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Hydrolysis of phospholipid monolayers by human spermatozoa. Inhibition by male contraceptive gossypol

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Monomolecular films of phospholipid were used to study the interaction of intact human spermatozoa with model membranes. Exclusively with negatively charged phosphatidylglycerol monolayers rapid penetration of spermatozoa into the monolayer with subsequent hydrolysis of the lipid was triggered by the addition of 5 mM calcium into the medium. The results suggest the localization of a calcium-dependent phospholipase A_2 at the outer acrosomal or plasma membrane of human spermatozoa with its active site exposed to the external environment. Preincubation of the cells with 100 μM gossypol completely abolished the ability of human spermatozoa to hydrolyze or penetrate monolayers of phosphatidylglycerol. The inhibition of the phospholipase activity by gossypol may contribute to the unknown contraceptive mechanisms of this non-steroidal male antifertility agent.

Fertilization involves a sequence of several coordinated membrane fusions initiating in sperm acrosome reaction and leading eventually to the fusion of sperm and egg membranes [1]. The essential role of extracellular calcium has been established in triggering and maintaining many of the membrane fusion processes in fertilization, like sperm acrosome reaction, sperm attachment and penetration into zona pellucida and sperm-egg fusion [2,3]. Although the mechanism of the calcium-induced cell fusion is unresolved a calcium-dependent phospholipase A_2 has recently been indicated to be involved in two important membrane fusion processes, exocytosis of synaptic vesicles and membrane resealing during nerve regeneration [4,5]. Also mammalian sperm have been shown to possess phospholipase activity which is essential for the ability of the sperm to undergo the acrosome reaction and fertilization [6–9].

In this study we assayed the interaction of intact human spermatozoa with monomolecular films of phospholipids under conditions where the composition and the physical state of the lipid can be exactly controlled [10]. Motile human spermatozoa did not interact with monolayers of phosphatidylcholine, phosphatidylethanolamine or with mixed monolayers of phosphatidylcholine/cholesterol at molar ratio of 1 : 1 while surface pressure was varied from 10 to 40 mN/m, either in the absence or presence of 5 mM calcium. Instead we observed an apparently specific interaction of human spermatozoa with negatively charged phosphatidylglycerol monolayers where the presence of 5 mM calcium was mandatory

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(Fig. 1). When spermatozoa were injected under a phosphatidylglycerol monolayer at a surface pressure of 15 mN/m there was an initial increase in surface pressure reflecting the penetration of the cells into the film [11]. After a lag time of 2 min

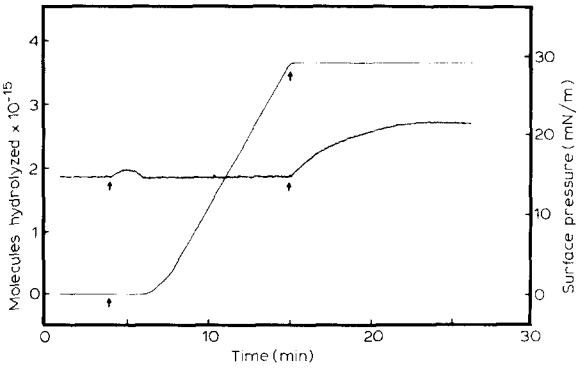

Fig. 1. Penetration and hydrolysis of monomolecular films of 1,2-didodecanoyl-*sn*-glycero-3-phosphoglycerol by human spermatozoa. Human sperm was collected from healthy human donors. Prior to use spermatozoa were washed three times with Krebs-Ringer bicarbonate buffer by repeatedly suspensing the cells in buffer followed by sedimentation at low speed centrifugation. After the washing procedure 90% of the spermatozoa remained microscopically motile. Cells were stored at 37°C and used within 6 h of isolation. The monolayer experiments were performed with a KSV 2200 Surface Barostat (KSV-Chemicals Oy, Valimotie 7, Helsinki, Finland). A key-type zero-order Teflon trough [26] was employed to measure the hydrolysis of phosphpolipid monolayers at constant surface pressure of 15 mN/m. The subphase consisted of 20 mM Tris-HCl buffer at pH 7.4 containing 0.9% (w/v) NaCl and 5 mM $CaCl_2$. The subphase of the reaction compartment was magnetically stirred at 250 rpm and thermostated to 25°C with an immersed glass coil connected to a circulating waterbath. 1,2-Didodecanoyl-*sn*-glycero-3-phosphoglycerol was prepared by phospholipase D-catalyzed transphosphatidylation [27] from 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (Sigma), and was > 98% pure by analytical high-pressure liquid chromatography. Monolayers were spread with a Agla micrometer syringe from chloroform solutions of the lipid. The reaction was started with the addition of 29 million human spermatozoa with a Hamilton microsyringe into the subphase of the reaction compartment (arrow at 3 min). The penetration of the monolayer by spermatozoa was monitored by the increase in surface pressure of the film. During subsequent hydrolysis of the monolayer the surface pressure was maintained constant at 15 mN/m by continuously replacing the lipid being hydrolyzed with new molecules from the reservoir compartment with the aid of KSV 2200 Surface Barostat. Addition of 6 mM EDTA (second arrow at 15 min) completely abolished the hydrolysis but did not affect the membrane-penetrating activity as revealed by the continuous increase in surface pressure.

typical for phospholipases, the 1,2-didodecanoyl-*sn*-glycero-3-phosphoglycerol molecules were rapidly hydrolyzed by the spermatozoa generating soluble products. The liberation of free fatty acids was verified on thin-layer chromatography. Addition of 6 mM EDTA completely abolished the hydrolysis but did not affect the membrane-penetrating activity as revealed by the continuous increase in surface pressure. The critical threshold pressure for the penetration of human spermatozoa into the phosphatidylglycerol monolayer was 22 mN/m, judged as described [11] by defining the initial surface pressure above which there is no increase in surface pressure (data not shown).

