EGP, Lyso-derivatives from CGP and EGP, respectively. **, BSA and *, A23187 present in the medium.

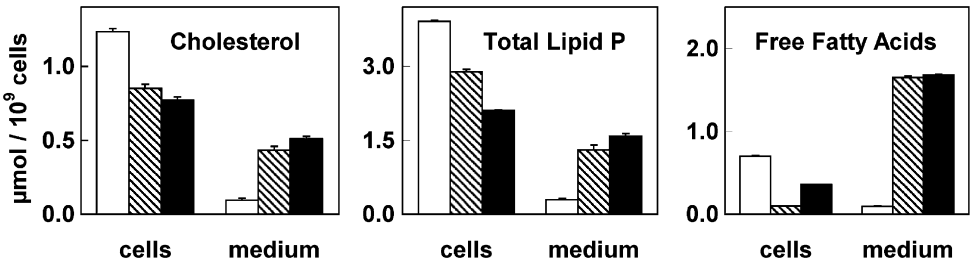

Fig. 3. Total amounts of cholesterol, lipid phosphorus, and free fatty acids in rat spermatozoa (and in the corresponding media) after incubations for 5 h in with no additions (N), in the presence of bicarbonate and albumin to induce capacitation (C), and in sperm thus capacitated after adding the calcium ionophore A23187 and incubating for 30 min to induce the acrosomal reaction (AR) (white, hatched, and black bars, respectively).

Taking into account the total cellular amounts of the five phospholipids shown in Fig. 4, namely CGP, EGP, PA, lyso-CGP and lyso-EGP, the *percentage* ratios among CGP, EGP and the sum of the other three lipid *products* were as follows: CGP, 71, 72 and 62%; EGP, 23, 21 and 21%; and the rest, 5, 9, and 17%, in controls, capacitated and AR cells, respectively. This is consistent with CGP and EGP being reduced virtually in parallel during capacitation, but CGP undergoing the main reduction (with respect to the capacitation value) during AR. After AR an important part of lyso-CGP accumulated in the gametes as new components.

In the gametes, the FFA dropped precipitously (to one fifth) from control to capacitated gametes (Fig. 4), but increased again from capacitation to AR. Adding up the amounts recovered in cells and media, the total FFA produced increased 113% from control to capacitated and an additional 260% from capacitated to acrosome-reacted samples (i.e., a total of 5-fold increase in their levels from control to acrosome-reacted cells) (Fig. 4). In comparison, the sperm-associated DRG increased 89% from control to capacitated and more than twice from these to acrosome-reacted gametes (about 5-times between controls and AR).

3.4. Fatty acids of GPL and their metabolites

Fig. 5 shows that major fatty acids, representative of specific molecular species, contributed differentially to the decreased GPL and increased metabolites depicted in Fig. 4.

After capacitation, and with respect to controls, 22:4n-9 was the fatty acid reduced relatively the most in CGP, followed by 22:5n-6 (−37% and −21%, respectively) (Fig. 5). In EGP, 20:4n-6 and 22:5n-6 were the fatty acids reduced the most (−26% and −30%, respectively)

Consistently, after capacitation 22:4n-9 and 22:5n-6 were the main fatty acids recovered as FFA, mostly in the BSA-containing medium. Adding up cells + medium amounts of each of the different FFA depicted in Fig. 5, the one produced most actively during capacitation was free 22:4n-9, followed by free 22:5n-6 and 20:4n-6 (694%, 521% and 422% increase with respect to controls, respectively).

The lyso-CGP and lyso-EGP present in the medium were not analyzed, but those collecting in cells after capacitation were mostly species containing saturated and monoenoic fatty acids (Fig. 5), with virtually unchanged levels of PUFA, strongly suggesting, i) that a form of phospholipase of the A_2 type was activated during capacitation; ii) that CGP and EGP were its main substrates, and iii) that this activity was mostly responsible for the increased production of the FFA massively effluxing to the medium, mostly polyunsaturated.

After AR, all GPL were additionally reduced below their capacitation levels (Fig. 5), with CPG again showing the largest quantitative drop. In this lipid, species with 22:5n-6 decreased −21% from controls to capacitation and −35% and from capacitation to completion of AR, respectively.

