We report for the first time that phospholipase D activity in sea urchin spermatozoa can be regulated by a component of egg jelly known to induce an acrosome reaction. The fucose-sulfate glycoconjugate (FSG) of egg jelly that induces an acrosome reaction in spermatozoa caused a 4-fold accumulation of \[^{3}H\]phosphatidic acid. Addition of FSG causes coordinated changes in ionic fluxes and cyclic nucleotide metabolism in spermatozoa (2, 3, 7). During the acrosome reaction, there is net Na\(^{+}\) influx, Ca\(^{2+}\) influx, H\(^{+}\) efflux, and K\(^{+}\) efflux (8–12). Concomitantly, intracellular pH is transiently increased by 0.16 pH unit (13–15), and the plasma membrane is transiently hyperpolarized, followed by a depolarization of approximately 30 mV (16–18). In addition, FSG or unfractionated egg jelly have been shown to elevate cyclic AMP concentrations (5, 19–22), increase adenylate cyclase activity (5, 23), stimulate cyclic AMP-dependent protein kinase activity (24), increase phosphoprotein phosphatase activity (25), and stimulate phosphorylation of sperm histone (26, 27). Induction of the acrosome reaction requires extracellular Ca\(^{2+}\) (28, 29) and is inhibited by Ca\(^{2+}\) channel antagonists that bind with high affinity to sites on the sperm plasma membrane (30, 31).

In addition to increases in the activity of enzymes involved in cyclic nucleotide metabolism, previous reports have suggested that phospholipase activity is increased during the acrosome reaction in sea urchin and mammalian spermatozoa (6, 32–34). When exogenous phospholipid substrates were incubated with egg jelly-treated spermatozoa, lysophosphatidylcholine, lysophosphatidic acid, and free fatty acids were produced, possibly by a phospholipase A mechanism (32). Additionally, egg jelly treatment of sea urchin spermatozoa increased the concentrations of free fatty acids (6).

We reported previously that FSG caused increased in \[^{3}H\]inositol phosphate accumulation in sea urchin spermatozoa (35). In addition to inositol phosphates, phosphatidylinositol hydrolysis may generate other potential second messengers in the phospholipid membrane, including 1,2-diacylglycerol and phosphatidic acid (36). We now report that phosphatidic acid concentrations increase in FSG-treated cells more rapidly and to a greater extent than diacylglycerol. In many cells that undergo increased phosphatidylinositol turnover in response to external stimuli, elevations in phosphatidic acid have been attributed to the sequential action of phospholipase C and diacylglycerol kinase (36, 37).

Inactivation of diacylglycerol kinase (36, 37) and phosphatidic acid concentrations have been inhibited by Ca\(^{2+}\) channel antagonists that bind with high affinity to sites on the sperm plasma membrane (30, 31).

Prior to or during fertilization, spermatozoa must undergo an acrosome reaction (1). In sea urchins, the acrosome reaction is induced upon contact of spermatozoa with the jelly layer of the egg and involves exocytosis from an apical vesicle, called the acrosomal granule, and protrusion of the vesicular membrane at the end of a rapidly polymerized actin filament (2, 3). A component of egg jelly that induces an acrosome reaction has been purified and characterized as a fucose-sulfate glycoconjugate (FSG) (4–6). Addition of egg jelly or the purified FSG causes coordinated changes in ionic fluxes and cyclic nucleotide metabolism in spermatozoa (2, 3, 7). During the acrosome reaction, there is net Na\(^{+}\) influx, Ca\(^{2+}\) influx, H\(^{+}\) efflux, and K\(^{+}\) efflux (8–12). Concomitantly, intracellular pH is transiently increased by 0.16 pH unit (13–15), and the plasma membrane is transiently hyperpolarized, followed by a depolarization of approximately 30 mV (16–18). In addition, FSG or unfractionated egg jelly have been shown to elevate cyclic AMP concentrations (5, 19–22), increase adenylate cyclase activity (5, 23), stimulate cyclic AMP-dependent protein kinase activity (24), increase phosphoprotein phosphatase activity (25), and stimulate phosphorylation of sperm histone (26, 27). Induction of the acrosome reaction requires extracellular Ca\(^{2+}\) (28, 29) and is inhibited by Ca\(^{2+}\) channel antagonists that bind with high affinity to sites on the sperm plasma membrane (30, 31).

