Polyphosphoinositide-derived Diacylglycerol Stimulates the Hydrolysis of Phosphatidylcholine by Phospholipase C during Exocytosis of the Ram Sperm Acrosome

EFFECT IS NOT MEDIATED BY PROTEIN KINASE C

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In ram spermatozoa, treatment with the ionophore A23187 and Ca$^{2+}$ led to an increase in total diacylglycerol mass and to exocytosis of the acrosomal granule. If sperm cells were prelabeled with [3H]palmitic acid, stimulation with A23187/Ca$^{2+}$ resulted in the generation of [3H]diacylglycerols with a mixture of saturated and unsaturated fatty acids. When cells were prelabeled with 1-O-[3H]octadecenglycerophosphocholine, stimulation led to the generation of [3H]alkylacylglycerol. No rise in [3H]diacyl- or [3H]alkylacylphosphatidic acid was detected under these conditions. Moreover, no changes in the mass of phosphatidic acid have been previously noted under similar conditions. Thus, these results indicate that diradylglycerols are generated via phospholipase C (PLC). Increases in diradylglycerols were paralleled by rises in monoacyl- or monoalkylglycerols labeled at position 1, but not in free [3H]palmitic acid or [3H]octadecanol, implying that, unlike somatic cells, spermatozoa catabolize diradylglycerols via a 2-diacylglycerol lipase. Activation of PLC appears to be effected by polyphosphoinositide-derived diacylglycerol exposure to Mg$^{2+}$, a cation known to inhibit phosphoinositide hydrolysis, resulting in less PLC activity upon stimulation, and addition of exogenous 1,2-diacylglycerols enhanced the enzyme's activity. However, 1,3-diacylglycerol and alkylacylglycerol also stimulated PLC activity, suggesting that the effect is unlikely to be mediated via protein kinase C. Since diradylglycerols are known to be essential in the molecular sequence leading to membrane fusion in mammalian spermatozoa, these results suggest that their generation via PLC constitutes a fundamental event during acrosomal exocytosis in response to physiological agonists.

Exocytosis in spermatozoa involves a single, large secretory granule (the acrosome) and constitutes an essential step during fertilization because it releases or exposes enzymes that enable the sperm cell to penetrate the egg vesicles. Acrosomal exocytosis is triggered by oocyte-associated factor(s), such as progesterone (1-3) or the zona pellucida glycoprotein ZP3 (4-6), but it can also be stimulated by treatment with ionophores and Ca$^{2+}$ (7-9).

Recent studies have found that treatment with A23187/Ca$^{2+}$ or progesterone results in a rapid hydrolysis of polyphosphoinositides by phosphoinositidase C (10, 11), which appears to be essential for exocytosis (10, 12). Stimulation of spermatozoa with A23187/Ca$^{2+}$ also leads to a considerable increase in the mass of diacylglycerol (DAG) (13, 14). However, since the mass of polyphosphoinositides in sperm membranes (15, 16) is considerably less than that of the DAG generated, other phospholipid sources and pathways for DAG generation are likely to be involved. Moreover, since sperm membrane phospholipids vary in their acyl, alkyl, or alkenyl composition (15, 17, 18), a variety of diradylglycerol (DRG) molecular species are likely to be generated.

In previous work, we have examined whether the phospholipase D [phosphatidic acid (PA) phosphohydrolase] pathway is involved in DRG generation in mammalian spermatozoa because this seems to be the major route for DRG formation in invertebrate sperm cells (19). We have found that although a phospholipase D is activated upon stimulation with A23187 and Ca$^{2+}$, this activation is slow and occurs well after the early and considerable rise in DRGs (20), so its contribution to the DRG pool seems negligible. Moreover, this pathway does not appear to make any significant contribution to the sequence of events underlying exocytosis because exposure of sperm cells to reagents that either reduce the amount of PA available to the PA phosphohydrolase or that inhibit the phosphohydrolase directly do not affect the proportion of cells that undergo exocytosis (20).

