

# Isolation and Partial Characterization of the Plasma Membrane of the Sea Urchin Egg

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**ABSTRACT** The cell surface complex (Detering et al., 1977, *J. Cell Biol.* 75, 899-914) of the sea urchin egg consists of two subcellular organelles: the plasma membrane, containing associated peripheral proteins and the vitelline layer, and the cortical vesicles. We have now developed a method of isolating the plasma membrane from this complex and have undertaken its biochemical characterization. Enzymatic assays of the cell surface complex revealed the presence of a plasma membrane marker enzyme, ouabain-sensitive  $\text{Na}^+/\text{K}^+$  ATPase, as well as two cortical granule markers, proteoesterase and ovoperoxidase. After separation from the cortical vesicles and purification on a sucrose gradient, the purified plasma membranes are recovered as large sheets devoid of cortical vesicles. The purified plasma membranes are highly enriched in the  $\text{Na}^+/\text{K}^+$  ATPase but contain only very low levels of the proteoesterase and ovoperoxidase. Ultrastructurally, the purified plasma membrane is characterized as large sheets containing a "fluffy" proteinaceous layer on the external surface, which probably represent peripheral proteins, including remnants of the vitelline layer. Extraction of these membranes with KI removes these peripheral proteins and causes the membrane sheets to vesiculate. Polyacrylamide gel electrophoresis of the cell surface complex, plasma membranes, and KI-extracted membranes indicates that the plasma membrane contains five to six major protein species, as well as a large number of minor species, that are not extractable with KI. The vitelline layer and other peripheral membrane components account for a large proportion of the membrane-associated protein and are represented by at least six to seven polypeptide components. The phospholipid composition of the KI-extracted membranes is unique, being very rich in phosphatidylethanolamine and phosphatidylinositol. Cholesterol was found to be a major component of the plasma membrane. Before KI extraction, the purified plasma membranes retain the same species-specific sperm binding property that is found in the intact egg. This observation indicates that the sperm receptor mechanisms remain functional in the isolated, cortical vesicle-free membrane preparation.

The sea urchin egg has been the object of much study in the areas of molecular and developmental biology during the last several decades. Recently, this cell type has gained increasing importance in studies in cell-cell adhesion, exocytosis, and other membrane-associated events. It is clear that the sea urchin egg provides an excellent model for these studies of more general biological interest, as well as for studies related to developmental and reproductive biology per se.

Despite the interest in this cell type, the cell surface of the echinoderm egg has received little attention and is poorly understood at the biochemical level. The available information indicates that the egg is surrounded by a morphologically

"typical" plasma membrane, coated on the outer surface with a highly developed array of external peripheral proteins collectively termed the vitelline layer. The vitelline layer has been studied in some detail (9, 10, 22, 31, 32). It is known to contain glycoproteins, one or more of which may be involved in sperm-egg adhesion (9, 23, 32), and is involved in formation of the fertilization envelope after the egg is fertilized. The cytoplasmic face of the plasma membrane has associated with it arrays of specialized secretory granules, the cortical vesicles, which undergo exocytosis upon fertilization.

Earlier work in this laboratory resulted in the isolation of a cell surface complex consisting of the plasma membrane, with

its associated vitelline layer, and the cortical vesicles, which remain tightly adherent to the inner surface of the plasma membrane (6). This complex is capable of inducing the acrosome reaction in sperm and binding them species specifically (5), two processes that are essential early steps in the fertilization of the intact egg. The complex also retains the ability to generate a fertilization envelope in response to  $\text{Ca}^{2+}$  treatment, which is apparently identical to that formed by the egg upon fertilization. As part of our ongoing studies of the cell surface complex and sperm-egg adhesion (23), we have now subfractionated the cell surface complex, isolated the egg plasma membrane, and determined a number of its basic structural and biochemical features.

## MATERIALS AND METHODS

### *Animals and Gametes*

The sea urchin *Strongylocentrotus purpuratus* was obtained from Pacific Biomarine Laboratories, Venice, Calif. and maintained as previously described (6). Freshly collected eggs were suspended as a 10% vol/vol suspension and adjusted to pH 5.5 to solubilize the jelly coat. The eggs were then washed twice with 10 vol of ice-cold Ca- and Mg-free artificial seawater (seawater C; (6)), and the cell surface complex as prepared as described (6) except that a 40-ml homogenizing vessel was used to homogenize the 10% vol/vol suspension of dejellied eggs. 30 ml of the homogenate was then diluted to 120 ml with seawater and treated as described earlier (6). To reduce proteolysis, the seawater C solutions contained the protease inhibitor, Trasylol (FBA Pharmaceuticals Corp., New York), at a concentration of 10 kallikrein inactivator units/ml. After the cell surface complex was isolated, 10  $\mu\text{l}$  of diisopropylfluorophosphate (0.5 M in isopropanol) was added per 0.5 ml of suspension to inhibit the cortical vesicle protease.