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Experimental Procedures

Chemicals—Carrier-free \[^{32}P\]orthophosphate (\[^{32}P\]) was obtained from ICN Radiochemicals (Irvine, CA). \[^{3}H\]1-O-alkyl-2-lyso-glycerol-3-phosphorylcholine (53 Ci/mmole), \[^{3}H\]alkyl-lyso-PC, \[^{3}H\]1-O-alkyl-2-lyso-glycerol-3-phosphorylcholine; \[^{3}C\]di-octanoyl phosphatidylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPS, guanosine 5'-O-(3-thio)triposphosphate; Gpp(NH)p, 5'-guanylylimidodiphosphate.

\[^{3}H\]Dioctanoyl phosphatidylcholine (di-Cs-PC) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). \[^{3}C\]Dioctanoyl phosphatidylcholine (di-Cs-PC) was prepared by chemical synthesis.\[^{3}H\]1-O-alkyl-2-lyso-glycerol-3-phosphorylcholine (53 Ci/mmole), \[^{3}H\]alkyl-lyso-PC, and \[^{3}C\]di-octanoyl phosphatidylcholine were synthesized by the method of Meek and Green (42). \[^{3}H\]Dioctanoyl phosphatidylcholine (di-Cs-PC) was prepared by chemical synthesis.\[^{3}H\]1-O-alkyl-2-lyso-glycerol-3-phosphorylcholine (53 Ci/mmole), \[^{3}H\]alkyl-lyso-PC, and \[^{3}C\]di-octanoyl phosphatidylcholine were synthesized by the method of Meek and Green (42). \[^{3}H\]Dioctanoyl phosphatidylcholine (di-Cs-PC) was prepared by chemical synthesis.\[^{3}H\]1-O-alkyl-2-lyso-glycerol-3-phosphorylcholine (53 Ci/mmole), \[^{3}H\]alkyl-lyso-PC, and \[^{3}C\]di-octanoyl phosphatidylcholine were synthesized by the method of Meek and Green (42). \[^{3}H\]Dioctanoyl phosphatidylcholine (di-Cs-PC) was prepared by chemical synthesis.
was synthesized from 3(3'ringO)octanoic acid (Du Pont-New England Nuclear) and glycerol-3-phosphocholine (free base from Sigma) by trifluoroacetic anhydride (Pierce Chemical Co.) catalysis (42). ATP, luciferin-luciferase reagent, hexokinase, bovine kidney affinity-purified alkaline phosphatase, dioctanoyl phosphatidic acid, dioc-tanoyl phosphatidylcholine, and 1,2-dioleoylglycerol were purchased from Sigma Chemical Co., St. Louis, Mo. The incubation medium for phospholipase D reaction contained 200 mM NaCl, 20 mM imidazole, 2 mM MgCl₂, 0.2 mM CaCl₂, 0.1% BSA, 0.1 mM ben zamidine, 0.1 mM EDTA, 100 mM Tris (pH 7.4), 100 mM HEPES, pH 7.4, and 100 mg of spermatozoa (wet weight)/ml. Lipids were identified by comparison of relative mobilities with those of standards and by specific sprays (48).

The mass of diacylglycerol in micrograms was converted to nanomoles by the formula 1 pg = 1.61 nmol based on the dioleoyl form of diacylglycerol; the mass of phosphatidic acid in micrograms was converted by the formula 1 µg = 1.29 nmol based on the dioleoyl form of phosphatidic acid (Na⁺ salt).

Choline and Choline Phosphate Assay—One ml reaction mixtures of spermatozoa (25 mg, wet weight) were stopped in 3.75 ml of chloroform/methanol (1:2) and the aqueous fractions of a Bligh and Dyer extraction preserved as described (44). Samples were lyophilized and resuspended in 1.0 ml of 100 mM Tris, pH 9.2. Aliquots (50 µl) were treated with alkaline phosphatase (0.05 unit, bovine kidney affinity-purified) for 30 min at 37°C to convert choline phosphate to choline (38) or left untreated. Alkaline phosphatase was inactivated by boiling treated samples in water at 100°C for 5 min. Choline concentrations were determined by enzymatic assay with [32P]ATP and choline kinase according to Haubrich and colleagues (51). Standard curves were made by adding choline to seawater and treating reference samples identical to experimental samples. Choline phosphate concentrations were calculated as the difference between alkaline phosphatase-treated and untreated samples (38).

Radiolabeling of Spermatozoa with [32P]Orthophosphate and Determination of [32P]ATP Specific Activity—S. purpuratus spermatozoa were washed and resuspended at a concentration of 125 mg (wet weight)/ml and incubated with [32P]orthophosphate (0.25 mCi/ml) for 4 h at 15°C. The cells were washed and 0.5- or 1.0-m1 aliquots used in reaction mixtures with FSG. Incubations were terminated with 10% trichloroacetic acid to determine [γ-32P]ATP specific activity or with chloroform/methanol/HCl to measure phosphatidic acid mass. Phosphatidic acid was described as phosphatidylcholine containing 10-20 mg of charcoal, vacuum filtration, and extraction with pyridine/ethanol/water (1:5:4) (52, 53). Nucleotide extracts were spotted on polyethyleneimine cellulose thin-layer plates, rinsed in 100% methanol to remove interfering substances (54), and developed in 1 M LiCl. Radioactive spots were located by autoradiography, and the area co-migrating with [32P]ATP standard was scraped and quantified by liquid scintillation counting. ATP concentrations in each sample were measured by luciferin-luciferase assay using a Chemglow II luminescence meter (SLM Instruments, Urbana, IL) (55).