Another mechanism for DRG generation in cells involves the direct action of phospholipase C (PLC) on membrane phospholipids. To ascertain whether this enzyme is responsible for the major DRG increase seen upon stimulation of mammalian spermatozoa and to gain insight into the regulation of this enzyme during exocytosis, we have now used various labeling protocols to quantify changes in DRGs when Ca$^{2+}$ entry is stimulated with A23187. We have chosen this experimental approach because earlier findings clearly indicated that in sperm from all invertebrate and vertebrate taxa studied so far, activation of phospholipases takes place after Ca$^{2+}$ entry, re-

1 The abbreviations used are: DAG, diacylglycerol; DRG, diradylglycerol ("radyl" refers to acyl, alkyl, and alkenyl substituents); DG, diglyceride; SU-DAG, saturated/unsaturated DAG (diacylglycerol) with a saturated fatty acid in position 1 and an unsaturated fatty acid in position 2; DS-DAG, disaturated DAG (diacylglycerol with saturated fatty acids in positions 1 and 2; PLC, phospholipase C; PC, phosphatidylcholine; PA, phosphatidic acid; [3H]alkyllyso-PC, 1-O-[3H]octadecyl-sn-glycerol-3-phosphocholine.

2 Although phosphoinositidase C is also a PLC in this paper, we refer to PLC as the enzyme that hydrolyzes phospholipids other than the polyphosphoinositides.
Phospholipase C and Sperm Acrosomal Exocytosis

gardless of the stimuli used to challenge sperm cells (see Ref. 20). The labeling protocols we used have allowed us to analyze changes in two different DRG species, namely 1,2-DAG and 1-alkyl-2-acylglycerol.

The results of this study indicate that phosphatidylcholine (PC) hydrolysis via PLC is the major source of DRGs, that PLC is regulated by Ca²⁺ and by phosphoinositide-derived DAG, and furthermore, that, unlike somatic cells, spermatozoa catalyze PC-derived DRGs via a lipase that deacylates first the fatty acid in position 2 of the glycerol backbone.

MATERIALS AND METHODS

Reagents—[9,10-³H]Palmitic acid (specific activity of 47–52 Ci/ mmol) was obtained from Amersham International (Amersham, United Kingdom) or Du Pont (Stevensage, UK). [1,2-³H]Palmitic acid (specific activity of 57 mCi/mmole) and 1-O-¹¹⁷⁷Ο-tocadeyl-sn-glycero-3-phosphocholine (¹¹⁷⁷Ο-Hallykyl-P,PC; specific activity of 164–171 Ci/mmole) were obtained from Amersham International. The ionophore A23187 was purchased from Calbiochem (Nottingham, UK). Polyvinyl alcohol (type II), polyethylene glycol compound, EDTA, and EGTA were from Sigma (Poole, UK). Lipids were from Sigma, Serdary (Ontario, Canada), or Novabiochem (Nottingham, UK). Polyphosphoinositides were kindly provided by Dr R. F. Irvine of this Institute.

Preparation, Labeling, and Treatment of Spermatozoa—The standard timed incubation medium used consisted of 142 mm NaCl, 2.5 mm KOH, 10 mm glucose, and 20 mm Hepes, adjusted to pH 7.55 at 20 °C with NaOH; a medium consisting of 222 mM sucrose in place of the NaCl was used for washing spermatozoa (21). Both media contained 1 mg of polyvinyl alcohol/ml and 1 mg of polyethylene glycol/ml and had an osmolality of 305 mmosm/g.

Ejaculated ram spermatozoa were separated from seminal plasma by dilution and washing through sucrose medium (22). Labeling was routinely carried out by incubating washed spermatozoa (~1.5 × 10⁷/ml) for 60 min at 37 °C in ~5 ml of saline medium containing 5 μCi of [³H] or [³C]palmitic acid/ml or 3 μCi of [³H]Hallyyl-P,PC/ml. Before stimulation, spermatozoa were washed through sucrose medium (400 × gmax, for 5 min and 1000 × gmax for 10 min) and resuspended in saline medium.

