Biochimica et Biophysica Acta, 944 (1988) 1–12
Elsevier

BBA 74120

Sea urchin sperm head plasma membranes: characteristics and egg jelly induced Ca^{2+} and Na^+ uptake

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(Received 19 October 1987)
(Revised manuscript received 30 May 1988)

Key words: Sperm; Plasma membrane; Phospholipid composition; Ion permeability; Egg jelly; Acrosome reaction; (Sea urchin)

Sea urchin sperm respond to egg factors with changes in the ionic permeability of their plasma membrane. It has been previously shown that plasma membranes isolated preferentially from sea urchin sperm flagella respond to egg jelly increasing their Ca^{2+} and Na^+ uptake (Darszon et al. (1984) Eur. J. Biochem. 144, 515–522). However, the egg jelly induced acrosome reaction occurs in the sperm head, and there is evidence for an heterogeneous distribution of plasma membrane components within the various regions of this cell. We here report a method for purifying sperm head membranes using positively charged beads according to Jacobson ((1977) Biochim. Biophys. Acta 471, 331–335). Under the transmission electron microscope these membranes appeared homogeneous and apparently free of internal membranes. The yield of the preparation was 0.9% of the total protein in the sperm homogenate. The preparation contained less than 5% of the mitochondrial marker cytochrome oxidase, and 10% of the total DNA/mg protein. Surface labeling with ^{125}I indicated a 2.5–3-fold enrichment in specific activity of the head membranes with respect to whole sperm. The SDS band pattern and the lipid composition of this preparation were different from those of isolated flagellar membranes. Phosphatidylcholine was higher in the head membranes, while phosphatidylserine and phosphatidylethanolamine were lower. The head membranes displayed a 1.7–2.3-fold higher Ca^{2+}-ATPase activity and a 2.5-fold lower Na^+/K^+-ATPase activity, than the flagellar membranes. These results are consistent with a heterogeneous distribution of membrane components along the sea urchin sperm plasma membranes. Isolated head membranes sonicated in the presence of soybean phospholipid liposomes responded to egg jelly with a species-specific increase in Ca^{2+} and Na^+ uptake. As in whole sperm, Ca^{2+} uptake was inhibited by the Ca^{2+} channel blocker nisoldipine. A close analog of this compound, [^{3}H]nitrendipine, binds with high affinity to head membranes in a saturable, reversible manner, showing a K_d and B_{max} of 31 nM and 5.3 pmol/mg protein, respectively.

Abbreviations: SDS, sodium dodecyl sulfate; ASW, artificial sea water; 0Ca0Mg ASW, Ca^{2+}- and Mg^{2+}-free artificial sea water; PMSF, phenylmethylsulfonyl fluoride; DHP, dihydropyridine.

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Introduction

Sperm, as other cells, must maintain ionic concentration differences in order to perform essential functions and respond to environmental stimuli. In the sea urchin sperm the plasma membrane permeability to Ca^{2+}, H^+, Na^+ and K^+ plays an important role in the motility of the cell [1–3] and in the acrosome reaction [4–7]. How the transport of these ions is controlled and changed during the acrosome reaction remains unknown [8]. This later reaction is a prerequisite for fertilization and occurs within seconds of the sperm encountering a fucose rich polymer, which is the main component of the outer investment –'jelly'– of the egg [9]. The egg jelly-induced acrosome reaction triggers the exocytosis of a membrane bound vesicle (acrosome), the formation of an acrosomal tubule [10], the stimulation of cyclic nucleotide metabolism [11], and the exposure to the extracellular space of lytic enzymes [12,13], a material that binds the sperm to the egg [14,15] and new membrane.

Among the best known mechanisms which regulate internal ionic composition are the Na^+/K^+-ATPase [16], Ca^{2+}-ATPase [17] and exchangers for $Na^+:H^+$ [18], $Na^+:Ca^{2+}$ [19] and $Cl^-:HCO_3^-$ [20]. A Ca^{2+}-ATPase has been found in plasma membranes of boar sperm heads and flagella, and Na^+/K^+-ATPase activity in flagellar plasma membranes [21]. A Ca^{2+}-ATPase has been measured in plasma membrane vesicles isolated from ram spermatozoa [22]. Potassium transport, linked to a plasma membrane Na^+/K^+-ATPase has been thought to be involved in the hamster sperm acrosome reaction [23]. A $Na^+:Ca^{2+}$ exchange activity has been reported in membrane vesicles isolated from ram sperm flagella [24]. In contrast, less is known about the presence of cation-dependent ATPases in the plasma membrane of the sea urchin sperm. Recently, a Na^+/K^+-ATPase activity has been reported in a sea urchin sperm plasma membrane preparation [25], and in whole sperm of this species it has been shown that this enzyme participates in the regulation of intracellular pH [26].

In the present paper we report a method to obtain a plasma membrane fraction derived from sea urchin sperm heads. Positively charged beads were used [27] to obtain the head plasma membrane from sperm that had their flagella removed. We have compared the basic lipid and protein composition and the activity of Ca^{2+}-ATPase and the Na^+/K^+-ATPase of these membranes with those derived preferentially from the sperm flagella [28,29].

We have also studied the effect of egg jelly on Ca^{2+} and Na^+ uptake into vesicles prepared from sperm head membranes and lipids. Additionally the presence of Ca^{2+} channels in these membranes was explored assaying the sensibility of the Ca^{2+} uptake to the Ca^{2+}-channel blocker nisoldipine [30] and the binding to the membranes of its close analog [^{3}H]nitrendipine. Some of these results appeared previously in abstract form [59].

