THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 25, pp. 18151–18161, June 22, 2007

Very Long-chain Polyunsaturated Fatty Acids Are the Major Acyl Groups of Sphingomyelins and Ceramides in the Head of Mammalian Spermatozoa*

Received for publication, January 25, 2007, and in revised form, March 28, 2007 Published, JBC Papers in Press, March 28, 2007, DOI 10.1074/jbc.M700709200

Natalia E. Furland[‡], Gerardo M. Oresti[‡], Silvia S. Antollini[‡], Andrés Venturino[§], Eduardo N. Maldonado[‡], and Marta I. Aveldaño[‡1]

From the [‡]Instituto de Investigaciones Bioquímicas de Bahía Blanca, Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional del Sur, 8000-Bahía Blanca, Argentina and the [§]Laboratorio de Teriogenología, Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional del Comahue, 8300-Neuquén, Argentina

Very long-chain (C24 to C34) polyunsaturated fatty acids (VLCPUFA) are important constituents of sphingomyelin (SM) and ceramide (Cer) in testicular germ cells. In the present paper we focused on the SM and Cer and their fatty acids in spermatozoa and their main regions, heads and tails. In bull and ram spermatozoa, SM was the third most abundant phospholipid and VLCPUFA were the major acyl groups (~70%) of SM and Cer. In rat epididymal spermatozoa the SM/Cer ratio was low in the absence of and could be maintained high in the presence of the cation chelator EDTA, added to the medium used for sperm isolation. This fact points to the occurrence of an active divalent cation-dependent sphingomyelinase. Bull and rat sperm had an uneven head-tail distribution of phospholipid, with virtually all the VLCPUFA-rich SM located at the head, the lower SM content in the rat being determined by the lower sperm head/tail size ratio. Most of the SM from bull sperm heads was readily solubilized with 1% Triton X-100 at 4 °C. The detergent-soluble SM fraction was richer in VLCPUFA than the nonsoluble fraction and richer in saturated fatty acids. Cer was produced at the expense of SM, thus decreasing severalfold the SM/Cer ratio in rat spermatozoa incubated for 2 h in presence of the sperm-capacitating agents, calcium, bicarbonate, and albumin. The generation of Cer from SM in the sperm head surface may be an early step among the biochemical and biophysical changes known to take place in the spermatozoon in the physiological events preceding fertilization.

In a number of mammals including humans a series of very long-chain polyunsaturated fatty acids (VLCPUFA),[2] *i.e.* n-6 and n-3 tetraenoic, pentaenoic and hexaenoic fatty acids with up to 32 or 34 carbon atoms, depending on the species, was characterized in the sphingomyelin (SM) from testis and spermatozoa (1, 2). In the testis of various mammals, we focused on the fatty acids of the ceramide (Cer), a lipid molecule with which SM bears a close precursor-product relationship, showing that SM and Cer species containing VLCPUFA are a specific feature of cells of the spermatogenic lineage (3). Because these testicular cells are predecessors of spermatozoa, the question arose as to the quantitative importance of these molecules in spermatozoa, where they could play a role in sperm functions related to fertilization.

Transit through the epididymis is a crucial phase in sperm maturation. Spermatozoa exiting the testis are immotile, unable to bind to eggs and to undergo the acrosomal reaction *in vitro* in response to commonly used stimuli. By the time they reach the region of cauda epididymis, sperm cells have acquired their progressive motility and their ability to bind, penetrate, and fertilize eggs (4). One of the questions we addressed was whether epididymal maturation gives rise to spermatozoa with a larger or a smaller proportion of SM and Cer containing these VLCPUFA as opposed to other fatty acids.

Spermatozoa are functionally regionalized cells. Sperm-oocyte interactions are head-related functions, energy production for motion is a main role of mitochondria located in the midpiece, and motility is a function associated with the contractile proteins located to the tail. We next addressed the distribution of VLCPUFA-containing species of SM and Cer in sperm tails and heads and employed a nonionic detergent, Triton X-100, to gain information on the behavior of the lipids present in the plasma membrane of sperm heads, focusing on SM and its fatty acids.

As a prerequisite to fertilization, mammalian spermatozoa must undergo the time-dependent priming process known as capacitation, as a result of which sperm change their motility patterns and migrate to the site of fertilization, in the process becoming competent to undergo the acrosomal reaction. This reaction entails a specific fusion event in the spermatozoal head whereby the plasma membrane of the apical region and the outer membrane of the underlying acrosome fuse at many

* This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas, Secretaría General de Ciencia y Tecnología Universidad Nacional del Sur, and Agencia Nacional de Promoción de la Ciencia y la Tecnología, Argentina. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: INIBIBB, Centro Regional de Investigaciones Básicas y Aplicadas de Bahía Blanca, CONICET, CC 857, 8000, Bahía Blanca, Argentina. Tel.: 54-291-4861666, Ext. 138; Fax: 54-291-4861200; E-mail: avelda@criba.edu.ar.

[2] The abbreviations used are: VLCPUFA, very long-chain polyunsaturated fatty acids; Cer, ceramide; SM, sphingomyelin; SMase, sphingomyelinase; PL, phospholipid; PBS, phosphate-buffered saline; KR, Krebs-Ringer; GC-MS, gas chromatography-mass spectrometry; CGP, choline glycerophospholipid; EGP, ethanolamineglycerophospholipids; DPG, diphosphatidylglycerol; ros, rod outer segment(s); PC, phosphatidylcholine.

points, creating vesiculations and fenestrations that enable exocytosis of the acrosomal constituents. The release of hydrolytic enzymes is one of the factors facilitating penetration of the egg coat, thus allowing the post-acrosomal membrane of a spermatozoon to merge with the oolemma. The molecular mechanisms underlying these processes have been studied in great molecular detail over the years (see Refs. 5–8 for reviews).

Capacitation increases sperm plasma membrane lipid disorder, reducing the stability of such membrane in preparation for the hyperactivated motility and membrane fusion events that follow (8). One of the first lipid changes shown to occur in the spermatozoal membrane in this context is a significant loss of cholesterol (9). More recently, the normal transbilayer asymmetry of four phospholipids (PL) including SM in the sperm plasma membrane was shown to change as a result of capacitation (10). The basic facts that SM has a high affinity for cholesterol, that both lipids are abundant in the sperm plasma membrane, and that this membrane has an active neutral sphingomyelinase (SMase) activity (11) capable of producing Cer *in situ* suggest that the SM-Cer pair may well be involved in sperm membrane functions. Given the increasing importance being accorded to SM and its hydrolysis in the generation of biologically active, and membrane disturbing, membrane-bound metabolites, especially Cer, investigation of SM in regard to sperm functions is of obvious interest.

Exposure of human spermatozoa to an exogenous SMase abbreviates by several hours the loss of sterols and sperm capacitation, measured as the ability of sperm to acrosome (react in response to progesterone), incubation of sperm with C6-Cer mimicking in part the SMase effects (12). The acrosomal exocytosis triggered by Ca^{2+} in the presence of a calcium ionophore is enhanced after incubation of boar spermatozoa with the analogue C2-Cer and with a ceramidase inhibitor, the results suggesting that Cer may be involved in the mechanisms underlying the acrosomal reaction (13). The nature of the endogenous SM and Cer of spermatozoa and how the SM/Cer ratio is affected during normal sperm functioning remain to be investigated.

In this work we studied SM and Cer, focusing on their fatty acids, in bull and ram spermatozoa isolated from fresh semen and in rat spermatozoa isolated from cauda epididymis. Beyond the exceptionality of their fatty acids, study of these lipids showed that conspicuous modifications in the SM/Cer ratio occur not only when spermatozoa are incubated in capacitating conditions, but even spontaneously when spermatozoa are isolated after a few simple steps. This biochemical information is of interest to both biophysicists and gamete physiologists, because the *in situ* conversion SM → Cer is likely to result in consequential changes in sperm membrane physicochemical properties and functions.

EXPERIMENTAL PROCEDURES

Spermatozoa—Freshly ejaculated semen was collected from fertile Shorthorn bulls in a local breeding station, transported to the laboratory in a Dewar glass at 25 °C, and rapidly centrifuged at room temperature at 600 × *g* to separate the seminal plasma. The cells were then gently suspended in phosphate-buffered saline (PBS) (95 mM NaCl, 2.7 mM KCl, 1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and centrifuged; this procedure was repeated twice to remove traces of seminal plasma (and its lipids) from sperm samples. Semen from Australian Merino rams was collected during the reproductive season and treated similarly. Rat spermatozoa were obtained from 4–5-month-old Wistar rats.

For rat spermatozoa isolation, caudal epididymi were excised, freed of their fat pad and blood vessels, and transferred to small dishes containing PBS. A few incisions were made with a scalpel blade in the sperm-rich area of each epididymis. The organs thus treated were gently incubated for 15 min at 34 °C to allow release and diffusion of spermatozoa and then removed. The spermatozoa were obtained from the supernatants by centrifugation at 600 × *g*. They were then resuspended in 65% (w/v) sucrose at 4 °C and purified in a discontinuous density gradient, made up of 70 and 75% (w/v) sucrose in PBS. These steps decreased the yield but increased the purity of the epididymal spermatozoa samples, in comparison with the procedures used in an earlier report (14). When present, the metal-ion chelator EDTA was incorporated at a concentration of 2.5 mM in the medium used for sperm isolation, including the initial PBS and the sucrose solutions. It was necessary to pool spermatozoa isolated from the epididymi of several rats to obtain sufficient material for analysis of SM, Cer, and their fatty acids.