Exocytosis of the sperm acrosome was induced by treating cells with Ca²⁺ (3 mm) and the ionophore A23187 (1 μm) in saline medium at 37 °C; occurrence of exocytosis was monitored by phase-contrast microscopy in glutaraldehyde-fixed samples (7).

Lipid Analyses—For quantification of changes in DRGs, reactions were stopped at various intervals after the beginning of A23187/Ca²⁺ treatment by addition of chlorform/methanol (1:2, v/v), and lipids were then extracted according to Bligh and Dyer (22). To measure changes in phospholipids, lipids were extracted essentially as described previously (13, 14), except that reactions were stopped with 10% (w/v) perchloric acid (23).

Lipids were separated by thin layer chromatography on Silica Gel 60-coated glass plates (0.25-mm thickness) or plastic sheets (0.2-mm thickness) (E. Merck, Darmstadt, Germany). For DAG mass quantification, neutral lipids were separated in the solvent system toluene/diethyl ether/acetic acid (98:2:1, v/v) (20) using 1,2-dimyristoyl-sn-glycero-3-phosphocholine (1,2-DAG) as internal lipid standards; (b) plates (5712, Merck) were developed twice in the solvent system n-hexane/diethyl ether/acetic acid (70:30:1, v/v) using 1,2-dioleoyl-rac-glycerol, 1,3-dioleoyl-glycerol, arachidonic acid, and 1-monooleoylglycerol as internal standards; or (c) plates (5712, Merck) were developed twice in the solvent system consisting of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100, v/v) (27) using 1,2-dipalmitoylphosphatidic acid, 1,2-dioleoyl-rac-glycerol, 1,3-dioleoyl-glycerol, arachidonic acid, and 1-monooleoylglycerol as internal standards. After development, plates were allowed to dry. Lipid spots were visualized by staining with naphthYLEVULINE and scraped off, and the radioactivity in each was determined by liquid scintillation counting.

Results—The results of this study indicate that phosphatidylcholine (PC) hydrolysis via PLC is the major source of DRGs, that PLC is regulated by Ca²⁺ and by phosphoinositide-derived DAG, and furthermore, that, unlike somatic cells, spermatozoa catalyze PC-derived DRGs via a lipase that deacylates first the fatty acid in position 2 of the glycerol backbone.

Phospholipids were separated on 10 × 10-cm plastic sheets pretreated by spraying with 1% (w/v) potassium oxalate, activated by heating at 110 °C for 10 min, and developed in a two-dimensional system in which the first solvent was chloroform/methanol/0.25% aqueous ammonia (85:10:5, v/v) and the second solvent consisted of chloroform/methanol/0.25% aqueous ammonia (40:40:5, v/v). The plates were air-dried briefly, and the various spots were detected by autoradiography using Fuji RX film. Using the autoradiographs as templates, the individual spots were scraped off, and the radioactivity in each was determined by liquid scintillation counting.

Statistics—Results are means ± S.E. Significance of results was examined using Student's t test or analysis of variance. Values of p < 0.05 were regarded as statistically significant.

RESULTS

Changes in 1,2-DAG Mass—Treatment of unlabeled ram sperm with A23187 (1 μm) and Ca²⁺ (3 mm) resulted in a rapid increase in the mass of 1,2-DAG, which peaked at 2.5 min and then declined slightly at 5 min (Fig. 1A). When cells were stimulated with A23187 alone, the increase in DAG mass was delayed, and it reached maximal values at 5 min (Fig. 1A). No accumulation of 1,2-DAG took place if 1 mm EGTA was added together with A23187 (data not shown). Treatment of spermatozoa with A23187/Ca²⁺ resulted in acrosomal exocytosis, which started at 5 min and reached maximal values by 30 min (Fig. 1B). No exocytosis occurred if cells were stimulated with A23187 in the absence of added Ca²⁺. Thus, DAG generation preceded the initiation of visible exocytosis.