### *Preparation of Plasma Membranes*

A typical preparation began with 0.5–0.8 ml of packed cell surface complex. This was suspended in 10 vol of 1.0 M sucrose adjusted to pH 8.0 with KOH and containing 10 U/ml of Trasylol. The cell surface complex was pelleted in a clinical centrifuge and then resuspended in 100 vol of this sucrose solution. The suspension was gently homogenized in a glass homogenizer with a Teflon pestle (VWR Scientific, Inc., San Francisco, Calif.) until the cortical vesicles were completely detached, as determined by phase-contrast microscopy. This procedure produced large sheets of plasma membrane, as well as smaller membrane fragments and free cortical vesicles. The membrane sheets were separated from the cortical vesicles and small membrane fragments by centrifugation in a Sorvall SS34 fixed angle rotor (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) at 5,000 rpm for 20 min at 5°C. The supernate was discarded, and the pellet, consisting largely of the membrane sheets, was resuspended in 100 vol of the sucrose solution by gentle homogenization. This suspension was again centrifuged at 5,000 rpm for 20 min. The resulting pellet, largely depleted of cortical vesicles, was then suspended in the sucrose solution and layered on a discontinuous sucrose gradient containing 6 ml of 40%, 6 ml of 60%, 8 ml of 70%, and 8 ml of 78% sucrose. After centrifugation in a Beckman SW-27 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 12–16 h at 72,000 g, the large membrane sheets were present as a single band, whereas the residual cortical vesicles were found in the pellet.

### *Extraction of Plasma Membranes with KI*

To determine which components of the plasma membrane were actually integral membrane proteins, we attempted to extract only peripheral proteins associated with the plasma membrane. The membrane sheets obtained as described above were adjusted to 30% sucrose and pelleted by centrifugation at 100,000 g for 30 min. The pellet was resuspended in 10 ml of 0.5 M KI containing 5 mM HEPES buffer, pH 7.5, mM EGTA, pH 7.5, and Trasylol, 10 U/ml. The membranes were extracted with this buffer for 3 h with gentle homogenization every 30 min. The suspension was then layered on a discontinuous gradient of 7 ml of 20%, 8 ml of 40%, 8 ml of 60%, and 6 ml of 70% sucrose. The purified membranes recovered from the sucrose gradient were diluted fivefold with 0.9% saline and centrifuged at 72,000 g for 1 h in a SW-27 rotor to pellet the membranes.

### *Analytical Procedures*

Before analysis, membrane-containing fractions were diluted fivefold with 0.9% NaCl and centrifuged at 100,000 g for 60 min. Protein was determined on

lipid-extracted or TCA-precipitated samples by two independent methods. Samples were neutralized, if necessary, with 0.1 M NaOH, then adjusted to 100  $\mu\text{l}$ , made 10% wt/vol in sodium dodecyl sulfate (SDS), and boiled for 5 min or until all material was solubilized. For protein determinations by fluorescamine assay (30) or by the assay of Lowry et al. (14), samples were adjusted to 1 ml with distilled water (final concentration of SDS, 1%). In both assays, bovine serum albumin standards were made to 1% wt/vol in SDS before use. These two assays agreed very closely in the determinations of membrane protein.

To determine membrane phospholipids, we washed membranes a second time with 0.9% NaCl before extraction. Phospholipids were then extracted by the procedure of Folch et al. (8) and stored in  $\text{CHCl}_3$  under nitrogen at  $-20^\circ\text{C}$ . Lipid phosphorus was determined by the method of Bartlett (2). Individual phospholipids were identified by thin-layer chromatography of Silica-G plates (VWR Scientific Inc., San Francisco, Calif.) using three different solvent systems: (A) chloroform:methanol:water (65:25:4); (B) chloroform:methanol:ammonia (65:25:5); and (C) a two-dimensional system using chloroform:methanol:ammonia (65:25:5) in the first dimension and chloroform:acetone:methanol:acetic acid:water (30:40:10:10:5) in the second dimension. Lipids were visualized by iodine vapor, and each spot was scraped from the plate for quantitative analysis of lipid phosphate after perchloric acid digestion (2). Individual phospholipids were identified by comparison with authentic standards, and recoveries calculated for each phospholipid standard were used to correct for losses during extraction and chromatography.