Sperm Heads and Tails—To separate sperm heads from tails, spermatozoa from freshly ejaculated bull semen or from the epididymi of several rats were gently suspended in PBS containing 1 mM phenylmethylsulfonyl fluoride, and the samples were sonicated at 30-s intervals using a model 250 Branson sonifier (15). The resulting mixture of heads and tails was pelleted at 600 × *g* at 4 °C, resuspended in 65% sucrose, and loaded onto a step gradient made up of 70 and 75% (w/v) sucrose in PBS-phenylmethylsulfonyl fluoride. The tubes were centrifuged at 104,000 × *g* at 4 °C for 60 min. The head fraction was collected as a pellet. The fraction at the 65–70% sucrose interface containing the tail-rich fraction was collected, resuspended, and purified by a further centrifugation (104,000 × *g*, 4 °C, 60 min) on a 65–75% sucrose gradient. The purity of the fractions was verified by light microscopy.

Detergent Solubilization of Head Phospholipids—A bull sperm head fraction prepared as just described was solubilized with a nonionic detergent to study the fatty acids of the SM present in the detergent-soluble and nonsoluble fractions. The following procedure was chosen in view of its successful application to the study of the localization of enzymes in human spermatozoal parts (16) and because it has been reported to completely remove the plasma membrane from whole, intact spermatozoa (17). Briefly, the pellet of sperm heads was suspended and homogenized in a hypotonic solution (58.5 mM NaCl, 4.8 mM KCl, 1 mM $MgCl_2$, 58.5 mM KH_2PO_4, and 50 mM Tris-HCl, pH 7.4), containing 2 mM phenylmethylsulfonyl fluoride. The resulting homogenate was centrifuged at 40,000 × *g* for 15 min to separate the sperm cytosolic fraction. The pellet was resuspended in 1% Triton X-100 (Sigma) prepared in the same saline buffer. The suspension was agitated gently for 10 min and then centrifuged at 40,000 × *g* for 15 min. This produced a detergent-soluble supernatant and a detergent-insoluble pellet. A temperature of 4 °C was maintained throughout.

Incubations of Rat Spermatozoa—Spermatozoa were isolated from the epididymi of a dozen rats in PBS containing or lacking EDTA, pelleted at 600 × *g*, and washed once in the same medium to reduce in the latter group possible divalent cations from the epididymal tissue. After discarding the supernatants, the cells were gently resuspended in a Krebs-Ringer (KR)-based medium (95 mM NaCl, 2.7 mM KCl, 1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, and 5.5 mM glucose, pH 7.4) in three different conditions. The cells that had been isolated in media containing EDTA were resuspended in KR containing 2.5 mM EDTA. The cells that had not been in contact with this chelator were divided into equal groups, pelleted, and resuspended in KR containing 2 mM $CaCl_2$, 25 mM $NaHCO_3$, and 0.4% fatty acid-free bovine serum albumin (Sigma), a medium classically used to stimulate sperm capacitation (18) or KR not containing these three factors. The three groups of cells were incubated for 2 h at 37 °C under a 5% CO_2, 95% air atmosphere. After incubations, aliquots were fixed and stained with Coomassie Blue for assessment of acrosomal status as described by Larson and Miller (19). Sperm counting was carried out using a Neubauer hemocytometric chamber.

Lipid Separation—Most of the solvents used in this work were high pressure liquid chromatography grade (JT Baker; UVE, Dorwill, Argentina). After preparation of lipid extracts, centrifugation, and partition (20), the organic phases containing the lipids were recovered, and the solvents were evaporated under N_2. The aliquots were taken for measuring total lipid phosphorus content in samples (21). For analysis of phospholipid class composition, aliquots of the extracts were spotted on high performance TLC plates (Merck) and resolved using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by volume), after presaturating the plates with the solvent vapors for 15 min (22). After separation, the phospholipid spots were located with the aid of iodine vapors, scraped from the plates, and quantified by phosphorus analysis (21).

To isolate Cer for fatty acid analysis, lipid extracts from spermatozoa or their parts were spotted (as bands) on TLC plates (500 μm, silica gel G) under N_2, along with commercial standards (Sigma). Ceramides were resolved using chloroform/methanol/ammonia/water (90:10:05:0.5, by volume) (3) as solvent. This solvent was run up to the middle of the plates, dried under N_2, and followed by a run up to the top of the plates with hexane/ether (80:20, by volume), to separate other neutral lipids (not described here). The silica support containing the total polar lipids remaining at the origin of these plates was collected for preparative isolation of SM. The PL were eluted by three successive washings of this lipid-laden silica with chloroform/methanol/water (1:1:0.2, by volume). The eluates were collected, and the solvents then were partitioned by the addition of 0.8 volumes of water. The organic phases were dried under N_2 and subjected to TLC to isolate SM, using chloroform: methanol:acetic acid:0.15 M NaCl (50:25:8:2.5, by volume) (23) as solvent, in this case without presaturating the tank atmosphere with the solvent vapors.

Lipid bands were located under ultraviolet light after spraying the TLC plates with 2′,7′-dichlorofluorescein in methanol, exposing them to NH_3 vapors in a closed tank, and then spraying with water. The zones containing SM and Cer were scraped into tubes for further elution, performed as detailed above. The eluted SM and Cer were taken to dryness and treated (under N_2) with 0.5 N NaOH in anhydrous methanol at 50 °C for 10 min to remove any potential lipid contaminant with ester-bound fatty acids. After alkaline treatment, chloroform and 0.5 N HCl were added to the methanol, the organic phase was rapidly recovered and dried, and the lipids were separated again by TLC.

Fatty Acid Analysis—The fatty acid composition of SM and Cer was determined by gas-liquid chromatography of their fatty acid methyl ester derivatives. These were prepared by warming the dry lipid samples and placing them overnight at 45 °C in Teflon®-lined screw capped tubes in the presence of 0.5 N H_2SO_4 in anhydrous methanol (24) (under N_2). Methyl heneicosanoate was added as an internal standard for quantitative analysis (2 μg/sample to measure Cer fatty acids). Before GC, the fatty acid methyl ester samples with the internal standard were routinely purified by TLC (using hexane/ether, 95:5, by volume) on silica gel G plates that had been previously washed (with methanol/ethyl ether, 75:25, by volume). The methyl esters were located after spraying the plates with 2′,7′-dichlorofluorescein, scraped into tubes, and recovered after thoroughly mixing the silica support with methanol: water:hexane (1:1:1, by volume), carrying out three successive hexane extractions.

The VLCPUFA of SM and Cer were identified by procedures and criteria detailed in previous work, including GC-MS. The VLCPUFA of ram spermatozoa have been characterized in detail by GC-MS by Poulos *et al.* (1). The major VLCPUFA of bull SM and Cer were the same as those characterized in ram and were also identical to the VLCPUFA previously characterized by GC-MS in bovine retina rod outer segment phosphatidylcholine (25). Those of rat spermatozoa were the same as those previously identified by GC-MS in the SM of rat testis (2) and in SM and Cer of rat seminiferous tubules (3).

For fatty acid composition analysis, a Varian 3700 gas chromatograph and a Varian Star Chromatography Work station (version 4.51) were used. The instrument was equipped with two (2 mm × 2 m) glass columns packed with 15% SP 2330 on Chromosorb WAW 100/120 (Supelco, Inc. CA) and two flame ionization detectors, operated in the dual-differential mode. The column oven temperature was programmed from 150 to 230 °C at a rate of 5 °C/min. This final temperature was then held constant for 30 min (rat) to 50 min (bull and ram) per run to allow VLCPUFA enough time to emerge from the column. Injector and detector temperatures were 220 and 230 °C, respectively, and N_2 (30 ml/min) was the carrier gas.

The fatty acid compositions of SM and Cer are expressed on a weight percentage basis in accordance with the described procedures. SM and Cer amounts are expressed as the sum of their respective fatty acid amounts, with no corrections for the presence of the sphingoid base in the two lipids or of phosphorylcholine in SM. SM/Cer ratios were calculated by dividing the sums of their respective total fatty acids (proportional to mole ratios). To simplify the presentation of the fatty acid data in the figures, the acyl groups of SM and Cer are grouped into saturated, monoenoic, dienoic, and very long-chain polyenoic fatty acids and appear as their sums.

TABLE 1

Fatty acid composition of sphingomyelin and ceramide from testis and spermatozoa

The results are expressed as percentages of the total fatty acids (% wild type). VLCPUFA, sum of polyunsaturated fatty acids with 24–34 carbon atoms.