Changes in Labeled DRGs—Incubation of ram spermatozoa with radioactive palmitic acid led to a time-dependent incorporation of label into the fatty acid pool, phospholipids, 1,2-DAG, and 1,3-DAG (linear during 180 min; data not shown). The polyphosphoinositides were not labeled under these conditions.

Preliminary experiments showed that separation of labeled lipids in the solvent n-hexane/diethyl ether/acetic acid (the solvent system usually employed to resolve neutral lipids) failed to reveal clear changes in labeled 1,2-DAG upon stimulation with A23187/Ca²⁺. This could relate either to the fact that spermatozoa have high basal levels of DAG (15–17, 29), which may obscure small increases in labeled DAGs generated as a result of phospholipid hydrolysis, or to the possibility that various DAG pools are changing simultaneously upon stimulation. DAGs found in unstimulated sperm cells have saturated fatty acids in positions 1 and 2 (15, 17, 29). However, sperm phospholipids have saturated fatty acids only in position 1 and unsaturated fatty acids in position 2 (15, 17, 29), and therefore, the DAGs generated by phospholipid hydrolysis will have the
Changes in [3H]SU-DAG, [3H]DS-DAG, and [3H]alkyl-DG after treatment of ram spermatozoa with A23187 (1 μM) in absence or presence of Ca2+ (3 mM). Spermatozoa prelabeled with [3H]palmitic acid (A and B) or [3H]alkyllyso-PC (C) were stimulated for various periods of time, and the lipids were then extracted and resolved in the solvent system chloroform/methanol/acetic acid (A and B) or hexane/diethyl ether/acetic acid (C). Results (means ± S.E.) from duplicate assays carried out on two (A and B) or three (C) occasions are shown. ⊙, A23187/Ca2+; △, A23187 alone; ⌐, Ca2+ alone (control).

**Figure 2.**

Changes in phospholipids after treatment of labeled ram spermatozoa with A23187/Ca2+.

Spermatozoa were prelabeled with [3H]palmitic acid and stimulated with A23187 (1 μM) and Ca2+ (3 mm) for various times; lipids were extracted and separated by two-dimensional thin layer chromatography, and the radioactivity in each spot was counted. Results are means ± S.E. from three different experiments.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>0 min*</th>
<th>1 min</th>
<th>5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/10^6 spermatozoa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>10,349 ± 228</td>
<td>8317 ± 77</td>
<td>8532 ± 298</td>
</tr>
<tr>
<td>PS</td>
<td>647 ± 48</td>
<td>633 ± 129</td>
<td>439 ± 11</td>
</tr>
<tr>
<td>PE</td>
<td>515 ± 122</td>
<td>439 ± 11</td>
<td>836 ± 350</td>
</tr>
<tr>
<td>PI</td>
<td>1689 ± 509</td>
<td>1514 ± 543</td>
<td>651 ± 102</td>
</tr>
<tr>
<td>PA</td>
<td>4832 ± 350</td>
<td>4588 ± 319</td>
<td>4461 ± 519</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>1987 ± 324</td>
<td>1697 ± 350</td>
<td>2014 ± 106</td>
</tr>
<tr>
<td>Lyso-PC</td>
<td>1782 ± 631</td>
<td>2638 ± 744</td>
<td>614 ± 101</td>
</tr>
</tbody>
</table>

*Incubation time.

**Table I**

- Different from 0 min (p < 0.05).
- Different from PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.
- Different from 1 min (p < 0.02).

