Running title: phospholipase A and PAF in eggs

Phospholipase A activities and Platelet activating factor production in membrane density gradient fractions of sea urchin eggs at fertilization or artificial starfish egg activation

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ABSTRACT

High basal activities of Phospholipase A_2 (PLA), not requiring stimulation, are present in sea urchin egg homogenate total pellet fractions as well as in all density gradient fraction pellets before and after fertilization. PLA activity can start divergent signal pathways from the splitting of phosphatidylcholine (PC) into fatty acid and lysophosphatidylcholine (LPC), second messenger products which can initiate or inhibit further enzyme reactions. LPC can also be used in lipid remodeling when groups are put back onto the C2 of LPC. The remodeled lipids can change the character of the membrane properties if the acyl chains added are longer or shorter or more or less unsaturated. Fluorescent alkyl-phosphatidylcholine (Platelet-activating-factor analog) substrate used in the PLA assay showed lysoplatelet-activating-factor (LPAF) as well as PAF were produced. PAF can be made from LPAF using acetyl-CoenzymeA-acetyltransferase (LPAF-AT). PAF is only made by membranes after cells are activated by 1- methyladenine in starfish or are activated by sperm in sea urchins. PAF production by PLA and LPAF-AT is also enhanced in the presence of Ca++ or cytosol in sea urchin and starfish eggs. The PAF produced is an autocoid lipid signal important in reproduction and through its receptor may aid gamete union.

INTRODUCTION.

Membrane lipid alterations by phospholipase A (PLA) can produce modified membrane properties, second messengers, lipid intermediate products that activate some and inactivate other enzyme cascades. Membrane lipid components are involved in the processes of fertilization [1]; [2]: recognition [3], adhesion [4]; [5], and fusion of membranes [6]; [7] [8]; opening of ion channels, influx and release of calcium [9] [10]; secretion of acrosomes [11] ; [12] ; [13]; [14] and cortical granules [15]; [16]; membrane cycling [17].

Phospholipase assays [18] revealed PLA activity on sea urchin and starfish egg fractions before and after fertilization or artificial activation under conditions also meant to expose control mechanisms. Factors that enhance the phospholipase A (PLA) production of lyso-PC or lyso-platelet-activating factor (LPAF) also produce arachidonic acid. The reactions modify the membrane lipid content and their products effect other pathways. Factors inhibiting or activating constitutive enzymes must also have their own controlling factors to allow the enzyme to work as needed. PAF is a fluid phase mediator of interaction-adhesion, [4] and PAF-like lipids (fragmented PC) are released in vesicles and stimulate neutrophils adhesion. [19]

Outstanding as the product of the only major change in *in vitro* enzyme activities detected here at activation is a secondary product in Phospholipase assays, a phospholipid (1-alkyl, 2-acetyl-glycerol-3-phosphocholine), also known as PAF. PAF is also produced *in vitro* from Bodipy PC by starfish dissociated follicle cells, eggs stimulated by 1-methyladenine and sperm as well as by sea urchin zygote membranes after fertilization. PAF is shown here to artificially activate the egg cortical reaction.

PAF is an autocoid lipid whose 7-pass-G-protein-associated receptor [20] is important in many kinds of cells [21]. Enzymes known to associate with or be activated by PAF receptor as a multivariant multienzyme complex [22] in various cells include: PI3kinase (lipid kinase), Phospholipases- D, -C, -A (PLD, PLC, PLA), protein kinase C (PKC), GTPase, tyrosine kinase, and MAPKinase [23] [22]. On the other hand, cAMP production [24] and resultant PKA activation can be inhibited by PAF. Activation of receptor also causes Ca^{++} concentration increase by influx and release [25] [26]. Products of these activated enzymes could explain many previously unexplained effects seen at fertilization. Since no single sperm receptor on eggs has been found to initiate or be critical for all of the activation events [27], a cluster of receptor interactions [27] [28], together with PAF-PAFR, are herein proposed as a possible means of sperm activation, penetration of the egg envelopes, engulfment by the egg, and disabling apoptosis mechanisms. These hypotheses presented are testable in the future with current technology.

Partial activation of eggs occurs following either influx of calcium due to calcium ionophore A23187 [29] or pH increases due to NH4 treatment [30] or application of PAF which stimulated PAF receptors to open Ca^{++} channels [26] and activate remodeling and signaling pathways.

The PAF produced is an autocoid lipid signal important in reproduction and through its receptor may aid gamete union as well as activation of phospholipases A,C, and D, phosphatidylinositol-3-phosphate kinase, GTPase, opening Ca++ channels, and activating protein kinase C to phosphorylate many cell proteins. The results implicate previously overlooked enzyme activation, production of lipid-ligand, which could initiate ligand-receptor and receptor-enzyme associations and activations crosstalk involved in sperm and egg interactions. Negotiation with other egg receptors about which pathways to activate or deactivate amongst those for proliferation, differentiation and prevention of apoptosis is suggested.

METHODS

MEMBRANE PREPARATION. Sea urchins eggs were obtained from *Strongylocentrotus purpuratus* (purchased from Marinus, LA, CA) by injection of 0.6M KCl for gamete shedding, fertilization accomplished as in [31]. *Asterina miniata* (collected by Marinus) had gametes extracted from dissected gonads and eggs were treated with 1-MA, then fertilized as in [32].

Unfertilized and fertilized eggs (checked under microscope to insure fertilization, with no fertilization membrane removal) collected progressively from the same batch of eggs at times after sperm addition (10 min unless labeled otherwise), were rapidly cooled and sedimented at 15,000 rpm, sea water was decanted, and the pellet maintained in ice to halt development. Samples were prepared for flotation gradients with sucrose, step density gradient all w/w (65%, 40%-contains homogenate sample with sucrose added, 30%, 22.5 % , 0.2M sucrose-Mg++ homogenizing medium) fractions collected as before [31]**.** Other samples used PLD Homogenizing Medium, modified from that previously used for Phospholipase D (PLD) studies on cultured *Hela* cells (5 volumes of 100mM KCl, 5mM $MgCl_2$, 1 mM ATP, 10mM benzamidine, 25 mM Tris Cl pH 9.6) (modified after [33]), for homogenization using the highspeed microattachment on a Sorvall Omnimixer at top speed on ice for 2 min. Whole homogenate pellets were isolated after centrifugation at 4^o C at 100,000 g for 20 min or some homogenates adjusted with sucrose were placed on gradients identical with the sucrose ones above.

ACTIVATION AGENTS: A23187 50 µg/ml; ammonium chloride (NH4) 10 mM in sea water, pH 9.0 [30] and PAF 0.5mM in sea water or with 0.5% BSA. Membrane density gradient fractions from such treated cells were prepared for comparison to controls.

ENZYME ASSAYS:All enzymes were assayed in the same basic mixture (in either total membrane pellets or density gradient fractions) using PLD ASSAY MIX (150mM NaCl, 25 mM Hepes, pH 7, 5 mM EDTA, 1 mM EGTA, 1 mM DTT) similar to that used for PLD in yeast membranes [18]. 40 µl of membranes (2 mg/ml wet weight/volume) suspended by teflon homogenizer was placed in 200 µl microfuge tubes and 4 µl of 2 mM octylglucoside (detergent to suspend lipid unless otherwise noted), containing 1mM Bodipy-PC (or other substrate) added. To start the reaction 2 µl ethanol (or n-butanol) was added and tubes were vortexed. Certain samples had other alcohols or no alcohol added or final concentrations of 0.4 M glycerol. To test for agents controlling the enzymes, 1 µl 0.28M Ca^{++} or 4 µl added 100,000g, 30 min supernate [34] from egg or embryo homogenate or 0.1mM sodium orthovanadate, [22] were added. The 0 time samples were placed on ice and the rest were incubated for 40 min (or other specified time) at 35^o C or room temperature where noted, 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. Remaining samples were frozen, to be placed on TLC with different solvents a few days later. Plates were run in saturated newly prepared paper lined chambers using chloroform:methanol:water: acetic acid 45:45:10:1 [18]. Kodak 35mm Techpan Film [18] and Kodak Technidol developer captured the visible fluorescent image from longwave UV exposure of plates in a TLC illuminator through a plastic UV filter. Images were

digitized by a Microtek 35mm slide scanner, then using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) R_f values and densities were measured on a MacIntosh Power PC computer.

