Membrane and lipid dynamics at fertilization in sea urchin eggs

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ABSTRACT

Membrane reorganization at fertilization results in dynamic lipid proportions. Detection of changes in phospholipid content was accomplished by analysis of proton NMR spectra before, and 15m after, fertilization. Evidence for possible participation of certain lipid pathway enzymes in these changes was investigated. The assay reaction mixture utilized as substrate fluorescent platelet activating factor analog, a lipid similar to phosphatidylcholine. With that substrate, membrane lipid remodeling enzymes, Phospholipases A and D, are detected in the density gradient fractions before and after fertilization. PLD and PA-phosphohydrolase that can alter or remove phospholipid head groups and PLA_2 and acyltransferases that can alter the properties of the acyl chains of the lipid such as chain length and degree of saturation are present before and after fertilization. Control mechanisms for such enzymes *in vivo* for alteration of lipid content such as release of Ca++, alteration of pH, or by altered vesicular transport or state of cytosol control proteins were investigated *in vitro*. Protein and lipid components found in common between membrane density fractions were uncovered.

INTRODUCTION

Membrane lipid study is important for understanding development because lipids are involved in defining the interface and defining the functions of various proteins segregating or attaching to hydrophobic domains specific to the lipid proportions. The content of the membrane and its association with the cytoskeleton and various G-proteins determines the surface area, shape, and the kinds of extensions and attachments of cells [1]. Lipid changes are also essential to vesicular transport and membrane cycling between cellular compartments [2]. Membrane modifications or signal transduction with lipid second messengers use component lipids of the membrane itself as substrates to produce new lipid intermediates. Detecting changing content of membrane lipids in development has been limited [3]; [4]; [5]. Glycosphingolipids and gangliosides have been described in sea urchin eggs of various species[6]; [7]; [8]; [9].

Phospholipase C activity and lipid second messenger products Inositol-tris-phosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol-bis-phosphate (PIP2) has been reported [10] [11] [12] . These products are involved in Ca++ release and temporary activation of PKC. Previous work has shown Phospholipase C (PLC) activation is important in eggs for short term signaling controlled by receptor occupation, with PI-PLC converting phosphatidylinositol bisphosphate (PIP2) to products inositol(1,4,5)P3 (IP3) and diacylglycerol (DAG) (reviewed in [13]). However, those products are short-lived [10] [14]. Products of PI3kinase, a phosphatidylinositol lipid kinase have also been reported [15]; [16] [14]; [17]. These enzymes are not further studied here.

The purpose of this study was to test the hypothesis that membrane lipid is altered, for a longer term, by other enzymes when directions of cell metabolism change or cell molecular superstructure is reorganized at fertilization, prior to cell behavior changes. The results of membrane lipid remodeling is presented, as seen in lipid pattern transitions during normal fusion of gametes. Lipid content was tested by analysis of proton NMR spectra of all four density gradient membrane fractions of sea urchin egg homogenates, comparing them before and after fertilization. All four fractions were studied instead of concentrating on the usual one density gradient fraction designated “the membrane.” There is no single cell fraction that contains the entire population of cell membranes during all stages of early development, as shown by enzyme or other protein markers ([18]; [19];[20];[21]). Membrane cycling and receptor stimulation leads to related kinds of membranes with different densities due to different attachments of

cytoskeleton, extracellular matrix, adapters, G-proteins, enzymes.

Long-term membrane alterations have been studied structurally but not biochemically. Longer term signaling can be dependent on DAG not made by PLC, for example PKC activation relies on DAG from Phospholipase D(PLD)activity found during division and differentiation [22] [23]. The membrane remodeling pathways utilize both PLD and Phospholipase A_2(PLA)and other enzymes which utilize their products. The activities of these enzymes is examined here *in vitro* utilizing fluorescent choline lipid substrates to reveal changes in enzymatic modifications of PC in membranes at activation.

METHODS

Sea urchins, ***Strongylocentrotus purpuratus*** (purchased from Marinus, Long Beach, CA) were injected for gamete shedding, fertilization accomplished, the fertilization membranes (FM) removed postinsemination [24].

MEMBRANE PREPARATIONS: Membrane fractions were prepared as previously published from this lab [24]. Homogenization was with the high speed micro-attachment on a Sorvall Omnimixer at top speed on ice for 2 min. Sucrose step density gradients were all w/w (65%, 40%-contains sample with sucrose added, 30%, 22.5 % , 0.2M sucrose-Mg++ homogenizing medium), run at 25,000rpm in a Beckman SW25 rotor for 20 hr or more. Fractions were collected from the bottom and membranes were pelleted after dilution with 0.01M tris pH 8 and centrifugation at 4^{o} C at 100,000 g for 1 hr. Pellets were then weighed and Folch extracted [25]; [5] with sonication with chloroform, methanol, water in steps [24];[26] .

PROTON NMR OF LIPID EXTRACTS. Proton NMR spectra of membrane extracts were collected as previously published from this lab [24];[27]. Spectra were analyzed using a Fortran computer algorithm, LIPICK to determine lipid content from peak areas and lipid relative quantitation was by algorithm ANALS1 [28].

SURFACE LABELS. Ruthenium red (0.2 mg/ml in sea water for 46 min in the refrigerator at about 8^{o} C) was used to label cell surfaces in embryos, after FM removal. Embryos were washed and density gradients prepared as above.

ENZYME ASSAYS: A PLD assay similar to that detecting PLD in yeast [29] was used. Membrane pellets from gradient fractions (2 mg/ml wet weight/volume) were suspended by teflon homogenizer in PLD assay mix (150mM NaCl, 25 mM Hepes, pH 7, 5 mM EDTA, 1 mM EGTA, 1 mM DTT) [29]. 40 µl was placed in 200 µl microfuge

tubes and 4 µl of 2 mM octylglucoside (a detergent to suspend lipid), containing 1mM Bodipy-PC added. Some samples contained calcium (1 µl of 0.28M). To start the reaction tubes were vortexed with or without 2 µl ethanol added. The samples were incubated for 40 min at 35 ^{o}C, then all were placed on ice. Lipids were not extracted; 5 µl samples from all tubes were pipetted directly onto the origin line 1 cm from the bottom of 10x10 cm HPTLC G60, non-fluorescent indicator plates (EM Reagents)and air dried [29]. Plates were run in saturated newly prepared paper lined chambers using chloroform:methanol:water: acetic acid 45:45:10:1 [29]. Kodak 35mm Techpan Film [29] and Kodak Technidol developer were used to record the visible fluorescent image from long wave UV exposure of plates in a TLC illuminator through a plastic UV filter. Images were digitized by a Microtek 35mm slide scanner.

ATPASE :4.5 ml assay mix (22 mM tris pH 6.9, 3.3 mM MgCl2, 150 mM NaCl, 33mM KCl, 3MM ATP), 0.1 ml fraction, incubated at 37 degreesC with or without 1mM ouabain, aliquots taken at 0,4,8min to TCA 2.5% final, filter. To 0.7 ml filtrate add 0.56 ml sodium molybdate (77.5 mM in 2.5M H2SO4, add.14 ml SnCl2 (1/200 dilution of a 40% w/v SnCl2 in conc HCl) read 5 min at 600nm. ELECTROPHORESIS: 10%SDS PAGE gels, 2%stacking gel. Samples contained SDS, DTT and bromphenol blue [30] [31]. Staining was by Coomassie blue R250. Biorad high and low molecular weight standards were used.

MATERIALS.