Sources of DRGs—To identify possible sources of DRGs, spermatozoa were labeled with [3H]palmitic acid, [3H]alkyllyso-PC, or [3H]alkyl-DG. As expected, a time-dependent incorporation of label into alkyl-PC, the diglycerides, and PA was found. When spermatozoa were prelabeled with [3H]alkyllyso-PC or [3H]alkyl-DG were treated with A23187/Ca2+, a considerable generation of [3H]alkyl-DG was observed (Fig. 2C), with a time course similar to that seen for [3H]SU-DAG. Exposure to A23187 alone resulted in a slower generation of [3H]alkyl-DG. No rises in [3H]alkyl-DG were seen after treatment with A23187/EGTA (data not shown).

**Sources of DRGs—**To identify possible sources of DRGs, spermatozoa were labeled for 60 min with [3H]palmitic acid, washed, and stimulated with A23187/Ca2+. At different times after the beginning of treatment, reactions were stopped, and the lipids were extracted and resolved using a two-dimensional thin layer chromatography system. Lipid spots were detected by autoradiography. Treatment with A23187/Ca2+ caused a significant increase in label mainly in PC (Table I). A slight decrease in label was also seen in phosphatidylserine, but it was not significant for phosphatidylinositol. No significant changes were detected in lyso-PC or PA. These results therefore indicate that PC constitutes the major substrate for PLC.

**Metabolism of DRGs—**1,2-DAG can be phosphorylated to PA by a kinase or sequentially deacylated by a DAG lipase, which usually removes the fatty acid in position 1, and then by a monoacylglycerol lipase, which removes the fatty acid in position 2. Both DAG kinase and DAG lipase seem to have poor affinity for substrates with 1-alkyl substituents (30).

To understand which pathways are used to metabolize DRGs in spermatozoa, we quantified putative DRG metabolites generated after A23187/Ca2+ treatment. In spermatozoa prelabeled with [3H]palmitic acid or [3H]alkyllyso-PC, the rise in [3H]SU-DAG or [3H]alkyl-DG was not accompanied by changes in [3H]PA (Fig. 3, A and B), which suggests a lack of phosphorylation of either DRG. On the other hand, increases in [3H]monoacyl- and [3H]monoaoylglycerol accompanied the rises in [3H]SU-DAG and [3H]alkyl-DG, respectively (Fig. 3, A and B). However, no changes in free [3H]palmitic acid or [3H]octadecanoylglycerol were seen. Since, under these two conditions, the label is located in the substituent in position 1 of the glycerol backbone, these results indicate that sperm DRG lipase acts first on the fatty acid present in position 2.

**Effect of Mg2+ on DAG Generation—**Stimulation of ram spermatozoa with A23187/Ca2+ induces a rapid activation of phosphoinositide C and the concomitant generation of some 1,2-DAG (10, 13). We have therefore investigated whether phosphoinositide C activation is related to the activation of PLC. This was done by stimulating spermatozoa with A23187 in the presence of Mg2+ because this cation is known to inhibit A23187-induced phosphoinositide hydrolysis via phosphoinositidase C (10). Results revealed that when spermatozoa were treated with A23187 (1 μM) and Mg2+ (3 mM), the generation of DAG was reduced when compared with cells treated with A23187 alone (Table II). If, however, sperm cells were first...
find that the alkylacylglycerol there was no reduction in DAG levels. This indicates, first, that before the peak in DAG mass levels, but after the majority

phosphoinositidase C action on the polyphosphoinositides. Results was quantified by densitometry after Coomassie Blue staining. Results

PLC itself is not affected by Mg2+ and, second, that PLC activation may be partly modulated by DAG generated through

lipids were extracted and resolved using the upper phase of the solvent system ethyl acetate/2,2,4-trimethylpentane/acetic acid/water. Results

