Phospholipid methylation in starfish spermatozoa is linked to sperm chemoattraction
(chemotaxis/gamete recognition oocyte)

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ABSTRACT The mechanism whereby ovarian peptides cause sperm attraction was studied in the starfish. Phospholipid methylation and protein-O-carboxyl methylation, reactions linked to chemotactic responses in a variety of systems, were studied in starfish sperm. When sperm were preincubated with [methyl-3H]methionine and then exposed to the attractant, a rapid drop in radioactivity occurred in the phospholipid fraction. Methylated phospholipids decreased by 90% in the first 2 sec; however, no change was observed in endogenous methylation of protein carboxyl groups. The effect on phospholipid methylation was dose dependent, with a 40% reduction in radioactive phospholipids in sperm occurring with the minimal amount of attractant necessary to obtain a positive response in a sperm attraction bioassay. Attractants from species of starfish with little or no cross-reactivity in the bioassay had a limited effect on phospholipid methylation. The transmethylase inhibitor, homocysteine, caused a marked decrease in the accumulation of methylated phospholipids under basal conditions, which was correlated with as much as a 50-fold increase in sperm sensitivity to the attractant. The addition of chemoattractant resulted in a reduction in the amount of all individual methylated phospholipids, but the amount of phosphatidylethanolamine relative to the other methylated phospholipid decreased by a factor of 4 after stimulation. Homocysteine had the same effect. The reduction in methylated phospholipids by attractants suggests that phospholipid methylation is linked to the mechanism of action of these peptides. Methylation of phospholipids may play a role in the rapid desensitization of sperm cells to the attractant, which would be required for the orientation of the spermatozoa in the gradient of ovarian peptide.

Sperm chemotaxis has been demonstrated in Asteroidea, a major class of echinoderms (1). The substance responsible for the attraction is produced by the ovaries and is of low molecular weight, highly polar, sensitive to proteases, and stable to heat and acid (T. Punnett and R.L.M., unpublished data). The affected spermatozoa swim up a gradient of the chemoattractant, which is specific at least at the family level in starfish. This response leads to more efficient and appropriate sperm–egg interaction in the open sea, where fertilization normally takes place in these animals. However, the mechanism by which these attractants exert their effect on sperm is not known.

Transmethylase reactions are involved in a variety of chemotactic responses (for review, see ref. 2). In bacteria, membrane-associated proteins are methylated by a protein-carboxyl methyltransferase as a requirement for chemotaxis (3, 4). Many cells such as cellular slime molds (5), guinea pig macrophages (6), and rabbit neutrophils (2) exhibit changes in the phospholipid composition of the plasma membrane consistent with the activation of phospholipid methyltransferases after exposure to specific attractants. Because of the well-known involvement of transmethylese reactions in signal transduction, we studied whether phospholipid or protein-carboxyl methylation were linked to sperm attraction in the starfish. We report here that ovarian extracts trigger a dose-dependent decrease in the accumulation of methylated phospholipids that parallels their sperm-attracting activity.

MATERIALS AND METHODS

Chemicals. L-[methyl-3H]Methionine (70–85 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. L-Homocysteine, phosphatidylcholine, ethanolamine, phosphatidyl-N-monomethylthanolamine, phosphatidyl-N,N-dimethylethanolamine, and arachidonic acid were purchased from Sigma. 3-Deazaadenosine was purchased from Southern Research Institute (Birmingham, AL).

Cell Preparation and Sperm Chemotaxis Assay. The starfish Pycnopodia helianthoides and Pteraster tesselatus were collected by scuba diving and dredging in the waters surrounding the Friday Harbor Marine Laboratory of the University of Washington. Ovaries, dissected free of the body, were extracted in 4–5 times their volume of 95% ethanol and dried. Aliquots of this extract, diluted in seawater, possessed potent sperm-attracting activity.

Semen, obtained from pricked whole testes, was kept in seawater at 4°C. The attractant bioassay was performed as described by Miller (1) with freshly diluted semen. Briefly, attractant was introduced into the edge of a drop of seawater containing swimming sperm. The attraction of sperm to the tip of the pipette was taken as an indication of a positive bioassay. Multiple dilutions of each attractant preparation were assayed to determine the minimal amount necessary (μg of protein per ml of seawater) to produce a positive response in the bioassay.