The identification of phosphatidylinositol was confirmed by gas-liquid chromatographic analysis of the inositol recovered after hydrolysis. After thin-layer chromatography, the spot containing phosphatidylinositol was recovered from the plate, and the silica gel was extracted twice with 2 ml of chloroform:methanol:water (10:10:3). The extract was dried under vacuum and hydrolyzed in 0.5 ml of 2 N HCl under nitrogen at  $100^\circ\text{C}$  for 48 h. The hydrolysate was dried under vacuum and acetylated by heating in acetic anhydride:pyridine (1:1) at  $100^\circ\text{C}$  for 1 h. The acetylated derivatives were chromatographed on a column containing 3% SP-2340, 0–200 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.). Recovery of inositol was estimated by carrying a known amount of authentic phosphatidylinositol through the entire procedure; 20% of the inositol was recovered. The ratio of inositol to  $\text{PO}_4$  in the isolated membrane lipid was found to be 0.37.

Total cholesterol in the lipid extract was determined after saponification as described by Dittmer and Wells (7).

### *Enzyme Assays*

Ouabain-sensitive sodium-potassium ATPase activity was determined under the following conditions: 150 mM NaCl, 50 mM HEPES buffer, pH 7.2, 20 mM KCl, 3 mM  $\text{MgCl}_2$ , 3 mM ATP, with or without 1 mM ouabain, at  $25^\circ\text{C}$ .  $\text{P}_i$  released was determined by the method of Marsh (15). Peroxidase activity was measured by the method of Herzog and Fahimi (10). Proteoesterase activity was measured as described earlier (6) using [ $^3\text{H}$ ]-*n*-tosyl-L-arginine methyl ester ([ $^3\text{H}$ ]TAME) (Amersham Corp., Arlington Heights, Ill.) as a substrate. Preliminary experiments were performed to ensure that each enzyme activity was measured under conditions where product formation was linearly dependent on enzyme concentration.

### *SDS Polyacrylamide Gel Electrophoresis*

Washed membranes were precipitated with ice-cold 10% TCA. The pellet was neutralized with 0.1 M NaOH and extracted twice with 5 ml of diethyl ether. The sample was then solubilized and electrophoresed according to the discontinuous system of Laemmli (13). A 3% acrylamide stacking gel was used, and the running gel was poured as a linear gradient from 5 to 20% acrylamide. After electrophoresis, the gel was fixed in 10% TCA and stained with Coomassie Blue or by the periodic acid-Schiff technique.

### *Electron Microscopy and Sperm-binding Assay*

The membrane pellets were prepared for electron microscopy as previously described (6).

To test the ability of the plasma membrane sheets and KI-extracted membranes to bind sperm, we prepared membrane sheets as described above except that the treatment with diisopropyl fluorophosphate was omitted. After purification on a sucrose gradient, half of the sample was extracted with KI as described above. Both the purified plasma membranes and the extracted membranes were washed with 40 ml of 0.9% NaCl and resuspended in artificial seawater buffered with 10 mM Tris-HCl at pH 8.0 (TBS).

Sperm (200  $\mu\text{g}$  of sperm protein) were diluted to 0.5 ml with TBS containing homologous egg jelly (200 nmol fucose/ml) for 30 s to induce the acrosome reaction (12), or with TBS as a control. Either 20  $\mu\text{g}$  of the control (unreacted) sperm or of the egg jelly-treated sperm was then added to a suspension of plasma membranes (7–10  $\mu\text{g}$  protein) and allowed to bind for 30 s. At the end of this

time, the reaction was stopped by addition of a few drops of 6% glutaraldehyde in artificial seawater.

## RESULTS

### Isolation of the Egg Plasma Membrane

The cell surface complex (Fig. 1*a*) is composed of two subcellular organelles; the plasma membrane with its associated vitelline layer and the cortical vesicles. Initial attempts to separate the cortical vesicles by exposure to various conditions of pH, high ionic strength, chaotropic agents such as KI or KBr, and urea met with limited success. Whereas some of these conditions could release up to 50% of the cortical vesicles, none were effective enough to be used in isolation of the plasma membrane. However, when the cell surface complex was suspended in 1 M sucrose under conditions resulting in a 1,000fold dilution of the ions originally present, virtually all of the cortical vesicles were released by gentle homogenization. After this treatment, the plasma membranes were separated from the