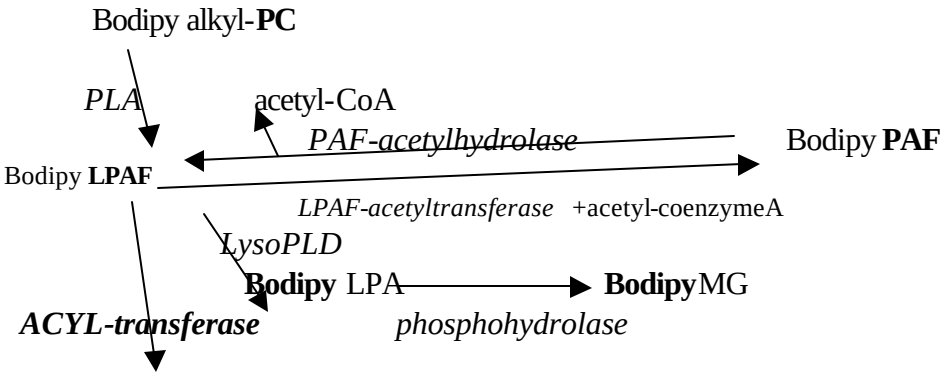
MATERIALS

Molecular Probes Bodipy or other fluorescent compounds were used as phospholipase substrates: BodipyPC (BPC) is PAF analog- D3771-alkyl C1 labeled, C2 10 carbon acyl chain used in most of the assays; Bodipy Fl C11 -lysoPAF (D-3772) with label alkyl on C1; PA- D3805-C2 labeled Bodipy Fl C5, C1 acyl 16 carbon, diammonium salt HPA; BPAF-A3773 Bodipy Fl C11-PAF, with alkyl Bodipy chain on C1 and acetyl group on C2; D7707-alkyl C1 labeled, C2-acyl-16 carbon chain; bisPC is B7701 bis Bodipy Fl C11-PC.

RESULTS

ENZYME ACTIVITY IN UNFRACTIONATED HOMOGENATE PELLETS. Direct detection of phospholipase A enzyme activities used an assay with fluorescent alkyl-PC substrate (Bodipy PAF analog), and Bodipy-containing fluorescent products (Fig.1) .

FIGURE 1 COMMON DETECTABLE PRODUCTS OF BODIPY PC 3771 (PAF ANALOG) IN SEA URCHIN EGGS



PLA products are mainly in the bottom half of the TLC plates below substraate in total homogenate membrane pellets (Fig.2).

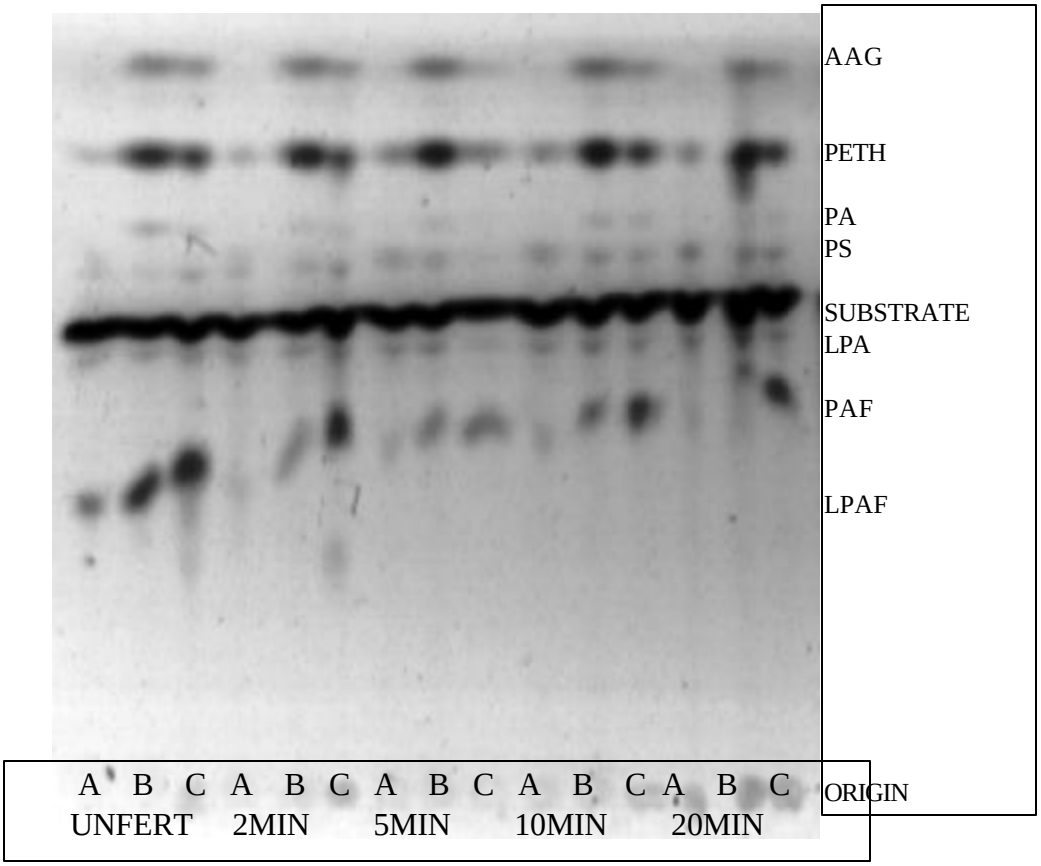
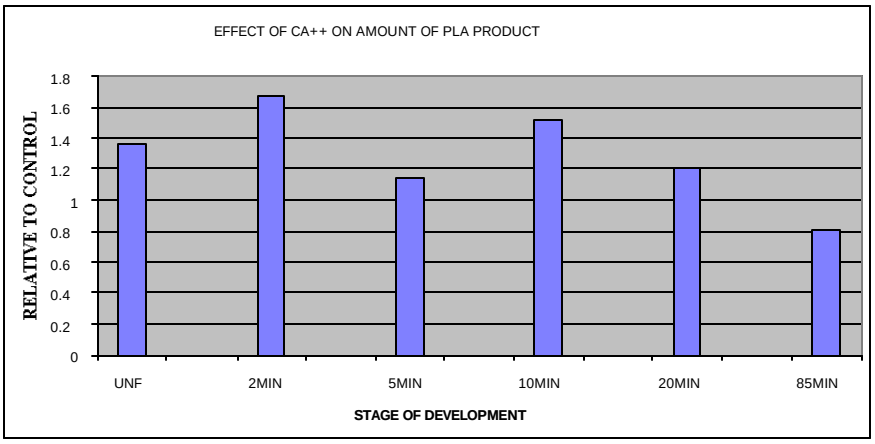


FIGURE 2. PHOSPHOLIPASE AND OTHER LIPID PRODUCTS FROM WHOLE HOMOGENATE PELLETS

Figure 2. Effect of time after fertilization and Ca^{++} on enzymatic products of Bodipy PC (PAF analog 3771): Using BodipyPC as substrate LPAF is made by PLA; PAF is made from LPAF by acetyltransferase; AAG is made from PA by PA Phosphohydrolase; PA and Peth are made by PLD; MG is made from LPA by PA Phosphohydrolase; LPA is made from LPAF by LPLD in sea urchin egg homogenate pellets. PLD homogenate total membranes collected from unfert and at set times after fertilization and PLD assay run. 0 is control at zero time before incubation at 36 degrees C for 45 min. Ethanol is in all samples, Ca^{++} in those marked. TLC plate run in chl:meth:acetic acid:water 45:45:1:10. Spots are labeled at level of standards migration.



Density analysis of plate 2 using NIH Image is shown above. Zero represents the activity of the control Lane B without calcium.

PLA_2 produces lysoPC from PC or in this case where we are using alkyl PC as substrate, the products are lysoplatelet-activating factor (LPAF) and fatty acid (FA, from removal of C2 acyl chain). But FA is not fluorescent since the Bodipy label is on C1. Above the axis is stimulation, below is inhibition of enzymes making those products in lane C.

PLA_2 product LysoPAF is seen below the alkyl-PC substrate on the plate (Fig 2) in all stages from unfertilized to 20 min after fertilization. PLA_2 lytic activity was enhanced by Ca++, and LPAF was then converted by another enzyme, LPAF-AT, to PAF (Fig 2). That was revealed through the change in the TLC R_f value for the LPAF product of PLA_2. LPAF can be rapidly acetylated with acetylCoA to make PAF [35] which moves further up the TLC plate, or alternatively, LPAF can be acylated by enzymes of the ER to make PC [33] which moves even further up the plate. The faster moving TLC spots on the right side of the plate, after stimulation, represented the conversion of LPAF to PAF, which occurred every time the PLD homogenizing medium was used to break the cells as in this case instead of sucrose homogenizing medium. Some PLA activity occurs in the Unfert control without Ca^{++}, but no PAF-AT is seen since it requires the added Ca^{++} until 2 min after Fert (no earlier time was tried) though added Ca^{++} stimulated both LPAF-AT and PLA even further until 20m. So calcium can determine which direction the reactions will go on the map in Fig 1. PLD products including Peth and PA appear above the substrate on the plate in the total homogenate post-centrifugation pellets when ethanol was present and will be dealt with in a separate publication.