Molecular Probes fluorescent BodipyPC (BPC) PAF analog- D3771-alkyl C1 labeled, was used as default phospholipase substrate, but other substrates were used. PAF -A3773 Bodipy Fl C11-PAF, with alkyl Bodipy chain on C1 and acetyl group on C2; PA-D3805-C2 labeled, C1 acyl 16 carbon; acyl-C1 PC labeled on C2; D7707-alkyl C1 labeled, C2-acyl-16 carbon chain; B7701 bis Bodipy Fl C11-PC, labeled on both C1 and C2, LPAF(D-3772) .

Deuterated methanol (99.8% purity) (obtained in 0.5 ml vials), Chloroform-d1 ($CDCl_3$, 99.8 atom %) and tetramethylsilane (TMS) were purchased from Aldrich Chemical Co. or Stohler Isotope Chemicals.

RESULTS

Density gradient separations prepared from homogenates of unfertilized (Unfert) and fertilized (Fert) are shown in Fig. 1A).

FIGURE 1A

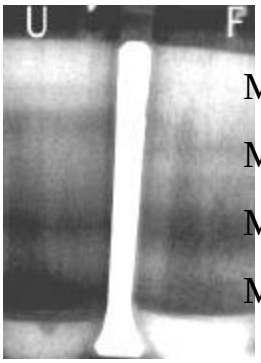


FIG1.A. Sucrose density flotation gradients of sea urchin egg homogenates from normal unfertilized and fertilized eggs after 23,000 rpm in swinging bucket rotor for 20 hours.

The entire density gradient contents were collected in four fractions, going from the most dense M1 to the least dense M4. Table 1 compares the distribution of ouabain sensitive Na+K+ATPase in fractions of unfertilized (Unfert) and fertilized (Fert) eggs.

TABLE 1. DISTRIBUTION OF ATPASE IN GRADIENT

fraction	total ATPase	Mg++ ATPase ouabain	ouab sen Na+K+ATPase	
unfertM1	0.88	0.57	0.31	
unfertM2	2.40	2.03	0.37	
unfertM3	1.77	3.71-stim		
unfertM4	1.05	0.87	0.18	
fertM1	1.04	1.27-stim		
fertM2	0.78	ATPase reverses		
fertM3	2.47	11.40-stim		
fertM4	0.00			

units:ugP/mg protein/min

All fractions of Unfert except for M3 contained ouabain sesntive Na+K+ATPase. However all fractions of fertilized eggs except M2 lacked inhibition by ouabain, and in fact were stimulated in its presence. M2 phosphate release reversed itself in the middle of the timed assay. Changes in ATPase activity at fertilization have been previously reported [32]; [20]. Altered enzyme

localization was seen for ATPase after fertilization from density of 1.23 to 1.14 [19].

Fertilization in sea urchins initiates obvious signs of membrane cycling: cortical granule secretion, followed by endocytosis [33]. Further evidence of membrane cycling has been seen in eggs where ATPase disappears from the surface of sea urchin [24] and *Xenopus* eggs at maturation and cleavage [34].

In fertilized eggs the surface marker ruthenium red, applied to Fert cells before fractionation was most concentrated in

M1,M2 (Fig. 1 B,C

B. Density gradient from ruthenium red treated FERT (RR).

C. Fractions M1-M4 collected from the bottom of the tube, so M1 is the most dense and the most stained with RR.

Previous investigations have shown that particles found in (density>1.24) of M1 would include cortical granules [35]; [18] or plasma membranes with attached corticle granules [36] from unfertilized eggs. Pellets of this fraction also contain easily visible pieces of fertilization envelope in our gradients when it was not removed before fractionation. M2 (density 1.23-1.18) is where unfertilized plasma membranes with Na+K+ATPase (density 1.23) have been localized [19] and ruthenium red is also seen in the current study, though less than in M1, in the pellets of this fraction. This suggests more surface membrane is in these fractions at this stage and less in M3 and M4 pellets that were light pink (Fig.1B, C). Ruthenium red not only binds to surface carbohydrates, but can also bind to Ca^{++} release channels on the ER [37]. The pink stain distributed in the fractions could be due either to different surface membrane domains with different densities or to endocytosis of surface dye into vesicles or a small amount of redistribution of dye at homogenization. M3 (density 1.18-1.13) would include fertilized plasma membranes with Na+K+ATPase (density 1.14) [19] and yolk platelet membranes [35] or cortical granule membranes [38] which are both found at the 25%/40% boundary; ER in *L .pictus* [39]. M4 (density 1.13-1.06) is where Golgi, ER and other vesicles

localize which have been shown to coat sperm chromatin to make male pronuclear envelope in *L. pictus* [39].

LIPID CONTENT OF MEMBRANES IN DENSITY GRADIENT FRACTIONS M1-M4 CHANGES DURING ACTIVATION

Lipid contents of M1-M4 density gradient fractions from unfertilized (Unfert) and fertilized (Fert) were calculated from Proton NMR spectra (compared in Fig. 2) (Appendix Table 1) and revealed that they are very different from each other, particularly in the glycerolipid head group protons shown from 3.2 to 3.8 ppm where different peaks are seen in the Fert fractions.

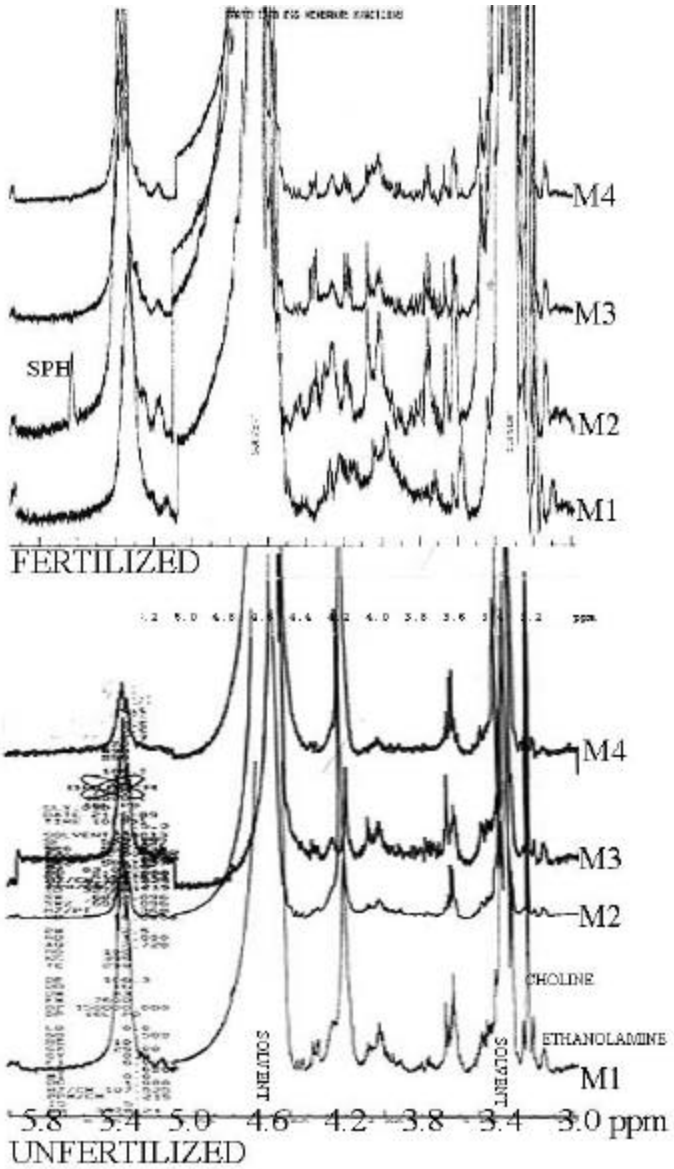
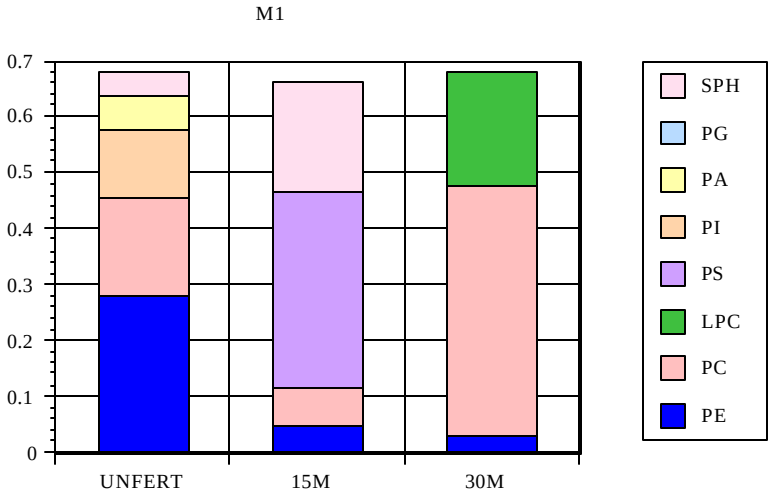