Purification of Attractant. Extracts of ovaries from two species of starfish were dissolved in seawater and applied to columns of XAD-7 Amberlite resin (Rohm and Haas), desalted by flushing the columns with distilled water, and eluted with methanol. The alcoholic eluate was flash-evaporated and lyophilized. This material was applied to and eluted from a Sephadex G-50 column (1.5 × 34 cm) (Pharmacia) with distilled water at a flow rate of 1.0 ml/min. The fractions containing the highest attractant activity were pooled and lyophilized. Samples from Py. helianthoides were dissolved in distilled water at 1 mg/ml; 100–400 μl were applied to an Alltech C18 HPLC column and eluted with a linear gradient from 0% to 100% acetonitrile and from 0.07% to 1.0% CF3COOH in water for 20 min (flow rate, 1.5 ml/min). The fractions were lyophilized, resuspended in 84x766

3589

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distilled water or seawater, and tested for their effect on phospholipid methylation and sperm attraction as described below. Fractions with attractant activity from several runs were pooled and stored at -20°C. This permitted biological studies on a preparation (purified 2000-fold) which showed activity in the sperm attraction assay at 0.1 μg/ml. Other preparations with lower specific activities were also tested in this study.

**Assay for Sperm Phospholipid Methylation.** Sperm were diluted in seawater at a concentration of 10⁷ cells per ml, allowed to regain motility for 30 min at 15°C, and then preincubated with 10 μCi of [³²P]methionine in aliquots of 0.2 ml. Preliminary studies showed a linear increase in the incorporation of radioactivity into phospholipids and proteins that plateaued after 30 min (results not shown). At 30 min, attractant was added to 50 μl of seawater, and the suspension was incubated at 15°C for up to 5 min. The reaction was stopped by the addition of 1.5 ml of cold 10% CCl₃COOH and centrifuged at 5000 × g for 10 min. The supernatant was discarded, and the pellet was resuspended and centrifuged once more in CCl₃COOH. The pellet was extracted in 2 ml of chloroform/methanol/10 M HCl, 2:1:0.01 (vol/vol/vol), mixed in a Vortex for 2 min, and kept at room temperature for 1 hr. The organic mixture was washed with 1 ml of 0.1 M KCl in 50% methanol and mixed several times; the aqueous phase was aspirated. This was followed by a second wash with 1 ml of the KCl/methanol solution pre-equilibrated with the organic mixture. Aliquots of the organic phase were transferred to counting vials, dried, and assayed for radioactivity.

To determine the degree of methylation of individual phospholipids, 1 ml from the organic phase was transferred to a glass tube, dried under nitrogen, and resuspended in chloroform/methanol, 2:1 (vol/vol). After addition of internal standards including arachidonic acid, phosphatidylincholine, phosphatidylethanolamine, phosphatidylmonomethyl ethanolamine, and phosphatidyldimethylethanolamine, the samples were applied to high-efficiency silica gel plates (Supelco, Bellefonte, PA) and developed with the solvent system: propionic acid/propanol/chloroform/water, 2:3:2:0.6 (vol/vol) in an Eastman Chromagram-Developing Apparatus. After visualizing phospholipids with iodine vapors, radioactivity was measured either from 2-mm bands removed from the entire silica gel plate or from the individual phospholipid spots, which were scraped into scintillation vials and dissolved in 12 ml of Aquasol (New England Nuclear).

**Total Phospholipid Synthesis.** Sperm were incubated for 30 min with sodium [³²P]phosphate (New England Nuclear; 5 μCi per 10⁷ sperm). Phospholipids were extracted as described above and [³²P] was measured.

**Protein-Carboxyl Methylation Assay.** The effect of the attractant on endogenous protein-carboxyl methylation was assayed as described for phospholipid methylation, but the final CCl₃COOH pellet was hydrolyzed in alkaline buffer, and the radioactive methanol was extracted for measurement of protein methyl esters as described previously (7).