DENSITY GRADIENT FRACTIONS: PHOSPHOLIPASE A and LPAF-ACETYL-COA ACETYLTRANSFERASE

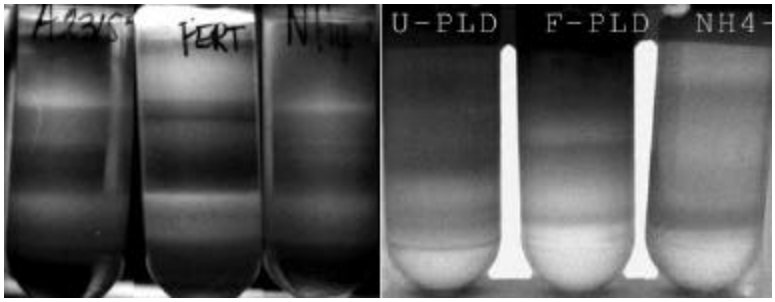
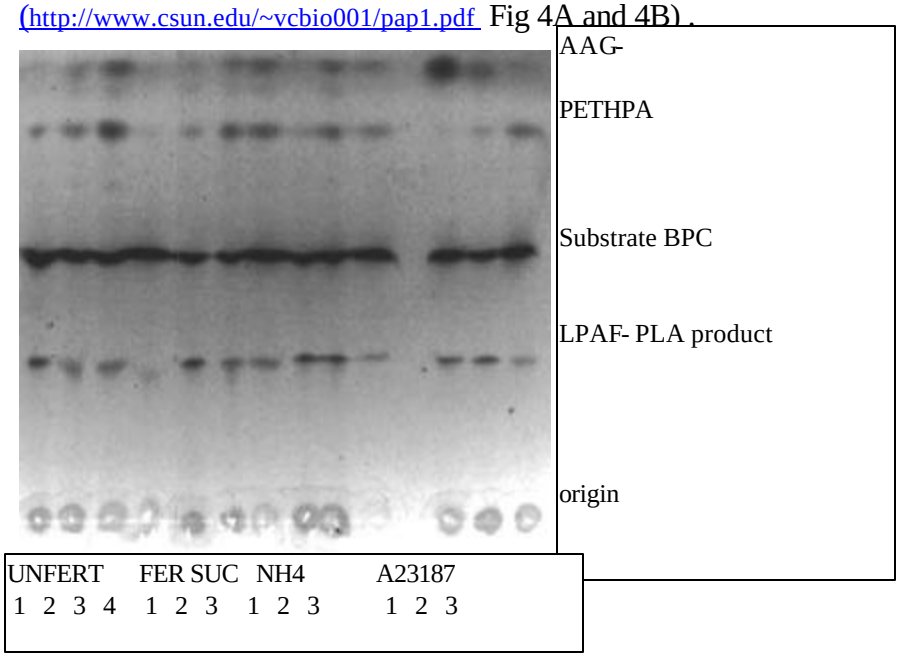


FIGURE 3 Density gradients of homogenates of eggs:A23187 activated, fertilized, ammonia activated, and unfertilized. Sucrose homogenates, the three on the left, PLD homogenates, the three on the right.

PLA2 is active in all Unfert and Fert and ammonia activated and A23187 activated sucrose density gradient fractions as seen in the first paper of this series (http://www.csun.edu/~vcbio001/pap1.pdf Fig 4A and 4B) .

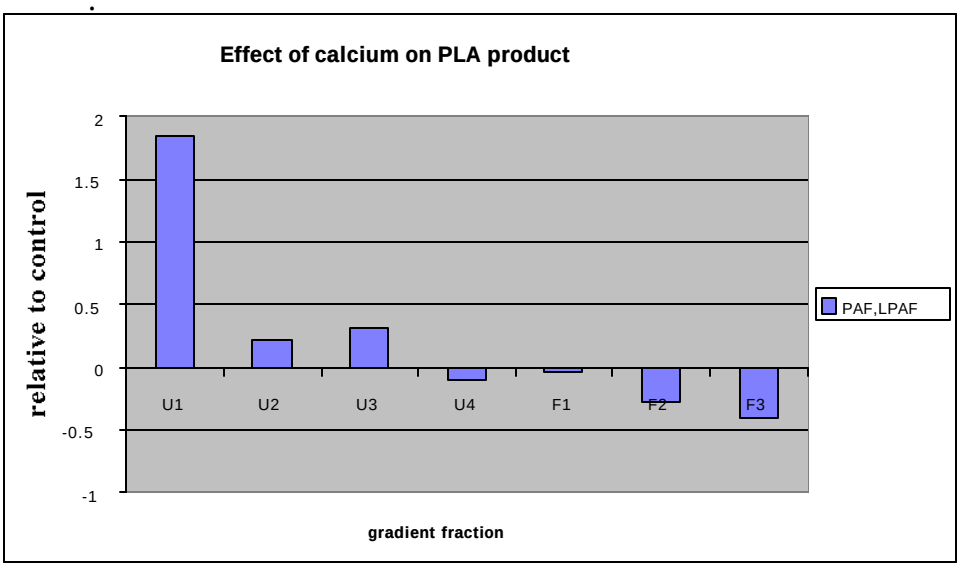


There is a difference in Ca^{++} sensitivity between enzymes of various membrane fractions of unfertilized and fertilized density gradients (Fig. 4). The values are relative to controls without Ca^{++} which have the value 0 (Fig.5). PLA is highly activated by Ca^{++} in Unfert M1 and not inhibited in any fraction, but Fert fractions M2-3 were inhibited by Ca^{++}. This may be evidence for more than one kind of PLA or their associated molecules present in the different pellets. In pellets of Unfert M1 and Fert M1, especially with Ca^{++} present, or in F1,F3 controls with no ethanol (Fig 4) the PLA product LPAF became converted to PAF after fertilization or in Ca^{++} as the result of acetylation of LPC by LPAF-acetyltransferase

The formation of PAF by LPAF-AT is not as dramatic in the fractions as it is in the whole homogenate pellet (compare Figs. 2 with 4 (replicated here) of the other). That could be due to either a better isolation from the supernate because of the dilution in the gradient, or some factors are working together in the whole pellet. What activity there is is restricted mainly

to M1. The presence of control factors in the supernate would explain this difference. Whole homogenate had no PLA enzyme activity.

FIGURE 3B. COMPARISON OF DENSITY GRADIENT FRACTION PRODUCTS OF ENZYME ACTIVITIES



The same PLD homogenizing medium that showed the PAF production in sea urchin whole homogenate pellets and effects of added supernate showed similar PAF production effects but no PLA effects in starfish supernates (which must contain many cofactors, small G proteins and actin-binding proteins). Previous work showed that after injection of fluorescent DiI into immature starfish eggs, membrane sheets were seen after 1MA treatment, associated with yolk platelets forming "shells" which disappeared at first polar body formation, and after sperm were added, evidence for ER fragmention was noted within 1 min. [36]. Previous studies have shown that ATP (present in the PLD homogenizing medium) could activate the LPAF acetyl-CoA-acetyltransferase in the pellet isolated using that procedure, but not as much in the sucrose homogenate density gradient fractions. Fig 9 shows products from the PLD assay mix, using starfish egg pellets alone in the C- control- wells and together with the various supernates in the S wells. Presence of LPAF, shows that PLA is constitutively active at all stages. LPAF was more obviously converted to faster moving PAF by the pellet from eggs that have been activated by 1-methyladenine (1MA) which is a radial nerve hormone used to reinitiate maturation divisions. After the addition of sperm, even more PAF is made converting all the substrate present, but in eggs from 30 min later not all LPAF is converted to PAF. There is no activation of PLA at fertilization as in sea urchins.

Fig 9. PLD is not active in starfish eggs and PLA and Acetyltransferase are activated by 1MA in the assay conditions. PLD assay mixes were run using Bodipy PAF analog 3771 and starfish egg fractions. A S1=UNTREATED SUPERNATE; S2=1MA TREATED SUPERNATE; S3=SPERM 30 MIN LATER SUPERNATE; S4=ANOTHER 30MIN LATER SUPERNATE