Materials and Methods

Materials

Sea urchins (*Strongylocentrotus purpuratus* and *Lytechinus pictus*) were obtained from Pacific Bio-Marine Labs. (Venice, CA) and kept in a temperature-controlled instant ocean aquarium. Spawning was induced by intracoelomic injection of 0.5 M KCl. Artificial sea water (ASW) was prepared to contain 486 mM NaCl, 10 mM KCl, 54 mM $MgCl_2$, 10 mM $CaCl_2$, 2.4 mM $NaHCO_3$ (pH 8). Ca^{2+}- and Mg^{2+}-free artificial sea water (0Ca0Mg ASW) was as above except that $CaCl_2$ and $MgCl_2$ were not added.

Materials were purchased from the following sources: ouabain, valinomycin, monensin, A23187, soybean trypsin inhibitor, Tris, PMSF, DNAase, ATP, heparin, DNA, cytochrome *c* and soybean phospholipids (lecithin IIId) (Sigma Chemical Co., St. Louis, MO); $^{45}CaCl_2$, $^{22}NaCl$ and $Na^{125}I$ (Amersham, Buckinghamshire, U.K.); [^{3}H]nitrendipine (New England Nuclear, Boston, MA); iodogen (Pierce, Rockford, IL); Dowex 50W-XZ and Affi-gel 731 (Bio-Rad Labs, Richmond, CA). All other reagents were of the highest quality commercially available.

Methods

Sperm head membrane preparation. All steps were carried out at 4°C. Dry sperm (1 ml) were washed twice by diluting them 15-fold in 0Ca0Mg ASW and centrifuging at 3000 × *g* for 10 min. The

flagella were broken off from sperm by passing the sperm suspension ten times through a 21 gauge hypodermic needle, as originally described by Gray and Drummond [31]. Subsequently the flagella were separated by centrifugation ($480 \times g$ for 5 min) and the procedure repeated with the pellet which contained heads and whole sperm.

Both supernatants were used to obtain flagellar membranes according to Cross [28] and as modified by Darszon et al. [29]. Two fractions were obtained at the end of the procedure which we have called Top and Mid membranes. Mid membranes were used in most experiments due to their higher yield.

The head suspension in 0Ca0Mg ASW (2 ml) was incubated with 2 ml Affi-gel suspension (250 mg/ml in 0Ca0Mg ASW) for 15 min. Subsequently, 15 ml of 0Ca0Mg ASW containing heparin (40 U/min) was added in order to block free cationic groups on the beads. Following a 15 min incubation, the suspension was centrifuged at $120 \times g$ for 5 min and the pellet resuspended in 15 ml Tris-HCl (pH 8) containing 0.1 mM EGTA, 0.2 mg/ml soybean trypsin inhibitor, 0.1 mM PMSF and 0.2 μg/ml DNAase (lysis medium). The suspension was vortexed every 3–4 min and after 15 min centrifuged at $120 \times g$ for 10 min. The pellet was washed twice with the lysis medium (without DNAase) resuspended in 0.25 ml of 0Ca0Mg ASW and then sonicated for 3 min in a bath sonicator (Branson Heat Systems Ultrasonics, Plainview, NY, power output 100 W). The head membrane fraction was obtained in the supernatant after centrifugation at $3000 \times g$ for 10 min. Membranes (2–2.5 mg/ml) were stored frozen at −80 °C until used. From the total sperm proteins, 0.9% was recovered on the average in the head membrane preparation.

^{125}I labeling of intact sperm. Dry sperm (1 ml) were suspended in 20 ml ASW containing 0.4 mM PMSF, 0.2 mg/ml soybean trypsin inhibitor, 0.1 mM EGTA (pH 7.6). This sperm suspension was reacted with 2 mCi of $Na^{125}I$ in a glass flask coated with 500 μg of iodogen. After 15 min, the sperm were transferred to a clean flask and washed three times with 15 ml 0Ca0Mg ASW by centrifugation ($2500 \times g$, 10 min).

ATPase activity measurements. 40–50 μg of membrane protein were incubated for 15 min at 20 °C with 150 mM NaCl, 5 mM KCl, 20 mM imidazole (pH 7.8). Thereafter, 2.5 mM ATP and 2.5 mM $MgCl_2$ were added (0.5 ml of total medium reaction) and incubation continued for 1 h. The reaction was stopped by addition of 0.1 ml of 10% trichloroacetic acid. After centrifugation of 0.1 ml of 10% trichloroacetic acid. After centrifugation, 0.1 ml of the supernatant was used to determine phosphate released by the Fiske-SubbaRow method [32]. ATPase activity was expressed as nmol of P_i/h per mg protein. The difference between the total ATPase activity and the activity determined in the presence of 4 mM ouabain represented the Na^+/K^+-ATPase. The Ca^{2+}-ATPase was measured using the same protocol, but the medium contained 2.5 mM $CaCl_2$, 2.5 mM ATP and 20 mM imidazole, at pH 7.8.

Lipid extraction and analysis. The membrane lipids were extracted according to Folch et al. [33]. The pure lipid solutions were evaporated under a stream of nitrogen and the dried samples dissolved in a known volume of benzene. Phospholipids were analyzed by thin-layer chromatography [34]. Thin-layer chromatoplates (20 × 20 cm) were coated with silica gel-ammonium sulfate (41 g silica gel G 60 + 4.5 g $(NH_4)_2SO_4$). The resolving solvent system for the phospholipid analysis was chloroform/methanol/acetic acid/water (170:40:12:10, v/v). Chromatograms were developed heating the plates in the oven at 180 °C for 30 min. Determination of lipid phosphorus was carried out with the Fiske-SubbaRow reagent [32]. Cholesterol was estimated as reported by Brodie et al. [35].