Fatty acid	Bull testis		Bull spermatozoa		Ram spermatozoa	
	SM	Cer	SM	Cer	SM	Cer
14:0	0.65 ± 0.09	0.37 ± 0.09	0.21 ± 0.05	0.27 ± 0.24	0.40 ± 0.01	0.44 ± 0.05
14:1	0.12 ± 0.03	0.07 ± 0.07	0.06 ± 0.01	0.08 ± 0.03	0.15	0.08 ± 0.04
15:0	0.35 ± 0.07	0.52 ± 0.20	0.09 ± 0.03	0.07 ± 0.02	0.20 ± 0.01	0.29 ± 0.15
15:1	0.10 ± 0.03	0.08 ± 0.11	0.03 ± 0.02	0.01 ± 0.01	0.09 ± 0.01	0.16 ± 0.06
16:0	40.55 ± 2.32	22.42 ± 2.86	10.23 ± 1.61	5.55 ± 0.80	14.36 ± 0.28	4.28 ± 1.03
16:1	0.55 ± 0.39	1.45 ± 0.18	0.26 ± 0.13	0.34 ± 0.08	0.76 ± 0.06	1.35 ± 0.30
17:0	1.56 ± 0.16	0.97 ± 0.08	0.18 ± 0.03	0.14 ± 0.07	0.31 ± 0.04	0.26 ± 0.06
17:1	0.10 ± 0.06	0.55 ± 0.37	0.04 ± 0.02	0.11 ± 0.09	0.15 ± 0.03	0.17 ± 0.06
18:0	7.96 ± 0.63	9.45 ± 1.32	1.51 ± 0.31	6.28 ± 1.98	5.11 ± 0.03	3.76 ± 1.61
18:1	0.71 ± 0.41	4.67 ± 0.92	0.21 ± 0.10	0.49 ± 0.08	1.15 ± 0.03	1.64 ± 0.14
19:0	0.34 ± 0.08	0.55 ± 0.70	0.05 ± 0.03	0.09 ± 0.04	0.16 ± 0.01	0.16 ± 0.01
18:2	0.17 ± 0.13	1.78 ± 2.36	0.03 ± 0.02	0.08 ± 0.06	0.44 ± 0.02	0.40 ± 0.12
20:0	1.02 ± 0.13	0.98 ± 0.96	0.61 ± 0.20	1.80 ± 0.11	3.39 ± 0.04	2.57 ± 1.07
20:1	0.04 ± 0.03	0.34 ± 0.15	0.02 ± 0.01	0.04 ± 0.03	0.04	0.05 ± 0.02
21:0	0.26 ± 0.03	0.09	0.05 ± 0.02	0.05 ± 0.01	0.08 ± 0.01	0.09
22:0	6.06 ± 0.43	4.71 ± 0.32	2.63 ± 0.76	4.18 ± 0.42	2.02 ± 0.01	1.19 ± 0.25
22:1	0.44 ± 0.04	0.46 ± 0.27	0.16 ± 0.04	0.40 ± 0.15		0.18 ± 0.03
23:0	2.91 ± 0.23	2.46 ± 0.28	0.45 ± 0.10	0.46 ± 0.14	0.09	0.11
23:1	0.94 ± 0.13	1.57 ± 0.50		0.24 ± 0.05	0.07	0.06
24:0	9.20 ± 0.49	11.63 ± 1.73	2.02 ± 0.69	1.76 ± 0.45	0.48 ± 0.02	0.62 ± 0.01
24:1	16.11 ± 2.08	16.62 ± 3.14	1.88 ± 0.39	2.71 ± 0.75	1.08 ± 0.05	0.64 ± 0.02
24:2	0.30 ± 0.04	0.34 ± 0.34	1.10 ± 0.17	2.03 ± 0.48	0.10 ± 0.03	0.06 ± 0.01
26:0	1.31 ± 0.31	1.04 ± 0.43	0.88 ± 0.06	0.57 ± 0.07	0.14 ± 0.05	0.05 ± 0.04
24:4n-6	0.35 ± 0.06	0.41 ± 0.36	0.20 ± 0.05			0.01
24:5n-6	0.69 ± 0.12	0.81 ± 0.39	0.22 ± 0.06	0.22 ± 0.08	0.02	0.04 ± 0.02
24:5n-3	0.15 ± 0.05	0.20 ± 0.27	0.24 ± 0.08	0.11 ± 0.11		0.01
26:3 + 24:6	0.25 ± 0.13	0.52 ± 0.44	3.59 ± 0.36	0.09 ± 0.04	0.20	0.24
26:4n-6	0.14 ± 0.14		0.28 ± 0.05	5.16 ± 0.22		0.07 ± 0.01
26:5n-6	0.24 ± 0.09	0.14	0.36 ± 0.08	0.33 ± 0.09	0.27 ± 0.09	0.20 ± 0.10
26:5n-3	0.00 ± 0.00	0.48 ± 0.03	0.03 ± 0.03			
28:3 + 26:6	0.03 ± 0.01	0.01 ± 0.04	0.91 ± 0.05	0.51 ± 0.07	0.02	0.01
28:4n-6	0.87 ± 0.21	1.95 ± 0.27	18.79 ± 0.37	24.67 ± 0.60	2.05 ± 0.04	2.91 ± 0.23
28:5n-6	0.08 ± 0.05	0.12 ± 0.13			0.10 ± 0.01	0.15 ± 0.08
28:5n-3	0.30 ± 0.14	0.47 ± 0.13	1.17 ± 0.18	1.71 ± 0.23	1.71 ± 0.06	1.92 ± 0.50
28:6n-3	0.01 ± 0.00	0.04 ± 0.06	0.09 ± 0.05			
30:4n-6	0.24 ± 0.09	0.44 ± 0.23	13.84 ± 1.92	8.86 ± 0.72	0.78 ± 0.01	0.83 ± 0.05
30:5n-6	0.02 ± 0.01	0.19 ± 0.09	3.42 ± 0.74	3.53 ± 0.81	0.07 ± 0.01	0.14 ± 0.16
30:5n-3	0.09 ± 0.05	0.19 ± 0.10	0.95 ± 0.18	0.60 ± 0.22	0.72 ± 0.01	0.71 ± 0.08
30:6n-3	1.30 ± 0.14	2.90 ± 0.52	4.28 ± 0.99	4.73 ± 0.99	13.56 ± 0.06	15.40 ± 1.52
32:4n-6			0.70 ± 0.18	0.17 ± 0.06		
32:5n-6	0.02 ± 0.01	0.03 ± 0.02	6.64 ± 2.03	4.54 ± 1.68	0.08 ± 0.02	0.08 ± 0.04
32:6n-3	2.75 ± 0.43	6.92 ± 0.61	16.09 ± 2.65	13.72 ± 2.84	40.62 ± 0.10	51.11 ± 2.26
34:5n-6	0.23 ± 0.32		1.88 ± 0.82	0.89 ± 0.15		
34:6n-3	0.46 ± 0.16	1.05 ± 0.14	3.59 ± 0.48	2.44 ± 0.29	9.03 ± 0.07	7.56 ± 0.12
VLCPUFA	8.23 ± 1.34	16.49 ± 2.00	77.29 ± 2.85	72.27 ± 2.90	69.23 ± 0.10	81.39 ± 4.90

RESULTS

SM and Cer of Whole Spermatozoa—After inspection of all major polar and neutral lipid classes separated by TLC, SM, and Cer were found to be the only lipids to contain VLCPUFA in spermatozoa. We unexpectedly found that VLCPUFA were *major* fatty acid components of both sphingolipids in fresh spermatozoa from adult, fertile bulls (≥70%) (Table 1). Ram spermatozoa, analyzed for comparison with bulls, had even higher percentages of VLCPUFA in the two lipids. In rams, there was a net predominance of n-3 hexaenoic VLCPUFA, the three major fatty acids of SM and Cer being 32:6n-3, 30:6n-3, and 34:6n-3 in agreement with the GC-MS tracings shown in Ref. 1. In bulls, the same VLCPUFA occurred, although a higher percentage of VLCPUFA of the n-6 series than in rams were observed.

SM amounted to 13–15% of the total PL present in bull (Fig. 1) and ram spermatozoa, thus the 70–80% VLCPUFA in this lipid class represents values ranging from 9 to 12% of the total fatty acids of bull sperm cells. Because Cer had a similarly high percentage of VLCPUFA, and the SM/Cer ratio was in the order of 2.5 for the spermatozoa whose fatty acids are shown in Table 1, the sum of SM + Cer contributed a total of ~12–15% of VLCPUFA to the *total* fatty acid weight of whole bull and ram spermatozoa, a far from negligible amount.