FIG2. NMR spectra of a)bottom- UNFERT M1-4 b) FERT M1-4, all starting from M1 on the bottom, between 3.0 and 6.0 ppm. Protons near double bonds have peaks from 5.36-5.7. Protons from the glycerol backbone have peaks from 4.12-5.25. Characteristic peaks are seen at 3.28 for choline, 3.18 for ethanolamine, 5.7 for sphingosine [27].

Peaks indicative of protons peculiar to certain lipids (choline or ethanolamine or sphingosine) or parts of phospholipids (head or glycerol region) or next to double bonds [27] are indicated in the figure. Such peaks are used to calculate the proportions of lipids, degree of saturation. Another region of the spectrum (0-3.0 ppm, not shown) is used to calculate acyl chain length and amount of cholesterol present [28].

Comparison of the relative concentrations of lipids from gradient fractions was made between Unfert, Fert 15m, and Fert30m to show the changes resulting from fertilization (Appendix Table 1). Standard deviations are high in samples where a particular lipid is not found in all of the repeat extract samples (so number of samples tested (N) is greater than the number with lipid detected (#) in Appendix Table 1), for example in 15m samples, suggesting changes are occurring rapidly at that time. A more visual representation of the phospholipid data showing their dynamic nature collectively is shown in Fig 3, though the table is necessary for the details.



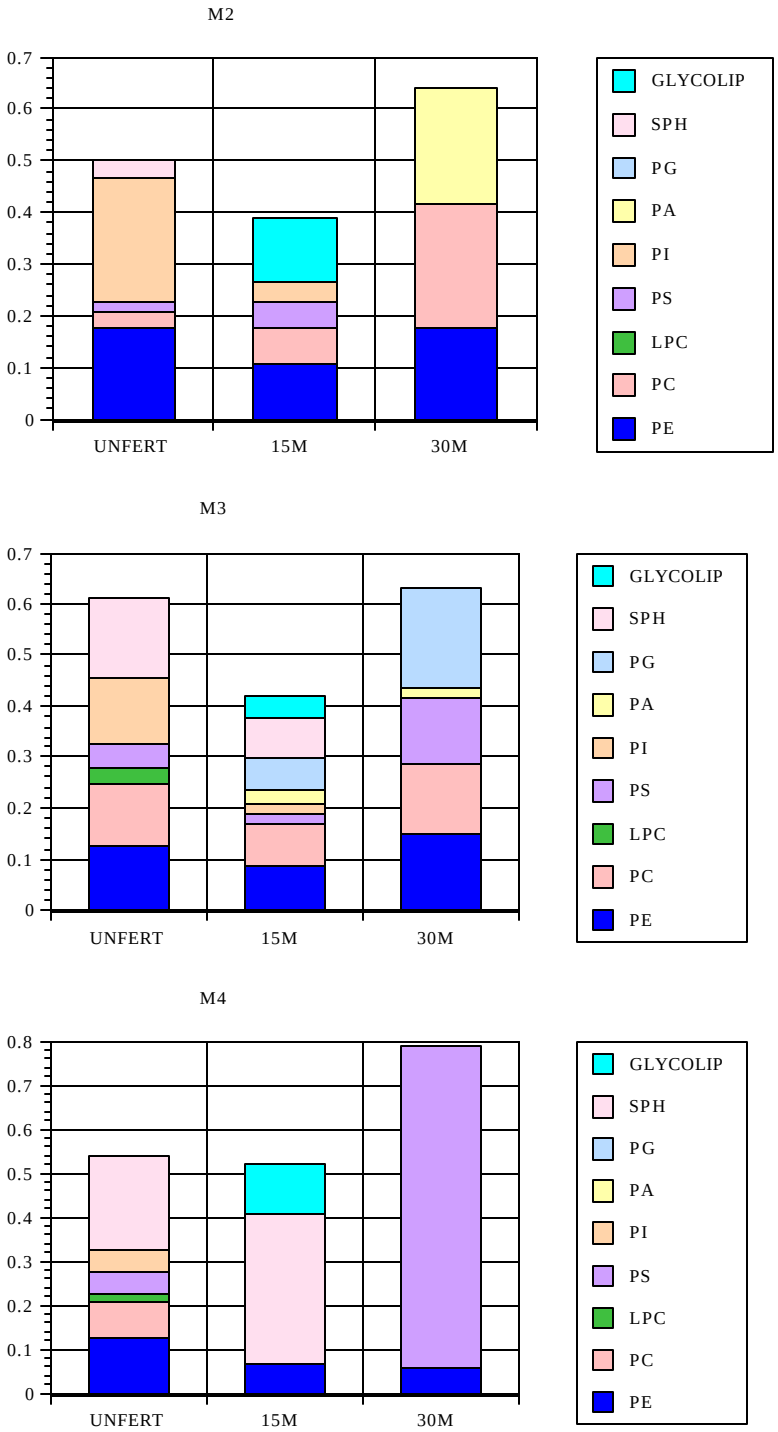


Fig 3. Comparison of PHOSPHOLIPID proportions from M1-4 fractions of fertilized with unfertilized eggs. Shown in each graph: CONTROL UNF, FERT-15M control, FERT-30m control. Details of lipid content can be found in Appendix Table 1. Lipid abbreviations in list of abbreviations.

At Fert 15m in M2, M3 a major dip is seen in the NMR spectra phospholipids in Fig. 3 (as well as by increased CH/PLIP ratio

in Table 2), which serve as phospholipase substrates. M1 just has different Plipids (but most of the lytic products for PLA and PLC or PLD are not increased) compared to Unfert membranes.

The greatest changes occurring (Appendix Table 1, Fig. 3) in major individual lipids **15 min** after fertilization are: **M1**: decreases in Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and increases in Phosphatidylserine (PS) or sphingomyelin (SM) (possible PLD –PC base exchange products- see diagram of lipid products). **M2**: Decrease in Phosphatidylinositol (PI). Diglyceride (DG) (PLC or PLD-PA-Phosphohydrolase (PAPH) product) is found in only half the cases and its average is not changed at 15m. **M3**: changes from PI and PE with either PC or SM in Unfert to PE and PC or phosphatidylglycerol (PG) (possible PLD base exchange product) in 2/3 Fert. **M4**: The major lipids PE and SM are not changed at 15 min.