The apparently specific hydrolysis of phosphatidylglycerol was confirmed by comparing the rate of degradation of a fluorescent substrate 1-palmitoyl-2-(pyren-1-yl)hexanoyl-*sn*-glycero-phosphoglycerol to analogous compounds with phosphatidylcholine or phosphatidylethanolamine as their polar head group (Table I). The positional specificity of the enzyme for the *sn*-2 ester-bond was verified as the total hydrolysis of 1-triacontanoyl-2-(pyren-1-yl)hexanoyl-*sn*-glycero-3-phosphocholine by human spermatozoa produced only pyren-1-yl hexanoic acid and the corresponding 1-triacontanoyl-2-lyso-*sn*-glycerophosphocholine as judged by thin-layer chromatography. As the apparent specificity for the interaction of human spermatozoa with phosphatidylglycerol model membranes remains enigmatic it is interesting to note that a phospholipase A_2 specific for the hydrolysis of monolayers of phosphatidylglycerol has recently been purified from intestine by Verger et al. [12].

TABLE I

HYDROLYSIS OF DIFFERENT FLUORESCENT PHOSPHOLIPID ANALOGS BY HUMAN SPERMATOZOA

The degradation of 1-palmitoyl-2-(pyren-1-yl)hexanoyl-*sn*-glycero-3-phosphoglycerol (PPHPG) and the corresponding analogs of phosphatidylcholine (PPHPC) and phosphatidylethanolamine (PPHPE) by human spermatozoa was assayed as described in Ref. 28 using from 80 to 235 million spermatozoa per ml. The activity is presented as pmol free fatty acids produced per min per 10^6 spermatozoa.

Substrate	PPHPG	PPHPE	PPHPC
Activity	48.1	2.8	1.9

Calcium is involved in initiating the sperm acrosome reaction resulting in the exocytosis of acrosomal granules into the extracellular space within a relative long time of 2 to 4 h [3]. In our control experiments the preincubation of human spermatozoa for 5 min in the presence of 10 mM $CaCl_2$ did not increase the small amount of phospholipase A_2 released from the cells as compared to control spermatozoa (data not shown). Thus the observed momentaneous calcium-induced interaction and subsequent hydrolysis of monolayers of phosphatidylglycerol by human spermatozoa was apparently not due to liberation of a soluble phospholipase A_2 from the acrosomal granules into the medium. Rather, the results suggest the enzyme to be membrane-associated with its catalytic and calcium-binding site exposed to the membrane surface.

The mechanism of the calcium-induced membrane fusion has remained unresolved (for a review see Ref. 13). Recently a calcium-activated phospholipase A_2 has been postulated to be intimately involved in two important membrane fusion processes, resealing of a ruptured nerve membrane and exocytosis of neurotransmitter granules [4,5]. Action of a phospholipase A_2 results in a rapid production of fusogenic 1-acyl-lysophospholipids and *cis*-unsaturated fatty acids [14–16]. Particularly the conically-shaped lysophospholipids promote membrane fusion by inducing a change of the planar bilayer configuration of phospholipids into a more globular micellar shape [17,18]. The adoption by the membrane lipids of the conformation of hexagonal II (H_{II}) phase or of the intermediate lipidic particles is considered pivotal for fusion of biological membranes [19]. Accordingly, the action of a calcium-activated phospholipase A_2 exposed to the spermatozoal membrane (outer) surface could promote the membrane fusion events during the acrosomal reaction and sperm-oocyte interaction in fertilization. The cytotoxic lysophospholipids generated by phospholipase A_2 could subsequently be removed by the action of sperm lysophospholipase [7].

Gossypol, a polyphenolic triterpene isolated from cottonseed oil, has prompted wide renewed interest since it was reported to be a promising male non-steroidal antifertility agent [20]. Apart from reports on its inhibitory effect on spermatozoal metabolism and membrane-bound enzymes [21–25], the cellular mechanism of its action is not well understood. When we preincubated human spermatozoa with 100 μM gossypol for 5 min, the ability of the cells to interact and hydrolyze monolayers of phosphatidylglycerol was completely abolished (Table II). This effect might be due to a perturbance in the sperm membrane structure induced by this highly surface active compound able to penetrate into phospholipid monolayers up to surface pressure exceeding 40 mN/m (Vainio, P., Thurén, T., Wichmann, K. and Kinnunen, P., unpublished results). The observed inhibition of the calcium-activated phospholipase A_2 of human spermatozoa by gossypol may be one means by which this non-steroidal antifertility drug for men exerts its action at the cellular level.

TABLE II

INHIBITION OF THE PHOSPHOLIPASE ACTIVITY OF HUMAN SPERMATOZOA BY GOSSYPOL

14.5 million human spermatozoa were incubated in 1 ml of Krebs-Ringer bicarbonate buffer at 25°C for 5 min in the absence and presence of 10 μM and 100 μM gossypol (2,2′-binaphthalene-8,8′-dicarboxaldehyde-1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl). Thereafter the cells were washed three times in Krebs-Ringer bicarbonate buffer. Only trace amounts of phospholipase activity were liberated into the supernatants. After the washing procedure the cells were resuspended into 1 ml of Krebs-Ringer bicarbonate buffer and assayed for hydrolysis of phosphatidylglycerol monolayers as described in the legend to Fig. 1.

Spermatozoa	Controls	Gossypol treatment	
		10 μM	100 μM
Activity remaining (% of controls)	100	23	0

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