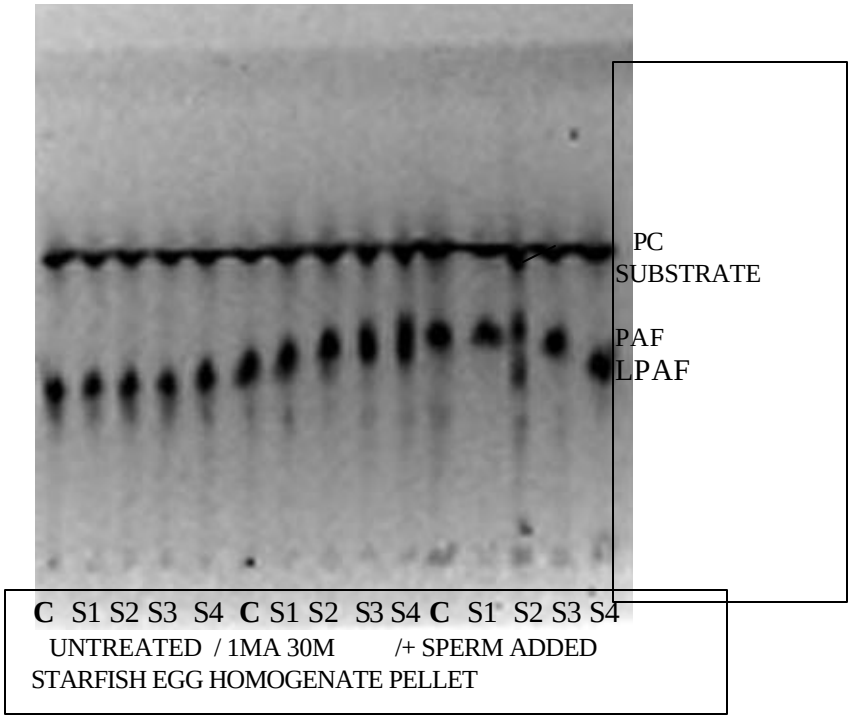
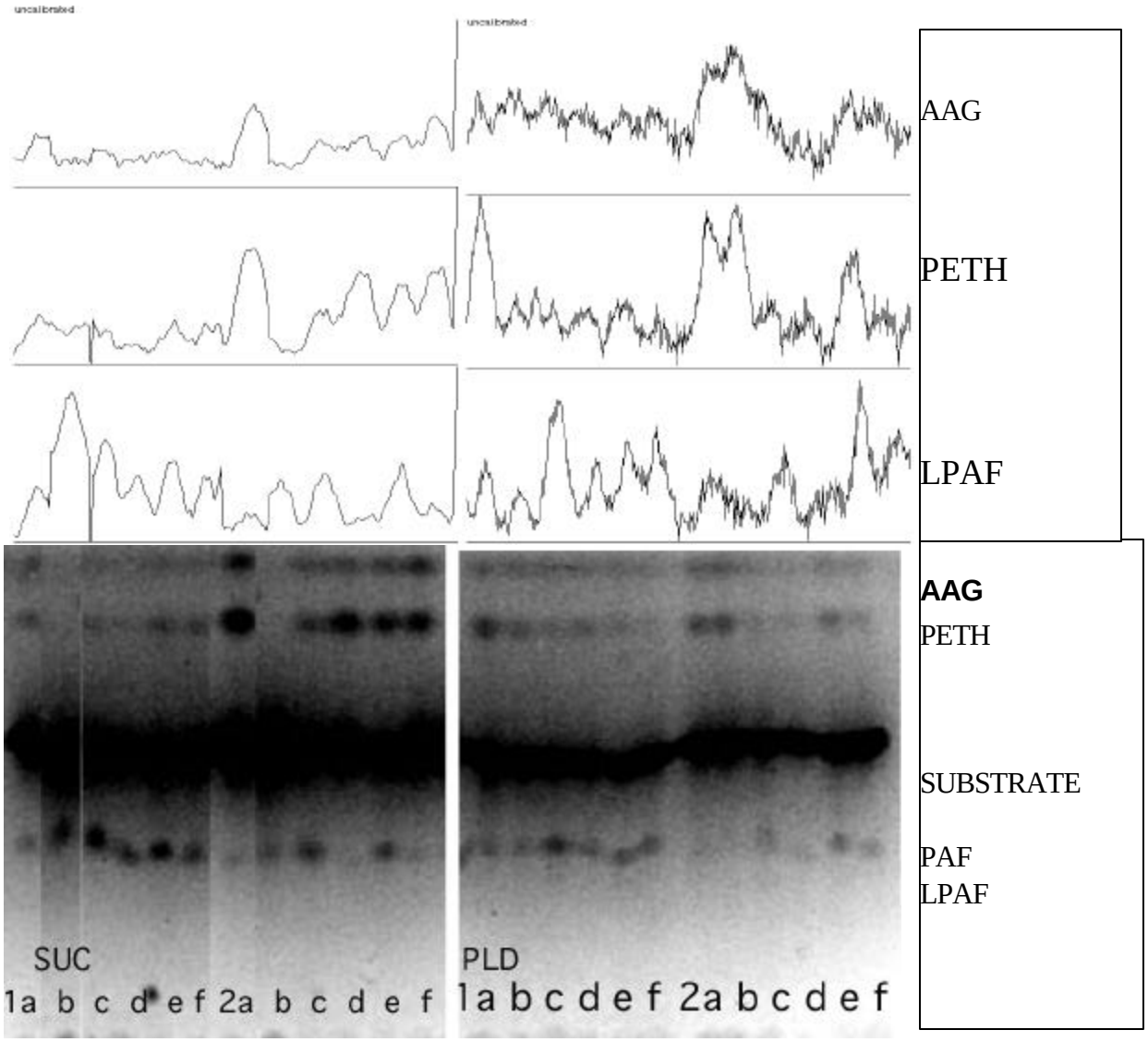


FIGURE 9. ACETYLTRANSFERASE BECOMES ACTIVE AFTER 1- METHYLADENINE AND SUPERNATE CAN ALTER THE ACTIVITY.

The effects of post-centrifugation supernates (S1-S4) on the pellet enzymes, when compared to controls without supernate added , reveals their effects are only on the activation of LPAF-AT or PAF-AH and are not all alike (Fig. 9). The supernates have different effects on the pellets from the three different stages of activation. Unfert super (S1) does little to any pellet, unlike the sea urchin data. Unfert sea urchin eggs have completed meiosis and starfish unfert have not even started theirs, so they are not at equivalent stages. All supers effect pellets of 1MA treated or fertilized eggs. The addition of supers from homogenates after 1 MA treatment (S2-4) increase the proportion of PAF/LPAF probably by activating LPAF acetyltransferase in the 1MA pellet. The same supers from Fert30m (S3) and Fert60m (S4) homogenates had the opposite effect in Fert30min pellet, a decrease of PAF probably due to conversion back to LPAF by an acetylhydrolase activated in the pellet which was not there in the pellets of earlier stages. Changes in both supernates and pellets must have occurred after 1MA and sperm treatment.

Addition of supernate to sea urchin enzyme assays showed some factors effecting PLA were present. Unf PLD supernate is special because it is the only one that activates M1 and M2 fraction PLA activities as well in both sucrose and PLD homogenate types , and activates LPAF-AT in suc M1. Fert PLD supernate does not activate the PLD homo M1,M2 PLA in those fractions. Fert PLD Sup does however activate M1 PLA from suc homogenates. LPAF-AT production of PAF is only in sucrose M1 and is turned on by Ca^{++} and UnfPLD sup. The supernates mimic the calcium effect somewhat on the M1 but the inhibition and activation by the supernates has a more complicated explanation including how the membrane fractions and supernates were prepared and whether they are Unfert or Fert. The supernates do not supply the enzymes, as shown by activation with adding only calcium. To try to summarize these results, Table 1 presents a matrix solution of some hypothetical activating and inhibiting

factors in the supernates which combine with corresponding response elements for them in the membranes to influence the enzymes.



Control of Fert M1-M2 density gradient fraction enzymes (40ul) by 4 ul various supernates, all have ethanol; the ones on the left are M1-M2 membranes from sucrose homogenate separation, on the right are M1-M2 from PLD homogenate mix:**a-f, contain in that order-a)control, no supernate, b)no sup,+ added Ca++, c) +supernate from Unf PLD whole homogenate; d) + supernate from Fert PLD whole homogenate; e) +supernate from Unf sucrose whole homogenate, f) + supernate from Fert PLD whole** homogenate+ATP. Tracings at the top are of densities going across the rows of three of the lipid products: AAG on top, Peth next row, LPAF bottom row; obtained by NIH Image. Conditions for TLC the same as in Fig 2.