The specific activity of the γ-PO₄ of [32P]ATP was estimated by the transfer of the terminal phosphate of ATP to glucose by hexokinase (56).

Radiolabeling of Spermatozoa with [3H]Glycerol—Spermatozoa were washed and then incubated at 125 mg (wet weight)/ml with [3H]glycerol (25 µCi/ml) for various times up to 90 min at 15°C. Within 5 min of incubation, radioactivity from [3H]glycerol was incorporated in lipids, including lysophosphatidic acid, phosphatidic acid, and diacylglycerol, and after 90 min, in additional phospholipids including phosphatidylcholine (data not shown). In experiments testing the effects of FSG on de novo synthesis of phosphatidic acid, spermatozoa were washed and resuspended for 10 min. Radiolabeling of Spermatozoa with [3H]Alkyl-lyso-PC—A phosphatidylcholine pool (1-O-alkyl-2-acyl-glycerol-3-phosphorylcholine) in spermatozoa was radiolabeled using [3H]O-alkyl-2-lyso-glycerol-3-phosphorylcholine ([3H]alkyl-lyso-PC) (40). Aliquots of [3H]alkyllysophosphatidylcholine was evaporated to dryness under N₂, resuspended with water at the original volume, and sonicated in a water bath at 4°C. Spermatozoa (125 mg (wet weight)/ml) were then incubated 4-6 h at 15°C with 1 µCi/ml of [3H]alkyl-lyso-PC, washed, and resuspended at 100 mg (wet weight)/ml in seawater. Approximately 750 cpm/mg (wet weight) were incorporated into phosphatidylcholine. Spermatozoa incubated under these conditions retained the ability to undergo an acrosome reaction induced by FSG.

Assay of the Acrosome Reaction—The percent of cells that had undergone an acrosome reaction (defined as the presence of an acrosomal process) was determined by scanning electron microscopy. Spermatozoa were fixed in 2% glutaraldehyde for 1 h, centrifuged at 1000 x g for 30 min, resuspended in 100 µl of seawater, and aliquots were placed on poly-L-lysine-treated coverslips overnight at 4°C. The coverslips with cells attached were then treated with osmium tetroxide, dehydrated in step gradients of ethanol, and critical point-dried with liquid carbon dioxide. Coverslips were mounted on 200 mesh Cu grids with Pd (Technics Hummer) and viewed on a Hitachi S-500 scanning electron microscope at 20 kV. At least 100 cells were counted to determine the percent acrosome-reacted cells.

In Vitro Assay of Phospholipase D in Spermatozoa Membranes—Phospholipase D activity was measured with 50 µl of packed spermatozoa homogenized with 100 µl of buffer; ice for 10 s, three times, with a buffer containing 250 mM NaCl, 20 mM benzamidin, 1 mM EDTA, and 20 mM HEPES, pH 7.0. The homogenate was centrifuged at 1,000 x g for 15 min and the super-
natant fraction centrifuged 100,000 x g for 15 min. The pellet from the final centrifugation was resuspended in fresh buffer (10 ml) and used as a particulate fraction for the in vitro assay. Reaction mixtures contained 0.6 mM [14C]dil-C8-PC (0.93 mCi/mmol), 50–100 μg of protein of the particulate fraction, and variable concentrations of Ca2+ and Mg2+. Reactions were stopped with chloroform/methanol/HCl, and lipid extracts were spotted on 20 x 10-cm Silica Gel 60 F-254 thin-layer plates and developed in ethyl acetate/isooctane/acetic acid (45:12:5:10). TLC plates were scanned for radioactivity with a Bioscan System 200 Imaging Scanner (Bioscan, Inc., Washington, DC). Bands co-migrating with [14C]dioctanoyl phosphatidic acid were re-extracted and the radioactivity measured by scintillation counting. Protein was measured by the Bradford method (57). The critical micelle concentration of di-C8-PC is 0.38 mM (58).

Data Presentation—Data shown represent the mean of duplicate samples except where indicated in the figure legend. Each experiment was performed two to four times.

