Isolation of plasma membranes from ram spermatozoa by a two-phase polymer system

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Summary. The plasma membranes of ram spermatozoa were disrupted in a hypotonic EDTA medium and isolated by using a two-phase polymer system of dextran–polyethyleneglycol. The plasma membranes obtained were of a relatively high degree of purity (~70%) as judged by electron microscope observations and measurements of the marker enzymes alkaline phosphatase, ATPase and AMPase. The activity of succinate cytochrome C reductase, a marker of mitochondrial membranes, was very low.

Introduction

Reports in the literature of the isolation of plasma membranes from sperm cells are very limited. Morton (1968) disrupted bovine epididymal spermatozoa by using a colloid mill followed by centrifugation in a sucrose density gradient and obtained a yellow band in the 40% sucrose zone which was identified by light microscopy as cell membrane fragments possessing hexokinase activity. Zahler & Doak (1975) treated bovine spermatozoa with a hypotonic sucrose solution, homogenized and centrifuged them in a sucrose density gradient and succeeded in isolating the outer acrosomal membranes. The uppermost band of the gradient was identified as plasma membranes on the basis of its high alkaline phosphatase activity, but was strongly contaminated by cytoplasmic droplets. Goranov, Bratanov & Dimitrova (1975), in an investigation of antigen properties of plasma membranes, isolated a crude membrane fraction. Those investigations, however, were not directly connected with the isolation, purification and characterization of sperm plasma membranes. A detailed method for the isolation of plasma membranes of boar spermatozoa was described by Lunstra, Clegg & Morr (1974). The spermatozoa were fragmented by low-intensity sonification and ultracentrifugation in a discontinuous sucrose gradient. The plasma membranes were identified by light and electron microscopic characteristics. Later Esbenshade & Clegg (1976) characterized the plasma membrane proteins electrophoretically.

In all investigations listed so far, the methods employed to disrupt the cells have been physical ones such as homogenization and sonification which affect other cell structures as well, thus creating conditions for contamination of the plasma membrane preparations by membranes of other organelles. Furthermore, the technique of ultracentrifugation in sucrose density gradients requires expensive ultracentrifuges.

In the present investigation our aim was to isolate the plasma membranes of ram spermatozoa by using mild conditions of treatment.

Materials and Methods

Preparation of the two-phase polymer system. The polymer system consisted of 5.5 g dextran 500 (Fluka A.G., Busch, Switzerland) and 4.2 g polyethyleneglycol (Fluka) dissolved in 90 ml 0.1 M-phosphate buffer, pH 6.5, with constant stirring. Another 100 ml buffer were added and the mixture was transferred to a separating funnel and kept at 4°C until the system separated completely into two clear phases. The lower phase (~44 ml) and the upper phase (~56 ml) were placed in beakers and kept at 4°C.

Isolation of the membranes. The semen was obtained in February–March from rams of the Friesian breed by collection into an artificial vagina. The scheme of the isolation procedure is shown in Text-fig. 1. About 5–10 min after collection each ejaculate (0.5–1.0 ml) was centrifuged at 1200 *g* for 10 min at ambient temperature. The supernatant was discarded and the sperm sediment was suspended (to 40 times the volume of the ejaculate) in cold 2.5 mM-EDTA solution, adjusted to pH 7.4 with NaOH. The suspension was stirred in an ice bath for 30 min, centrifuged at 2600 *g* for 10 min at 4°C and the sediment (*S*₁) was preserved. The supernatant was centrifuged at 9500 *g* for 15 min at 4°C and the sediment (*S*₂) was kept while the

Text-fig. 1. Flow diagram of the isolation procedure for sperm plasma membranes.

supernatant was discarded. To each sediment (S_1 and S_2) were added 5.6 ml of the upper phase of the polymer system. After careful mixing by successive passage into and out of a pipette with a narrow orifice, 4.4 ml of the lower phase were added. The mixture was centrifuged at 6000 g for 30 min at 4°C. The membranes separated at the boundary between the two phases as a distinct layer (narrower for S_1) and the impurities sedimented. The membrane layers from the two tubes (capacity 26 ml) were withdrawn by suction using a syringe (capacity 2 ml) with a stainless-steel needle. The membrane layers were combined, suspended twice in 20 ml 2.5 mM-EDTA solution, pH 7.4, and centrifuged at 9500 g for 15 min at 4°C. The resulting sediment was suspended in 1 ml 50 mM-Tris-HCl, pH 7.4. The sediments, containing 'stripped' cells and impurities, obtained after centrifugation with the two phases were also combined and suspended in 2.5 mM-EDTA, pH 7.4, centrifuged at 9500 g for 15 min at 4°C and finally suspended in 30 ml 50 mM-Tris-HCl, pH 7.4.