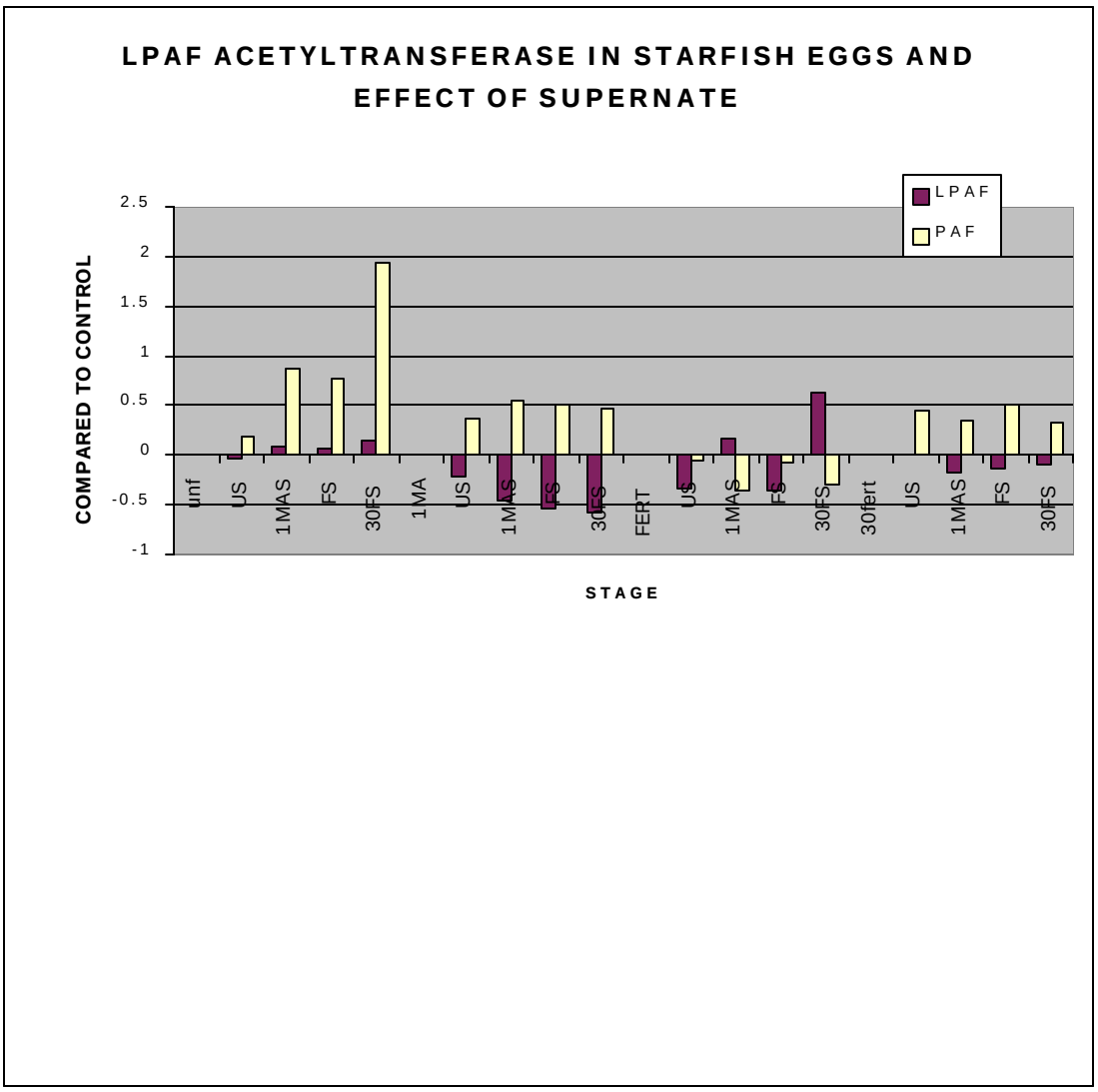
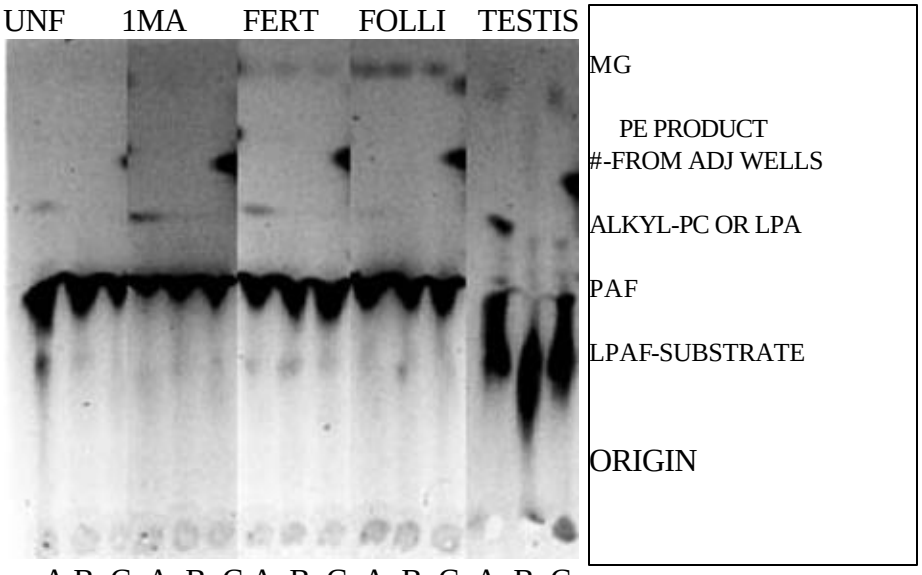

LPAF ACETYLTRANSFERASE IN STARFISH EGGS AND EFFECT OF SUPERNATE
LPAF
PAF
COMPARED TO CONTROL
2.5
2
1.5
1
0.5
0
-0.5
-1
unf
US
1MAS
FS
30FS
1MA
US
1MAS
FS
30FS
FERT
US
1MAS
FS
30FS
30fert
US
1MAS
FS
30FS
STAGE

FIGURE 10A



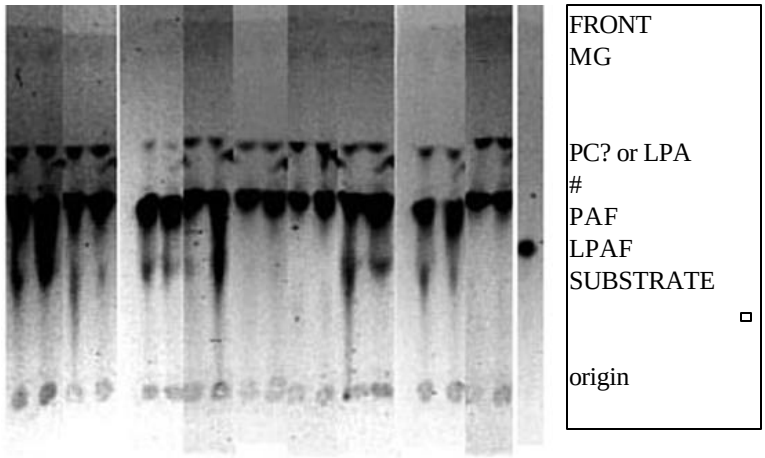


FIGURE 10B CONVERSION OF LPAF TO PAF BY ACETYLTRANSFERASE AND THE EFFECT OF SUPERNATE, TEMPERATURE AND DETERGENT

Fig 10 shows more direct proof of the production of PAF *in vitro* by Acetyl-CoenzymeA-acetyltransferase (EC 2.3.1.67) (AT) in the remodeling pathway by membranes of starfish eggs after 1MA treatment. Using Bodipy LPAF as substrate in the presence of acetyl-CoA, instead

of relying on PLA_2 to make it, shows that both Ca^{++} and vanadate activate the Acetyl-CoenzymeA-acetyltransferase in untreated Unfert eggs moving all fluorescence up the plate (Fig. 10a). After treatment with 1MA, most products are increased even in controls. This could lead to the conclusion that 1MA is altering something responding to Ca^{++} and activating the enzymes and these effects are on the AT (since PLA_2 is not detected in these plates since its substrate is not present). When cells are incubated with ATP or other nucleotides, PAF is formed. A purine Receptor with G protein is involved [37]. Perhaps 1-methyladenine is activating such a receptor.

The lysoPLD product LPA seen in the A lanes is decreased with Ca^{++} present (B lanes) or in vanadate (C lanes). Vanadate inactivates protein phosphatase so that phosphorylated proteins are not dephosphorylated [23] and LPAF-AT activity is increased . LysoPLD works only on alkyl lipid [38]. The PAF enzymes are also present in follicle cells removed from the eggs, and they are mostly activated by both Ca^{++} and vanadate, as in the untreated egg (Fig. 10A). In isolated testis, the kinetics of the enzyme are different since not all of the substrate is converted to PAF, even less with Ca^{++} and vanadate, compared with the eggs and follicle cells (Fig.10A). Even at zero time of incubation and at room temperature the Fert pellet LPAF-AT rapidly converts LPAF to PAF (Fig 10b, 23C). Addition of Fert supernate to the mixture (37C+sup) shifts the equilibrium at 37C so that in the mixtures without detergent (A lanes), the substrate is impressively all converted to PAF by 5 m and in detergent (B lanes) by 25 m compared to 45 m with no sup. In some cases PC is formed from LPAF. When ATP is present, acyl transfers onto LPAF are favored rather than acetyl transfers, resulting in PC not PAF. The acetyltransferases are activated by phosphorylation, though inhibited by MgATP [39].