Other changes at 15 min which influence membrane properties (shown in Appendix Table 1) include high CH/PLIP that switches from fraction M4 in UNFERT to M2 and M3 at 15 m post fertilization, remaining high only in M2 at 30m. Cell plasma membranes have high ratios. The incorporation of CH-rich cortical granules [18][33 is expected to increase CH as seen in M2, M3 after fert.

After 30 min, (Appendix Table 1) M1: PC comes back and LPC (a phospholipase A product) appears. M2: PC increases and PA appears (a phospholipase D product). M3: PLA product FA (at top) increases in M2 and PG and PS increase the most. M4: SM is replaced by a high concentration of PS (possible PLD base exchange product). This is not a complete list of possible changes since only 13 possible lipid metabolic components were in the NMR spectrum solution matrix [Sparling, 1990 #185] for getting the relative concentrations. The main identifiers of the lipids in proton spectra are the head group protons, which can be passed from lipid to lipid, especially by transphosphatidylation [40] [41], or the head groups can be lost to detection as they become split off and water soluble, not be seen in lipid extracts, ***i.e.*** choline-P, choline, IP3, ethanolamine, serine. The dramatic decrease in PC (in M1) and PI (in M1-M4) seen after fertilization without increase in Plipase products implicates such pathways.

COMMON DETECTABLE PRODUCTS OF BODIPY PC 3771 (PAF ANALOG) IN SEA URCHIN EGGS

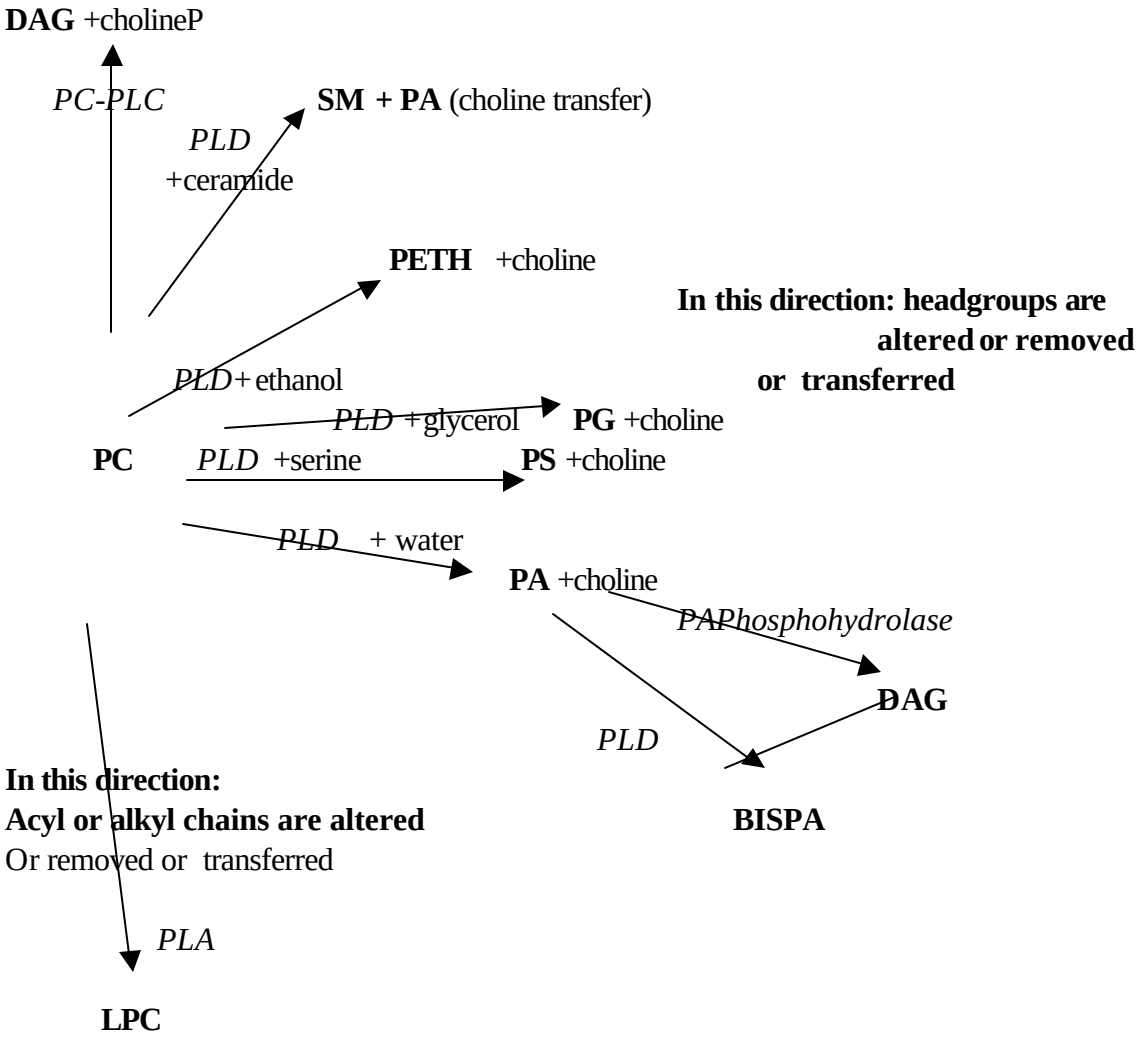
Changes in relative concentrations of the lipids are detected in each individual fraction from the same sample of eggs over time, during development, in possible response to

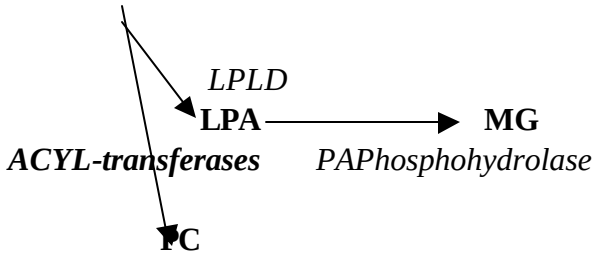
stimuli encountered at fertilization. This happens even though each experiment was done with the combined eggs from three or more females to try to exclude individual variations. Even with such a gross separation, some changes can be consistently seen after fertilization. The enzymes must be tightly controlled by many factors in time and space. New proteins don't have to appear in order to cause the changes, enzymes can be present and able to be activated and inactivated at any time.

To sort out which enzymes are active, we have to know which products are due to particular enzymes in the reaction mixture (see diagram).

Fig. 6 COMMON PRODUCTS OF PC IN MEMBRANE REMODELING

Detectable lipids are shown in bold, enzymes in italics





PLD. PLD activity exchanges the -OH from water for the choline head group on PC to make PA and can also change the relative phospholipid proportions by base exchange [40]; [41]. PLD transphosphatidylation (PLDTP) exchanges molecules other than water, which also have -OH groups for choline on PC. For example, PLDTP with PC plus glycerol forms PG [41] or serine forms PS [42], and PC with ethanol forms phosphatidylethanol, the indicator of PLD activity [41] [29]. PLD produces phosphatidylethanol from Bodipy PC and ethanol . The reaction with ethanol was linear for an hour in yeast [29].

Fig. 4 shows the products of PLD (P-ethanol) and PLA (LPC) are made in all of the density gradient fractions both before and after fertilization, though they are more concentrated in fractions M2 and M3.