**RESULTS**

**Effect of Attractor on Methylated Phospholipid and Endogenous Protein-Carboxyl Methylation in Spermatozoa.** Since the biological response of sperm motility to the attractant takes place almost instantaneously (1), the effect of partially purified starfish attractant on phospholipid methylation in sperm was measured over a period of 20 sec. High concentrations of the highly purified attractant (5 μg/ml) resulted in an 85% decline in the [³²P]methylated phospholipids in spermatozoa within 2 sec (Fig. 1). Longer incubation times, up to 5 min (not shown), did not further change the quantity of radioactive phospholipids present in these cells. In subsequent experiments, the effects of the attractant on spermatozoa were determined after 3 sec. A study was then performed that indicated that the minimal concentration of attractant needed to inhibit phospholipid methylation was similar to that which also showed attractant activity (Table 1). This drop in methylated phospholipid was dose dependent for each attractant preparation and occurred without a change in total phospholipids. The maximal effect was achieved at a concentration 3-fold greater than that required for minimal activity.

**To determine whether the attractant activity was associated...**

![Figure 1](image-url)

**FIG. 1.** Effect of a partially purified chemotaxtactant on phospholipid methylation in spermatozoa from *P. helianthoides* starfish. Sperm cells (2 × 10⁹ per 0.2 ml) were preincubated with [methyl-³²P]methionine for 30 min and then exposed to attractant at a final concentration of 5 μg/ml. The reaction was stopped at different time intervals, and the methylation of total phospholipid was determined as described. The values are the means of triplicate determinations ± SEM.

<table>
<thead>
<tr>
<th>Attractor concentration, μg/ml</th>
<th>Bioassay response*</th>
<th>Radioactivity in phospholipid, cpm/10⁷ sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[³²P]</td>
<td>[³²P]</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>3050 ± 280</td>
</tr>
<tr>
<td>0.02</td>
<td>0</td>
<td>3100 ± 290</td>
</tr>
<tr>
<td>0.06</td>
<td>0</td>
<td>2080 ± 185†</td>
</tr>
<tr>
<td>0.1</td>
<td>+</td>
<td>1530 ± 230†</td>
</tr>
<tr>
<td>0.2</td>
<td>+</td>
<td>850 ± 157†</td>
</tr>
<tr>
<td>0.3</td>
<td>+</td>
<td>900 ± 170†</td>
</tr>
<tr>
<td>0.4</td>
<td>+</td>
<td>1200 ± 280†</td>
</tr>
<tr>
<td>0.5</td>
<td>+</td>
<td>1150 ± 230†</td>
</tr>
</tbody>
</table>

- Sperm suspensions in a total volume of 0.2 ml (20 × 10⁹ cells) were incubated in seawater with [³²P]methionine or sodium [³²P]phosphate for 30 min as described, and 50 μl of attractant dilutions were added for 3 sec. Total phospholipids were extracted as described. Radioactivity values are means of triplicate determinations ± SEM.
- *A positive bioassay response was the accumulation of sperm at the tip of the pipette used to introduce the attractant into the sperm suspension.

*P < 0.01 versus control with no attractant. The attractant preparation used in these studies was purified by sequential ion exchange and gel filtration chromatography and reverse-phase HPLC as described.
ated with the substance affecting phospholipid methylation, individual fractions of ovary extracts obtained after HPLC were assayed (Fig. 2). The two activities comigrated; only fractions with attractant activity exhibited an effect on phospholipid methylation.

Since female starfish strongly attract sperm only from the same species, ovarian extracts from two species with weak cross-reactivity in the bioassay were examined for their effects on phospholipid methylation (Fig. 3). The two extracts tested had both been purified by ion exchange and gel filtration. Spermatozoa from Py. helianthoides responded to homologous ovarian preparations with a dose-dependent reduction of phospholipid methylation and positive sperm attraction. When exposed to extracts from Pt. tesselatus ovaries, a much greater concentration of attractant was required, resulting in a shift of both dose–response curves to the right (Fig. 3 Upper). Conversely, when sperm from Pt. tesselatus were exposed to homologous attractant, as little as 0.2 μg/ml altered motility and decreased methylated phospholipid. A 10-fold higher concentration of attractant from Py. helianthoides ovary was necessary to produce a similar effect on these sperm (Fig. 3 Lower).

Since protein-carboxyl methylation is believed to be involved in the regulation of motility in mammalian spermatozoa, this reaction was investigated for its possible role in the attraction of starfish sperm. After incubation with [3H]methionine, almost no protein methyl esters were formed either during the preincubation period (10 pmol of [3H]methionine per 10⁷ sperm) or after exposure of the cells to saturating concentrations of attractant over a 5-min period.