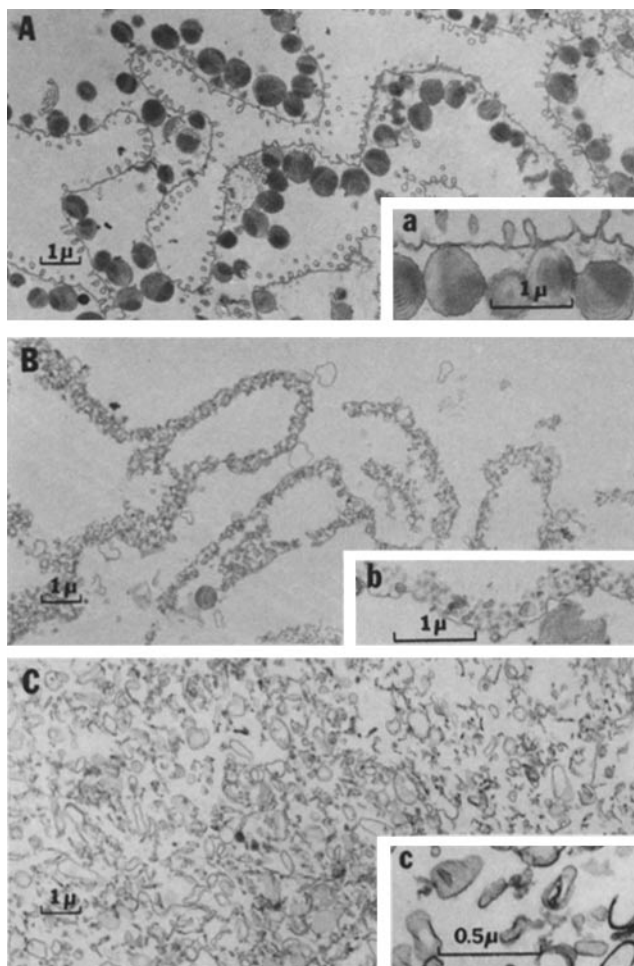


FIGURE 1 Ultrastructure of the sea urchin egg plasma membrane fractions. (A) Cell surface complex composed of large sheets of plasma membrane with vitelline layer on the outer surface and cortical vesicles on the inner surface.  $\times 7,100$ ; inset,  $\times 15,000$ . (B) Plasma membranes purified by centrifugation on a sucrose gradient. The membranes are present as large sheets. At higher magnification (inset) the "fluffy" layer is evident. A residual cortical vesicle has been selected in this figure to demonstrate sidedness.  $\times 7,100$ ; inset,  $\times 15,300$ . (C) KI-extracted plasma membranes. Membranes treated in this way form vesicles, and the fluffy coat present before extraction is absent.  $\times 7,100$ ; inset,  $\times 41,300$ .

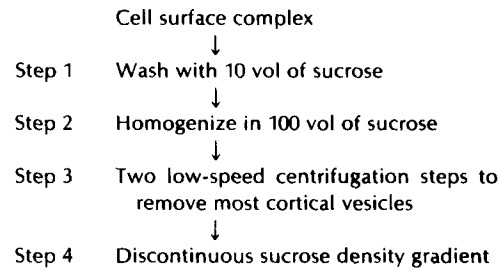


FIGURE 2 Procedure for isolation of the sea urchin egg plasma membrane. See Materials and Methods for a detailed description of these steps.

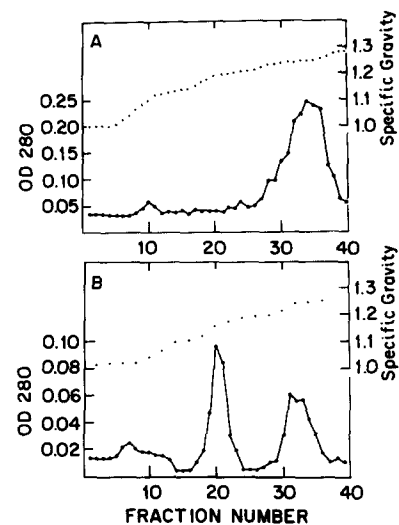


FIGURE 3 Purification of egg plasma membranes by centrifugation on a discontinuous sucrose gradient. (A) Plasma membranes were loaded on the gradient after step 3 (Fig. 2) and centrifuged at 72,000  $g$  for 12 h. Fractions were collected, the absorbance at 280 nm was determined, and the fractions were examined by phase-contrast microscopy for the presence of membrane material. Most of the membranes sedimented as a single band. (B) Plasma membranes purified as in A were extracted with 0.5 M KI and centrifuged on a discontinuous sucrose gradient as described in Materials and Methods. The membranes now sediment into two bands. The band with a specific gravity of 1.2 contains most of the vesiculated membrane material. The second, denser band contains some membrane sheets and residual cortical vesicles that were not removed by the above treatments.

vesicles by centrifugation. A schematic representation of this purification scheme is presented in Fig. 2.

Ultrastructural analysis of samples at various points during the procedure indicated that the cortical vesicles were ruptured during or soon after the detachment process. However, the lamellar contents remained inside the "opened" vesicles; these vesicles could be recovered from the supernate at step 3. The plasma membranes obtained at step 3 were in the form of large sheets that could be separated from any residual cortical vesicles by sucrose density gradient centrifugation (step 4). The elution profile from such a gradient is presented in Fig. 3*a*. Electron microscope examination (Fig. 1*b*) of the membrane sheets recovered from the gradient indicated the presence of a "fluffy" layer, probably proteinaceous, associated with the external surface of the membranes (i.e., the surface opposite to that to which an occasional cortical granule can be seen still bound; see Fig. 1*b*, inset). Ultrastructural examination of the cell surface complex after suspension in 1.0 M sucrose suggests