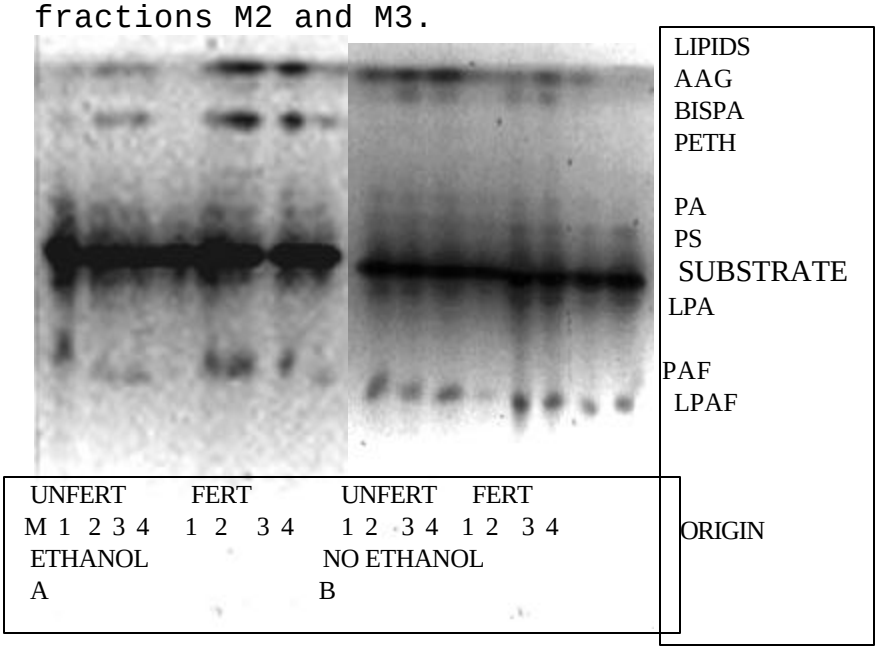


FIGURE 4 A and B Phospholipase assays, effect of ethanol on lipid products made using Bodipy alkylPC by sea urchin egg fractions. Comparison of sucrose density fractions M1-M4 in Unfert and Fert 10m. Ethanol is in all samples of the gel on the left in A, but not on the right in B. TLC plate run in chl:meth:acetic acid:water 45:45:1:10, with origin at the bottom. Spots are labeled on the right at level of standards migration.

Fig. 5 A. Comparison of the effect of ethanol presence as seen in Fig. 6 on the Unfert and Fert phospholipases, inhibiting AAG in all but Fert M4 and LPAF production in all but in Unfert M1 or Fert M3.
B. the effect of calcium presence on the Unfert and Fert phospholipases, activating PLA in M1-3 in Unfert, inhibiting PLA and PLD in Fert M2-3.
Density of spots on plates in Fig. 6 was quantified by NIH Image and plotted by Excel. Zero represents the value of the control without ethanol.

Figure 5a

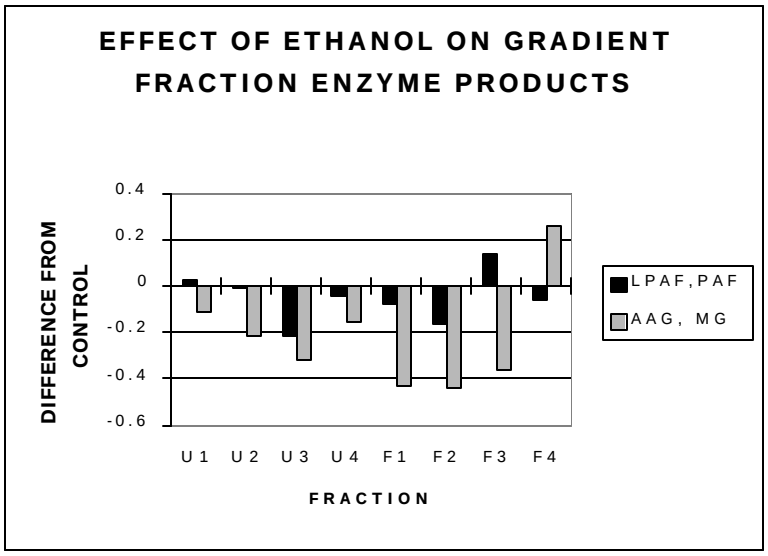
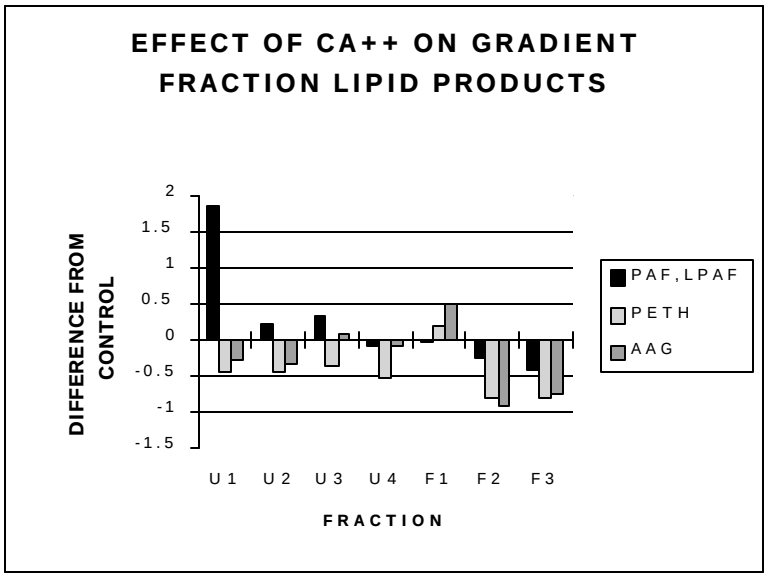


Figure 5b



Figs. 5 and 6 show that Ca^{++} inhibits PLD products but not PLA under our conditions.

Effect of calcium and ethanol on phospholipases.