**Effect of Transmethylase Inhibitors and Attractant on Phospholipid Methylation.** The involvement of phospholipid methylation in the attraction response was further studied by using two well-known inhibitors of the transmethylase reaction. The results in Table 2 depict the effects of l-homocysteine and 5-deazaadenosine on basal (nonstimulated) methylation in spermatozoa. l-Homocysteine produced a marked reduction (ID₅₀, 50 μM) in methylated phospholipids, whereas no change was observed with 3-deazaadenosine. l-Homocysteine (100 μM) was as effective as saturating amounts of attractants (2 μg/ml) in reducing the accumulation of methylated phospholipids. Since l-homocysteine and attractant had a similar effect on phospholipid methylation, the effect of the inhibitor on sperm motility was next examined.

**Table 2. Effect of transmethylase inhibitors on phospholipid methylation in starfish spermatozoa**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inhibitor dose, μM</th>
<th>[3H]Phospholipids, cpm per 10⁷ sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>3300 ± 180</td>
</tr>
<tr>
<td>Attractant, (2 μg/ml)</td>
<td>0</td>
<td>650 ± 120*</td>
</tr>
<tr>
<td>Inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homocysteine</td>
<td>10</td>
<td>3005 ± 120</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2550 ± 130*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1930 ± 110*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>650 ± 90*</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>250 ± 75*</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>85 ± 50*</td>
</tr>
<tr>
<td>3-Deazaadenosine</td>
<td>10</td>
<td>3010 ± 150</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3200 ± 120</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3338 ± 170</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3175 ± 120</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>3050 ± 75</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>3006 ± 180</td>
</tr>
</tbody>
</table>

Sperm suspensions were incubated with [methyl-3H]methionine for 30 min in the presence and absence of different concentrations of transmethylase inhibitors. Control suspensions were incubated with attractant as described in Fig. 1. The values are means ± SEM of triplicate determinations from two experiments.

*P < 0.01 versus control.

**Fig. 2.** Copurification of attractant and phospholipid methylation-stimulating activities. A sample of ovarian extract enriched in attractant activity after purification on Amberlite and Sephadex G-50 was subjected to HPLC as described. The fractions were assayed for their effects on sperm phospholipid methylation and attraction. (Upper) Percentage inhibition of phospholipid methylation. (Lower) Absorbance at 254 nm (---); attractant activity (number of serial half dilutions of the preparation necessary to obtain a negative response) (......).
the inhibition of phospholipid methylation shown in Table 2 correlated with a linear decrease in the amount of attractant necessary for a positive response (Fig. 4). This effect was dose dependent; with the maximal concentration of L-homocysteine tested (175 μM), there was a 50-fold increase in sperm sensitivity to the attractant as compared to the controls.

Effect of L-Homocysteine and Attractant on Incorporation of the [3H]Methyl Groups into Individual Phospholipids. When phospholipids from spermatozoa incubated with [methyl-3H]methionine were fractionated on silica gel, similar amounts of radioactivity were incorporated into phosphatidylycholine, phosphatidyl(dimethylethanolamine), and phosphatidylmonomethyl ethanolamine (Fig. 5). Homocysteine treatment of sperm resulted in a 50% inhibition of 3H incorporation in all phospholipid moieties. The attractant had the same effect, but it also markedly reduced the amount of phosphatidylmonomethyl ethanolamine relative to the other phospholipids. This latter effect is shown in Fig. 6 as a function of increasing concentrations of attractant. A gradual increase in the ratio of phosphatidylmonomethyl ethanolamine to total phospholipid was observed until ovarian extract was added in an amount sufficient to produce sperm attraction; thereafter, an increase in attractant resulted in a decrease in relative phosphatidylmonomethyl ethanolamine concentration.

The effect of L-homocysteine on relative accumulation of phosphatidylmonomethyl ethanolamine was next studied. After a 30-min preincubation with the inhibitor, aliquots were assayed for the amount of labeled phosphatidylmonomethyl ethanolamine (Fig. 7). Homocysteine produced a dose-dependent accumulation, which resulted in an increase in the ratio of phosphatidylmonomethyl ethanolamine to total methylated phospholipids. (Figs. 5 and 6). This effect on phosphatidylmonomethyl ethanolamine was the opposite of that produced by the attractant.