Enzyme characteristics. The suspensions obtained were tested for the activities of Mg^{2+} (Na + K)-ATPase (EC 3.6.1.3), 5'-AMPase (EC 3.1.3.5) and alkaline phosphatase (EC 3.1.3.1) as markers of plasma membranes, and of succinate cytochrome C reductase (EC 1.3.99.1) as a marker of mitochondria and mitochondrial membrane contamination. The enzyme activity was related to 1 mg protein. The ATPase activity was determined on the basis of liberated inorganic phosphate (P_i). The sediment and membrane suspensions (0.05–0.2 ml) containing 10–100 µg protein depending upon the fraction (about 10 µg from the membranes and 100 µg from the sediment) were incubated for 1 h at 37°C in a medium of 50 mM-Tris-HCl, 5 mM-MgCl₂, 100 mM-NaCl, 20 mM-KCl and 5 mM-ATP-disodium salt, pH 7.4, in a final volume of 1 ml.

The activity of 5'-AMPase (5'-nucleotidase) was determined on the same principle in a medium containing a certain amount of the suspension (30–200 µg protein) in 50 mM-Tris-HCl, 10 mM-MgCl₂, 5 mM-5'-AMP, pH 7.4, in a final volume of 1 ml.

After the incubation in an ice bath for 5 min the enzyme reaction was stopped by adding 0.25 ml perchloric acid and 0.25 ml 1.0 M-KCl. The mixture was centrifuged at 2600 g for 10 min at 4°C and P_i was determined by the method of Chen, Toribara & Warner (1956).

The alkaline phosphatase was determined in a reaction mixture containing a certain amount of the suspension (100–200 µg protein) in a medium of 1 M-Tris and 15 mM *p*-nitrophenylphosphate, pH 10.2, in a final volume of 2 ml. The changes in optical density were read from a kinetic curve registered at 400 nm, 25°C, with a recording double-beam spectrophotometer (Specord UV-VIS, Carl Zeiss, Jena, GDR). The activity of succinate cytochrome C reductase was determined on the basis of changes in optical density at 550 nm resulting from the reduction of cytochrome C in a mixture of a certain amount of the suspension (0.1–0.2 ml, 100–200 µg protein, in 50 mM-Tris-HCl, pH 7.4), 5 mM-KCN, 75 mM-sodium succinate, 0.44 mM-cytochrome C and 50 mM-phosphate buffer, pH 7.4, in a final volume of 2 ml. The changes in optical density were read from a kinetic curve registering the reaction velocity at 25°C. Protein was determined with the method of Lowry, Rosebrough, Farr & Randall (1951).

Electron microscopy. The material (spermatozoa and membrane sediment) was fixed in 2.5% (w/v) glutaraldehyde in 0.1 M-phosphate buffer, pH 7.4, containing 10% (w/v) sucrose and post-fixed in 1% (w/v) OsO₄ in the same buffer for 2 h. After dehydration in absolute ethanol and embedding in epoxy resin, sections were stained with uranyl acetate, followed by lead citrate (Keyhani & Storey, 1973).

Results

The electron microscope patterns showed that sperm cells not treated with hypotonic EDTA solution were completely preserved (Pl. 1, Fig. 1). The treatment, however, stripped the spermatozoa of their membranes, without inflicting damage to the mitochondrial apparatus (Pl.

1, Fig. 2). The isolated plasma membranes were predominantly of vesicular form. Nevertheless, compact formations with a non-lamellar structure were also observed, indicating contamination with material that did not originate from the plasma membranes (Pl. 1, Fig. 3).

The determinations of the marker enzymes proved a 6–11-fold higher activity of the markers in the membrane preparation compared to the total activity of the 'homogenate' (Table 1). There was no increase in succinate cytochrome C reductase.

Table 1. Enzyme activities ($\mu\text{mol/h/mg}$ protein) in the various fractions of treated ram spermatozoa

Enzyme	No. of ejaculates	Total activity*	Sediment	Membranes	Increase of activity (x)	Yield of the enzyme (%)
$\text{Mg}^{2+}(\text{Na} + \text{K})\text{-ATPase}$	9	3.61 ± 0.56	2.9 ± 0.45	29.5 ± 4.86	8.2	22
5'-AMPase	3	1.74 ± 0.13	1.5 ± 0.11	10.3 ± 0.57	5.9	16
Alkaline phosphatase	5	0.18 ± 0.01	0.13 ± 0.01	2.0 ± 0.22	11.1	30
Cytochrome C reductase	4	1.66 ± 0.45	1.7 ± 0.34	0.35 ± 0.24	0.21	0.6
Protein (%)	7	100	97.3 ± 0.46	2.7 ± 0.46	—	—

Values are mean $\pm$ s.e.m. for the no. of ejaculates indicated.