Polyacrylamide gel electrophoresis. Samples containing a final concentration of 0.625 M Tris-HCl (pH 6.8), 2% SDS and 3% mercaptoethanol were electrophoresed in slab gels (9 cm) using a 7.5–15% polyacrylamide gradient and 0.1% SDS according to Laemmli [36]. The gels were fixed and stained for protein with Coomassie blue. Molecular weight standard proteins were myosin (205000), phosphorylase B (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20000) and lactalbumin (14400). Gels which contained a radioactive sample were soaked in 1 M sodium salicylate for 30 min, dried and used for fluorography.

Electron microscopy. Head membranes were centrifuged at 100000 × *g* for 1 h and the pellet fixed for 1 h with 2.5% glutaraladehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature. Thereafter, the pellet was postfixed in 1% osmium tetroxide in the same buffer, and dehydrated exposing it to solutions with increasing concentrations of ethanol up to 100%. The preparation was embedded in epon 812, sectioned, and stained with uranyl acetate and led citrate, and observed on a Zeiss EM 10 (Zeiss Co., Ober Kochen, F.R.G.) electron microscope.

Ca^{2+} and Na^+ uptake into vesicles. Vesicles for uptake were prepared by sonicating a 1 : 1 mixture of head membranes (150–300 μl in 0Ca0Mg ASW at 2–2.5 mg protein/ml) and liposomes. The liposomes were made in 0Ca0Mg ASW from partially purified soybean phospholipids (10 mg/ml) as described by Darszon et al. [29]. The reaction mixture contained: 100 μl of vesicles for uptake, 1 μCi of $^{45}Ca^{2+}$ or $^{22}Na^+$, non radioactive Ca^{2+} (10 mM final concentration) and 0Mg ASW to adjust the final volume to 260 μl, considering the addition of either: egg jelly (4.7 μg of fucose equivalents), an equivalent volume of Mg^{2+}-free artificial sea water to the control or antibiotic (1 μl in ethanol of 0.19 mM A23187 or 1 mM monensin for $^{45}Ca^{2+}$ or $^{22}Na^+$ experiments, respectively). In some experiments valinomycin (5 μM) was present to colapse the membrane potential, possibly generated by the uptake. Triplicate columns were used, each with a 60 μl aliquot of the reaction mixture as described previously [29].

Nitrendipine binding to isolated plasma membranes. The equilibrium binding assays were carried out as follows: In a final volume of 0.175 ml, membrane protein (20–30 μg/assay) from three different membrane preparations, was incubated at 20–22°C in 10 mM Hepes, (pH 7.5), 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 mg/ml bovine serum albumin, containing the indicated concentration of [^{3}H]nitrendipine (87 Ci/mmol). Incubations were carried out for 60 min in the dark to prevent the breakdown of nitrendipine, which can occur at shorter wavelengths. The purity of nitrendipine was checked by thin-layer radiochromatography on silica gel GF. The binding reaction was stopped by centrifugation at 90000 rpm for 2 min in an airfuge (Beckman). The pellets were superficially washed with water and dissolved in 2% SDS. Samples were added to 10 ml of Tritosol and counted in a Beckman liquid scintillation counter. Corrections were made for quenching and counting efficiency. Nonspecific binding (65–80%) was determined adding 10^{-5} M unlabeled nitrendipine and was subtracted from the total binding to yield the specific binding. The assays were conducted in triplicate at each concentration. Data were plotted and constants were calculated according to Scatchard [37].

Other assays. Cytochrome *c* oxidase was measured in 100 mM KCl, 20 mM Tris-HCl (pH 7.4), in the presence of 0.02% Triton X-100 using reduced horse cytochrome *c* (9 μM) as substrate [38]. Cytochrome *c* was previously reduced with ascorbate (10 mM) and dialyzed 12 h against 100 volumes of 20 mM Tris-HCl (pH 7.4) to eliminate the excess of reductant. Oxidation of cytochrome *c* was followed at 550–540 nm in a dual wavelength Aminco DW-2a spectrophotometer using 1-cm path cells. The activity of cytochrome *c* oxidase considered was sensitive to 5 mM KCN.

The DNA content in sperm head membranes was determined by the diphenylamine method [39]. DNA from 'calf thymus' was used as a standard.

Egg jelly was prepared as previously described [29]. Protein was determined by the method of Lowry et al. [40], with bovine serum albumin as standard.

Results

Electron microscopy and protein composition of the head plasma membranes

Fig. 1 shows a representative electron micrograph of the isolated sperm head membranes (see Materials and Methods). The preparation contains mostly vesicular membranous material apparently free of mitochondrial and DNA contamination (see below for further discussion). A few microtubules araising probably from disrupted flagella could be observed. Curiously, some of the membranes formed multilayered vesicles.

The Coomassie blue-stained electrophoretic pattern of whole sperm, head membranes and mid and top flagellar membranes, is compared in Fig. 2A. The bands present in the flagellar preparations were very similar to those described by Cross

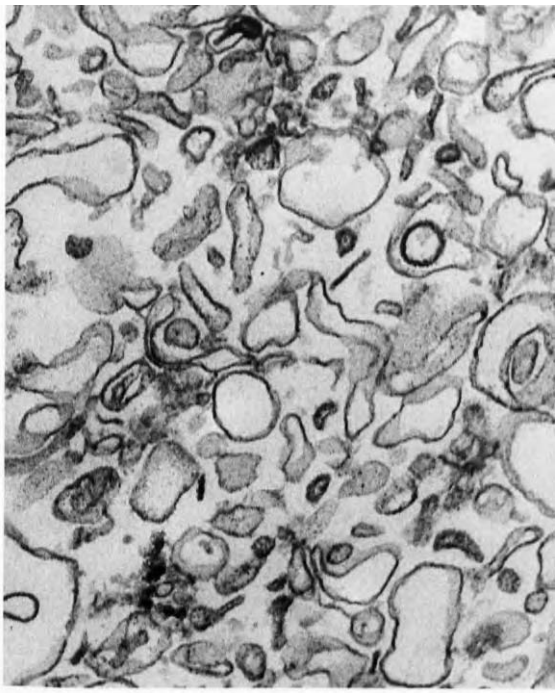

Fig. 1. Electron micrograph of thin-sectioned head plasma membranes. Membranes were pelleted by centrifugation (90000 × g) and treated for electron microscopy as described under Materials and Methods. Magnification: 31500×.