FIGURE 11

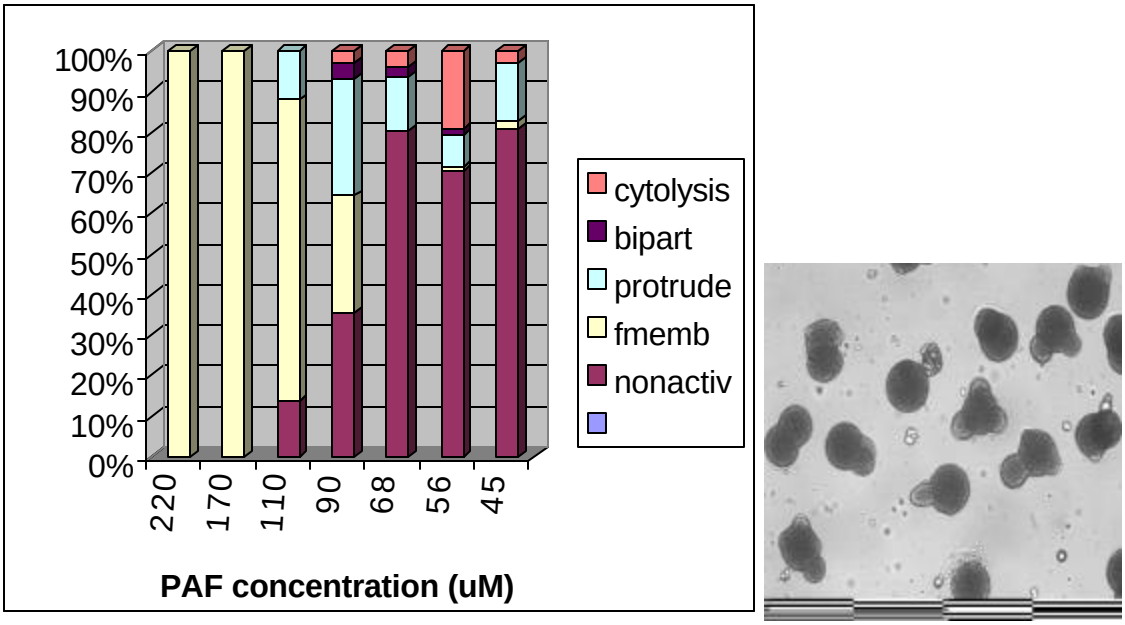


Fig. 11a shows the effects of PAF suspended in sea water on one batch of unfert sea urchin eggs. Eggs are all activated above 200 µM, but below that some eggs without the cortical reaction show large protrusions Fig 11b. In the six batches of eggs tested, all were activated but

cytolysed above 500 μM, then a cutoff of activation at about 100 μM but with differing degrees of cell protrusions between 50 and 500 μM.

In artificial activation the agents prevent some of the normal elements of membrane cycling so the lipid changes due to that are also prevented. Ionophore induces exocytosis and NH4 prevents it. NH4 [40, 41] and the Ca^{++} levels in the ionophore may also prevent endocytosis. Inhibition of the cortical granule reaction (as with NH4) inhibits the endocytosis until much later [42]so it is not likely that vesicular transport occurs right after NH4 activation. These artificial activation changes in membrane cycling alter lipid content and membrane density of affected fractions. Ammonia (NH4) treated eggs have altered internal pH, late activation events, no exocytosis and calcium ionophore (A23187) treated have altered IP3 , early events, exocytosis.

DISCUSSION

Membranes are not digested down to small products by phospholipases *in vivo*, but are only changed by a small percent to cause changes in cell behavior. There are certainly mechanisms for many lipid interconversions present, since sea urchin fractions have high basal levels of enzyme activities with alkylPC substrate producing: Peth, PA, LPA, PG, LPC, bisPA, AAG, Pbut, PAF, LPAF, AAG, and MG. The enzymes must be tightly controlled by many factors in time and space but cells have to be ready to rapidly alter membrane properties and produce second messengers. New proteins do not have to appear in order to cause the changes and enzymes can be present and able to be activated and inactivated at any time. Most factors regulating enzymes are controlled by receptor signals and include phosphorylation which in turn is controlled by another factor; being bound to the membrane, near the enzymes or receptors doing the phosphorylation. Other regulatory factors are amount of free Ca^{++}, ATP, and bound inhibitor or activator proteins.

CONTROL OF ENZYMES:

Effects of phosphorylation. Vanadate treatment inhibits protein tyrosine phosphatase that results in: augmented phosphorylation of proteins and loss of active PAF receptors [23]; activated PLD [43]; lack of DAG production because of inhibition of PAPH [38], and increased production of PAF through activation of LPAF acetyltransferase [44]. Effects of phosphorylation were tested and verified here by vanadate stimulation of LPAF-AT production of PAF. The PKC family has some members that stimulate PLD by phosphorylation in response collectively to Ca^{++}, PS, and DG. Other isoforms of PKC do not require Ca^{++} or DG and some do not require kinase activity but activate PLD just by the presence of regulatory domain pieces of PKC.

Effects of calcium. The PLD, PLA and PAPH enzymes found in the four membrane density gradient fractions do not all have the same sensitivities to Ca^{++} or ethanol. Such differences suggest there may be alternate forms of each [45], or different associated regulatory molecules such as calmodulin and calmodulin sensitive kinases and a possible change of state after the Ca^{++} release in each fraction after fertilization. Assay conditions are artificial as both EDTA and EGTA are present in the mixture so that altered divalent cation content is masked. In macrophages phospholipases are also found in several membrane fractions, with Ca^{++} dependent ones associated with plasma membrane, ER, mitochondria [46].

Presence or absence of activators or repressors (or indirect effectors) can affect the enzymes as discovered in other studies with Ca^{++}, alcohol [18] ; glycerol, ATP and Mg^{++}[47]; some lipids: PMA without Ca^{++} [48] or phorbol ester and bradykinin [33] effect PLD, and oleate stimulates a PLD, but the stimulus is inhibited by PIP2 (3.5 uM) or PA (200 uM). When PLC breaks down PIP2, this PLD is disinhibited [49].

CYTOSOL EFFECTS. Changes in both supernates and pellets must have occurred in this study after 1MA treatment in starfish and addition of sperm in both sea urchins and starfish. Cytosol factors include complicated sets of inhibitors but no activators of PLD and activators but not inhibitors of PLA. This could involve binding of cytosolic proteins to membranes or the cytoskeleton, the binding of Ca^{++} to proteins, phosphorylation of proteins, dimerization of proteins, binding to receptors or adapters after a stimulus, so that adding more stimuli through the cytosol from a later stage can have an effect on pellets only before that effect normally

occurs. Brain cytosol contains an inhibitor of PLD on all stimulation pathways- ARF-GTP and PMA, that also inhibits PLC [50]. Cytosolic proteins control PLD [51], either in an ATP-dependent or ATP-independent manner in other systems. They include GTP-dependent ARF and RHO [52]; PKC family which can bind to PLD.

The cytosol inhibitors of PLD must be present to regulate the constitutive activity as well as to promote activation after stimulation by a change in their state. Gelsolin, a nucleotide binding protein, copurifies with PLD and activates PLD only when no nucleotide triphosphate is present . Actin rearrangements are coordinated through this physical association of gelsolin and PLD [53]. Gelsolin can work synergistically together with PIP2, suggesting multiple pathways of regulation *in vivo* [54][55] [56] [57]. Such changes would be expected during the many events of activation, most involving cytoskeletal changes. Long term signaling that is dependent on DAG, such as PKC activation, relies on PLD activity found during division and differentiation [58] [59]. Further study of mechanisms of control in eggs is needed.

Earlier work [15]; [60] suggested PLA had a connection to the sea urchin egg cortical reaction. The present study suggests that may be by providing the substrate for PAF production by LPAF-AT. PAF would then stimulate a whole series of responses including the cortical reaction.

FUNCTIONS OF PAF AND LPAF-ACETYLTRANSFERASE IN ACTIVATION

Against the background of consistent constitutive PLD, PLA, PAPH activity which must be held in check by cytosol components, a stark contrast is seen in the progressive changes in LPAF-acetyltransferase after 1MA or sperm activation of eggs and the changing responses of the enzymes to supernates which also may have changed components. What is LPAF-AT accomplishing by producing PAF?

PAF effects occur through activation of a PAF receptor (PAFR), which has SH_3 (Sarc-homology 3) domains for binding to and affecting the cytoskeleton; SH_2 (Sarc-homology 2) domains for binding to effector molecules (PLC, GTPase activation protein-GAP, or PI3K, PLD, PLA all of which have SH_2 groups which can bind to tyP on the receptor helping them to become phosphorylated as well). PAFR stimulation can come from autocoid secretion of PAF onto the same cell surface or from secretion by the nearby cells. PAFR overexpression causes abnormal reproduction, bronchial hyperactivity, and tumors in skin [20]. In general, an early event in the PAF signal is phosphorylation of the associated enzymes by the receptor or nearby src tyrosine kinases [22]. Then activation of those associated enzymes leads to products causing further domino effect changes. The receptor together with bound lipid utilizing enzymes would be a membrane alteration machine as well as a producer of lipid second messengers.

For example on B cells, ty phosphorylation of PLCγ1 by src (Fyn, Lyn- cytosolic) kinases is maximal within 2 min; autophosphorylation of the receptor within 2 min; p85 regulatory subunit of PI3K activated within 10m max. After activation of PLC, there is increased free Ca^{++}, activation of PKC, then protooncogenes are expressed. PIP_3 made by PI3K induces autophosphorylation of PKCδ necessary for mitogenic activation in oocytes. Ty-kinase inhibition decreases these effects . Associated enzymes make clusters of proteins; heterogeneous multienzyme conglomerates [22]. Exposure to TGF-β on B-cells causes upregulation of PAF receptor gene. Increased transcription is due to a responsive element -44

to-17 to start, which facilitates binding of nuclear factors. PAF causes polyPI breakdown, Ig secretion, modulation of the cytokine TNF-β production in B-cells [61].