The fatty acid profiles in Fig. 5 support the view that the lyso-CGP and also an important part of the FFA produced after the AR, both remaining in the gametes, could have arisen from hydrolysis of CGP.

The finding that the polyenoic fatty acids mostly responsible for the important DRG increase after AR were 22:5n-6 and 22:4n-9 (Fig. 5) suggests that an important part of these DRG may have originated in those CGP that remained in the cells after capacitation.

3.5. SM and Cer and their fatty acids

The amounts of *total* SM decreased significantly with respect to controls after incubation of spermatozoa in conditions leading to

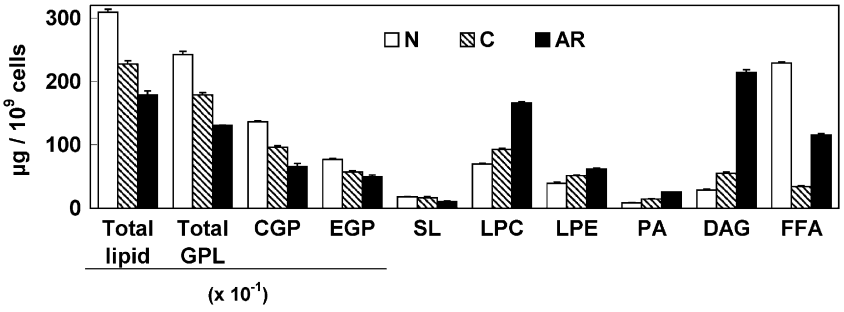

Fig. 4. Amounts of major lipids of rat spermatozoa and of some of the metabolites produced after incubation in control (N, no additions), capacitation (C), and acrosomal reaction-inducing media (AR). The figures are expressed as µg of total fatty acids present in each ach lipid, as determined after lipid separation and fatty acid analysis by GC. GPL, total glycerophospholipids; CGP and EGP, choline and ethanolamine glycerophospholipids respectively; SL, seminolipid; PA, phosphatidic acid; LPC, LPE, abbreviated forms of Lyso-CGP and Lyso-EGP, respectively; DAG, diradylglycerols (mostly diacylglycerols); FFA, free fatty acids.