[28] and Podell and Vacquier [48]. Apparently the mid membranes are contaminated with proteins from the axoneme, tubulin (55000) mainly [28]. The tubulin band was much weaker in the top membranes indicating that they are purer. The 14 most prominent bands in the head membranes from several preparations had an apparent molecular weight of: 230000, 149000, 135000, 94000, 90000, 88000, 81000, 47000, 43000, 40000, 38000, 36000, 26000 and 24000. The high molecular weight bands (230000, 149000 and 135000), although present at a significantly lower concentration than in flagellar membranes, were reproducibly detected. The head preparation had much less than 55000 band, which is consistent with it being tubulin from the flagellar axoneme. There were five distinct bands (47000, 43000, 40000, 38000, 36000) in the 50000–36000 region, which were enriched with respect to whole sperm and not distinguishable in the flagellar membranes, with the exception of a 40000 band.

The differences between the head and flagellar membrane electrophoretic patterns could reflect heterogeneous distribution of plasma membrane proteins in the sea urchin sperm, although possible contamination of the head membranes with organelles must be considered. Less than 5% of the total sperm activity of cytochrome *c* oxidase, a membrane protein of the mitochondrial respiratory chain, was found in the head membranes. In addition, the DNA/mg protein content of this preparation was below 10% of that found in whole sperm (see Materials and Methods). Considering that the acrosome vesicle represents only 0.06% of the total sperm volume [41], these results suggest that the major bands observed in the gels of the head preparation, are proteins associated to the head plasma membrane.

Surface labelling

To further establish that our head membrane preparation was derived from the sperm head plasma membrane, sea urchin sperm were surface labeled by radio-iodination (see Materials and Methods) and head and flagellar membrane isolated from them. Fig. 2B shows a representative autoradiography of the labelled species analyzed on SDS-polyacrylamide gels. Sperm showed four major bands of labeled proteins with apparent molecular weights of 182000, 136000, 84000 and 64000. This pattern has been shown to correspond to external labeling [28]. In agreement with Cross, we found that the same protein bands appear labelled in the flagellar membranes. Although the head and flagellar membranes display different protein electrophoretic patterns, their ^{125}I surface labeling pattern was the same (Fig. 2B). The head membrane preparation showed a 2.5–3-fold increase in their ^{125}I specific activity with respect to whole sperm. These results indicate that the head membrane preparation is enriched in plasma membrane components.

Lipid composition

In order to further characterize the flagellar and head membranes their lipid composition was determined and compared. Table I shows the phospholipids present in both preparations. The top and mid membranes had a similar phospholipid composition and because of this they were