TABLE I  
Enzyme Activities of the Cell Surface Complex and the Purified Plasma Membranes

Preparation	Na/K ATPase		Peroxidase		Proteoesterase	
	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity
Cell surface complex	135 (1)	13,500	0.195 (1)	19.50	$5.4 \times 10^5$ (1)	$5.3 \times 10^8$
Plasma membranes	1,148 (8.5)	4,420	0.014 (0.07)	0.054	$0.3 \times 10^5$ (0.06)	$0.01 \times 10^8$

The specific activity of the ouabain-sensitive,  $\text{Na}^+/\text{K}^+$  ATPase activity is expressed as nanomoles of released  $\text{P}_i$  per milligram of protein per hour. The specific activity of the peroxidase is expressed as the change in absorbance units (at 465 nm) per milligram of protein per minute. The specific activity of the proteoesterase is expressed as counts per minute of  $[^3\text{H}]\text{CH}_3\text{OH}$  released from  $[^3\text{H}]\text{TAME}$  per milligram of protein per hour. Values in parentheses represent the relative specific activities.

TABLE II  
Purification and Properties of the Cell Surface Complex and of the Plasma Membrane Fraction before and after KI Extraction

Preparation	Total protein	Total phospholipid	$\mu\text{g}$ Protein/ $\mu\text{g}$ phospholipid	Buoyant density
	$\mu\text{g}$	$\mu\text{g}$		$\text{g}/\text{cm}^3$
Egg homogenate	141,000			
Cell surface complex	6,520 (100%)	650 (100%)	10.0	>1.3
Sucrose-treated membranes	867 (13%)	309 (47%)	2.8	1.25
KI-extracted membranes	160 (2.5%)	123 (19%)	1.3	1.12

These data represent the results of a typical plasma membrane preparation. The cell surface complex is considered to be the starting material, and the numbers in parentheses represent the recovery of lipid and protein relative to it.

that the fluffy layer represents peripheral proteins, some of which may be part of an altered, disorganized vitelline layer.

### Comparison of the Cell Surface Complex and the Plasma Membrane

To assess the extent of purification of the plasma membranes after removal of cortical vesicles, we assayed the cell surface complex and the purified membranes for marker enzymes for the plasma membrane and the cortical vesicles. The results, shown in Table I, indicate that the plasma membrane enzyme, ouabain-sensitive  $\text{Na}^+/\text{K}^+$  ATPase,<sup>1</sup> and, as previously reported (6), two cortical granule enzymes, proteoesterase and ovoperoxidase, are readily detectable in the cell surface complex. Furthermore, it is apparent that further purification of the plasma membrane per se by removal of the cortical vesicles leads to an almost 10-fold increase in the specific activity of the plasma membrane marker enzyme. Also, it is clear that the purified plasma membrane has minimal contamination with cortical vesicle components, at least as assessed by the presence of the cortical vesicle enzymes, because only very low levels of proteoesterase and ovoperoxidase are detectable. Thus, although 33% of the plasma membrane marker  $\text{Na}^+/\text{K}^+$  ATPase is recovered in the purified plasma membrane, <0.3% of the two cortical granule enzymes are present in the preparation.

Some additional characteristics of the cell surface complex and the plasma membrane are presented in Table II. The cortical vesicles and other components removed from the cell surface complex by treatment in sucrose account for a considerable amount of the protein in the complex because the purified membrane sheets are less dense ( $1.25 \text{ g}/\text{cm}^3$ ) and have a much lower ratio of protein to lipid (2.8). Even so, the egg plasma membrane is unusually dense and protein rich, probably because of the presence of the vitelline layer and other peripheral proteins that compose the fluffy layer seen in Fig. 1 b.

<sup>1</sup> The reason why this ATPase could not be detected in our earlier study (6) is unknown.

To characterize the peripheral and integral proteins, we treated the purified plasma membranes with the chaotropic agent, KI, which would be expected to solubilize peripheral membrane proteins. This could be done either after step 3 (Fig. 2) or after the density gradient centrifugation. This treatment caused the membrane sheets to vesiculate, and when centrifugation through a discontinuous sucrose density gradient was carried out two bands of membranes were resolved (Fig. 3 b). The lighter band (buoyant density 1.12), accounting for most of the membranes, consists of vesicles that are essentially free of the fluffy layer (Fig. 1 c). The heavier band (buoyant density 1.25) consists of small amounts of membrane in both sheet and vesicle form, as well as membrane fragments bound to residual cortical vesicles. This material was discarded. As seen in Table II, the membranes recovered after extraction with KI are significantly less dense than the native plasma membrane and have a lower ratio of protein to lipid, indicating that substantial amounts of peripheral proteins have been removed.