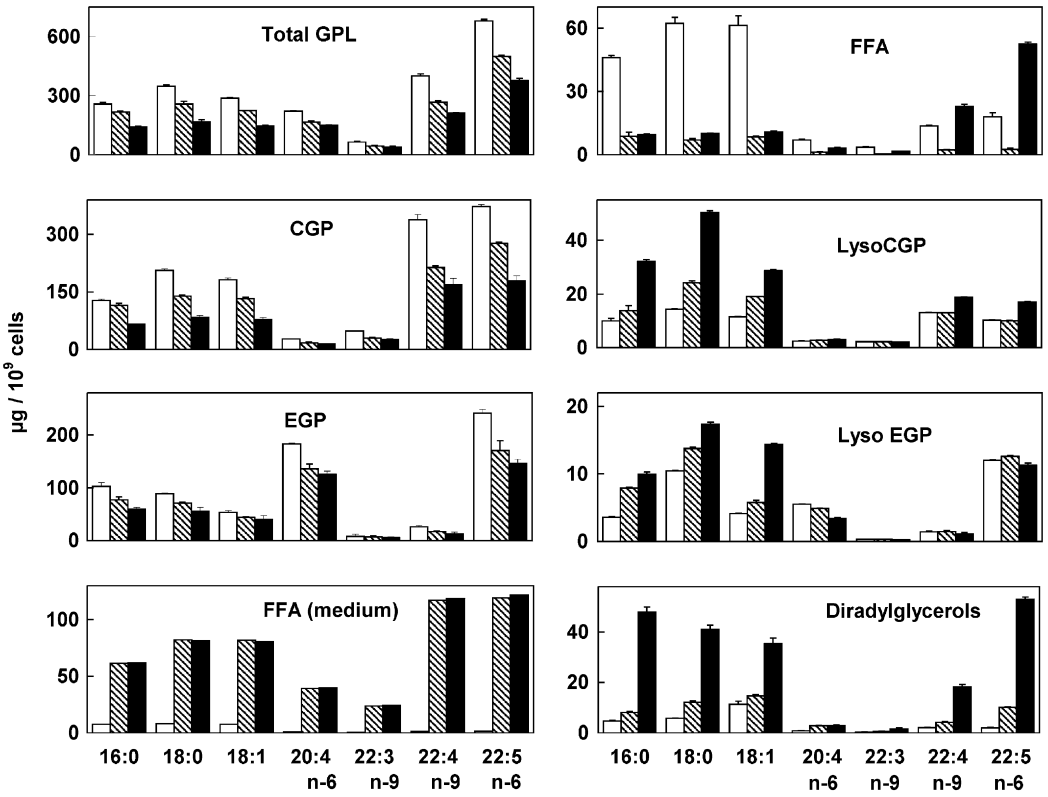

Fig. 5. Amounts of representative fatty acids contributing to the changes that occur in some of the lipids whose total fatty acids are depicted in Fig. 4. The bars represent the values in spermatozoa incubated in control, capacitation, and AR-inducing conditions (white, hatched, and black bars, respectively).

capacitation (−27%) but much more so between capacitation and AR (−67%) (Fig. 6). The decrease in total SM was, in relative terms, the largest change of all phospholipid classes surveyed in this study, a total of −75% drop from control to acrosome-reacted cells (compared to a total CGP and EGP relative decrease of −43% and −36%, respectively).

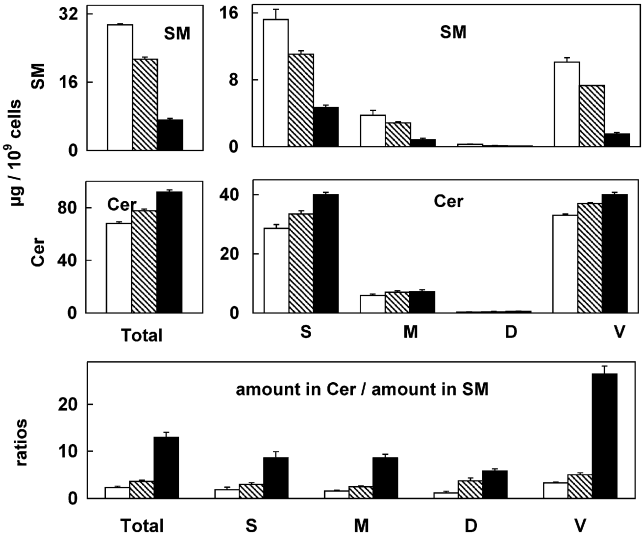

Fig. 6. Amounts of sphingomyelin (SM) and ceramide (Cer) fatty acids after incubation in control, capacitation, and AR-inducing conditions (white, hatched, and black bars, respectively). S, M, D, and V, stand for the groups of fatty acids representing different molecular species of SM and Cer, containing saturated, monoenoic, dienoic and very long chain polyenoic fatty acids (VLCPUFA), respectively. The "V" represents the sum of VLCPUFA (non-hydroxy plus 2-OH VLCPUFA). In the lower panel, the *ratios*, amount in Cer/amount in SM for each of the above fatty acid groups are compared.

Considering the amounts of major groups of fatty acids of SM (Fig. 6), representative of main groups of molecular species, there were apparent differences between the effects of capacitation and AR. The groups containing saturated (S), monoenoic (M), dienoic (D), and very long chain polyenoic (V) fatty acids were reduced to similar extents from controls to capacitated cells (nearly −28%). By contrast, from capacitated to acrosome-reacted cells, the former 3 groups were reduced between −60 and −70% whereas the group containing VLCPUFA decreased more, nearly −80%. This represents a total decrease of about −85% i.e., a virtual disappearance, of the VLCPUFA-containing species of SM from control to acrosome-reacted cells. The ratio, amount in Cer/amount in SM for each group of fatty acids (Fig. 6) underscore the relatively more active hydrolysis of the SM species with VLCPUFA when compared to other species in AR.