* Estimated on the basis of the specific activity of the sediment and the membrane fraction as well as on their percentage ratio.

Discussion

These ultrastructural and enzymic studies show that the membranes obtained by this method were of a relatively high degree of purity. Although alkaline phosphatase activity could be partly due to contamination with the seminal plasma enzyme, absorbed to the plasma membranes, we feel, like Zahler & Doak (1975), that it can be used as a convenient marker. The alkaline phosphatase activity and the ATPase activity showed about 11–8-fold increase, which indicated an adequate degree of membrane purification. The succinate cytochrome C reductase activity of the membrane fraction was far weaker and that proved a minimal mitochondria and mitochondrial membrane contamination, as supported also by the morphological data. It is possible that there was contamination by outer acrosomal membranes, but a suitable marker of acrosomal membranes to indicate the degree of contamination has not yet been found. Zahler & Doak (1975) investigated an enzyme marker for outer acrosomal membranes of bovine spermatozoa and measured acrosin, hyaluronidase and D-L-fucosidase activity, but these were inappropriate because of low enzyme activity in the isolated membranes and a lack of enzyme build up in the latter. Since Srivastava, Munnell, Yang & Foley (1974) showed that hypotonic treatment of ram spermatozoa affected only minimally the outer acrosomal membranes we assumed that the isolated plasma membranes in our experiments were only slightly contaminated by acrosomal membranes. Neville (1975) estimated that if there was a 15-fold purification of a membrane marker and the activity of the mitochondrial marker in the membrane preparation was 0.3, then a purification of about 75% would be achieved. In this investigation the activity of alkaline phosphatase increased 11-fold, that of ATPase 8-fold, and that of succinate cytochrome C reductase was reduced to 20%, so it can be accepted that about 70% of the isolated fraction is composed of plasma membranes.

On the basis of the distribution of the enzyme activity of the membrane markers it can be calculated that the membrane yield is 16% in relation to the AMPase, 22% according to the ATPase activity and 30% for the alkaline phosphatase. However, we consider that the purity of the membrane fraction and the degree of the yield cannot be reasonably judged on the basis of the AMPase activity because although many investigators consider it a typical enzyme of the plasma membrane it has been established that in many cells it is also localized in the membrane

Fig. 1. Electron micrograph of an intact spermatozoon, showing the complete preservation of the plasma membrane. $\times 9000$.

Fig. 2. Electron micrograph of spermatozoa treated in EDTA hypotonic medium. The sperm membranes are stripped but the mitochondria are structurally intact. $\times 15\,000$.

Fig. 3. Electron micrograph of isolated membranes. $\times 18\,000$.

of the different cell organelles to a considerable degree (Neville, 1975). Even the activity of the other enzyme markers cannot be used as an absolute criterion of the purity and yield, because isolated plasma membranes form closed semi-permeable vesicles most often with the outer side turned inwards so that a considerable part of the membrane enzyme might remain masked (De Pierre & Karnovsky, 1973). We suggest that part of the sperm plasma membrane has remained attached to the cells because considerable marker enzyme activity was found in the sediment although such activity may also be due to membranes separated but 'caught' by the stripped cells and to the impurities produced by membranes sedimenting with the cell residues during their centrifugation in the two-phase system.

The S_1 membrane pellet is much smaller (about 5 times) than the S_2 pellet obtained in the present system and this stage can be omitted. There does not appear to be any difference between the membranes obtained in the S_1 and S_2 pellets for the ATPase activity at least.

Compared to existing methods, our method for the isolation of membranes by using a hypotonic EDTA medium for the disruption of plasma membranes and centrifugation in a two-phase polymer system has several advantages: (1) it does not require an ultracentrifuge; (2) the polymer aqueous solutions exert a stabilizing effect on biological structures because of the low surface tension of the boundary between the two phases (0.001–1.0 $\mu\text{N}/\text{cm}$ compared with 10–200 $\mu\text{N}/\text{cm}$ for non-polymers) where the distribution is selective and the cell organelles are not damaged; (3) the separation depends not only on the size of the particles and their density but primarily on their surface properties, i.e. surface area, structure, nature and charge of the groups located on the surface, which in turn increase the selectivity of the method (Albertson, 1971).

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