A review of the literature on phospholipases and PAF and gametes shows their participation is universal in reproduction. PAF Effects on sperm. PAF increases sperm motility at 10^{-8} to 10^{-9} M, but it is toxic at higher concentration [62]). PAF causes the acrosome reaction [63] and PAFR antagonist BN52021 slows the sperm motility (*S. intermedius)* [62]. PAF at 10-6 M increased capacitation and sperm-egg interaction in mice [64]. There are PAFR on human sperm ([65]) which activate both PCspecific-PLC and PI-PLC to aid in fusion of membranes of sperm acrosome and PM [62]. Sperm jelly receptors cause activation of sperm phospholipases ([66]). In human sperm A23187 or progesterone stimulated PAF production and newly synthsized H3-PAF is found in the supernate, so released [67].

PAF and eggs and embryos. PAF receptors are found in 2-cell human embryo which release Ca^{++} when PAF is added with a 4x increase in intracellular Ca^{++} in human two-cell stage embryos [68] and in other cells measured with fura-2. Control of the number of receptors may be at the level of translation since PAFR transcripts are detected in late mammal two cell stage all the way to blastocyst and low level maternal RNA for PAFR is detected in unfertilized and fertilized eggs[69]. In the current study $2x10^{-4}$ M PAF caused the cortical reaction. It is not suggested that PAFR is the only receptor involved at fertilization because in some species PAF antagonist BN52021 did not prevent fertilization even though it slowed the sperm motility (*S. intermedius*) though some PAF antagonists did prevent fertilization in rabbit [70]. PAF causes altered cell adhesion [71]), In the current study, the starfish follicle cells, eggs and testis could all make PAF, so cell interactions are entirely possible, and mechanisms controling the enzymes are very important. More human sperm entered and decondensed in eggs treated with 10^{-14} M PAF [Angle, 1993 #327]; PAF enhanced survival and implantation of *in vitro* fertilized embryos [72]; [69]; [64]. Calcium ionophore A23187 can artificially activate sea urchin eggs, including the cortical reaction. A23187 activates cytoplasmic PLA by causing it to attach to membrane in leukemic cells [73] and activates LPAF-AT and causes PAF production in neurons [74] and spleen [75]. In this study PAF itself can cause the cortical reaction, so it is also probably involved in the A23187 response. PAF causes massive degranulation with secretion of histamine and serotonin secretion by platelets with inflammation resulting [23], activated secretion of cytokines [76], so it may cause the cortical reaction in eggs.

> There is a difference in Ca^{++} sensitivity between enzymes of unfertilized (Fig 8)and fertilized density gradient fractions (Figs. 6,7) and artificially activated (Fig. 7).

MISSING PHOSPHOLIPASE PRODUCTS IN ARTIFICIAL ACTIVATION. Artificially activated eggs have altered membrane cycling. Ionophore induces exocytosis and NH4 prevents it. NH4 [40, 41] and the Ca^{++} levels in the ionophore may also prevent endocytosis. Inhibition of the cortical granule reaction (as with NH4) inhibits the endocytosis until much later [42] so it is not likely that vesicular transport occurs right after NH4 activation. These artificial activation changes in membrane cycling alter lipid content (myref) and membrane density of affected fractions. The artificially activated eggs have Plipase enzyme activity in most fractions, so it doesn't seem to be a case of missing enzymes, but just as in the normal fractions the control mechanisms must be what are changed.

Related PAF effects on other cells. PAF is a chemoattractant affecting motility of immune cells ([77]). There is association of PLC, PLD, PI3K, PLA with PAFR which results in their phosphorylation in B cells [22]. PAFR antagonists SCH37370, 3H-Web2086 inhibit the PAF effect in other cells. Both Ca^{++} influx and release are influenced by the antagonists [26]. Activation of phospholipases, kinases and transcription factors, calcium channel opening is caused by PAF stimulation [78]. PAF induces 86Rb influx and efflux in B lymphoblastoid cells, so it activates a K+ channel due to PAF Ca++ mobilization [79]. Three PKCs move from the cytosol to the membrane after stimulation with PAF within 30 sec, and then are returned to cytosol in 10 min. PKC-α is important in regulation of PAF stimulation of PLD.[80]. PAF formation can be controlled by phosphorylation of AT by an acetyltransferase-calmodulin dependent kinase or a cAMP dependent kinase (PKA), as well as by protein phosphatase which removes the P [81]. External PLD (such as found in sperm), makes PA in the fibroblast PM external leaflet (which could happen on egg) and then it is internalized or hydrolzed to DAG, and PKC is translocated [82].

RECEPTORS WITH LIPID MODULATING EFFECTS FOUND ON GAMETES

These related studies, together with the many sperm-egg receptor models which do not seem to work individually alone, led to review of possible receptors involved besides PAF receptor which could have a effect on the lipids.

A) Epidermal growth factor receptor. EGFR and EGF ligand cause tyrosine phosphorylation of receptor-associated PLD2 in kidney fibroblasts [83], EGF receptors occur on bovine eggs [84] . When human EGFR were synthesized in Xenopus eggs, EGF caused activation of the eggs [85]. Fertilin extracellular protein on mouse sperm binds to sperm receptor on egg. Fertilin has several domains: metalloprotease, disintegrin, EGF-like and fusion domains [86].

B) Integrins are found in sea urchin eggs [87] [27].; Fertilin is on sperm and has integrin binding domains [88]as well as a fusion peptide domain which induces fusion of vesicles with negatively charged lipid [7] which could be involved in cell adhesion and fusion. Fertilization is inhibited by anti-integrin antibodies, both adhesion and fusion. Vinculin (140 and 150 kDa) , actinin (85 kDa) and talin (210 kDa) are present in the cytosol as well for signalling by integrin-ligand ([88] [27]). Co-stimulation of integrin and sperm receptor could stimulate metabolism and phagocytosis of sperm by egg as in leucocytes. Multiple pathways for activation by large multimolecular complexes are known [27].

C) The 350kDa sperm receptor may be a complex of proteins not amenable to dissociation. Rapid transient phosphorylation is seen at Fert [89]. Sperm receptor or binding protein from sea urchin eggs is soluble in SDS, 350kDa protein stains with phosphotyrosine antibody at 1 min to 5 min [90]. Certain domains on the protein can prevent fertilization if sperm are treated with it first [91].

D) Intercellular membrane adhesion occurs between two mouse gametes at the fusion step mediated by MHCII located at posterior region of mouse sperm head attaching to CD4 on egg PM which then gets Ty kinase attached to the cytoplasmic domain of CD4 on egg [92]. The mode of activation would be like CD4 on helper immune cells reacting with MHCII surface molecules of B cells or macrophages and this causes a response of

the helper cells which secrete growth factors which affect attached B cells which then secrete or macrophages which then do endocytosis and destruction.

NEW HYPOTHESIS OF FERTILIZATION INCLUDING MULTIPLE RECEPTORS: There is control at activation through cell interaction involving having several kinds of receptors on eggs and sperm: PAFR on both eggs and sperm, with its ligand PAF; EGFR with ligand-domain-containing-proteins with *notch* or EGF domains; CD4-like proteins with the MHCII-like proteins; integrin with fertilin which has disintegrin as well as EGF domains; sperm egg jelly receptor; egg envelope sperm receptor; having them all engaged by corresponding ligands, then sending signals for changes in activity collectively due to cross-talk and modulation. None of the receptors alone can cause or prevent all phases of fertilization.

Survival factors such as ligands for surface receptors are common for maintenance of cells in tissues and organs, but eggs and sperm are released from tissue associations away from such growth factors. The collective array of occupied receptors at fertilization would turn off the apoptosis machinery engaged if there is no gamete fusion, and zygotes would start the proliferation mode. There is evidence that the machinery for apoptosis is present in eggs [93]and sperm [94] and is used during gamete differentiation in the gonads for eliminating imperfect gametes or those damaged by drugs. Such machinery in *Xenopus* egg cytoplasm is used as an *in vitro* apoptosis test system [93]. PAF autocoid stimulation of its receptor and other receptors work together in immune cells. Anti Ig M antibodies in lymphoblastoma deleted 30-40% of cells in 24 hr, but 10^{-7} M PAF decreased the number of apoptotic B cells, as shown by annexin label which attaches to PS exposed on apoptotic cells. Abrogation of apoptosos did not require *de novo* protein synthesis [95]. PAF can prevent apoptosis of nerve cells and aid in neurotransmission [74], PAF receptor inhibits cAMP production.