RESULTS

Stimulation of Phosphatic Acid and Diacylglycerol Accumulation—Reactions of spermatozoa with or without FSG were terminated at varying times with chloroform/methanol/HCl (100:200:1) and chloroform extracts obtained as described under "Experimental Procedures." When the phospholipids were chromatographed and quantified, the most apparent changes were in the concentration of phosphatidic acid, not diacylglycerol. Although concentrations of diacylglycerol gradually increased up to 2-fold, FSG induced rapid increases in phosphatidic acid, up to 10 times higher than in unstimulated cells (Fig. 1). Monovalent and divalent cation ionophores that induce an acrosome reaction and concomitant Ca2+ influx, gramicidin S (30 μM) (Fig 1) and ionomycin (10–50 μM) (not shown), also increased concentrations of phosphatidic acid and diacylglycerol. The concentrations of the major phospholipids, phosphatidylcholine, phosphatidyethanolamine, phosphatidylserine, phosphatidylinositol, and cardiolipin, were not altered significantly in FSG-treated cells (data not shown). Basal concentrations of 1,2-diacylglycerol were 42 ± 7 pmol/mg (wet weight) (mean ± S.D.; n = 5 separate experiments); basal phosphatidic acid concentrations were 25 ± 11 pmol/mg (wet weight) (mean ± S.D.; n = 14 separate experiments). Concentrations of phosphatidic acid in FSG-stimulated cells were as high as 200 pmol/mg (wet weight) and could account for 3–4% of the total phospholipids in the cell.

Verapamil Inhibition of FSG-induced Increases in Phosphatic Acid—Ca2+ channel antagonists inhibit the FSG-induced acrosome reaction, elevations in cyclic AMP (5), and increases in [3H]inositol 1,4,5-trisphosphate accumulation (35). Fig. 2A shows the concentration-dependent inhibition by verapamil of phosphatidic acid increases induced by FSG. The concentration of verapamil that caused one-half maximal inhibition (IC50) of FSG-induced increases in phosphatidic acid was approximately 25 μM, similar to an IC50 of approximately 30 μM for FSG-induced elevations of cyclic AMP and [3H]inositol 1,4,5-trisphosphate reported previously (35). Increases in diacylglycerol were also inhibited by verapamil (not shown). In addition, the effects of verapamil were rapidly reduced when it was added a few seconds after FSG (Fig. 2B). When added 15 s, or later, after FSG, verapamil had no effect on phosphatidic acid increases measured after 2 or 5 min of incubation, consistent with the hypothesis that an initial Ca2+ influx after FSG addition triggers rapid activation of enzymes within the cell, including phospholipase(s).

Choline and Choline Phosphate Concentrations—Some Ca2+-mobilizing hormones have been shown to stimulate hydrolysis of phosphatidylcholine in addition to phosphatidylinositol (59). To test for possible turnover of phosphatidylcholine during the acrosome reaction, spermatozoa were incubated with FSG for 15 s to 5 min, and the squeous phase of Bligh and Dyer extractions were assayed for choline and choline phosphate (see "Experimental Procedures"). As shown in Fig. 3, choline concentrations were elevated 5-fold by FSG, whereas choline phosphate concentrations were not significantly changed. The rates of turnover of choline and choline phosphate are not known, however, and the possibility that choline phosphate is rapidly converted to choline cannot be excluded.

Phosphorylation of Diacylglycerol and de Novo Synthesis of Phosphatic Acid—Phosphatidic acid has been thought to be formed by the phosphorylation of diacylglycerol by diacylglycerol kinase during the turnover of phosphoinositides (36, 37). To test whether elevations of phosphatic acid in spermatozoa were due to phosphorylation of diacylglycerol, spermatozoa were incubated with [32P]orthophosphate to label intracellular ATP as described under "Experimental Procedures." In control experiments, the specific activity of intracellular [γ-32P]ATP did not significantly change when [32P]-radiolabeled spermatozoa were treated with FSG for up to 5 min (not shown). The specific activity of phosphatidic acid rapidly decreased upon addition of FSG (Fig. 4), suggesting that the increases in phosphatidic acid during the acrosome reaction did not result from phosphorylation of 1,2-diacylglycerol. To test for possible de novo synthesis of phosphatic
cubated for replicate determinations. To study activity of phospholipase D in vivo, spermatozoa were incubated with [3H]alkyl-lyso-PC (40). After incubation with [3H]alkyl-lyso-PC for 6–8 h (see "Experimental Procedures"), an aliquot of spermatozoa (20 mg, wet weight) contained approximately 100–150 cpm in [3H]phosphatidic acid. FSG caused a 4-fold accumulation of [3H]phosphatidic acid and a similar increase in the concentration of phosphatidic acid (Fig. 8). Furthermore, concentration-response curves for elevations of phosphatidic acid mass and specific activity changes in spermatozoa previously radiolabeled with [32P]orthophosphate as described under "Experimental Procedures." At time 0, FSG (10 μg fucose/ml) (O) or an equal volume of water (●) were added. Reactions were terminated at the indicated times with chloroform/methanol/HCl (100:200:1) and the mass (A) and specific activity (B) of phosphatidic acid measured in duplicate incubations according to "Experimental Procedures." In control experiments, the specific activity of intracellular 1γ[32P]ATP was approximately 450 cpm/nmol and did not significantly change in incubations up to 5 min with FSG (not shown).