An analysis of the cell surface complex and the plasma membrane by SDS polyacrylamide gel electrophoresis is shown in Fig. 4. The cell surface complex contains at least 18 major polypeptide species, as well as many minor ones. Six of these major species stain positively with the periodate-Schiff procedure and are probably glycoproteins. The purified plasma membrane sheets obtained upon removal of cortical vesicles are depleted in several polypeptide species of ~450,000, 180,000, 110,000, 100,000, 65,000, and 33,000 daltons that are apparent in the cell surface complex; presumably, these proteins represent cortical vesicle components. Of the proteins remaining associated with the membrane, 14 exist as major components; a large number of minor components are also present.

When the membrane sheets were subsequently extracted with KI, several additional proteins of 55,000, 50,000, and 32,000 daltons, as well as several bands between 26,000 and 10,000 daltons, were removed. Although the number of major proteins in the KI-extracted membranes was considerably reduced (five to six major bands), a large number of minor proteins were now visible. To rule out the possibility that

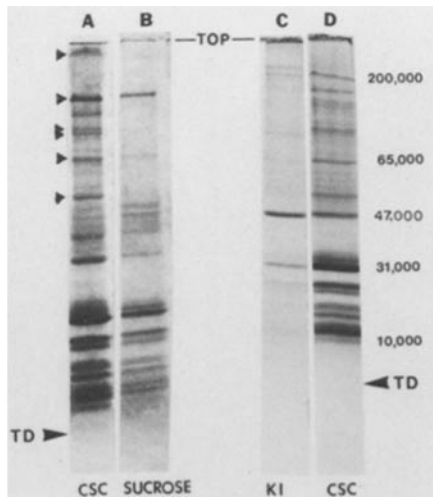


FIGURE 4 Sodium dodecyl sulfate-polyacrylamide gradient (5–20%) gel electrophoresis of the cell surface complex and plasma membrane fractions. Lanes A and D are preparations of the cell surface complex. The approximate molecular weights of some of the components are represented in lane D. The small arrows (lane A) indicate bands that were periodate-Schiff positive. Lane B represents plasma membranes purified on sucrose density gradient. Several major polypeptide components of the cell surface complex are greatly reduced in this fraction; 450,000, 180,000, 110,000, 100,000, 65,000, and 33,000 daltons. Lane C represents the purified plasma membranes obtained after extraction with 0.5 M KI. Several components present in lane B have been selectively removed, leaving six to eight major membrane components, as well as a large number of minor species associated with the plasma membrane. TD represents the tracking dye. Gels A and B and gels C and D were run at different times, and the migration distances were different, as shown by the position of TD. Proteins applied: A and D, 125  $\mu$ g; B, 100  $\mu$ g; C, 60  $\mu$ g.

proteolysis (27) or ovoperoxidase-mediated cross-linking was responsible for the changes in the relative abundance of certain polypeptides described above, we tested for a time-dependent change in the polypeptide profile of the cell surface complex after homogenization in sucrose. After a 20-h incubation, we found that, except for an increase in the intensity of the 200,000-dalton band, no other changes in the polypeptide profile were detectable. We conclude that proteolysis or cross-linking is not responsible for the change in the abundance of selected proteins observed as the membranes are purified.

### Lipid Analysis

A determination of phospholipids and cholesterol was performed on samples of KI-extracted membranes. The results, shown in Table III, indicate that phosphatidylethanolamine was the predominant phospholipid, followed by phosphatidylinositol and phosphatidylcholine. All the other phospholipids listed were detected at only very low levels. Cholesterol was found to be a major component of the plasma membrane.

### Sperm Binding

An earlier report demonstrated that the cell surface complex could induce the acrosome reaction in sperm and subsequently bind them species specifically (5). These findings suggest that the cell surface complex contains sufficient residual jelly coat to induce the acrosome reaction, as well as sperm receptors. In view of these findings, it was of interest to examine the purified

plasma membranes with respect to these properties of the cell surface complex. As shown in Fig. 5a, homologous sperm, in which the acrosome reaction had not been previously induced, did not bind to the plasma membrane sheets. However, if the acrosome reaction was previously induced in the sperm, extensive binding to the membrane was observed (Fig. 5b). This indicates that the purified membrane sheets retain receptors for sperm but probably have lost the residual jelly coat necessary for inducing the acrosome reaction in the sperm. Heterologous sperm from *Arbacia punctulata* did not bind to the membrane sheets, even when the acrosome reaction was preinduced by soluble jelly coat from *A. punctulata* (Fig. 5c and d). Thus, the species-specific nature of the sperm-binding event inherent in the intact egg and the isolated cell surface complex is retained in these purified membranes. Sperm adhesion to eggs is thought

TABLE III  
Lipid Analysis of the Purified and KI-Extracted Plasma Membranes

	nmol lipid/ 100 $\mu$ g protein
Phosphatidylethanolamine	61.0
Phosphatidylinositol	25.3
Phosphatidylcholine	16.2
Phosphatidylserine	<2.5
Sphingomyelin	<2.5
Phosphatidic acid	<2.5
Cardiolipin	Not detectable
Cholesterol	86.0

Values have been corrected for recovery. The ratio of inositol to P<sub>i</sub> in the phosphoinositide is 0.37, but we have not determined the proportions of mono-, di-, and triphosphoinositides by chromatographic means. Several other lipids not containing phosphate were detected by TLC and have not been identified.