Effect of Mg2+ on DAG mass accumulation in A23187-treated ram spermatozoa

Spermatozoa were stimulated with A23187 (1 μm) in the absence or presence of Mg2+ (3 mM). Reactions were stopped after 5 min; lipids were extracted and separated by thin layer chromatography; and DAG mass was quantified by densitometry after Coomassie Blue staining. Results are means ± S.E. of duplicate assays carried out on three occasions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1,2-DAG after 5 min</th>
<th>μg/10^9 spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.13 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>A23187 alone</td>
<td>2.31 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>A23187/Mg2+</td>
<td>2.52 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>A23187 for 1.5 min; then Mg2+ added</td>
<td>3.26 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

* Different from control (p < 0.005)

† Different from A23187 alone (p < 0.02).

treated with A23187, and Mg2+ was added 1.5 min later (i.e. before the peak in DAG mass levels), but the majority (~90%) of the phosphoinositides have been hydrolyzed (10)), there was no reduction in DAG levels. This indicates, first, that PLC itself is not affected by Mg2+ and, second, that PLC activation may be partly modulated by DAG generated through phosphoinositidase C action on the polyphosphoinositides.

Effect of Exogenous DRGs on PLC—To examine further the possible regulation of PLC by DRGs, spermatozoa prelabeled with [3H]palmitic acid were exposed to various DRGs and then treated with A23187/Ca2+. The purity of 1,3-dioctanoylglycerol was examined by thin layer chromatography before the experiments. It could be argued, however, that 1,3-dioctanoylglycerol may be converted to the 1,2-isomer in solution or by spermatozoa and that the effect observed is, in fact, due to the action of 1,2-dioctanoylglycerol. We have ruled out these possibilities with the following experiments. First, when 1,3-dioctanoylglycerol or 1,3-dioleoylglycerol (with ~10% 1,2-isomer as internal reference) was incubated for 15 min at 37 °C in standard saline medium, no increase in 1,2-dioctanoylglycerol or 1,2-dioleoylglycerol was seen upon lipid extraction (22), separation by developing plates twice in n-hexane/diethyl ether/acetic acid (70:30:1, v/v), and staining with Coomassie Brilliant Blue (24, 25). Second, when these 1,3-DAGs were incubated with spermatozoa in saline medium (37 °C, 15 min), followed by centrifugation at 13,000 rpm for 7 min to sediment spermatozoa, no increase in the amount of 1,2-isomers in the supernatant was detected after lipid extraction, separation, and staining as described above. Third, when spermatozoa in saline medium were incubated with the permeant 1,3-dioctanoylglycerol (37 °C, 15 min), and sperm lipids were extracted, separated, stained as described above, and compared with lipids extracted from spermatozoa incubated without 1,3-dioctanoylglycerol, no increase in 1,2-dioctanoylglycerol was detected in the former. Finally, further evidence supporting the idea that spermatozoa do not appear to convert 1,3-DAG stems from the finding that stimulation with A23187/Ca2+ resulted in no changes in the levels of this metabolite. Thus, these observations indicate that the effect of 1,3-dioctanoylglycerol on stimulation of [3H]SU-DAG generation is authentic. Taken together, these results therefore suggest that DRG stimulation of PLC is not mediated via protein kinase C, and on the other hand, they lend support to the idea

† Separation of lipids by developing plates twice in n-hexane/diethyl ether/acetic acid (70:30:1, v/v) resolved three spots: (a) 1,2-dioctanoylglycerol, (b) 1,3-dioctanoylglycerol plus 1,2-DAGs with long fatty acyl chains (such as sperm 1,2-DAGs), and (c) 1,3-DAGs with long fatty acyl chains. Thus, changes in 1,2-dioctanoylglycerol were readily detected with this system.

* B. R. S. Roldan and T. Murase, unpublished observations.
that phosphoinositide-derived DAG modulates PLC activity. In addition, they suggest that DRGs generated via PLC could, in fact, exert a positive feedback on the enzyme's activity.