Stimulation of Phospholipase D Activity in Vivo during the Acrosome Reaction—A characteristic reaction catalyzed by phospholipase D is the transfer of the phosphatidyl moiety of phospholipids to nucleophilic alcohols (43, 60). In the presence of water, glycerol, or ethanol, phospholipase D-catalyzed breakdown of phosphatidylcholine produces phosphatidic acid, phosphatidylglycerol, or phosphatidylethanol, respectively, along with choline. This reaction, called transphosphatidylation, has recently been studied in vivo in hepatocytes stimulated with Ca2+-mobilizing hormones (39) and in (Met-Leu-Phe-stimulated HL-60 granulocytes (40). When spermatozoa were incubated in seawater containing 2.5% ethanol for 15 min, relatively small amounts of phosphatidylethanol were produced in reaction mixtures without FSG, whereas addition of FSG greatly potentiated phosphatidylethanol accumulation (Fig. 6). The ionophores, ionomycin (30 μM) and gramicidin S (30 μM), also caused accumulation of phosphatidylethanol in spermatozoa (not shown). In addition, when ethanol concentrations were varied from 0.1 to 2.5%, elevations of phosphatidylethanol were increased, whereas relative phosphatidic acid elevations were reduced (Fig. 7), suggesting a competitive transphosphatidylation reaction by phospholipase D.

To study activity of phospholipase D in vivo, spermatozoa were incubated with [3H]glycerol ("Experimental Procedures"). As shown in Fig. 5, addition of FSG did not affect the incorporation of radioactivity in phosphatidic acid from [3H]glycerol.

FIG. 2. Effects of verapamil on FSG-induced elevations of phosphatidic acid. A, spermatozoa (100 mg, wet weight) were preincubated for 2 min with various concentrations of verapamil. At time 0 (after verapamil), 10 μg of FSG (fucose/ml) (●) or an equal volume of water (○) were added. Incubation conditions were otherwise as described in the legend to Fig. 1. Reactions were terminated after 2 min and phosphatidic acid assayed according to "Experimental Procedures." Data shown represent the mean of duplicate reaction mixtures and are representative of two additional experiments. B, verapamil (100 μM) was added to reaction mixtures containing spermatozoa (100 mg, wet weight) and FSG (10 μg fucose/ml) at the times shown after addition of FSG (○). Control reaction mixtures contained FSG without verapamil (●). Reactions were terminated after 2 min and phosphatidic acid concentrations measured as described under “Experimental Procedures.” Data shown represent the mean of duplicate determinations.

FIG. 3. Concentration of choline and choline phosphate in FSG-treated spermatozoa. Spermatozoa (25 mg, wet weight) were added to reaction mixtures in the presence (+) or absence (−) of 10 μg of FSG (fucose/ml) and incubated for the times indicated. The aqueous phase of Bligh and Dyer extractions from each reaction mixture was assayed for choline (Cho) (○, □) and choline phosphate (Pcho) (▲, △) concentrations as described under “Experimental Procedures.” Each data point represents the mean of three reaction mixtures.
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FIG. 7. Effects of increasing concentrations of ethanol on phosphatidylethanol and phosphatidic acid accumulation stimulated by FSG. Sea urchin spermatozoa (100 mg (wet weight)/ml) were incubated with increasing concentrations of ethanol (0%, 0.1%, 0.5%, 2.5%) in seawater. After 15 min, FSG (10 μg fucose/ml) (+) or an equal volume of water (-) was added to 0.5-ml aliquots. After 2 min, reactions were stopped and the lipid extracts chromatographed and charred (see “Experimental Procedures”) to measure phosphatidylethanol (PtdEtOH) (○, ●) and phosphatidic acid (PA) (○, ●) concentrations. Data shown are mean of two reaction mixtures.

FIG. 8. Effects of FSG on radioactivity and mass of phosphatidic acid in cells previously radiolabeled with [3H]alkyl lysophosphatidylcholine. Spermatozoa were incubated with [3H]alkyl-lysophosphatidylcholine for 6 h to radiolabel spermatozoan alkyl-PC (“Experimental Procedures”). Cells were washed, and 10 μg FSG (fucose)/ml (○, ▲) or an equal volume of water (●, ○) were added to 1.0-ml aliquots (100 mg, wet weight). At the times indicated, reactions were terminated and aliquots of the lipid extracts chromatographed to determine the amount of radioactivity in [3H]phosphatidic acid (A) and the concentration of phosphatidic acid (B) as described under “Experimental Procedures.” Each data point represents the mean of duplicate determinations.