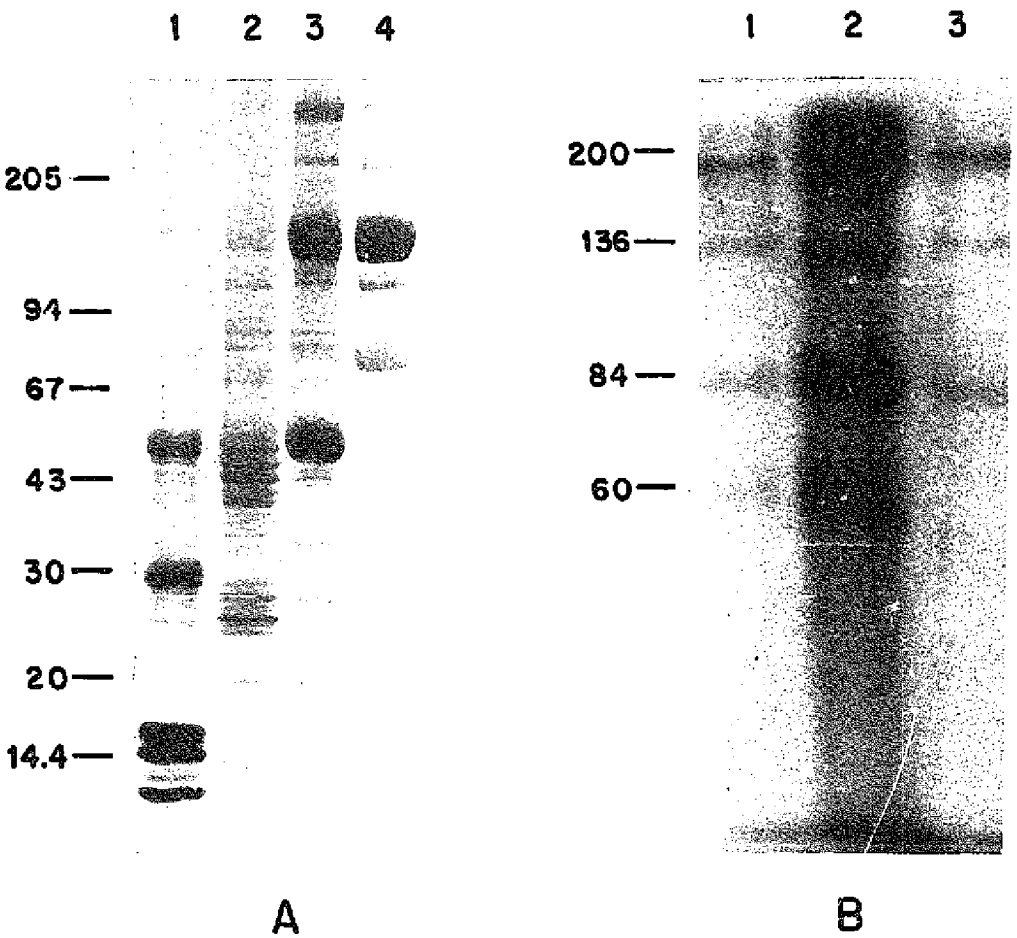

Fig. 2. Electrophoretic (A) and autoradiographic (B) profile... sperm (1A, 1B), head membranes (2A, 3B) and flagellar plasma membranes (3A Mid, 4A Top and 2B flagella). Electrophor... was performed in 7.5–15% acrylamide gels stained with Coomassie blue in A, and 7.5% acrylamide in B, with 80 μg, protein per lane. (B) The sperm were first ^{125}I labeled, then head and flagellar membranes were prepared, and the autoradiography was performed as described in Materials and Methods.

TABLE I

PHOSPHOLIPID CONTENT OF HEAD AND FLAGELLAR PLASMA MEMBRANES FROM *S. PURPURATUS* SEA URCHIN SPERM

Abbreviations are: SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; C/P, cholesterol-phospholipid ratio. Phospholipids were identified by thin-layer chromatography, using pure phospholipids as standards and measured as described in Materials and Methods. Results are expressed as the percentage (mean ± S.D., $n = 10$) of total phospholipids. Top and mid membranes had a very similar phospholipid composition and because of this were grouped and called flagellar membranes.

Membrane fraction	SM	PI	PS	PC	PE	C/P
Heads	17.7±1.7	15.3±1.6	17.3±0.8	29.3±5.6	17.9±2.4	0.30
Flagella	16.9±1.3	15.2±1.0	22.3±1.8	18.3±0.8	27.3±1.3	0.51

grouped in Table I and referred to as flagellar membranes. Differences were observed when the percentage of each phospholipid, with respect to total phospholipid, was compared in the two preparations. The head membranes contained less PS and PE and more PC, than the flagellar membranes. Cholesterol was also determined and the cholesterol to phospholipid ratio was higher in the flagellar membranes (0.51) than in the head preparation (0.3). Thus, the results illustrated in Table I, indicate a heterogeneous distribution of phospholipids and cholesterol through the sperm plasma membrane.

ATPase activity

The activity of the Ca^{2+}-, and Na^+/K^+-ATPases was determined in the head and flagellar preparations. The Na^+/K^+-ATPase has often been used as a plasma membrane marker [16]. This enzyme together with the plasma membrane Ca^{2+}-ATPase [17] plays an important role in maintaining ionic concentration differences in cells, which are crucial for physiological function. Table II shows that the top and mid flagellar membranes are 10-fold enriched in their Na^+/K^+-ATPase specific activity with respect to the whole sea urchin sperm homogenate. For reasons not understood the activity of this ATPase was variable within the 30 head preparations tested (391 ± 263 3 nmol P_i/h per mg protein), although

TABLE II

Na^+/K^+- AND Ca^{2+}-ATPase ACTIVITIES OF ISOLATED MEMBRANES FROM THE HEAD AND FLAGELLA OF *S. PURPURATUS* SEA URCHIN SPERM

The ATPase assays, as well as the preparation of membranes, were as described in Materials and Methods. The results are expressed as the mean ± S.D., and in parenthesis are the number of experiments. Similar results were obtained with *L. pictus* sperm.

Preparation	ATPase activity (nmol P_i/h per mg protein)	
	Na^+/K^+-ATPase	Ca^{2+}-ATPase
Whole sperm homogenate	84 ± 63 (12)	735 ± 312 (5)
Head membranes	[illegible] ± 263 (30)	2276 ± 1191 (5)
Mid membranes	[illegible] ± 586 (20)	793 ± 496 (6)
Top membranes	1196 ± 347 (6)	643 ± 578 (4)

the general band pattern was the same. The Na^+/K^+-ATPase activity in the head membranes was 2.5-fold less than that displayed by the flagellar membranes. On the other hand, the Ca^{2+}-ATPase was 2.8-fold lower in the flagellar than in the head membranes. In fact, the specific activities of the Ca^{2+}- and Na^+/K^+-ATPases in the head membranes were 3- and 4-fold higher than in whole sperm homogenates, as expected for a preparation which is enriched in plasma membranes. To discard possible contributions to the measured ATPase activities by the dynein ATPase from the sperm axoneme [41] and the mitochondrial ATPase, ENHA (1 mM) [43], a dynein inhibitor and oligomycin (1 μg/mg), a well known mitochondrial ATPase inhibitor were used. The Na^+/K^+- and Ca^{2+}-ATPase activities of the isolated membranes were not significantly modified by the presence of these inhibitors. These results suggest some regionalization of the Na^+/K^+- and Ca^{2+}-ATPases in the sperm plasma membrane.

Transport studies

We have shown that vesicles formed from flagellar membranes plus lipid respond to egg jelly increasing their Ca^{2+} (species specifically) and Na^+ uptake [29]. Since the acrosome reaction occurs at the sperm head, and the composition of the flagellar and head membranes is different, it seemed interesting to ask if head membrane vesicles would respond to jelly. As seen in Fig. 3, the uptake of $^{45}Ca^{2+}$ by vesicles formed from *S. purpuratus* head membranes cosonicated with soybean phosholipid liposomes (see Methods), was stimulated when egg jelly or the Ca^{2+}-ionophore A23187, were added. The antibiotic served as a control for the uptake assay and indicated that the vesicles constituted a compartment into which ions could be transported. Although the increase in Ca^{2+} uptake induced by jelly could be observed at the earliest point measured (1 min), the difference with the control was more evident at 15 min, therefore, the rest of the experiments were performed at this time. In 15 out of 16 experiments with head membranes from *S. purpuratus*, the addition of homologous egg jelly increased the Ca^{2+} associated to the vesicles, with respect to the control (0.1 ± 0.05% of total $^{45}Ca^{2+}$), by an average factor of 1.6 (99.5% confidence level, *t*-test).