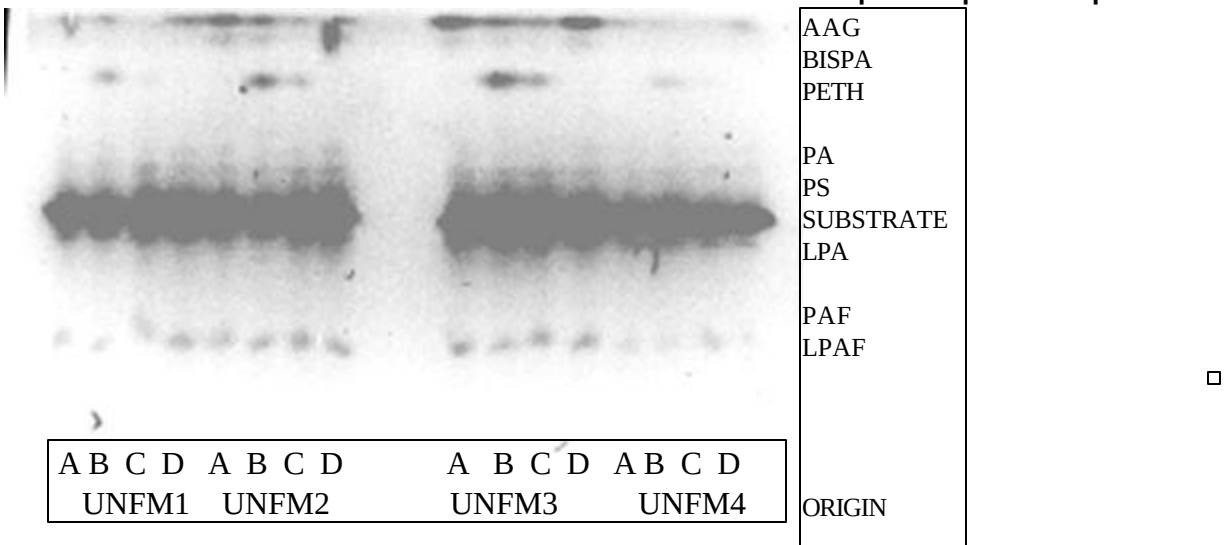
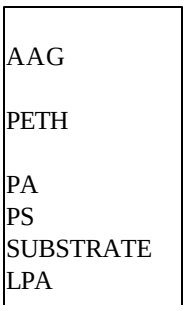
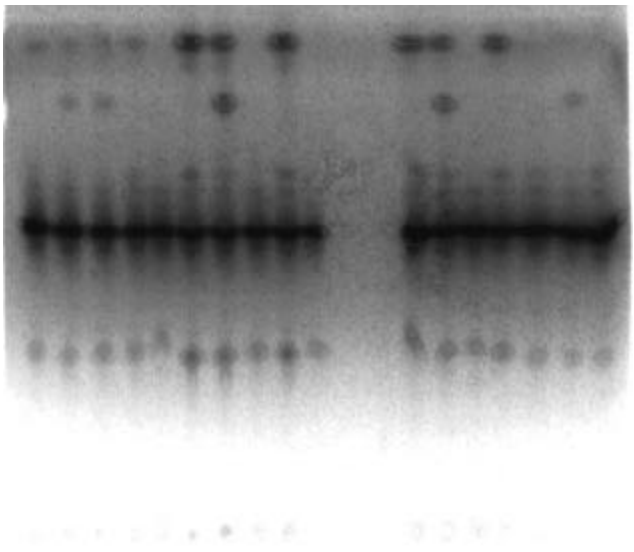


Fig 6A. UNFERTILIZED FRACTIONS A=CONTROL, NO ETHANOL; B=ETHANOL; C=ETHANOL+CALCIUM; D=SERINE, NO ETHANOL





FERT M1	FERTM2	FERT M3	FERT M4
A B C D E	A B C D E	A B C D	A B C

FIGURE 6.B. COMPARISON OF FERTILIZED SUCROSE DENSITY GRADIENT FRACTION PRODUCTS OF ENZYME ACTIVITIES A=CONTROL; B=ETHANOL; C=ETHANOL+CALCIUM; D=SERINE; E=SERINE + CALCIUM

PLA produces lysoPC and FA (from C2 acyl chain) from PC but only the LPC is fluorescent when the label is on C1. The spot above LPAF in Fig 6 has the same Rf as PAF. When alkylPC is used as in Fig. 6, PLA first produces LPAF, then if Ca++ is present AcetylTransferase (AT) can convert LPAF to PAF. PAF is formed in bovine cornea after injury if given LPAF and acetylCoA and is sensitive to cyclooxygenase and lipoxygenase inhibitors (87). The PAF only shows up after fertilization or addition of calcium suggesting the AT is controled by calcium release at fertilization.

PC specific-PLC might produce DAG+Choline-P from the substrate [45]. Evidence for PLC production of DAG and IP3 from PIP2 is not shown using our methods, since only fluorescent PC was in the reaction mixture, and PI-PLC would not be demonstrated in the assays. The DAG produced here is most likely not from PLC but from PA phosphohydrolase (PAPH) since fluorescent PA substrate allowed DAG formation in Fert preparations (Fig 7). PAPH converts PLD product PA to DAG (or AAG when alkyl-C1 PC is used) [29]; [43]; [44], and DAG kinase goes in the opposite direction in the presence of ATP[29] and they sometimes confuse the PLD and PLC activities. PLDTP can convert DAG plus PA to bisPA, removing two lipid messengers in that way (86).

The use of the alkylPC bodipy substrate was fortunate since various bodipy acylPC substrates did not serve well for either PLA or PLD (Fig 7).

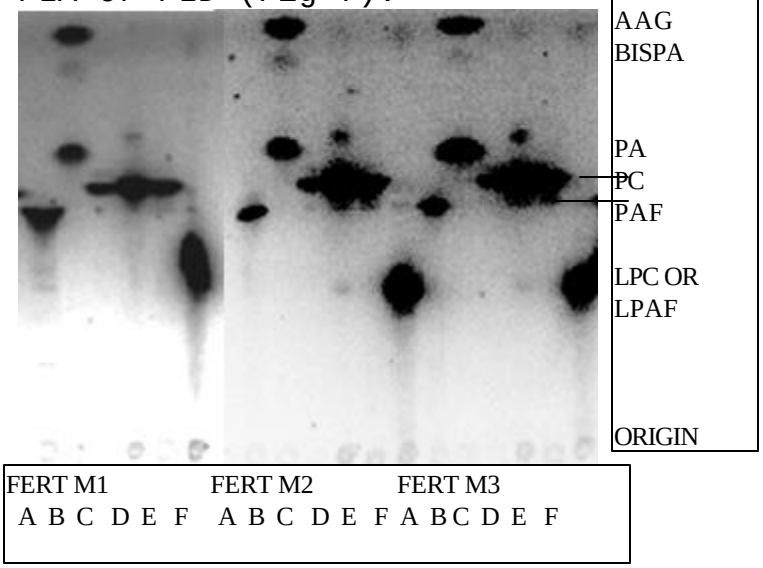
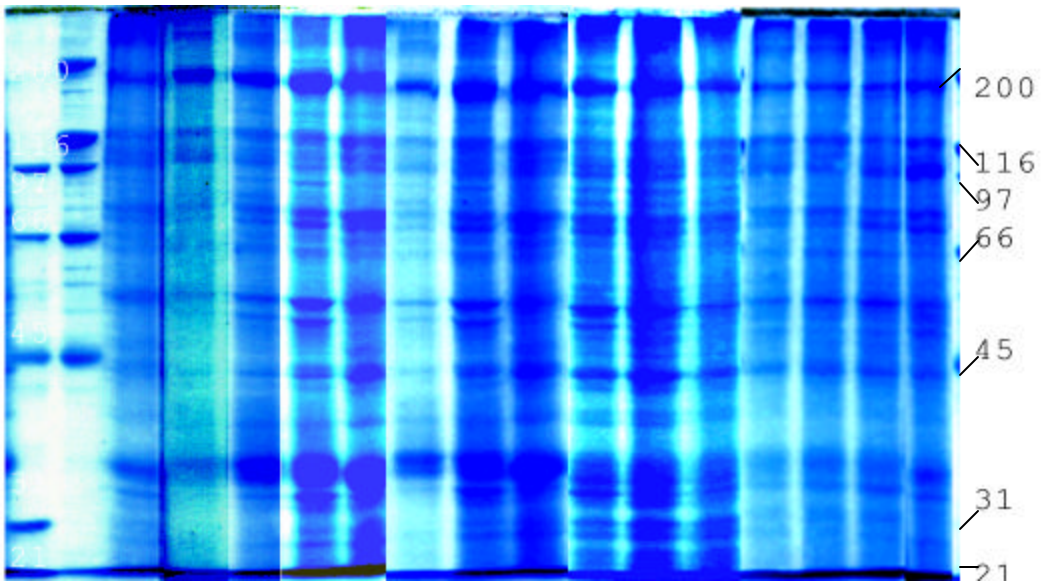


FIG7. Utilization of various fluorescent substrates by sea urchin egg enzymes. PLD assay mix was used, but different substrates were substituted in the detergent and alcohol was also present. TLC plate run in chl:meth:acetic acid:water 45:45:1:10. Spots are labeled at level of standards migration. Three Fert fractions M1-3 were each assayed with the different additives: **lane A**- PAF -A3773 Bodipy Fl C11-PAF, with alkyl Bodipy chain on C1 and acetyl group on C2; **lane B**- PA-D3805-C2 labeled, C1 acyl 16 carbon; **lane C** is acyl-C1 PC labeled on C2; **Lane D** is D7707- alkyl C1 labeled, C2-acyl-16 carbon chain; **lane E**-is B7701 bis Bodipy Fl C11-PC, labeled on both C1 and C2, **Lane F** is LPAF(D-3772) . Repeated runs showed similar results.