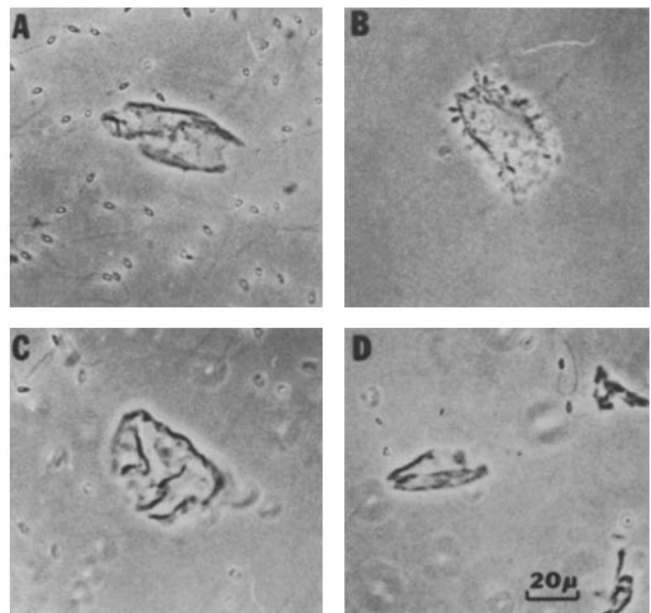


FIGURE 5 Sperm binding to purified plasma membranes. (A) *S. purpuratus* sperm that have not undergone the acrosome reaction (0% reacted) do not bind to the purified membrane sheets prepared from *S. purpuratus* eggs. (B) Many of the *S. purpuratus* sperm previously induced to undergo the acrosome reaction (90% reacted) bind to the membrane sheets. Neither unreacted, heterologous *A. punctulata* (C) sperm nor (D) reacted (57% reacted) *A. punctulata* sperm bind to the membranes, indicating that sperm binding to these purified membrane sheets is species specific.  $\times 333$ .

to be mediated by peripheral membrane components of the vitelline layer (8, 16, 21, 27). Because KI extraction of the isolated egg plasma membrane apparently removed all peripheral components, we tested the ability of *S. purpuratus* sperm to bind to KI-extracted membranes. We found that this egg plasma membrane preparation was essentially devoid of sperm binding capacity (data not shown). These results indicate that the cell surface complex contains both jelly coat and receptors for sperm, whereas the purified plasma membranes appear to contain the sperm receptor but no jelly coat. Extraction of the purified membranes with KI removes all traces of the vitelline layer and almost totally abolishes the ability of the membranes to bind sperm, presumably by solubilizing or denaturing the sperm receptors.

## DISCUSSION

This study has extended earlier work from this laboratory in which the cell surface complex was first described (6) and its functional properties examined (5). Our present objective was to isolate and characterize the egg plasma membrane per se. To accomplish this, we devised a method of detaching the cortical vesicles from the cell surface complex and recovering the plasma membrane. The membranes obtained are in the form of large sheets that are devoid of cortical vesicles but contain an amorphous fluffy layer detected ultrastructurally on the external surface. The results of biochemical studies clearly document the efficacy of the procedure used to remove the cortical vesicles from the plasma membrane. Whereas the cell surface complex contains the plasma membrane marker enzyme, ouabain-sensitive  $\text{Na}^+/\text{K}^+$  ATPase,<sup>2</sup> as well as the cortical vesicle enzymes, proteoesterase and ovoperoxidase, the purified plasma membrane has only trace levels of the latter two enzymes. Moreover, the specific activity of the plasma membrane marker enzyme in the isolated plasma membrane preparation is enhanced almost 10-fold over that found in the cell surface complex.

The fluffy layer observed in the purified plasma membrane may represent a disorganized form of the vitelline layer, as well as other peripheral proteins, but it is clear from the marker enzyme studies that there is little contamination with cortical granule contents. The protein hyaline is known to be tightly bound to the surface of fertilized eggs (see reference 26 for a review). In unfertilized eggs, there is evidence suggesting that hyaline is present both in cortical granules (1, 26) and on the cell surface (16). In view of these facts, hyaline is a potential contaminant of the purified plasma membrane. However, the binding of hyaline to the cell surface has an absolute requirement for  $\text{Ca}^{++}$  (1, 26). Because the isolation medium used for preparation of the cell surface complex is devoid of  $\text{Ca}^{++}$  and contains EDTA, and because all subsequent media used to isolate the purified plasma membrane are devoid of  $\text{Ca}^{++}$ , it is highly unlikely that hyaline is a significant contaminant in the purified plasma membrane preparation. Further work is now underway to more precisely define the components of the vitelline layer, cortical vesicles, and the fluffy layer of the purified membranes.