Fig. 6. Stimulation of phosphatidylethanol and phosphatidic acid accumulation by FSG in the presence of ethanol. Spermatozoa (100 mg (wet weight)/ml) were incubated with ethanol (2.5%, v/v) in seawater. After 15 min, 10 μg (fucose)/ml FSG (+) or an equal volume of water (−) was added to 0.5-ml aliquots of spermatozoa at time 0 and reactions terminated with chloroform/methanol/HCl (100:200:1) at the times shown. Phosphatidylethanol (PtdEtOH) (○, ●) and phosphatidic acid (PA) (○, ●) concentrations were measured according to “Experimental Procedures” in duplicate reaction mixtures.

Fig. 5. Effects of FSG on mass and radioactivity of phosphatidic acid in cells previously radiolabeled with [3H]glycerol. Spermatozoa were preincubated with [3H]glycerol for 15 min. In control experiments, radioactivity from [3H]glycerol was detected in lysophosphatidic acid, phosphatidic acid, and diacylglycerol within the first few minutes of the labeling period. Aliquots of radiolabeled spermatozoa (50 mg, wet weight) were added to seawater with (○, ▲) or without (●, △) FSG (10 μg fucose/ml). At the times shown, reactions were stopped and aliquots of the chloroform extracts chromatographed and charred to measure phosphatidylethanol (PtdEtOH) (○, ●) and phosphatidic acid (PA) (○, ●) concentrations. Data shown are mean of two reaction mixtures.

[3H]phosphatidic acid obtained from FSG-treated cells paralleled those for percent of cells that have undergone an acrosome reaction induced by FSG (Fig. 9). Radioactivity from [3H]alkyl-lysophosphatidylcholine (200-300 cpm/incubation), but incubation of spermatozoa with FSG for up to 5 min did not affect the accumulation of radioactivity in [3H]diacylglycerol (not shown). Last, when [3H]alkyl-lysophosphatidylcholine labeled spermatozoa were incubated in ethanol-containing seawater, FSG elicited the accumulation of both [3H]phosphatidic acid and [3H]phosphatidylethanol (data not shown).

To test for phospholipase D activity in vitro, particulate fractions from sea urchin spermatozoa were assayed with a radiolabeled phosphatidylcholine analog, [14C]di-C₄-PC, that can act as both a detergent and a substrate for phospholipase D as described under “Experimental Procedures.” Sea urchin spermatozoan particles could hydrolyze [14C]di-C₄-PC to produce [14C]phosphatidic acid or, in the presence of 0.1-10% ethanol, [14C]phosphatidylethanol (not shown). When as-
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DISCUSSION

Phospholipase D, once thought to be present only in plants, has been partially purified from rat brain and characterized in vitro in a variety of rat tissues (61, 62). Based on several lines of evidence, we report here that phospholipase D activity is present in spermatozoa and can be regulated by a component of egg jelly that induces an acrosome reaction. First, neither phosphorylation of diacylglycerol nor de novo synthesis from glycerol could account for increases in phosphatidic acid caused by FSG. Second, FSG caused phosphatidylethanol accumulation when ethanol was present in the extracellular medium. Third, in cells with endogenously radiolabeled phosphatidylcholine, FSG caused elevations in [3H]phosphatidic acid. Fourth, spermatozoan membranes hydrolyzed a [3H]phosphatidic acid analog in vitro to produce [3H]choline, but not choline phosphate, into the culture medium. These authors concluded that phorbol esters could stimulate turnover of phosphatidylcholine by phospholipase D. When tested on sea urchin spermatozoa, several concentrations of phorbol esters did not detectably affect phosphatidic acid concentrations.

The functions, if any, of phosphatidic acid and diacylglycerol in sea urchin spermatozoa are not known. 1,2-Diacylglycerol appears to act as a second messenger in many cells by virtue of its effects on protein kinase C (70). In mouse spermatozoa, addition of a diacylglycerol analog or phorbol esters alters the zona pellucida-induced acrosome reaction at an intermediate step (71). In sea urchin spermatozoa, we found that addition of several concentrations of phorbol esters and

FIG. 9. Elevation of [3H]phosphatidic acid and percent acrosome-reacted cells as a function of FSG concentration. Sea urchin spermatozoa were radiolabeled with [3H]alkyl-lyso-PC, washed, and various concentrations of FSG (0 to 10 μg fucose/ml) added to 1.0-ml reaction mixtures. After 2 min, reactions were stopped with either chloroform/methanol/HCl (100:200:1) (A) for measurement of [3H]phosphatidate (Δ) or with 2% glutaraldehyde in seawater (B) to determine the percent acrosome reacted cells (□) (see "Experimental Procedures").