The percentage of SM with respect to the total PL was almost twice as large in sperm as in testis (15 and 8% of the total PL, respectively). The percentage of VLCPUFA with respect to total SM fatty acids was 8-fold larger in sperm as in testis (70 and 8%, respectively; Table 1), this concentration obviously being an important feature of epididymal maturation. Spermatozoa isolated from *caput* and *cauda* epididymi (not shown) displayed intermediate values between these extremes, indicating that the VLCPUFA-rich SM of sperm originate in the testis and that part of these lipid species are conserved in the cells as they mature in the epididymis. The fact that SM gained importance with respect to other reflects the fact that more of the latter than of the SM was lost in the process of epididymal sperm remodeling, which entails a condensation of the cell (4). Similarly, the fact that VLCPUFA concentrated in SM at sperm maturation indicates this lipid lost most of its original saturated

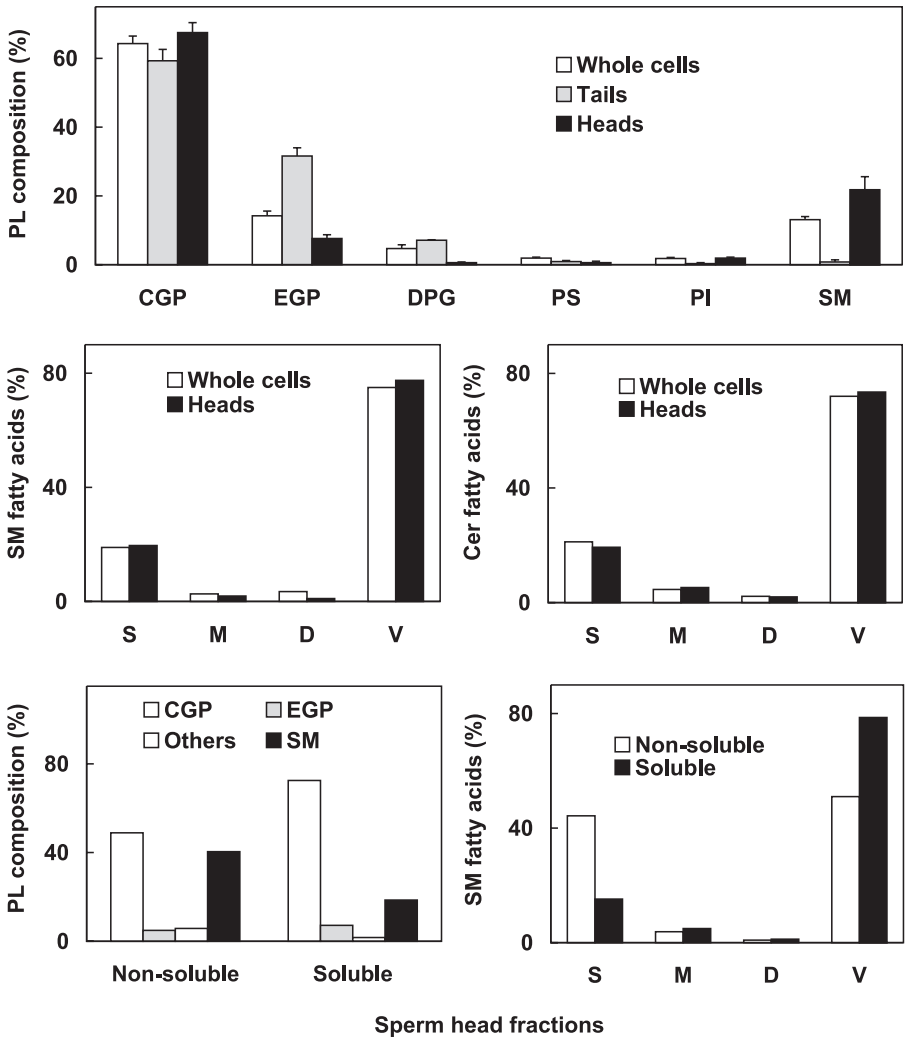

FIGURE 1. **SM, Cer, and their fatty acids in bull spermatozoa and main regions.** Sperm cells were obtained from fresh semen of fertile animals after removing the seminal plasma. Sperm heads and tails were separated by sonication followed by ultracentrifugation at 104,000 × *g* using a discontinuous sucrose gradient. *Top panel*, phospholipid composition. *Middle panels*, main groups of SM and Cer fatty acids. *Bottom panels*, phospholipid composition (*left*) and fatty acid composition of SM (*right*) of the fractions obtained by treating sperm heads with 1% Triton X-100 for 10 min at 4 °C and then centrifuging at 40,000 × *g*. *PS*, phosphatidylserine; *PI*, phosphatidylinositol; *S*, saturated fatty acids; *M*, monoenoic fatty acids; *D*, dienoic fatty acids; *V*, very long-chain polyunsaturated fatty acids.

and monoenoic fatty acids in the process. Epididymal remodeling of sperm lipid also qualitatively affected the fatty acids of SM, as suggested by the fact that 28:4n-6, from being just one of the VLCPUFA of bull testicular SM, became the major *N*-acyl group of mature bull spermatozoal SM.

The described difference between the fatty acid composition of testicular and spermatozoal SM was also observed in rat. Whereas the SM of rat testis had a series of tetraenoic and pentaenoic VLCPUFA up to 32:5n-6 (3), the SM of rat spermatozoa had much less pentaenes, with 28:4n-6 becoming the most prominent component of this group of fatty acids.

The spermatozoa of the three mammals examined had in common a significant amount of Cer with a fatty acid pattern similar to that of SM. A contrasting characteristic between large mammals and rats was that in the latter the percentage of VLCPUFA with respect to total fatty acids in SM and Cer was higher in testis (16 and 40%, respectively) (3) than in spermatozoa (Fig. 2) (~14% in both lipids). We were equally puzzled by the paucity in rat and the overabundance in bull and ram of VLCPUFA in sperm sphingolipids.

Sperm Head versus Tail SM and Cer—The phospholipid composition showed that the percentage of SM was significantly higher in the head than in the tail fraction of spermatozoa, both in bull (Fig. 1) and in rat (Fig. 2). In both cases the tail fraction had a barely detectable amount of lipid phosphorus in the zone of SM (less than 1% in the bull and 2% in rat), which explains the species-related difference described above. The spermatozoa of bulls, as in other large mammals such as the ram and the boar, is characterized by a quite massive, round-shaped head lodging a voluminous acrosome and a tail of a relatively much smaller volume. By contrast, in rat spermatozoa the weighty and very long tails markedly predominate over the really minute, hook-shaped heads, lodging a small, flat acrosome.

Other PL found to be unevenly distributed between sperm tails and heads in bulls and rats alike were the choline and ethanolamine glycerophospholipids (CGP and EGP) and diphosphatidylglycerol (DPG). In bull sperm head, SM was the second most abundant phospholipid (20%) after the major CGP, EGP being a distant third (Fig. 1). In rat sperm head, the major CGP was followed in order of importance by EGP and SM (Fig. 2).

In the tail the second most abundant phospholipid after CGP was EGP in both cases, and the third was DPG. That the latter was insignificant in the head and abundant in the tail agrees with the fact that this fraction contained the midpiece lodging the tail mitochondria.

Consistent with SM being mostly a sperm head-associated lipid, almost negligible in the tail, in bull the fatty acid composition of SM from whole sperm was very similar to that of sperm head SM, both having ≥70% of their fatty acids as VLCPUFA (Table 1 and Fig. 1). Just as with SM, most of the bull sperm VLCPUFA-rich Cer was located at the head.

In the rat, SM with high proportions of VLCPUFA was also a distinctive feature of the sperm head (Fig. 2). The small amount of SM contained in the tail was virtually devoid of VLCPUFA, having mostly saturated fatty acids. Consistently, the fatty acid composition of SM in whole rat sperm was intermediate between that of head and tail SM, constituted in this case by the sum of the 2% SM present in the