**DISCUSSION**

The ability of a cell to recognize and then move up a chemical gradient depends upon binding of an attractant to specific
receptors on the cell membrane. Although the signal transduction for chemotaxis in bacteria (8) and some eukaryotic cells (9–11) requires methylation of specific proteins, in others, such as molds (5) and macrophages (6), the methylation of phospholipids is involved. The experiments in the present study suggest that the latter reaction may be linked to the chemoattractant response of starfish sperm to ovarian extracts. A decrease in phospholipid methylation strongly correlated with attractant activity. Exposure to the sperm attractants produced time- and dose-dependent effects on both phospholipid methylation and attraction. The copurification of the agent responsible for attractant activity and the phospholipid effect also suggests a linkage between them. The association between changes in phospholipid methylation and sperm attraction was strengthened by experiments with two species of starfish in which ovarian extracts from one species stimulated phospholipid methylation in heterologous sperm only at the very high concentrations that also were required to produce sperm attraction. By contrast, studies with homologous extracts and sperm showed a strong correlation between biological effect on motility and phospholipid methylation at much lower attractant concentrations.

Analysis of individual phospholipids after exposure to the attractant revealed a marked decrease in the relative concentration of phosphatidylinomonomethylethanolamine. However, no accumulation of phosphatidylycholine or the dimethylated intermediate was observed, which suggests that they might be metabolized rapidly. Although no experiments were carried out to study further the metabolism of phosphatidylycholine in response to the attractant, a possible explanation for the decrease in this compound could be its degradation to water-soluble products. The hydrolysis of phospholipids by phospholipase C could result in the accumulation of lysophosphatidylycholine and this product would not be extracted and measured by the method used in this study.

Although inhibition of methylation blocks chemotactic response in macrophages (10) this is not the case for sperm attraction. In fact, in the presence of methylation inhibitors, the cells are sensitized to the attractant, thus amplifying its effect. These observations suggest that phospholipid methylation might be important in the recognition of the attractant. Sperm must be able to detect higher and higher concentrations of this molecule as they approach the egg. The ability of the spermatozoa to move up such a gradient can be explained by at least two possible mechanisms: the continuous accumulation of a second messenger due to the higher occupation of receptors for the attractant; or the modulation of receptor activity per se by decreasing the affinity or number of the receptors so that higher concentrations of attractant are required. Consistent with the latter postulate are the observations indicating that methylation of membrane phospholipids plays a role in both signal transduction (12, 13) and receptor modulation (14). In fibroblasts, the manipulation of the membrane composition of methylated phospholipids altered the binding properties of Con A (15). Furthermore, increasing the ratio of highly methylated to total phospholipids lowered the affinity of Con A for its receptor and increased the number of sites per cell. A similar mechanism could be involved in the recognition of the attractant gradient by spermatozoa. Low concentrations of this ovarian peptide would induce changes in receptor affinity and/or number so that, as the sperm approached the egg, higher concentrations of attractant would be required to sustain the intracellular signal.

If gradient recognition involved resistance to attractant this was linked with the attractant-induced decreases in the ratio of phosphatidylmonomethylethanolamine to total phospholipid observed in this study, then one might expect increased sensitivity to the ovarian peptide, with an increase in the phosphatidylmonomethylethanolamine/total phospholipid ratio. When this latter change was induced by preincubation of sperm with L-homocysteine; these cells were able to recognize a gradient, but at an attractant concentration 1/50th that for control cells. Membrane-modulated sensitivity to attractant would be a reasonable hypothesis, provided that this peptide did not stimulate second messengers, such as cyclic nucleotides. This was of particular importance because, in sea urchin, components of the egg jelly induced activation of motility (16) and caused changes in ion fluxes (17) and cyclic nucleotide metabolism (18, 19). By contrast, in starfish sperm, no significant change in cyclic AMP or cyclic GMP were detected with specific RIAs in cells exposed to L-homocysteine or attractant (results not shown).

The results of this study show that in starfish specific mobilization of sperm membrane phospholipids by ovarian extracts could be related to the mechanism of attraction between gametes, which results in efficient and specific fertilization in the sea.

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