Further research is required to establish the reason for the presence in spermatozoa of intriguingly high endogenous levels of ceramides with VLCPUFA (mostly 2-hydroxylated VLCPUFA), observed in spermatozoa incubated in the three conditions of this study, including controls (Fig. 6). This is a characteristic of rat spermatozoa, since it was also observed in fresh gametes isolated directly from the epididymis [21] as well as in round spermatids [31]. The amount of these "basal" ceramides did not change with incubations leading to capacitation or AR. Above this background, the *decreases* undergone by SM were equivalent to corresponding *increases* in the form of Cer, almost stoichiometrically. The sum, amount in Cer *plus* amount in SM was virtually the same in control, capacitated, and acrosome-reacted spermatozoa.

In conclusion, these results show that, i) the increased Cer were directly originated from the decreased SM; ii) the produced Cer

were not significantly modified further (e.g., to free fatty acid and sphingosine) after being produced; and iii) the species of SM containing VLCPUFA were preferentially involved in AR.

4. Discussion

The results in this study show that differential changes affect the lipids of rat spermatozoa during incubations that lead to sperm capacitation and acrosomal exocytosis. Capacitation was associated with a remarkable depletion of lipids from the gametes, not only involving the expected loss of cholesterol but mainly phospholipids. The major glycerophospholipid, CGP, played the main quantitative role in this decrease, being actively hydrolyzed into lyso-CGP and FFA, mostly free 22:4n-9 and 22:5n-6, which, as cholesterol, abandoned the cells and effluxed to the medium. After the AR, CGP and SM were the two phospholipid classes decreasing the most. In this case hydrolysis of CGP mostly resulted in the generation of considerable amounts of diglycerides (notably 22:5-rich DRG) on the one hand, and some additional lyso-CGP + FFA on the other. Also, hydrolysis of SM resulted in the generation of ceramides, remarkably VLCPUFA-rich Cer. The lipid composition thus changed considerably after completion of capacitation and AR, diglycerides and ceramides becoming important new components of spermatozoa that have undergone capacitation and the acrosome reaction, and are thus potentially apt ready to fertilize an oocyte.

4.1. Lipids and capacitation

During capacitation, rat spermatozoa fulfilled the criteria, over which there is general consensus, of effluxing an important proportion of the total cholesterol present in their plasma membranes to a medium containing an adequate acceptor, and concomitantly increasing the phosphorylation of specific proteins in tyrosine residues (PYP) [7,8,11]. Because the bulk of sperm tyrosine-phosphorylated proteins is generated during the course of capacitation, and most of such proteins are localized to the flagellum, PYP has been implicated in the hyperactivation of motility that is one of the hallmarks of sperm capacitation [32]. As observed with the fluorescent marker filipin, the loss of cholesterol affected the whole plasma membrane of the gametes. Based on size, volume, and surface area considerations, it can be inferred that the capacitation-related decreases in cholesterol, and probably also that of GPL, involved predominantly the sperm tails.

The fact that free 22:4n-9 and 22:5n-6 and lyso-CGP were main lipid products during capacitation suggests that an endogenous phospholipase of the A_2 type was responsible for the decreased cellular levels of choline GPL. Using a series of radioactively labeled lipid substrates, including phosphatidylcholine with [^{14}C]20:4n-6 at *sn-2*, this activity was previously demonstrated in ram spermatozoa [33].

An unexpected finding of this study was that abundant FFA and lyso-CGP were present in spermatozoa incubated for 5 h in the medium that had no additions. With such controls, the main effect of incubation in capacitation-supporting conditions (bicarbonate, albumin, calcium ions) was to deplete the sperm membranes of an important part of these two membrane-disturbing lipids. From this perspective, the accumulation of these GPL-derived metabolites could be regarded as factors *interfering* with capacitation *unless* an adequate lipid acceptor (e.g., BSA) is at hand in the surrounding medium to relieve the gametes from their presence.

As in most mammalian cells, in spermatozoa the choline-containing phosphatidylcholine and SM are located mostly on the outer, and the aminophospholipids phoshatidylethanolamine and phosphatidylserine mostly on the inner, leaflet of the plasma membrane [34,35]. Regarding capacitation, an early event that apparently precedes cholesterol efflux is a bicarbonate/CO_2-induced, protein kinase A-mediated, but Ca^{2+} and albumin (BSA)-independent, loss of this normally asymmetric transbilayer distribution of the major sperm membrane phospholipid (PL) classes [34]. This early PL "scrambling" was proposed to result in an augmented disorder in the lipid bilayer that is capable of inducing a cholesterol redistribution on, thus enabling cholesterol efflux from, the sperm plasma membrane [34]. Because BSA has no affinity for cholesterol in the absence of bicarbonate, these authors proposed that bicarbonate is the component of the medium that is mostly responsible for inducing the mentioned lipid reorganization in the sperm plasma membrane during capacitation, facilitating cholesterol exposure and fixation onto BSA, when it is present. Our observation that glycerophospholipid-derived FFA and lysophospholipids accumulated in the cells when incubated in a bicarbonate- and albumin-free medium, but were massively displaced from cells to a medium containing both during incubations leading to capacitation, adds a new element to consider in this sequence of events.