In Fig. 7 Fert M1 TLC slot F, lysoPAF is converted to PAF, as seen by the spot lengthening with incomplete separation of LPAF form PAF. Such a change is also seen in Fig 6A in Unfert M1 in the presence of Ca^{++}, slot C.

ELECTROPHORETIC SEPARATIONS of proteins from the gradient fractions are shown in Fig 8.

FIGURE 8



LOW,HI STDS U4 U4 U3 U2 U1 U3 U2 U1 F3 F2 F1 F3 F2 F1 F4 MW STDS

DENSITY GRADIENT FRACTION

Fig.8. Composite from four SDS PAGE gels, which were run simultaneously in series. There are duplicates for the four density gradient fractions of both Unfert and Fert 10m sea urchin eggs. Wells from left to right: **1.** low molecular weight standard, 2. high MW Std, **3.** Unfert M4, **4.** UnfertM4, **5.** UnfertM3, **6.** UnfertM2, **7.** UnfM1, **8.**UnfertM3, **9.** UnfertM2, **10.** UnfertM1, **11.** Fert M3, **12.** Fert M2, **13**. Fert M1, **14.** Fert M3, **15.** Fert M2, **16**. Fert M1, **17.** Fert M4. The edges of a HiMW std can be seen on the right where bands are labeled.

Fractions from two different dates for both Unfert and Fert are shown. There are so many bands extending across all the membrane fractions and only a few unique bands including: in Fert M1-43kDa, and in M1 and 2 both 33, 67, 212 kDa; in M2 and 3 both 85 kDa; in 3 -245 kDa and in M4 -257 kDa. There seems to be more of a 37 kDa protein in unfert fractions, and M1 and M4 are missing the band at 49kDa. The MW of PLA is 35 kDa [46]. Electrophoresis of the PM fraction in previous studies showed membranes were similar except for peptides of 280, 160, 120, and 25 kDa which are gone after fert [19].

DISCUSSION

No constant basic lipid content was found in this study to match a particular density gradient membrane fraction. Membranes from the same density gradient fraction and the same batch of eggs have different lipid content before and after fertilization. Different batches of eggs (or embryos) from the same stage produce membrane preparations from the same density, which vary in lipid content. That could be because many internal transitory signals are produced during the first 30 min of development that result in phospholipase activations, cytoskeleton remodelling and vesicle transport. However, some lipids are consistently found in all or most of the samples of a fraction and those will be the focus of this discussion. Similar variability was also found previously in the same four density gradient fractions from the three separated cell types at the 16 cell stage [24]. In dividing up the density gradient into only four fractions, there must be included in each fraction many membrane organelles, vesicles and cytomembrane domains.

POSSIBLE CAUSES OF LIPID CONTENT MODIFICATION IN MEMBRANES AT FERTILIZATION

Density gradient membrane lipid content changes are expected as a part of activation. The signal cascades result in enzymatic membrane remodeling lipid second messenger production and vesicular cycling after fertilization. The current study made no attempt to purify the cell membrane because there is no one fraction we can call “the membrane”. Membrane domains will change position in gradients as they cycle in and out of vesicles and their density changes with their content and coats as they move around the cell or associate with cytoskeleton [47] or remove protein from membranes by calcium-dependent proteases [48]. Most previous membrane studies of eggs have described one fraction identified as cell membranes, because it had the most of the marker they were using for it, and the study was from only one differentiated cell type at only one developmental stage or in one state of stimulation, leaving out discussion of the possible other fractions also containing the lower activities of the marker enzyme in question [24, 38, 39, 49, 50] [36][35][51][18]. Similar density gradient studies from adult cells have also located specific enzymes of lipid metabolism ([52] [53] [54], or different attached cytoskeleton or docking, snare proteins [55] but even then there is enough overlap that new affinity techniques have had to be developed to separate cell components[55]. They are all very dynamic during periods of cell activation, of setting cell polarity, and during cell division as occurs at fertilization.

An assortment of lipid enzymes can produce the changes seen here: PLA, PLD, PAPH are in all four density gradient fractions. Head groups can be changed or transferred by PLC and PLD, whereas PLA changes the hydrophobic chains (diagram in results). Cell activities after Fert can be explained by lipid changes in the membrane and *vice versa*.Many specific membrane alteration events after fertilization including cortical granule exocytosis [56]; [57] and microvilli extension in the first minute [58]; endocytosis, recycling the membrane occurring in a burst in sea urchin eggs 3-5 min after fertilization and extending until cleavage [59] ;[33]; assembly of sperm pronuclear membrane [39] leading to nuclear fusion proceeding by 20 min [60]; movement of acidic vesicles to the surface ([61]; calcium uptake by mitochondria [62]; rearrangement of the endoplasmic reticulum [63]; [64] and altered metabolism [3] must involve careful control of the membrane altering enzymes.

A high PE/PC ratio in M1 Unfert in this study and its reduction at Fert might make endocytosis more likely then. An increase in PE compared to PC reduced observed phagocytosis of beads or pinocytosis of horse radish peroxidase; high PC and unsaturated FA increased phagocytosis rate [65]. Altered membrane phospholipid composition and the level of unsaturated fatty acids and sterols in macrophages affects endocytosis rates in fibroblasts [65]. PS appearance seen in the current studies after PI disappears at fertilization is known to allow fusion protein to attach for exo-or endo-cytosis in another system [66].

A dynamic membrane lipid content seen here at fertilization also directly influences membrane properties such as: fluidity which is altered at fertilization [67]; [68]; the mobility of G-proteins and associated enzymes, the starting elements of the chains of second-messenger-activated kinases or phosphodiesterases, permeability [69]; lipid domain formation with proteins and receptors restricted to certain lipid domains [70], [71], and the conversion of membrane component lipid substrates to signals or anchors [22] [72] .

Part of the dynamics after fertilization could be due to yolk and its breakdown. Yolk contains storage of membrane lipids, which may be released during development for position specific adhesion [73]. Normally cells double their phospholipid mass to divide, G1 in the cell cycle sees 75% turnover of PC, which stops in S-phase which accumulates phospholipid. Eggs do not grow before division, and lack G1 in cleavage so any synthesis of lipid would have to be by the remodeling pathway, using yolk.

Direct enzyme assays for phospholipase activity showed the enzymes are widespread in the fractions before and after Fert

but only scattered phospholipase products are found there by NMR spectra analysis. These two observations have to be reconciled. The dramatic decrease in PC in M1 and PI in M1-M4 after fertilization, without increase in phospholipase products implicates: A.) more complicated lipid pathways such as the remodeling pathway (including PLA and LPAF-AT) [74] using up the products as soon as they are formed to make other lipids; B.) control of enzymes.