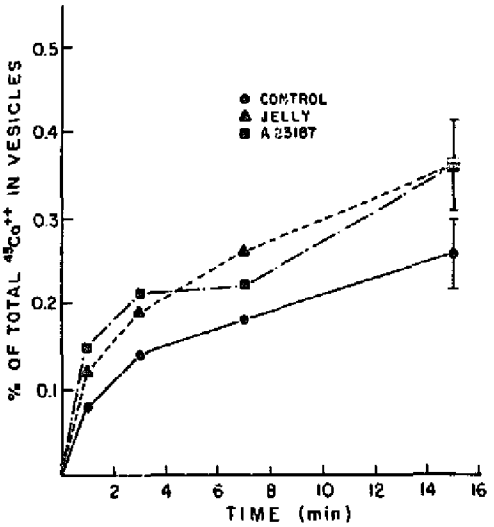

Fig. 3. Time course of $^{45}Ca^{2+}$ uptake by cosonicated *L. pictus* sperm head membranes and liposomes. $^{45}Ca^{2+}$ retention is expressed as the percentage of the total radioactivity in the reaction mixture. The assay was performed as described in Materials and Methods: adding 1 μl of a 2 mg/ml valinomcyin solution in ethanol to the reaction mixture. It was found that valinomycin stimulated $^{45}Ca^{2+}$ uptake by 15–30% in seven experiments, possibly by causing the membrane potential generated by the Ca^{2+} influx to collapse. The values are the average of at least three experiments performed in triplicate. The bars at 15 min indicate the standard deviation. (●) Control, (▲) Jelly and (■) A23187.

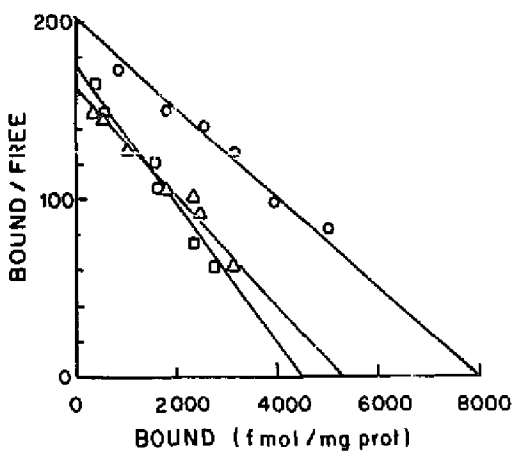

Fig. 4. Scatchard plot of [^{3}H]nitrendipine specific binding to plasma membranes of sea urchin sperm *L. pictus*. Mid (□), top (○) and head (△) membranes. Assays were carried out as described in Materials and Methods. Saturation binding experiments were carried out at different concentrations of [^{3}H]nitrendipine (2–60 nM) in at least three experiments performed in triplicate.

Previously, it was demonstrated that vesicles formed only of soybean phospholipids did not increase their Ca^{2+} or Na^+ uptake in response to egg jelly, and that jelly by itself did not carry a significant amount of Ca^{2+} or Na^+ through the columns used to determine uptake [29]. Head membrane vesicles formed from *L. pictus* sperm also displayed a statistically significant response to their homologous egg jelly increasing their Ca^{2+} content by a factor of 2.8 with respect to the control ($0.06 \pm 0.02\%$ of total Ca^{2+}, $n = 3$). Control experiments in the presence of oligomycin showed that there was no contribution of a mitochondrial minor contaminant to the jelly induced uptake of Ca^{2+} or Na^+ in the sperm head plasma membrane vesicles.

The egg jelly from *Arbacia punctulata* does not induce the acrosome reaction in *S. purpuratus* sperm [9], therefore, it was used to test the species-specificity of the jelly induced Ca^{2+} uptake in the head membrane vesicles. As shown in Table III, *Arbacia* jelly failed to induce significant Ca^{2+} uptake into these vesicles. This table also illustrates the inhibition of the jelly induced Ca^{2+} uptake caused by nisoldipine (1 μM), a potent blocker of Ca^{2+} channels [30] and of the acrosome reaction [7,25].

Since Na^+ uptake also accompanies the acrosome reaction [6], we assayed for the species-specific uptake of this ion in vesicles formed with *L. pictus* head membranes. In these experiments the antibiotic monensin, which carries out a $Na^+ : H^+$ exchange, was used to test the uptake assay (see Materials and Methods). The results in Table IV indicate that the homologous jelly induced a statistically significant increase in Na^+ uptake of these vesicles, while *Arbacia* jelly did not. Therefore, the jelly response of the vesicles formed from the sperm head membranes is similar to that of intact sperm in several respects: it involves a species-specific increase in Ca^{2+} and Na^+ uptake, and the increase in Ca^{2+} uptake is inhibited by nisoldipine.