The formation of these two membrane destabilizing metabolites on the sperm plasma membrane (presumably in the outer leaflet) could respond for the transbilayer phospholipid class reorganization (scrambling) that precedes the efflux of cholesterol. In the presence of bicarbonate/BSA, FFA are effluxed from the cells and are expected to be mostly bound to the acceptor protein. Through hydrophobic interactions, sperm cholesterol could be passively drawn by these FFA, which would explain in part the scarce binding of cholesterol by lipid-free BSA alone. In support of this interpretation, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), high-density lipoproteins (HDL), as well as the albumin fraction from serum, can act as acceptors of cholesterol from human capacitated spermatozoa [36].

4.2. Lipids and acrosomal reaction

Because capacitation primes spermatozoa to acrosome-react, the AR normally occurs in already capacitated germ cells and associated lipid changes. Conversely, during incubations in the (calcium containing) media that are required to induce capacitation, some of the gametes normally undergo spontaneously the AR. This in part occurred in this study, affecting about one third of the cells. Yet, it was clearly apparent that some of the reactions were far more active during the 30 min incubation elapsed to induce the AR than during the long 5 h required to achieve capacitation. This was the case for the reactions CGP → DRG and SM → Cer.

Sequential changes due to capacitation and AR affecting CGP could be observed thanks to the species of this lipid that contain 22:4n-9 and 22:5n-6. The highest production of 22:5n-6-rich DRG occurred during AR, suggesting that a phospholipase C (PLC), acting on the CGP that *remained* in the cells after capacitation, was strongly activated during AR.

The present data, focusing on sperm lipid products, agree with previous research showing that AR is associated with the activation of calcium-dependent sperm phospholipases (phospholipase C, D and A_2) [37]. Thus, when ram or boar spermatozoa are incubated with radio-labeled GPL in the presence of progesterone, ZP, or the calcium ionophore A23187, labeled diacylglycerols, diradylglycerols, some phosphatidic acid, lysophospholipids and free fatty acids are produced [33]. Moreover, two forms of PLC were shown to be activated sequentially during A23187-induced acrosomal exocytosis: a phophatidylinositol-4,5 bisphosphate-specific PLC generates definite diacylglycerol species (with a saturated fatty acid at *sn-1* and a polyunsaturated fatty acid at *sn-2*) that are capable of stimulating, later on, the production of DRG by a second PLC that

acts on CGP [38]. Among other isoforms of PLC (beta, gamma, delta), in mice and hamsters a sperm-specific cytosolic PLC-zeta has been discovered [39]. This PLC isoform was found to be located in the acrosomal region, as also observed in rat spermatozoa [40]. One of these PLCs could be the enzyme responsible for the accumulation of 22:5n-6-rich DRG observed in acrosome-reacted rat spermatozoa in the present study. Interestingly, the introduction of the soluble sperm PLC-zeta into an oocyte triggers egg activation, with Ca^{2+} oscillations and development into an embryo during fertilization.

Of particular interest with respect to AR was seminolipid, a comparatively minor but sperm-specific lipid that is involved in sperm-ZP binding [41]. Its amount per cell was virtually unchanged with capacitation, which is consistent with the fact that in this process seminolipid *migrates* to the equatorial region of the plasma membrane of the sperm head, a pre-requisite for the AR to proceed. The decrease observed after AR is consistent with the fact that this reaction leads to its partial de-sulfation [41].