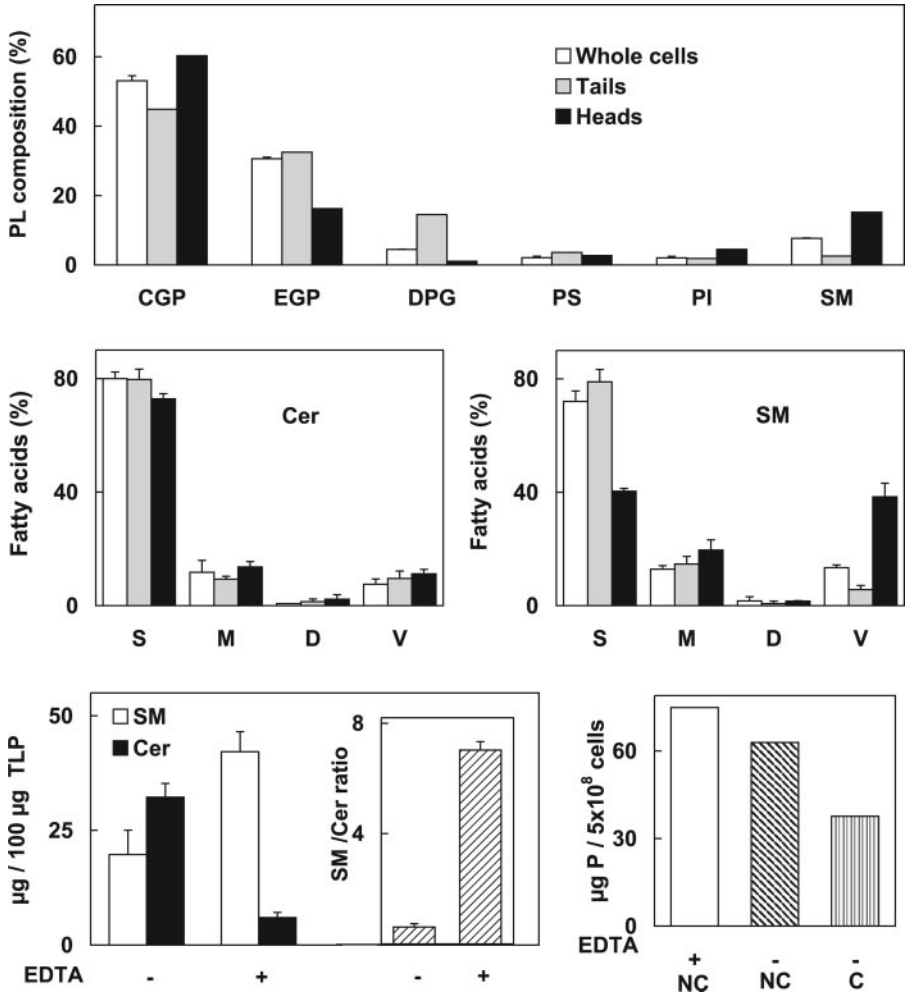

FIGURE 2. **SM, Cer, and their fatty acids in rat spermatozoa isolated from cauda epididymis and their parts.** Spermatozoa were isolated using sucrose gradients (4 °C), and the heads and tails were separated as described in the legend to Fig. 1, except that the medium contained 2.5 mM EDTA. *Top panel*, phospholipid composition; *middle panels*, main groups of Cer and SM fatty acids from spermatozoa sperm tails and sperm heads. The PL and fatty acids are abbreviated as in Fig. 1. In the *bottom panel*, the graph on the *left* compares the content of total sperm SM and Cer as obtained from epididymal spermatozoa in the absence (−) and in the presence (+) of EDTA in the media used for isolation. The *inset* shows the contrasting SM/Cer ratios that result. The *graph* on the *right* depicts the total amount of lipid phosphorus in a fixed amount of rat sperm cells after isolation and incubation in the presence of EDTA (+) and after isolation and incubation in its absence (−) in a medium containing calcium, bicarbonate, and albumin as capacitating agents (*C*) in comparison with the same medium lacking these three components (*NC*). Other details are as shown in Fig. 3.

(larger) tail PL fraction and the 15% SM present in the (smaller) head PL fraction.

Detergent Solubilization of SM Species from Bull Sperm Head—A fraction containing heads from bull spermatozoa was exposed to a hypotonic solution, the homogenate was centrifuged to separate the cytosolic fraction, and the pellet was solubilized with 1% Triton X-100 at 4 °C, a protocol expected to solubilize the plasma membrane from spermatozoa (16, 17). The presence of the detergent in the lipid extracts interfered with the analysis of Cer but did not hamper that of SM. Starting from a bull sperm head fraction containing approximately 8 mg of total phospholipid, this treatment solubilized most (83%) of this lipid. The major component of this major fraction was CGP, followed by SM (Fig. 1). Not only did the fraction of total phospholipid that remained nonsoluble (17%) differ in phospholipid composition (having relatively less CGP and more SM), but its phospholipid components differed in their fatty acid composition. The SM that remained in the nonsoluble lipid fraction was richer in saturated fatty acids and poorer in VLCPUFA than the large fraction readily extracted by the detergent, considerably richer in VLCPUFA than the former (Fig. 1).

SM/Cer Ratios—The close similarity in the fatty acid patterns of SM and Cer, whether from whole sperm or from sperm heads (Table 1 and Fig. 1), and the fact that the SM/Cer ratios were quite lower in whole spermatozoa (2.5:1) and sperm heads (3:1) than in the testis (20:1), suggested that part of the Cer of spermatozoa could have been formed from SM during isolation.

This became even more apparent in rat spermatozoa. When isolated from cauda epididymis using the same phosphate-buffered saline as that used for bull spermatozoa, rat sperm exhibited even lower SM/Cer ratios than those of bull (close to 1 and even lower, *i.e.* more Cer than SM on a molar basis, considering their amounts of fatty acids; see Fig. 2). Although this was an artifact of sperm preparation, this initial finding pointed to the important biochemical fact that the conversion SM → Cer is a highly sensitive and active reaction in rat epididymal spermatozoa, because nothing had been added to stimulate this reaction. Ascribing this to the possible activation of a cation-dependent SMase activity, we resorted to another artifact to obtain a "steady picture" of spermatozoal lipids: keeping the cells in medium containing 2.5 mM EDTA from the start.

In the presence of the metal-ion chelator, the SM/Cer ratio was severalfold larger than in the absence of EDTA in whole spermatozoa (Fig. 2), indicating that the "spontaneous" generation of Cer from SM had been successfully inhibited by this addition. This is why we decided to include EDTA in the medium used to isolate rat spermatozoa as well as in the medium used to separate sperm heads from tails for studying their lipid classes (Fig. 2). The addition of EDTA was a good resort to study lipid composition. We were able thereby to confirm, in rat, that SM was a more important component of the sperm head than of the sperm tail.

Cer Generation from SM in Conditions Favoring Sperm Capacitation and Acrosomal Reaction—Because 28:4n-6, the major and virtually the only VLCPUFA of rat spermatozoa SM

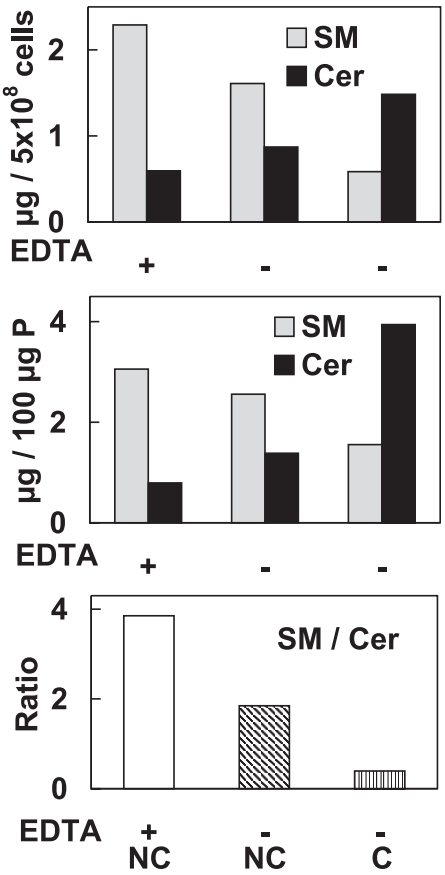

FIGURE 3. **Effect of incubation in capacitating and noncapacitating media on the amounts of rat spermatozoal 28:4n-6 *N*-acyl bound to SM and Cer.** After isolation in media containing (+) or lacking (−) EDTA, the cells were incubated for 2 h at 37 °C in a Krebs-Ringer medium containing calcium, bicarbonate, and albumin as capacitating agents (*C*), in the same medium lacking these three components (*NC*), and in the latter condition but containing EDTA. The results are expressed as µg 28:4 in SM or in Cer per a fixed amount of cells or per a fixed amount of total lipid phosphorus. *Bottom panel*, ratios between the amount of 28:4 in SM and the amount of 28:4 in Cer in the three conditions.

and Cer, was practically absent from these lipids in the tail, this fatty acid is a good marker of, and proportional to, the changes in sperm head SM and Cer. The amount per cell of sperm head-associated, 28:4-containing Cer increased, whereas that of 28:4-containing SM decreased almost equivalently, upon incubation of rat spermatozoa for 2 h in a medium containing the components required for capacitation (calcium ions, bicarbonate, and albumin) as compared with incubation in a similar medium that did not contain these three elements (Fig. 3). Staining with Coomassie Blue after incubation to determine the number of acrosome-reacted cells showed that, in the capacitated group, they amounted to 50%. Thus, the effect of incubation in a capacitation-inducing medium was to stimulate the conversion of a conspicuous proportion of the preexisting SM into Cer. On the basis of their main VLCPUFA, 28:4n-6, the conversion SM → Cer was almost like an equation, the sum 28:4 in SM + 28:4 in Cer per cell being just slightly lower in capacitating than in noncapacitating conditions.

The total amount of lipid phosphorus per cell (Fig. 2) decreased (−16%) from sperm isolated and incubated for 2 h in the presence of EDTA to cells isolated and incubated in its absence. From the latter as a control of incubation in noncapacitating conditions, a further important reduction (−40%) occurred as a result of incubation in the capacitating medium (Fig. 2). Thus, if one expresses the amount of SM and Cer fatty acids on the basis of a fixed amount of lipid P, the lower denominator enhances the changes (Fig. 3).

The SM/Cer ratio decreased from a value close to 4.0 in controls isolated and incubated in the presence of EDTA to close to 2.0 in cells isolated and incubated in the absence of EDTA, to approximately 0.4 in sperm incubated in capacitating conditions. Because Cer became the most important of the two sphingolipids, we can also say that the Cer/SM ratio increased from less than 0.25 to 0.5–2.5 in the conditions shown in Fig. 3.