Binding studies

Since the uptake of $^{45}Ca^{2+}$ into the head vesicles was sensitive to nitrendipine, we used [^{3}H]nisoldipine a close analog of nitrendipine, to study the presence of binding sites in our preparations. Ra-

TABLE III

SPECIES SPECIFICITY AND NISOLDIPINE SENSITIVITY OF $^{45}Ca^{2+}$ UPTAKE IN *S. PURPURATUS* HEAD VESICLES

Experiments were performed as indicated in Materials and Methods, when nisoldipine was used they were carried out in the dark using a red lamp. The values represent the mean ± S D. of the $^{45}Ca^{2+}$ retained by the vesicles, as the percentage of total radioactivity $(1–2)\cdot10^6$ cpm) applied to the column. [a] The % of $^{45}Ca^{2+}$ retained by vesicles treated with *Purpuratus* egg jelly (*P*Jelly) was higher than that of those treated with *Arbacia* jelly (*A*Jelly), or those treated with *Purpuratus* jelly plus 0.5 μM nisoldipine at the 99.5% confidence level.

$$A\text{Jelly}/C = \frac{\%\text{ of total cpm retained by vesicles plus } \textit{Arbacia} \text{ jelly}}{\%\text{ of total cpm retained by control}}$$

$$P\text{Jelly}/C = \frac{\%\text{ of total cpm retained by vesicles plus } \textit{Purpuratus} \text{ jelly}}{\%\text{ of total cpm retained by control}}$$

Arbacia jelly was kindly provided by Gary Ward and induced 90% of acrosome reaction in *Arbacia* sperm. The success index represents the number of experiments where *Purpuratus* jelly increased $^{45}Ca^{2+}$ uptake more than *Arbacia* jelly (b) or *Purpuratus* jelly plus nisoldipine (c) divided by the total number of experiments.

C	*A*Jelly	*A*Jelly/*C*	*P*Jelly	*P*Jelly/*C*	*P*Jelly + nisoldipine	A23187	*P* [a]	Success index
0.14 ± 0.08	0.14 ± 0.07	1	0.2 ± 0.11	1.43	–	0.26 ± 0.17	(*A*Jelly vs. *P*Jelly) < 0.01	8/8 [b]
0.1 ± 0.009	–	–	0.16 ± 0.02	1.6	0.08 ± 0.01	0.35 ± 0.04	(*P*Jelly vs. *P*Jelly + nisoldipine) < 0.01	4/4 [c]

diolabeled dihydropyridines (e.g. nitrendipine, nifedipine, PN200-110) have been shown to be useful for identification, localization and characterization of dihydropyridine receptors in isolated membranes [44–46]. In skeletal muscle, these receptors are localized specially in the transverse tubule [44] where the receptor has been shown to be closely related to a calcium channel [46]. Our results are consistent with the presence of receptors to [^{3}H]nitrendipine in the head and flagellar membranes. [^{3}H]Nitrendipine binds with high affinity in a saturable and reversible manner to our preparations. Scatchard analysis of the saturation isotherm revealed a single straight line in the

TABLE IV

$^{22}Na^+$ UPTAKE INTO *L. PICTUS* HEAD VESICLES

Experiments were performed as described in Methods. *C* = control (without jelly), the value is the mean ± S.D. ($n = 4$) of the $^{22}Na^+$ retained by the control vesicles, as the percentage of total radioactivity ($\approx 3\times10^5$ cpm).

$$A\text{Jelly}/C = \frac{\%\text{ of total cpm retained by vesicles plus } \textit{Arbacia} \text{ jelly}}{\%\text{ of total cpm retained by control}}$$

$$L\text{Jelly}/C = \frac{\%\text{ of total cpm retained by vesicles plus } \textit{Lytechinus} \text{ jelly}}{\%\text{ of total cpm retained by control}}$$

$$\text{Monensin}/C = \frac{\%\text{ of total cpm retained by vesicles plus monensin}}{\%\text{ of total cpm retained by control}}$$

The means *A*Jelly/*C*, *L*Jelly/*C* and Monensin/*C* were determined by calculating the ratios for each experiment. The % of $^{22}Na^+$ retained by the vesicles exposed to *Lytechinus* jelly was higher than that of those exposed to *Arbacia* jelly at the 99.5% confidence level (*t*-test). The success rate represents the number of experiments when *Lytechinus* jelly increased $^{22}Na^+$ uptake more than *Arbacia* jelly, divided by the total number of experiments.

C	*A*Jelly/*C*	*L*Jelly/*C*	Monensin/*C*	*P*(*A*Jelly vs. *L*Jelly)	Success index
0.02 ± 0.004	1.13 ± 0.18	1.4 ± 0.11	2.54 ± 1.1	< 0.05	4/4

concentration range tested, indicating the presence of a single population of binding sites. The K_d values obtained were 26, 38 and 31 nM and the B_{max} of 4.4, 7.9 and 5.3 pmol/mg protein for mid, top and head membranes, respectively. These results indicate that the distribution of nitrendipine receptors in the head and tail preparation is similar with no important differences in the dissociation constants.

Discussion

Various observations indicate that some components of the sea urchin plasma membrane are heterogeneously distributed between the head and the flagella [31,47]. Therefore it seemed useful to isolate plasma membranes from the sperm head to compare their properties and composition with those from the flagella.

Several methods have been reported to prepare plasma membranes from sea urchin sperm, but they have either utilized whole sperm, and therefore yield a mixture of membranes from the head and the flagella [48–50,25], or from the flagella [31,28,29]. Recently however, Toowicharanont and Shapiro [51], reported that membranes isolated from the head had a completely different protein composition from that of flagellar membranes. Here we report the preparation and partial characterization of a plasma membrane fraction derived from the head of sea urchin sperm using the method described by Jacobson [27] for other cells [52–55]. The sperm head suspension used as a starting material for the head membrane preparation showed under the phase-contrast microscope minimal flagellar contamination (five tails per one hundred heads). The isolated head membranes appeared relatively clean by electron microscopy. They contained less than 5% of mitochondrial contamination and almost no DNA.

The major protein bands observed in gels of flagellar membranes are present at a much lower concentration in head membranes. In contrast, the head fraction displays several bands in the lower molecular weight region (47000, 43000, 40000, 38000 and 36000) which are almost absent in the flagellar membranes. The bands that have been assigned to sperm histones are present at a very low concentration in the head membranes, indicating that most of the nuclear contamination has been removed, in agreement with the low DNA content found. Considering that the head and flagellar fractions have only minor contaminants, it can be said that the major differences in their SDS-PAGE band patterns indicate a heterogeneous protein distribution.