was proposed by Bennett and colleagues (63) to occur by phospholipase C hydrolysis of phosphatidylcholine and phosphatidylinositol, whereas phosphatidic acid increases were attributed to phosphorylation of diacylglycerol by diacylglycerol kinase. Phosphatidic acid accumulation during the acrosome reaction of sea urchin spermatozoa as shown here, however, appears to result from the activation of phospholipase D. Whether diacylglycerol elevations in sea urchin spermatozoa are due to phospholipase C or to dephosphorylation of phosphatidic acid by phosphatidate phosphohydrolase has not been determined.

The mechanism by which phospholipase D is activated during the acrosome reaction is not known. Elevation of phosphatidic acid concentrations by FSG appears to be linked to an influx of extracellular Ca++. When assayed in the presence of EDTA, detergent-solubilized phospholipase D from rat brain microsomes did not require added Ca++ for activity, but was stimulated 30-40% by 5 mM Ca++ or Mg++ (65). Purified phospholipase D from plants has an absolute requirement for Ca++ for activity (66, 67), and optimal concentrations of Ca++ were found to be proportional to the concentration of substrate (67). When tested in this study, phospholipase D activity from spermatozoan membranes was not significantly altered in vitro by the addition of Ca++ or Mg++.

Reports exist to suggest that phospholipase D activity can be regulated by a guanine nucleotide-binding protein (38, 39). GTPyS caused a time-dependent increase in phosphatidic acid and decrease in phosphatidylcholine concentration in liver plasma membrane preparations (38). Addition of ATP or ADP to membranes in the presence of submaximal concentrations of GTPyS also caused accumulation of phosphatidic acid, presumably by acting through P2-purinergic receptors coupled to a guanine-nucleotide-binding protein (38). Although sea urchin and mammalian spermatozoa contain guanine nucleotide-binding proteins (68, 69), we found no effect of GTP analogs, including GTPyS and Gpp(NH)p, on phosphatidic acid concentrations in spermatozoan plasma membrane preparations.

Another mechanism of regulation of phospholipase D may involve protein kinase C (41). Phorbol esters stimulate [3H]diacylglycerol and [3H]phosphatidic acid accumulation in REFS2 cells radiolabeled with [3H]myristic acid. When propranolol was added to inhibit phosphatidate phosphohydrolase, accumulation of diacylglycerol was blocked, whereas phosphatidic acid accumulation was unaffected. Furthermore, in cells radiolabeled with [3H]choline, phorbol esters caused release of choline, but not choline phosphate, into the culture medium. These authors concluded that phorbol esters could stimulate turnover of phosphatidylcholine by phospholipase D. When tested on sea urchin spermatozoa, various concentrations of phorbol esters did not detectably affect phosphatidic acid concentrations.

The functions, if any, of phosphatidic acid and diacylglycerol in sea urchin spermatozoa are not known. 1,2-Diacylglycerol appears to act as a second messenger in many cells by virtue of its effects on protein kinase C (70). In mouse spermatozoa, addition of a diacylglycerol analog or phorbol esters alters the zona pellucida-induced acrosome reaction at an intermediate step (71). In sea urchin spermatozoa, we found that addition of several concentrations of phorbol esters and

Previous investigations have reported increases in [3H]diacylglycerol and [32P]phosphatidic acid in human spermatozoa treated with A23187 to induce an acrosome reaction (63), and the presence of a phosphoinositide-specific phospholipase C activity in vitro (64). Diacylglycerol formation

was proposed by Bennett and colleagues (65) to occur by phospholipase C hydrolysis of phosphatidylcholine and phosphatidylinositol, whereas phosphatidic acid increases were attributed to phosphorylation of diacylglycerol by diacylglycerol kinase. Phosphatidic acid accumulation during the acrosome reaction of sea urchin spermatozoa as shown here, however, appears to result from the activation of phospholipase D. Whether diacylglycerol elevations in sea urchin spermatozoa are due to phospholipase C or to dephosphorylation of phosphatidic acid by phosphatidate phosphohydrolase has not been determined.

The mechanism by which phospholipase D is activated during the acrosome reaction is not known. Elevation of phosphatidic acid concentrations by FSG appears to be linked to an influx of extracellular Ca++. When assayed in the presence of EDTA, detergent-solubilized phospholipase D from rat brain microsomes did not require added Ca++ for activity, but was stimulated 30-40% by 5 mM Ca++ or Mg++ (65). Purified phospholipase D from plants has an absolute requirement for Ca++ for activity (66, 67), and optimal concentrations of Ca++ were found to be proportional to the concentration of substrate (67). When tested in this study, phospholipase D activity from spermatozoan membranes was not significantly altered in vitro by the addition of Ca++ or Mg++.