We interpret these results as indicating a conspicuous stimulation by the capacitating conditions of the endogenous phospholipases of spermatozoa, including an active SMase. Interestingly, diacylglicerols were also generated, apparently to a lower extent than Cer, both accounting in part for the lipid phosphorus loss from the cells (Fig. 2). In the present conditions, neither of these two neutral lipids appeared in measurable amounts in the medium. The medium contained significant amounts of albumin-bound free fatty acids and lysophosphatidylcholine, also contributing to the reduced lipid phosphorous.

The highest, steady-state value for the SM/Cer ratio was observed in cells not incubated and maintained with EDTA throughout isolation (Fig. 2), and the lowest was observed in cells incubated for 2 h in the capacitation-stimulating medium (Fig. 3). The importance of the isolation conditions in promoting a spontaneous generation of Cer from SM is clearly apparent and may be attributed to activation of the same SMase-catalyzed reaction stimulated in capacitation.

DISCUSSION

Although we did expect to find VLCPUFA in spermatozoal sphingomyelin (1–3), we were amazed at the extremely high percentages found in bull and ram sperm SM and to find easily measurable amounts of Cer with a similar fatty acid pattern to SM. In bull, the percentage of SM with respect to other phospholipids and the percentage of VLCPUFA with respect to other fatty acids in both SM and Cer increased from testis to spermatozoa, suggesting that VLCPUFA-containing sphingolipids have a role to play in mature spermatozoa. The finding that VLCPUFA-containing SM and Cer were specifically concentrated in the sperm head, the tails having minute amounts of SM with fatty acids other than VLCPUFA, was a first indication of their physiological importance. This result disclosed a phenomenon of lateral regionalization of a lipid in the plasma membrane of two functionally distinct macrodomains not often observable in other cells. In addition, the fact that most of the SM from bull sperm heads were readily soluble at 4 °C in a widely used nonionic detergent and that these SM were richer in VLCPUFA than the ones "left behind" in the detergent-resistant residue points to a phenomenon of membrane lipid lateralization rarely observable by simple chemical analysis.

In epididymal rat spermatozoa, the modest amount of SM and the modest proportion of VLCPUFA in this lipid compared with the spectacular SM of the large mammals, and especially compared with testicular SM and Cer in rat itself (3), were at

first disappointing. Attributing this to the possible presence of other cells from the epididymal tissue in the samples, we resorted to sperm preparations of higher purity; however, the results did not change substantially. Considering the large species-related morphometric differences between the proportions of heads and tails, the fact that both of these sphingolipids are also located at the head in rat sperm explains the discrepancy. Aside from the exceptionality of their fatty acids, the studies with rat spermatozoa show that sperm head SM can be easily converted into Cer when spermatozoa are incubated in capacitation-inducing media, putting SM and Cer together, right at the spot where important reactions occur in preparation for the life-relevant events involved in oocyte fertilization.

Lipids with VLCPUFA—From the structural point of view it is interesting to note a common feature of 28:4n-6 and 32:6n-3, the major VLCPUFA of sperm SM in the different animal species analyzed here; they have a long straight saturated chain segment of 13 carbon atoms between the first carbon, involved in the amide bond, and the first double bond of the series of methylene-interrupted *cis* double bonds contained in these fatty acids toward the methyl end (14 carbons in 32:5n-6, and 15 in 34:6n-3, the longest detected in rats and large mammals, respectively). Thus, in their natural setting, the whole series of double bonds is deeply embedded in the membrane, probably introducing a significant degree of physical "disorder" toward the middle of the sperm membrane bilayer. Because the other chain in SM is sphingosine, with 18 carbons, and considering that this sphingoid base serves the dual role of interfacial backbone of SM and nonpolar hydrocarbon chain (Refs. 26 and 27 and references therein), it follows that sperm head SM species have an extreme degree of mismatch in their hydrophobic chains, both in terms of length and degree of insaturation. Intermolecular chain mismatch is a general feature of most naturally occurring SM containing long, saturated and monounsaturated *N*-acyl chains, the sperm SM species representing an extension of this natural continuum of chain lengths and double bonds.

Two structural features of SM that enable it to provide a specialized membrane environment with a tendency to form microdomains are the presence 1) at the membrane surface of functional groups capable of functioning as donors (the hydroxyl group) and acceptors (the nitrogen in the amide bond) in hydrogen bond interactions with neighboring lipids and 2) deeply embedded in the membrane, of hydrophobic chains with marked length asymmetry (26, 27). By comparing the behavior in monolayers of pure SM with medium (C12–C14), long (C16–C18), and very long (C24–C26) acyl chains, it was shown that chain length alone has an important impact on interfacial properties (26). Also in monolayers, a homologous series of saturated SM with fatty acid lengths of C14–C24 interacted more strongly with cholesterol than a similar but (n-9) monounsaturated series, except for 22:1-SM and 24:1-SM, which behaved as their saturated counterparts (28). From this, the authors concluded that in C14–C20 monoenoic species of SM, the double bond proximal to the interface interferes the interaction with cholesterol, but in 22:1-SM and 24:1-SM does not, because it is located distal to the region of the interaction (28) (*i.e.* 13 and 15 carbon atoms away from the air-water interface). Thus, if the first double bond of the acyl chain is what matters, some of the VLCPUFA-containing SM of spermatozoa including 28:4n-6 and 32:6n-3 are likely to maintain a strong interaction with cholesterol despite their high number of double bonds.

Ceramides generated from the VLCPUFA-containing SM in sperm head plasma membrane are likely to differ in physical properties from those generated in membranes of other cells. In monolayers of dimyristoylphosphatidylcholine, two species of Cer, C-16:0 and C-24:1, differed markedly in their effect on the surface potential, in their tendency to form domains, and even in the morphology of such domains, raising the possibility that Cer with different acyl groups could serve different functions in cells (29).

The rod outer segments (ros) of retina photoreceptor cells are the only other mammalian cell membranes in which a phospholipid containing VLCPUFA of similar characteristics as the ones shown here to abound in sperm SM (*e.g.* 32:6n-3) has been described (25, 30, 31). The VLCPUFA are bound to the *sn-1* position of the glycerol backbone in the dipolyunsaturated phosphatidylcholines (PC) of ros, where the *sn-2* position is mostly occupied by 22:6n-3 (31). These PCs thus also present a considerable intramolecular length mismatch in their acyl chains. This characteristic has posed many still unanswered questions and speculation about VLCPUFA-containing phospholipids, such as how these fatty acids "accommodate" in the thickness of the membrane, how they interact with integral membrane proteins, and the nature of the lipid dynamics in the membranes in which they are found (31, 32).

One of the first attempts to characterize some of the properties of VLCPUFA-containing molecular species of PC isolated from ros was to study how they affect the thermotropic behavior of liposomes of two disaturated PC, dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine, as measured by fluorescence spectroscopy (32). To arrive at a more complete, accurate, and systematic characterization, additional physical techniques should be applied to the study of the physicochemical properties of VLCPUFA-containing species of ros PCs and sperm SM. One intriguing basic question that arises is why VLCPUFA occurrence is restricted to the only two membrane phospholipids that contain a phosphoryl-choline headgroup, namely PC and SM. The answer probably lies in the large cross-sectional area these very long and highly unsaturated fatty acids impose on the lipids. In view of the large proportion of VLCPUFA-containing species in bull and ram spermatozoa SM as opposed to the low amount of the species generally expected to occur (those with C16-C24 saturates and monoenes), elucidation of the nature of lipid-protein and lipid-lipid interactions in sperm membranes calls for a revisiting of concepts on naturally occurring sphingomyelins and opens up a number of avenues for future research in which sperm SM species could be used as models.

When disks obtained from the (SM-poor) retinal ros were solubilized with two nonionic detergents, octylglucoside and Emulphogene, the detergent-soluble and nonsoluble fractions differed in phospholipid composition, the former being significantly richer in PC than the latter (33). The present results show that another nonionic detergent, Triton X-100, solubi-

lized from bull sperm heads more of the total CGP and SM than of other phospholipids, resulting in two main fractions differing in PL composition. Most interestingly, whereas the major fraction of SM readily soluble in 1% Triton X-100 at 4 °C was rich in VLCPUFA, the minor amount of this lipid that "resisted" solubilization was relatively poorer in these fatty acids and richer in saturates. Several factors may have contributed to this result in the present circumstances. First, the cool temperature *per se* may have forced the saturated molecular species of SM to coalesce laterally as a consequence of a lipid phase transition, forming lipid aggregates by lipid-lipid interactions, thereby precluding their interaction with the detergent. At the same time, most of the SM with VLCPUFA were detergent-soluble, even at 4 °C, suggesting that they were in a physical state that facilitated detergent penetration into the membrane. A second factor that may explain the presence of two populations of SM differing in fatty acids in two lipid fractions also differing in PL composition is the occurrence of separated lipid domains in different anatomical regions of the sperm head, such as the apical plasma membrane *versus* the post-acrosomal region (34). A third possibility is that the saturated SM and other phospholipids in the detergent-insoluble fraction represent in part the lipid present in sperm midpieces plus tails, perhaps remaining as minor contaminants of the head preparations. A further possibility is that the two main lipid fractions we observed with 1% Triton X-100 at 4 °C, clearly distinct in lipid and fatty acid composition, are in some way connected with the detergent-resistant and detergent-soluble membrane fractions that represent actual lipid domains (known as "lipid rafts"), believed to exist in membranes. If this were the case, our results would be consistent with most of the species of sperm head SM (*i.e.* those containing VLCPUFA) not forming part of the detergent-resistant lipid domains.