We can conclude that the ^{125}I-labeled bands are plasma membrane proteins since iodogen basically labels superficially exposed tyrosine groups [56]. The isolated head membranes were enriched 2.5-fold in specific ^{125}I radioactivity with respect to whole sperm, which is consistent with a purification of the head plasma membrane. It is worthwhile noting that although the bands that are ^{125}I-labeled are the same in the head and flagellar membranes, the label enrichment is higher in the latter ones (7-fold) [28]. This is what would be expected, since the four bands labeled are present at higher concentrations in the flagellar membranes.

To further substantiate the differences between the two membrane preparations we determined their phospholipid and cholesterol content. It was found that the head membranes contained less PS and PE and more PC than the flagellar membranes. On the other hand, the cholesterol phospholipid ratio was higher in the flagellar preparation than in the head membranes. In addition, the head membranes had a higher Ca^{2+}-ATPase (1.7–2.3-fold) and a lower Na^+/K^+-ATPase (0.4-fold) activity than that found in flagellar membranes. The head and tail membranes prepared by Toowicharanont and Shapiro [51] had a similar Na^+/K^+-ATPase specific activity, in contrast to our results. This may be due to the different procedures used to isolate the membranes. However, their tail membranes contained significantly more cAMP and cGMP phosphodiesterases and guanylate and adenylate cyclases than the head membranes. The Ca^{2+} channel antagonist ^{3}H-D600 bound to the two preparations with a similar K_d, although the B_{max} for the tail membranes was twice that of the head membranes. Therefore, these observations support a heterogeneous distribution of membrane components through the sperm plasma membrane. However, the possibility that some of the differences found between the head and flagellar membranes could be due to contamination of the head plasma membranes by

internal membranes (nuclear), has not been completely discarded. To rule it out, it will be necessary to determine the lipid composition and the Ca^{2+}-ATPase activity of isolated nuclear membranes from sea urchin sperm.

It has been previously demonstrated that vesicles formed with flagellar membranes take up Ca^{2+} and Na^+ in response to egg jelly, the physiological trigger of the acrosome reaction [29]. In the present work we observed that egg jelly likewise stimulated Ca^{2+} and Na^+ uptake in vesicles prepared with head membranes in a species-specific manner. Moreover, Ca^{2+} uptake by the vesicles was inhibited by nisoldipine, which prevents Ca^{2+} entry associated with the jelly induced acrosome reaction in whole sperm [7]. The stimulation of Ca^{2+} and Na^+ uptake induced by the homologous jelly in the head membranes was similar (1.4-fold) to that found in the mid flagellar membranes [29].

In addition, [^{3}H]nitrendipine, a close analog of nisoldipine, binds to the head membranes with a K_d of 31 nM and B_{max} of 5.3 pmol/mg prot. The kinetic constants found in our preparation are consistent with reports in other systems [55]. Kazazoglou et al. [25] using plasma membranes from *S. purpuratus* sea urchin sperm and [^{3}H]verapamil as ligand, reported a large number of verapamil binding sites (about 600 pmol/mg protein) and a K_d of about 1 μM, in agreement with the ID_{50} for inhibition of the acrosome reaction. In this sense, our K_d for [^{3}H]nitrendipine binding also agrees with the ID_{50} for blocking $^{45}Ca^{2+}$ uptake and the acrosome reaction found by us in *L. pictus* [7]. However, it should be pointed out that we have observed marked differences in the sensitivity to various dihydropyridines among *L. pictus* sea urchins collected at different sites. Discrepancies between ID_{50} and binding constants occur in other systems where differences of 100--1000 have been found [57,58].

It is interesting that although the Na^+/K^+- and Ca^{2+}-ATPases, as well as some of the lipids and surface antigens [47], are heterogeneously distributed among the head and the flagellar sperm plasma membrane, the nitrendipine binding sites have similar characteristics and density in the two membrane regions. This later finding is consistent with the fact that egg jelly induces similar increases in Ca^{2+} uptake into vesicles from head and flagellar membranes.

Since the mechanism by which egg jelly triggers the sperm acrosome reaction is still unknown, it is difficult to evaluate the jelly responses we have observed in vesicles containing sperm membranes. However, our results together with those found in other sperm membrane preparations [8] apparently indicate that the isolated membranes contain the essential components required for jelly to trigger an increase on Ca^{2+} and Na^+ uptake.

The possibility that the increases in Ca^{2+} and Na^+ uptake we have observed could be a consequence of a jelly induced conformational change of the receptor which alters the permeability of the vesicles or their cation binding characteristics in a nonphysiological way must be considered. On the other hand, the fact that the vesicles do respond could indicate that the jelly receptor is itself a transport system which activates when jelly binds, triggering directly or indirectly a change in Ca^{2+} and Na^+ uptake.

Using planar bilayers containing sperm plasma membrane proteins [60], the patch clamp technique [61] and membrane potential measurements [62], we have established the presence of K^+ channels in the sea urchin sperm, as well as their participation in the acrosome reaction. There is growing evidence for the presence of Ca^{2+} channels in the sea urchin sperm plasma membrane [25,51,62,63], and for the involvement of K^+ channels in the responses triggered by speract [64].

The results in this study open the possibility of separately characterizing the ionic channels present in the head and flagellar sea urchin plasma membrane and their relationship with the physiology of this cell.

Acknowledgements

We thank Dr. Carlos Arguello for his assistance with the transmission electron microscope and Mrs. Martha Montes for her secretarial help. This study was supported by grants from CONACYT, the Organization of American States, COSNET and the World Health Organization.

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