PAF may also be involved in events prior to fertilization leading up to chemotaxis, adhesion and fusion of gametes. Precedent for such an hypothesis is set by immune cell interactions that utilize PAF and its receptor (PAFR) together with other receptors (thrombin, IgE, cytokine) to recognize, adhere to, interact with [19], and phagocytize cells. PAF has been shown to enhance ovulation [23]; oviduct egg transport [96]; sperm capacitation, acrosome reaction, sperm motility [12]; *in vitro* fertilization [Angle, 1993 #327]; survival of early embryos [69]; [72]; and implantation in mammals. In most of these documented cases, PAFR inhibitors prevent the reactions [97].

INVOLVEMENT OF RECEPTORS, PAF AND OTHER LIGANDS AND PHOSPHOLIPASES AT FERTILIZATION:

STEP 1. Effects on sperm at contact with egg jelly - Some PAF, which came from the egg, may have been caught in the egg jelly, causing chemotaxis of sperm, increasing sperm motility, leading sperm to egg, PAF causes the Ca^{++} effect in sperm, jelly receptors on sperm are activated by jelly and as a result phospholipases are activated, PLA produces LPAF which is converted to PAF by AT; PLC is activated to get acrosome reaction. PAF from sperm diffuses to egg or contacts egg.

STEP 2. Effects on eggs upon contact with sperm: PAF causes egg PAFR Ca^{++} channel to open in egg membrane. The membrane potential changes and secretion of cortical granules occurs. Then there is association of PLC, PLD, PI3K, PLA with PAFR-PAF complex which results in: phosphorylation of PI3K, so now there is PIP2 available as a substrate for PLC, at

the region where PLC becomes activated by phosphorylation; PLD activation by PLC products and resulting PKC activation. The egg has activated PLA which produces LPAF, then LPAF-AT makes PAF because AT is phosphorylated after Ca++ release by a calmodulin-dependent kinase, giving a prolonged autocoid effect. PLD products and PAF effects cause alteration of cytoskeleton and membrane and engulfment of sperm by egg.

STEP 3. Other receptors are activated at the same time- A.) EGFR binds sperm protein with EGF domain, activation of PLD2 B.) integrin binds sperm fertilin. There is also a role in adhesion at fert for these molecules as well as activation [27] [98]. C.) Activation of sperm receptor on eggs by sperm with activation of phospholipases, PI3K, Ca^{++} channels, and preparation for cell interaction includes modification of the cell membrane by phospholipases and proteases in preparation for fusion. PLC yields IP3 and more Ca^{++}, then DAG is sustained by PLD and PAPH. D.) CD4-like molecules on eggs binds MHC on sperm.

STEP 4. PKC prolonged activation due to the multireceptor complex stops apoptosis, initiates a proliferation signal. cAMP antagonists can reverse apoptosis resulting from a cAMP producing agonist and Bcl2 (an anti-apoptosis protein) expression is uneffected, so cAMP is important on its own in apoptosis regulation [99]. Apoptosis promoter Bax RNA is found in all stages of mouse eggs, but antagonist Bcl2 is only in zygotes. Expression may save them from apoptosis [100]. . Cells are protected from apoptosis by activation of Akt [101]. There is more activation of Akt when growth factors (like InsulinGF) activate PI3K, and PAF may do that activation here and be considered a growth factor considering its great effects on zygote survival. PAF's autocoid nature also supports prolonged protection from apoptosis. Kinases bound to the membrane by PIP2 could be released from the membrane by PLC breaking down the PIP2, then they would go to the cytoplasm to do kinase activities- *i.e.* inactivate glycogen synthase kinase and stimulate glycogen synthesis [101].

There are many avenues of research open for future work to determine the sequence of events in parts of the cell turning on and off enzymes for membrane modification, the inhibition of apoptosis, and the cooperation of receptors during early development.

ABBREVIATIONS
AAG alkyl-acyl glycerol
AT acetyl-Coenzyme A- acetyl-transferase
CE cholesterol ester
CEREB cerebroside
CH cholesterol
CHL chloroform
DAG diacylglycerol
ER endoplasmic reticulum
FA fatty acid
FERT fertilized
FM fertilization membrane
IP3 inositol (1,4,5) trisphosphate
LPAF lyso-PAF
LPC lysoPC
M1...M4 density gradient membrane fractions
Meth methanol
NTP nucleotide triphosphate
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
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
UNFERT unfertilized

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8. PAF dose response by sea urchin eggs. PAF was suspended in sea water with buffer.

added 30 min post 1MA, 4 is 30 min after that. C is the pellet from such homogenates and S is supernate. 40?l of suspended pellet had 4 ?l super added. TLC as in Fig 2. Repeated runs showed similar results.

10. Calcium and vanadate activate acetyltransferase. Substrate in assay mixture was LPAF in the presence of acetyl-coenzymeA and the product was PAF. The images are composite from several plates run at the same time and were run as in Fig 2.

A. LPA was also made probably by LPLD from LPAF when no calcium or vanadate were present. MG was made from LPA by PAPH in Fert eggs and follicle cells. Substrate LPAF is totally converted to products. Samples are in triplets: A is control, B is with calcium, C is with vanadate.

B. Acetyltransferase assays using fert starfish egg pellets and supernate, studying the effect of temperature, detergent, added supernate and time for assay. From left to right, the assays go in pairs, a and b, where a has none and b has detergent. Pair 1 room temp fert pellet 20m, Pair 2. 40 m; Next group of 4 pairs are 0, 5m, 25m, 45m pellet plus supernate run at 37 deg. Next group of three pairs are 5m,25m,45m of pellet alone run at 37 deg. There was no added Ca^{++}. On the right is the LPAF substrate by itself.

TABLE 1 Effects of supernates and calcium on density gradient fraction enzyme activities

+=activation; A= activator of PLA, P= activator of LPAF-acetyltransferase. AR-activator response element, CR= calcium responsive
element,

density fraction	ENZYME EFFECT		PLD M1		PLD M2		SUC M1		SUC M2
Supernate		**FACTORS**					**AR, P**		
UNF PLD SUP	PLA	**A,AR**	A,AR	+	A,AR	+	A,AR	+	A,AR
	LPAF-AT	**Y**					YP	+	
FERTPLD SUP	PLA	**A**	A		A		A,AR	+	A
SUCUNFERT SUP	PLA	**A,AR**	A,AR	+	A,AR	+	A,AR	+	A,AR
PLDFERT+ATP SUP	PLA	**A**					A,AR	+	A
CALCIUM	PLA	**CA^{++}**	CA^{++}		CA^{++}		AR, CA^{++}	+	CA^{++}
	LPAF-AT	**CA^{++}**	CA^{++}		CA^{++}		P, CA^{++}	+	CA^{++}

A+AR allows activation of PLAas does AR+ CA^{++};

TABLE 3

PRODUCT	ENZYME PRODUCING	POSSIBLE EFFECT OR FUNCTION AS A SIGNAL OR INVOLVEMENT IN REMODELING OF MEMBRANE
FA	PLA_2	Prostaglandin and lipoxygenase pathways: alter endocytosis and phagocytosis rates. After release of arachadonic acid get spreading, lamellipodia, filopodia, more with Ca^{++}than without. [102] Lots of arachadonic acid found in cortical granules [103]. FA cause translocation of PAPH from sol to mic [104]
LPA	PLA_2	PAP converts LPA to MG. PLD activation by lysoPA. The PAF antag did not affect PLD stimulation by LPA [105].
LPAF	PLA_2, AH	Substrate for making PAF or alkylPC. LPLD converts to LPA-alkyl [12]
LPC	PLA_2	Perturbs membrane properties. PLA product LPC can activate PLD in coronary endothelium [106] or macrophages [105].
MG	PLA_2,LPLD	PLA2 important in making fusigenic lipids-DAG, MG, PA, lysolipids [76].
PAF	Acetyl-CoA-acetyltransferase, CoA-indep-transacetylase	7-trans memb, g-protein receptor. Coupled to PLA2, PLC, PLD, MAPK, PI3K, ty kinases, inhibits adenyl cyclase. PAF induced IP3 production peak at 15 sec. Arachodonic acid in 15 min. Different Gprot for IP3 production and adenyl cyclase inhibition-pertussis insensitive to toxin for PLC and sensitive for ad cyc [20]. PAF fluid phase mediator of interaction-adhesion, degranulation, inflammation coagulation, neuronal death or differentiation, chemotaxis in monocytes, endothelium relaxant. Alter gel-liq crystal transition, stabilize lamellar phase, but make it more fluid. May be mechamism for making domains with higher conc that can perturb the membrane[4]. Increase sperm motility, acrosome reaction [62]. Increase successful spe sperm decondensation [107]. PAF leads to early response genes, cfos and c-jun are activated [108]
PC	Transacylation or	1-alkyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine from lyso

	acyltransferase	platelet-activating factor by the CoA independent transacylase [109]. Maj component, substrate for phospholipases. Cell cycle, in G1, PC turnover 75% [110]

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