In the present study, as indicated by their amounts, all of the ceramides produced during AR were formed *in situ* by an endogenous enzyme activity that uses SMs as substrates. An Mg^{2+}-dependent neutral sphingomyelinase has been characterized in the plasma membrane of rat spermatozoa [42] that could in part be responsible for the irreversible conversion SM → Cer observed in our study after the AR. There are just a few previous studies relating ceramides and AR. In human spermatozoa, the AR induced by progesterone is accelerated after exposure to an exogenously added sphingomyelinase or to C6-ceramide [43]. In porcine gametes, the AR induced by A23187 was also shown to be enhanced in the presence of added C2-ceramide [44].

As sperm SM is mostly located on the sperm head [28], so may be the Cer that is produced *from* such SM during the AR. Moreover, since most of the sperm SM faces the exoplasmic side of the plasma membrane [34], the generated Cer could also remain on this side of the bilayer. The ceramides that accumulate on the sperm membrane after completion of capacitation and AR could play a role in the creation of the appropriate surface physical properties on the sperm head that prompt these gametes for the next crucial event of fertilization: fusion between sperm and oocyte membranes upon binding to specific receptors. What such properties are remain to be established, but at least in model systems the effects of gradually changing the SM/Cer mass ratio from mostly SM to mostly Cer are dramatic [45].

In addition to biophysical considerations, the diglycerides and ceramides produced during capacitation and AR may act as lipid second messengers in spermatozoa. These two lipids are known to modulate, stimulating and inhibiting, respectively, protein kinase C, of which nine isoforms have been identified in rat testicular germ cells that change in amount and intracellular localization as cells develop into spermatozoa [46]. Going further, one could speculate that once a single capacitated, acrosome-reacted spermatozoon interacts with a metaphase II-arrested (MII) oocyte, the diglycerides and ceramides transported in acrosome-reacted sperm could play regulatory roles in the fertilized egg by interacting with appropriate egg protein transducers or effectors. In *Xenopus* oocytes, microinjection of Cer, or its generation through treatment with sphingomyelinase, are sufficient to allow oocyte maturation and its reentry into the meiotic cell cycle [47].

Recently it has been reported that follicular fluids normally contain a (cumulus cell-derived) acid ceramidase that improves the outcome (successful embryos, healthy births) when added to male-female gametes during in vitro fertilization techniques [48]. In vivo, only one spermatozoon is required to fertilize an oocyte out of the myriad of competent, acrosome-reacted spermatozoa that normally reach the surroundings of one female gamete. Taken together with the present results, this extracellular enzyme may be an important post-fertilization catabolic factor to inactivate/eliminate the superfluous (Cer-rich) spermatozoa that swarm to no avail in the vicinity of a successfully fertilized oocyte.

5. Conclusion

To summarize, whereas GPL-derived FFA were mostly produced during *in vitro* induced capacitation and were displaced (together with part of the cholesterol) from sperm membranes to an adequate acceptor in the environment, DRG and Cer were mostly produced during AR and stayed in sperm cells. In other words, whereas the capacitation-related events were dominated by a significant delipidation, the AR was characterized by the production of a series of (mostly neutral) lipid metabolites that collected in the lipid-depleted gametes.

A major challenge in assisted reproduction technologies is to mimic the natural conditions required to sustain oocyte and embryo survival after fertilization. In developing zygotes by intracytoplasmic sperm injection (ICSI), rat oocyte activation is accelerated, and subsequent embryonic development is improved, if (epididymal) rat spermatozoa are pre-treated with agents (lysolecithin, Triton X-100) that remove the acrosome and most of the plasma membrane [40,49]. Although the main concern in these studies was to eliminate the oocyte-disturbing sperm acrosome, these agents also cause a partial delipidation of the sperm cells employed. In part, this resembles what happens after rat sperm capacitation *and* AR induced *in vitro*, as shown here, although the quality of the sperm-associated neutral/polar lipids is likely to differ.

Acknowledgements

This work was supported in part by funds, granted to authors M.W. Fornés and M.I. Aveldaño, by Consejo Nacional de Investigaciones Científicas y Técnicas, Agencia Nacional de Promoción de la Ciencia y la Tecnología, Universidad Nacional del Sur y Universidad Nacional de Cuyo (Argentina). For the realization of part of this work, Dr. S. R. Zanetti was supported by a fellowship from PLISSER, Programa Latinoamericano de Investigación en Salud Sexual y Reproductiva.

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