In a carefully carried out isolation of detergent-resistant membranes from retinal ros membranes, Martin *et al.* (35) showed that the PC remaining in the detergent-resistant membranes was relatively richer in disaturated species of PC (called PC1 by the authors) and poorer in other two groups of PC species containing 22:6n-3 (called PC2 and PC3 by the authors). The fatty acids of the three groups of ros PC species that tend to separate by TLC (32) have been characterized, PC2 being rich in hexaenoic species of PC and PC3 being, precisely, the group of dipolyunsaturated PCs (25, 30–32) that contain a VLCPUFA at *sn-1* and 22:6n-3 at *sn-2* in ros (31). Two interesting coincidences between the present results and those in reference (35) are 1) that in both cases the detergent removed most of the (22:6-containing) PCs and 2) that VLCPUFA-containing ros PC (35), like the present VLCPUFA-containing sperm head SM, tended to be mostly removed by the detergent or excluded from the detergent-resistant membranes. The concept of lipid rafts evolving from the study of membrane models now awaits the development of new technical tools to arrive at a better understanding of the nature of lateral heterogeneity in biological membranes (36). The present results call attention to the fact that lipids in "non-raft" domains should not be disregarded.

SM/Cer Ratio in Spermatozoa—In previous work (3) the minor ceramides of testis, considerably rich in VLCPUFA, were envisaged mostly as metabolic precursors in the *de novo* biosynthetic route leading to the major (10–20-fold larger) VLCPUFA-containing testicular SM. In spermatozoa on the other hand, the close similarity in fatty acid composition between SM and Cer, and the low SM/Cer ratios compared with testis, suggest that part of the sperm Cer could have been produced by hydrolysis of SM. A neutral SMase activity has been characterized in the plasma membrane of spermatozoa (11) that could be responsible for this SM → Cer conversion.

Cholesterol efflux from the sperm surface was reported years ago (9) and has been confirmed by many researchers to be a key process in sperm capacitation. Physiologically, capacitation is a relatively slow maturation process in which cholesterol efflux is mediated by female genital tract protein factors whose role is to act as cholesterol acceptors (37). Concomitant with cholesterol efflux, or even preceding it, something must occur in the sperm membrane itself that facilitates exposure of this lipid to its extracellular acceptors. One of the changes that precede cholesterol efflux is the trans-bilayer movement of sperm membrane phospholipids (10), although the enzyme or factors promoting such translocation have not yet been identified. Given the high affinity generally agreed to exist between SM and cholesterol in biological membranes (26) and considering that SM and CGP are naturally located in the outer leaflet of the sperm membrane (10), we propose that the exit of cholesterol from the sperm head surface could be facilitated by a decrease in the amount of SM in the membrane by its hydrolysis into Cer. This simple, one-step removal of the phosphorylcholine headgroup from SM would release cholesterol molecules from their interaction with SM, thereby facilitating exposure to, for interaction with, the extracellular cholesterol acceptor macromolecules.

Our present finding that incubation of rat spermatozoa in capacitating conditions promotes the generation of a substantial amount of Cer in parallel with an almost equivalent decrease in the original amount of SM in just 2 h provides experimental support to the hypothesis, based on the observation of a sperm head-specific SM and Cer fatty acid, that the endogenous SM → Cer conversion probably plays a role in prefertilization events such as sperm capacitation and its natural continuation, the sperm acrosomal reaction. Experimental evidence for this already exists in the literature. First, human spermatozoa respond earlier to progesterone, and more cells are spontaneously acrosome-reacted, when exposed to an exogenous SMase or to an exogenously added C6 analogue of Cer (12), and second, the acrosomal exocytosis triggered by calcium in the presence of the calcium ionophore A23187 is enhanced when boar spermatozoa are incubated with a C2 analogue of Cer or with an inhibitor of ceramidase (13).

The proposed endogenous, SMase-catalyzed generation of Cer from SM could be of equal physiological importance to the production of free fatty acids, lysophospholipids, and diacylglycerols by activation of plasma membrane phospholipases, including phospholipase C (38) and phospholipase A_2 (39). In all three cases lipid catabolytes are produced that are potential destabilizers of the sperm membrane when generated *in situ*, increasing its fluidity and instability and promoting fusion events. Recently, cholesterol efflux during capacitation has been shown to be concomitant with altered sperm membrane lipid stability and to result in lipid and protein lateral

reorganizations, in the detergent-resistant membrane domains (rafts) obtained from boar (40), human (41), and mouse (42) spermatozoa.

Since the recognition that several of the physiological actions of Cer may take place through changes in the physical properties of membranes (43–47), work from many groups has shown that ceramides tend to coalesce laterally into Cer-enriched microdomains, to induce membrane fusion, and to trigger vesicle efflux from bilayers. A great deal of research in the biophysical sciences is currently focused on this area. In liposomes containing SM, the addition of ceramides, or their generation *in situ* by the addition of SMase, was shown to promote the transbilayer (flip-flop) motion of lipids (48). Although this latter study was oriented toward understanding mechanistic aspects of apoptosis, the same could occur in sperm capacitation, because the enzyme that produces Cer from SM is present in the spermatozoal membrane (10, 12), and phospholipid scrambling is an early event in sperm capacitation (10). *Bacillus cereus* SMase added to, or incorporated in, giant liposomes made of dioleoyl-PC and SM, results in rapid lateral phase separation of ceramides (capping), vectorial budding of the bilayer, and shedding of vesicles (49). This mechanistic explanation of what probably happens in the membrane of a cell undergoing apoptosis may also apply to the acrosomal reaction in spermatozoa, both resulting in the budding of fluid-filled, membrane-enclosed vesicles. In the acrosomal reaction, the endogenous generation of the destabilizing and highly fusogenic Cer could be an important force governing the membrane fusion events taking place in the sperm head, in which the plasma membrane fuses with the outer acrosomal membrane at many points, resulting in vesiculation of the fused membrane, budding, formation of fenestrations, release of the acrosomal content, and "denudation" of the sperm head, eventually covered just by the inner acrosomal membrane.

In the present work, the fact that sperm phospholipids were lost under incubation conditions favoring capacitation may be due to 1) the activation of phospholipases, including SMase, during capacitation and 2) the release of small vesicles from the membrane to the medium by those capacitated cells that had undergone the acrosome reaction. Coincidentally, an acrosome-like reaction induced by 2 h of incubation of cauda epididymal boar sperm with only albumin decreases the percentage of SM more than other PL in the plasma membrane isolated from these cells (50). Furthermore, the vesicles released to the medium from such acrosome-reacted sperm are richer in SM than the PL of the plasma membrane isolated from (whole) cells (51). This is consistent with the present results, taking into account that the (total) plasma membrane of spermatozoa is composed of the region overlying the head, concentrating most of the SM, and the region covering the tail, which virtually lacks SM.

Spontaneous Cer Generation in Sperm—The fact that ejaculated spermatozoa from bulls and rams and in particular the spermatozoa from rat epididymal cauda already had relatively low SM/Cer ratios in the starting material is consistent with the interpretation that a certain degree of sperm activation may have been stimulated by the conditions used for sperm isolation. In the case of ejaculated bovine spermatozoa, simply washing at room temperature (necessary to remove the seminal plasma and its lipids) could have removed from the sperm head surface those proteins that most likely keep the SM → Cer reaction momentarily inhibited after ejaculation. Important proteins among these latter could be some of the major proteins of the seminal plasma, originating in seminal vesicles and known to coat the sperm surface noncovalently bound to phospholipids that specifically contain a phosphorylcholine headgroup (*i.e.* CGP and SM) (52). These proteins normally prevent lateral movements of phospholipids, thereby stabilizing the sperm membrane. Study of these proteins over the years (37) has shown that precisely when they are released from the sperm surface, they facilitate sperm capacitation induced by protein factors present in oviductal and follicular fluids.

In the case of rat epididymal spermatozoa, aside from the possibility of having diluted and/or removed proteins (53) and other elements present in the intraluminal epididymal fluid whose purpose is preventing capacitation from occurring at an inopportune time and place, there was probably a certain amount of divalent cations (*e.g.* calcium, magnesium, and zinc) originating in the excised epididymal epithelium (*e.g.* extracellular fluids and small amounts of blood from cut capillaries) contacting the cells during their preparation as a possible cause of SMase activation. In addition, we introduced the stress of cooling the cells to 4 °C during their purification in sucrose density gradients. This low temperature may have induced lateral lipid domain separations from a (mostly) fluid membrane to a membrane containing patches of laterally segregated lipid domains. This phase separation may have allowed SM-SMase interaction in the fluid lipid domain, thereby decreasing SM and producing Cer as a new sperm membrane component. Independently of the mechanism responsible for this change, its importance lies in its permanence. Once produced, there is no way in mature spermatozoa to revert the conversion SM → Cer.