There is a high basal activity of both PLD and of PLA_2 available in all membranous fractions of sea urchin eggs before and after fertilization. Previous studies suggest PLA_2 is important in cortical granule release [75]. There was no indication of a phospholipase A or D appearance at fertilization, just the increase in activity by fertilization. Changes in activity by presence of ethanol which blocks PA formation or by presence of Ca++ suggests that calcium release at fertilization may ba a control mechanism. Prolonged activitation of PLD is also not indicated right after fertilization *in vivo* as shown by lack of increased PA consistently after fertilization, except in M2 and not until 30m. PLD is inhibited by Ca++ which is released *in vivo* at fertilization. DAG is already present in considerable amount (seen in DG+TG) in the Unfert egg M2 in half the cases, so any small changes, as in some 15m M3, would hardly be noticed. LPC from PLA is already in some UnfertM3 and M4 fractions with decreases in M3 in Fert. Retention of some signal lipids in the membrane would maintain it in a suspended state awaiting completion of the signal at cell fusion by removal of the products or regeneration of the system. These lipids signify the possible earlier activation signals in Unfert eggs, also detected at ovulation or maturation in starfish [20], *Chaetopterus,* where DG goes up at GVBD and then back up again at fertilization [76], and amphibian eggs exposed to progesterone [77]. PLD products act as accessories to signals that activate PLD or are important in remodeling the membrane, an essential step in some changes in cell behavior. For example, Ca^{++}, PA and DAG have important effects in membrane fusion/fission events [78]. Increased PG and PA are seen as important membrane components prior to muscle cell fusion [79]. They also may lead to further cascades of reactions in cells such as vesicle coat assembly, or calcium availability [80] which can lead to further developmental changes [13][81][82]. Long-term activation of the PKC/PLD pathway by long-term phorbol-ester treatment results in mitogenesis and alteration of morphology of chick embryo brain astrocytes resulting in increased vimentin [83].

The significance of phospholipase products is in their influence as well on transitory cytoskeleton assembly and

disassembly, activation of protein kinases and other enzymes associated with the membrane, and membrane fusibility, all important components in cell movement and division. PA and choline or other base exchange products, formed at times of enzyme activation, may be very important for cell change [66] and membrane trafficking [84] [70].

Future work will test these hypotheses about the causes and effects of membrane lipid changes during activation and early cleavage.

APPENDIX TABLE 1. EFFECTS OF EGG ACTIVATION ON MEMBRANE LIPID CONTENT.
Number (of experiments), # shows how many had the particular
lipid. REL CONC represents the % of total lipid, numbers in ()
are SD.

FRACTION M1		UNFERT		FERT15M		FERT30M
NUMBER	4		2		1	
EXTRACTS	#	REL CONC	#	REL CONC	#	REL CONC
PC	4	.18(.13)	1	.07(.11)	1	.45
LPC	0		0		1	.20
PI	1	.12(.23)	0		0	
PA	1	.06(.11)	0		0	
PE	4	.28(.31)	1	.05(.08)	1	.03
PS	0		1	.35(.50)	0	
SPH	2	.04(.05)	1	.19(.27)	0	
CER+SUL	1	.01(.03)	0		0	
CH+CE	4	.31(.20)	2	.33(.04)	1	.31
CH/PLIP		.45		.48		.45
SPH/PC		.24		2.61		0/.45
CHNLEN		16.39		16.12		15.49
UNSAT		1.20		.57		1.18
#LIPIDS		4.25		3.0		4
FRACTION M2		UNFERT		FERT15M		FERT30M
NUMBER	4		2		1	
PC	2	.03(.04)	1	.07(.09)	1	.24
PI	4	.24(.20)	1	.04(.06)	0	
PA	0		0		1	.22
DG+TG	2	.12(.14)	1	.11(.15)	0	
FA	0		0		1	.09
PE	4	.18(.13)	2	.11(.02)	1	.18
PS	1	.02(.04)	1	.05(.07)	0	
SPH	3	.03(.03)	0		0	
CER+SUL	0		1	.12(.17)	0	
CH+CE	4	.38(.10)	2	.52(.06)	1	.27
DOL+DOLP0	0		1	.07(.10)	0	
CH/PLIP		.76		1.38		1.08
SPH/PC		1.0		0/.07		0/.24
CHNLEN		19.06		23.78		20.12
UNSAT		1.59		1.06		1.45
#LIPIDS		5.5		4.5		5.0

FRACTION M3						
NUMBER	4		3		1	
PC	3	.12(.10)	2	.08(.09)	1	.14
LPC	1	.03(.07)	0		0	
PI	4	.13(.07)	1	.02(.04)	0	
PA	0		1	.03(.04)	1	.02
DG+TG	0		1	.03(.05)	0	
FA	0		1	.01(.02)	1	.07
PE	4	.13(.07)	3	.09(.03)	1	.15
PS	1	.05(.09)	1	.02(.03)	1	.13
PG	0		2	.06(.06)	1	.19
SPH	3	.15(.16)	1	.08(.14)	0	
CER+SUL	0		1	.04(.08)	0	
CH+CE	4	.39(.09)	3	.48(.07)	1	.30
DOL	0		1	.05(.09)	0	
CH/PLIP		.63		1.12		.49
SPH/PC		1.3		.95		0/.14
CHNLEN		19.75		22.98		22.4
UNSAT		1.43		.87		1.49
#LIPIDS		5.0		6.0		7.0
FRACTION M4						
NUMBER	3		2		1	
PC	1	.08(.14)	0		0	
LPC	1	.02(.03)	0		0	
PI	1	.05(.09)	0		0	
PE	3	.13(.04)	2	.07(.01)	1	.06
PS	1	.05(.08)	0		1	.73
SPH	2	.21(.19)	2	.34(.16)	0	
CER+SUL	0		1	.11(.16)	0	
CH+CE	3	.47(.14)	2	.48(.33)	1	.21
CH/PLIP		.89		.91		.27
SPH/PC		2.73		.34/0		0/0
CHNLEN		24.6		19.54		22.32
UNSAT		1.66		.92		1.36
#LIPIDS		4		3.5		3

ABBREVIATIONS
AAG alkyl-acyl glycerol
AT acetyl-Coenzyme A- acetyl-transferase
BPC, Bodipy-PC Fluorescent PAF analog- D3771-alkyl C1 labeled
CE cholesterol ester
CEREB cerebroside
CH cholesterol
CHL chloroform
CHN LEN chain length
DAG diacylglycerol
DB dextran blue
DOL dolichol
DTT dithiothreitol
EDTA ethylene diamine tetraacetic acid
EGTA
ER endoplasmic reticulum
FA fatty acid
FERT fertilized
FM fertilization membrane
GANG ganglioside
GLYCO glycolipid
HEPES
HPTLC high performance TLC plate
IP3 inositol (1,4,5) trisphosphate
LPAF lyso-PAF
LPC lysoPC
Meth methanol
M1...M4 density gradient membrane fractions
mito mitochondria
NTP nucleotide triphosphate
OUAB ouabain
PA phosphatidic acid
PAF platelet activation factor- 1 alkyl,2-acetyl glycero-phosphoryl choline
PAF AH PAF acetylhydrolase
PAPH PA phosphohydrolase
PC phosphatidyl choline
PE phosphatidyl ethanolamine
PG phosphatidyl glycerol
PI phosphatidyl inositol
PIP2 phosphatidylinositol bisphosphate
PKA protein kinase A
PKC protein kinase C
PLA phospholipase A
PLC phospholipase C
PLD phospholipase D
PLIP phospholipid

PS phosphatidyl serine
REL relative concentration
SD standard deviation
SM sphingomyelin
SPH sphingosine
SULP sulphatide
TG triglyceride
UNFERT unfertilized
UNSAT unsaturation

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