Extraction of the purified membranes with the chaotrophic agent, KI, solubilized this fluffy layer and yielded membranes recovered as vesicles that were relatively low in protein content,

<sup>2</sup> Another egg plasma membrane marker enzyme, adenyl cyclase (3), was also detected in the cell surface complex, but it proved too labile to be used reliably as a marker.

compared with plasma membranes from most mammalian cells (4, 21). Characterization of the protein components of the cell surface complex, and of the plasma membrane before and after KI extraction by gradient gel electrophoresis gave us much more information than the results obtained earlier with the SDS-urea system (6). The cell surface complex consists of at least 18 major protein components, as well as a large number of minor ones. Removal of the cortical vesicles by homogenization in sucrose results in the loss of several major polypeptide species, including three major periodate-Schiff-positive components that presumably are glycoproteins. Some of these proteins must represent components unique to the cortical vesicles. Schuel et al. (25) have provided histochemical evidence that sea urchin cortical vesicles are rich in acidic glycoproteins.

As mentioned above, extraction of the purified membranes with KI removed all traces of the vitelline layer, and analysis of the extracted membranes revealed that several of the major membrane-associated proteins were removed by this procedure. The picture that emerges from this type of analysis is that the cell surface complex contains a large number of major proteins that are not integral components of the plasma membrane per se. The cortical vesicles seem to account for 5–10 of these, including several high-molecular-weight glycoproteins. The vitelline layer and other peripheral components contain several of the remaining major species. After removal of these by KI extraction, the purified plasma membrane contains five or six major polypeptides, as well as a large number of minor components that were barely detectable in the cell surface complex.

One of the most important functions of the sea urchin egg surface is the recognition of, binding to, and fusion with sperm of the same species. The cell surface complex is known to retain the capability of inducing the acrosome reaction in homologous sperm and of binding these reacted sperm to the vitelline layer (5). The cortical granule-free plasma membranes prepared in the present study are capable of binding sperm only if the sperm are preinduced to undergo the acrosome reaction. This suggests that the jelly coat material that normally induces the acrosome reaction in the intact egg (28) has been removed during the isolation procedure. The fact that these membranes can bind sperm species specifically indicates that the sperm receptors are still intact. The receptor(s) for sperm are thought to reside in the vitelline layer (9, 24, 28), which persists in a modified form in the plasma membrane preparation as an amorphous fluffy layer. KI extraction removes this layer and essentially abolishes the ability of the membranes to bind sperm. Presumably, the receptor(s) have been solubilized by this procedure. The KI and other extraction procedures are currently being investigated as methods of solubilizing the sperm receptor in active form.

Determination of the lipid composition of the sea urchin egg plasma membrane revealed that, for a rather normal cholesterol to phospholipid molar ratio of ~1:1, this cell type has a rather atypical plasma membrane. Phosphatidylethanolamine was the major phospholipid, followed by phosphatidylinositol and phosphatidylcholine. This composition is quite different from that found in other plasma membranes, where phosphatidylcholine is the predominant phospholipid and phosphatidylinositol is only a minor component (19–21). Additionally, sphingomyelin, which is a major component of mammalian plasma membranes, was barely detectable in the egg plasma membrane.

Of particular interest is the high content of phosphatidylinositol, which in other types of plasma membranes comprises at most 3–9% of the total phospholipids (19–21, 29). For over 20 years it has been known that in a wide variety of secretory systems stimulation of the secretory event is accompanied by rapid breakdown and resynthesis of phosphatidylinositol (18). Also, it is clear that  $\text{Ca}^{++}$  plays a key role in both the secretory process and the breakdown of phosphatidylinositol. The sea urchin egg possesses thousands of secretory vesicles, the cortical vesicles, poised at the plasma membrane and ready to be discharged rapidly upon fertilization. The very high level of phosphatidylinositol in the egg plasma membrane may play an essential role in this massive exocytotic event. Indeed, early work from this laboratory (24) showed that there is rapid incorporation of inositol into phosphatidylinositol immediately upon fertilization of eggs. Clearly, the secretory response observed at fertilization bears striking parallels to the more classical secretory systems that have been studied (see reference 26 for review). Because of the massive, concerted nature of the exocytotic event in both the egg and the isolated cell surface complex, this system may prove most useful in studying the biochemical steps of secretion.

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