We found a way of avoiding this change in rat spermatozoa by including EDTA to inhibit any cation-dependent reaction right from the outset in the solutions used for sperm isolation. The result was a high SM/Cer ratio (Fig. 2) despite the contribution of the factors described. Washing spermatozoa rapidly after their isolation from the epididymis, thus reducing the time of contact with epididymal tissue fluids containing divalent cations (probably mostly calcium ions) improved the SM/Cer ratio (Fig. 3). We learned, from its inhibition or its prevention, that the SM → Cer conversion is highly sensitive and reactive in epididymal spermatozoa, because it was promoted by one or more of the conditions we were employing for their isolation. In view of the involvement of this conversion in sperm capacitation, we can say that our initial spermatozoa displaying a low SM/Cer ratio had partially undergone a spontaneous capacitation or a capacitation-like state, because no incubations of additions intended to induce such a reaction had been carried out. The high responsiveness of this reaction to cations was put into evidence when stimulated by incubations for 2 h at 37 °C, a condition resulting in a high percentage of acrosome-reacted cells and amounts of Cer clearly prevailing over those of SM.

Concluding Remarks—Measuring the main VLCPUFA of SM and Cer, we were able to observe changes in the proportions of

these lipids that may have implications for spermatozoal functions located at the head. Aside from the fatty acids these sphingolipids contain, of greater significance is the fact that if the SM-Cer balance is displaced toward the formation of Cer by stimulation of SM hydrolysis, consequential changes in the lipid organization and dynamics of the sperm plasma membrane may be expected. This hydrolysis may facilitate not only lateral but also transmembrane movements of other lipids, thereby promoting release of inhibitory proteins from the sperm surface, loss of cholesterol, changes in the activity of membrane-associated proteins, increases in ion permeability, and possibly many other changes. Researchers studying SM among the lipids of spermatozoa could benefit from the awareness that it has unique fatty acids and that it is easily converted into Cer. Whether the Cer generated in these cells is involved in the initiation of a chemical signaling pathway or is in itself a lipid produced to create a paramount biophysical change in the sperm plasma membrane remains to be established. Our results would appear to favor the second possibility in the case of VLCPUFA-containing SM and Cer. The uniquely long and highly unsaturated fatty acids contained in sperm head SM and Cer may be helpful markers in discerning the answer to this question.

REFERENCES

1. Poulos, A., Johnson, D. E., Beckman, K., White, I. G., and Easton, C. (1987) *Biochem. J.* **248,** 961–964
2. Robinson, B. S., Johnson, D. W., and Poulos, A. (1992) *J. Biol. Chem.* **268,** 1746–1751
3. Furland, N. E., Zanetti, S. R., Oresti, M. G., and Maldonado, E. N. (2007) *J. Biol. Chem.* **282,** 18141–18150
4. Williams, R. M., Graham, J. K., and Hammerstedt, R. H. (1991) *Biol. Reprod.* **44,** 1080–1091
5. Yaganimachi, R. (1994). in *The Physiology of Reproduction* (Knobil, E., and Neill, J. D., eds) 2nd Ed., pp. 189–317, Raven Press, New York
6. Baldi, E., Luconi, M., Bonaccorsi, M., and Forti, G. (2001) *Front. Biosci.* **5,** 110–123
7. Flesh, F. M., and Gadella, B. M. (2000) *Biochim. Biophys. Acta* **1469,** 197–235
8. Martinez, P., and Morros, A. (1996) *Front. Biosci.* **1,** 103–117
9. Davis, B. K. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 7560–7564
10. Gadella, B. M., and Harrison, R. A. P. (2000) *Development* **127,** 2407–2420
11. Hinkovska, V. T., Petkova, D. H., and Koumanov, K. S. (1987) *Biochem. Cell Biol.* **65,** 525–528
12. Cross, N. L. (2000) *Biol. Reprod.* **63,** 1129–1134
13. Murase, T., Imaeda, N., Kondoh, N., and Tsubota, T. (2004) *J. Reprod. Dev.* **50,** 667–674
14. Aveldaño, M. I., Rotstein, N. P., and Vermouth, N. T. (1992) *Biochem. J.* **283,** 235–241
15. Gitlits, V. M., Toh, B. H., Loveland, K. L., and Sentry, J. W. (2000) *Eur. J. Cell Biol.* **79,** 104–111
16. Horowitz, J. A., Toeg, H., and Orr, G. A. (1984) *J. Biol. Chem.* **259,** 832–838
17. Fisch, J. D., Behr, B., and Conti, M. (1998) *Human Reprod.* **13,** 1248–1254
18. Toyoda, Y., and Chang, M. C. (1974) *J. Reprod. Fertil.* **36,** 9–22
19. Larson, J. L., and Miller, D. J. (1999) *Mol. Reprod. Dev.* **52,** 445–449
20. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
21. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) *Lipids* **5,** 494–496
22. Holub, B. J., and Skeaff, C. M. (1987) *Methods Enzymol.* **141,** 234–422
23. Brown, E., and Subbaiah, P. (1994) *Lipids* **29,** 825–829
24. Christie, W. W. (1982) in *Lipid Analysis*, 2nd Ed., pp. 51–61, Pergamon Press, Oxford, UK
25. Aveldaño, M. I., and Sprecher, H. (1987) *J. Biol. Chem.* **262,** 1180–1186
26. Barenholz, Y. (2004) *Subcell. Biochem.* **37,** 167–215
27. Li, X., Smaby, J. M., Momsen, M. M., Brockman, H. L., and Brown, E. E. (2000) *Biophys. J.* **78,** 1921–1932
28. Ramstedt, B., and Slotte, J. P. (1999) *Biophys. J.* **76,** 908–915
29. Holopainen, J. M., Brockman, H. L., Brown, R. E., and Kinnunen, P. K. (2001) *Biophys. J.* **80,** 765–775
30. Aveldaño, M. I. (1987) *J. Biol. Chem.* **262,** 1172–1179
31. Aveldaño, M. I. (1988) *Biochemistry* **23,** 1229–1239
32. Antollini, S. S., and Aveldaño, M. I. (2002) *J. Lipid Res.* **43,** 1440–1449
33. Aveldaño, M. I. (1995) *Arch. Biochem. Biophys.* **324,** 331–343
34. Wolfe, C. A., James, P. S., Mackie, A. R., Ladha, S., and Jones, R. (1998) *J. Reprod.* **59,** 1506–1514
35. Martin, R. E., Elliott, M. H., Brush, R. S., and Anderson, E. E. (2005) *Ophtalmol. Vis. Sci.* **46,** 1147–1154
36. Jacobson, K., Mouritsen, O. G., and Anderson, R. G. (2007) *Nat. Cell Biol.* **9,** 7–14
37. Thérien, I., Bousquet, D., and Manjunath, P. (2001) *Biol. Reprod.* **65,** 41–51
38. Roldan, E. R. S., and Murase, T. M. (1996) *J. Biol. Chem.* **269,** 23583–23589
39. Roldan, E. R. S., and Vazquez, J. M. (1996) *FEBS Lett.* **396,** 227–232
40. Cross, N. L. (2004) *Biol. Reprod.* **71,** 1367–1373
41. van Gestel, R. A., Brevis, I. A., Ashton, P. R., Helms, J. B., Brouwers, J. F., and Gadella, B. M. (2005) *Mol. Hum. Reprod.* **11,** 583–590
42. Sleigh, S. B., Miranda, P. V., Plaskett, N. W., Maier, B., Lysiak, J., Scrable, H., Herr, J. C., and Visconti, P. E. (2005) *Biol. Reprod.* **73,** 721–729
43. Kolesnick, R. N., Goñi, F. M., and Alonso, A. (2000) *J. Cell. Physiol.* **184,** 285–300
44. Cremesti, A. E., Goñi, F. M., and Kolesnick, R. (2002) *FEBS Lett.* **531,** 47–53
45. Deleted in proof
46. London, M., and London E. (2004) *J. Biol. Chem.* **279,** 9997–10004
47. Yu, C., Alterman, M., and Dobrowsky, R. T. (2005) *J. Lipid Res.* **46,** 1678–1691
48. Contreras, F. X., Basanez, G., Alonso, A., Herrmann, A., and Goñi, F. M. (2005) *Biophys. J.* **88,** 348–359
49. Holopainen, J. M., Angelova, M. I., and Kinunen, P. K. J. (2000) *Biophys. J.* **78,** 830–838
50. Desnoyers, L., and Manjunath, P. (1992) *J. Biol. Chem.* **268,** 10149–10155
51. Nixon, B., MacIntyre, D. A., Mitchell, L. A., Gibbs, G. M., O'Bryan, M., and Aitken, R. J. (2006) *Biol Reprod.* **74,** 275–287
52. Nikolopoulou, M., Soucek, D. A., and Vary, J. C. (1986) *Arch. Biochem. Biophys.* **250,** 30–37
53. Nikolopoulou, M., Soucek, D. A., and Vary, J. C. (1986) *Lipids* **21,** 566–570