**DISCUSSION**

This study is the first to demonstrate that the activation of PLC is the main mechanism for DRG generation when mammalian spermatozoa are stimulated to undergo exocytosis, that PC is the main substrate for PLC, that both diacyl- and alkylacylglycerol are generated by PLC, that both phosphoinositide-derived DAG and PC-derived DRGs modulate PLC, and that this DRG effect is unlikely to be mediated by protein kinase C.

Several pathways exist in somatic cells for the generation of DRGs. They include the breakdown of polyphosphoinositides by a phosphoinositidase C or the hydrolysis of phospholipids other than the polyphosphoinositides either directly by phospholipase C or via the sequential action of phospholipase D and PA phosphohydrolase. Since membrane phospholipids can have a variety of substituents present in position 1 (i.e., alkyl, aryl, or alk-1-ene), and the total length of substituents in position 1 or 2 can also vary widely, hydrolysis of these phospholipids usually results in an array of DRG molecular species.

Our previous work showed that stimulation of spermatozoa with A23187 and Ca\(^{2+}\) induces a rapid hydrolysis of the polyphosphoinositides and a concomitant generation of DAG (10, 12). Quantification of DAG mass, however, revealed that other sources of DAG and additional enzymatic pathway(s) involved in its generation were likely to exist in spermatozoa (12-14). Recently, we have presented evidence against phospholipase D as a pathway for DRG formation (20), and we have therefore investigated to what extent PLC contributes to the generation of DRG.

The generation of DRGs was studied using three different experimental approaches: (a) measuring changes in total DAG mass; (b) prelabeling cells with [\(^{3}H\)]phospholipid to follow changes in diacylphospholipids, 1,2-DAG, and 1,2-diacyl-PA; and (c) prelabeling cells with [\(^{3}H\)]alkyllyso-PC to follow changes in alkyl-PC, alkylacylglycerol, and alkyl-PA.

The evidence gathered using these experimental approaches indicates very strongly that PLC activation is the main mechanism for DRG formation in mammalian spermatozoa. First, stimulation of spermatozoa with A23187 and Ca\(^{2+}\) led to a considerable increase in DAG mass; this rise is probably related to the breakdown of phospholipids other than the polyphosphoinositides because the latter represent only a very small proportion of sperm membrane lipid (15, 16). No changes in PA mass have been previously detected when spermatozoa were stimulated using a similar treatment (13). Second, in spermatozoa prelabeled with radioactive palmitic acid and stimulated with A23187/Ca\(^{2+}\), there was a rapid loss of label from PC and a parallel rise in labeled 1,2-DAG with a saturated fatty acid in position 1 and an unsaturated fatty acid in position 2 (SU-DAG), but no changes in labeled diacyl-PA. PC was found to be the major (if not the sole) source of DAG, and other potential sources (phosphatidylserine or phosphatidylethanolamine) showed little decrease in label. Third, experiments in which cells were prelabeled with [\(^{3}H\)]alkyllyso-PC revealed that alkyl-DG, but not alkyl-PA, rose significantly after stimulation with A23187/Ca\(^{2+}\). Thus, these results indicate clearly that in mammalian spermatozoa, DRG generation takes place mainly via PLC activity. This is a very different scenario from that reported for sea urchin spermatozoa, where the major pathway for DRG generation appears to involve the sequential action of phospholipase D and PA phosphohydrolase and where no PLC activity has been detected (19).

Despite the rise in DRG pools detected with the three experimental approaches described above, there was one DRG pool that experienced a different fate. Using a solvent system that allowed us to distinguish two different types of DAGs (i.e., 1,2-DAGs with saturated fatty acids in position 1 and unsaturated fatty acids in position 2 (SU-DAG) and 1,2-DAGs with two saturated fatty acids (DS-DAG)), we found that DS-DAG actually decreased upon stimulation with A23187/Ca\(^{2+}\). The fate and/or role of this DS-DAG is unknown, but one possibility could be that upon cell stimulation, DS-DAG enters a deacylation/reacylation pathway in which a saturated fatty acid in position 2 is replaced by an unsaturated one; the transient rise in labeled monoglycerides found upon sperm stimulation is consistent with this idea (and see below). This DAG could eventually contribute to increase the PC or phosphatidylethanolamine pool (30, 33).