Reports exist to suggest that phospholipase D activity can be regulated by a guanine nucleotide-binding protein (38, 39). GTPyS caused a time-dependent increase in phosphatidic acid and decrease in phosphatidylcholine concentration in liver plasma membrane preparations (38). Addition of ATP or ADP to membranes in the presence of submaximal concentrations of GTPyS also caused accumulation of phosphatidic acid, presumably by acting through P2-purinergic receptors coupled to a guanine-nucleotide-binding protein (38). Although sea urchin and mammalian spermatozoa contain guanine nucleotide-binding proteins (68, 69), we found no effect of GTP analogs, including GTPyS and Gpp(NH)p, on phosphatidic acid concentrations in spermatozoan plasma membrane preparations.

Another mechanism of regulation of phospholipase D may involve protein kinase C (41). Phorbol esters stimulate [3H]diacylglycerol and [3H]phosphatidic acid accumulation in REFS2 cells radiolabeled with [3H]myristic acid. When propranolol was added to inhibit phosphatidate phosphohydrolase, accumulation of diacylglycerol was blocked, whereas phosphatidic acid accumulation was unaffected. Furthermore, in cells radiolabeled with [3H]choline, phorbol esters caused release of choline, but not choline phosphate, into the culture medium. These authors concluded that phorbol esters could stimulate turnover of phosphatidylcholine by phospholipase D. When tested on sea urchin spermatozoa, various concentrations of phorbol esters did not detectably affect phosphatidic acid concentrations.

The functions, if any, of phosphatidic acid and diacylglycerol in sea urchin spermatozoa are not known. 1,2-Diacylglycerol appears to act as a second messenger in many cells by virtue of its effects on protein kinase C (70). In mouse spermatozoa, addition of a diacylglycerol analog or phorbol esters alters the zona pellucida-induced acrosome reaction at an intermediate step (71). In sea urchin spermatozoa, we found that addition of several concentrations of phorbol esters and

2 S. E. Domino, S. B. Bocckino, and D. L. Garbers, unpublished observations. Membranes were obtained by nitrogen cavitation or sonication in 0.5 M salt buffers and were isolated by sucrose density centrifugation.

3 S. E. Domino, S. B. Bocckino, and D. L. Garbers, unpublished observations.
cell-permeable diacylglycerols did not detectably affect the FSG-induced acrosome reaction or protein phosphorylation in spermatozoa radiolabeled with $^{32}$P.$^{,3}$

Intracellular fusion of the outer acrosomal and plasma membranes of spermatozoa during the acrosome reaction has been suggested previously to result from elevations of membrane destabilizing lysophospholipids (32-34, 72-74). An alternative mechanism of membrane fusion may involve Ca$^{2+}$ interactions with anionic phospholipids, including phosphatic acid, to promote nodulibar configurations in the lipid membrane and subsequent membrane fusion (75, 76). Phospholipid vesicles containing phosphatic acid and phosphatidylycholine undergo Ca$^{2+}$-induced fusion in vitro (77, 78). A possible role of phosphatic acid elevations in sea urchin spermatozoa, then, may be to promote, in the presence of Ca$^{2+}$, intracellular membrane fusion during the acrosome reaction.

In support of the potential importance of anionic phospholipids in membrane fusion, Bearer and Friend (79, 80) have studied the localization of anionic lipids in guinea pig spermatozoa using the antibiotic polymyxin B. Regions of the plasma membrane specialized for fusion during the acrosome reaction had higher affinity for polymyxin B over nonfusional regions and were perturbed in distinct areas. These authors noted that anionic lipid concentrations increased as the membrane became fusionally competent during capacitation and suggested local concentrations of anionic lipids may affect membrane stability.

A second membrane fusion event in fertilization is the intercellular fusion of the egg and acrosome-reacted spermatozoon. An adhesion protein, “bindin,” is exposed during the acrosome reaction and mediates the binding, and possibly fusion, of sea urchin gametes (81). When tested in vitro, bindin induced aggregation and fusion of mixed anionic and neutral phospholipid vesicles of phosphatidyserine and phosphatidylycholine, but did not affect vesicles of phosphatidyserine or phosphatidylycholine alone (82, 83). In addition, Ca$^{2+}$ increased the rate of vesicle fusion (83). If bindin acts as a fusion protein with specific phospholipid requirements, phosphatic acid may alter the anionic charge in the acrosomal membrane and the subsequent binding and fusion of the acrosome-reacted spermatozoon and the egg.

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REFERENCES

Phospholipase D Activation during Acrosome Reaction