Metabolism of DRGs can take place via phosphorylation by a kinase or deacylation by a lipase. Our results indicate that PC-derived 1,2-DAG and alkylacylglycerol do not appear to serve as substrates for a kinase because the rise in labeled DRGs was not accompanied by increases in labeled diacyl- or alkyl-PA. This is not entirely surprising because DAG kinase can show a marked substrate specificity, phosphorylating only phosphoinositide-derived DAG (34) due to the enzyme's preference for arachidonic acid-containing DAG (35). Sperm PC-derived DAG does not seem to have arachidonic acid in position 2 because generation of arachidonic acid-labeled DAG was not observed when spermatozoa prelabeled with this precursor were stimulated with A23187/Ca\(^{2+}\) (23, 36). Furthermore, our observation that the rise in labeled alkylacylglycerol was not accompanied by changes in labeled alkyl-PA is consistent with the fact that alkylacylglycerol is a poor substrate for this kinase (30).

While DAG kinase does not seem to use PC-derived DAG or alkylacylglycerol as substrate, DAG lipase seems to be responsible for removing a substantial proportion of the DRGs. A considerable increase in monoacyl- and monomonoalkylglycerol was detected upon stimulation of spermatozoa. This finding is remarkable because detection of monoacyl- or monomonoalkylglycerol, labeled at position 1, implies that activation of a 2-DAG lipase is taking place. This contrasts with results obtained in somatic cells, where a 1-DAG lipase removes first the fatty acid in position 1 (e.g., Refs. 37-40). No radioactive free fatty acid or fatty alcohol was found to rise in parallel to the monoglycerides, thus lending support to the idea that the sperm DAG lipase is not removing the acyl or alkyl chain in position 1 and, moreover, indicating that the monoglycerides are not further catabolized by a monoglyceride lipase, but rather that they may be reacylated.

We have attempted to understand how PLC could be regulated in spermatozoa. In somatic cells, PLC regulation is related to G proteins, tryrosine kinase phosphorylation, or protein kinase C or directly to changes in intracellular Ca\(^{2+}\) levels (41-43). In sperm cells, both Ca\(^{2+}\) and DAG modulated the enzyme's activity. PLC-mediated DRG generation took place when Ca\(^{2+}\) internalization was stimulated with A23187. The enzyme seems to require only micromolar levels of the cation because treatment with A23187 in the absence of added Ca\(^{2+}\) resulted in a decrease in DAG (1-25 pm Ca\(^{2+}\) in the extracellular space) (44) sufficient to induce both SU-DAG and alkylacylglycerol generation. This agrees with results that showed that no PLC activity was evident in the absence of Ca\(^{2+}\) in extracts from rabbit or bull spermatozoa, but that maximal enzyme activity occurred with low levels of Ca\(^{2+}\) (45). These results allow us to propose that PLC may represent the as yet unidentified, micromolar Ca\(^{2+}\)-requiring event that takes place in the sequence leading to acrosomal exocytosis (44).

Although ram sperm PLC is activated by micromolar Ca\(^{2+}\), this may not be sufficient for maximal PLC activation because...
inhibition of phosphoinositide hydrolysis (and hence generation of phosphoinositide-derived DAG) reduced PLC activity. When sperm cells were stimulated in the presence of Mg²⁺, which blocks breakdown of the phosphoinositides (10), a reduction of DAG generation was noticed. It is unlikely that the Mg²⁺ effect is due to a direct inhibition of PLC because although Mg²⁺ reduced DAG levels when added at zero time, this effect was not seen if Mg²⁺ was added 1.5 min after A23187 treatment (when 90% of the polyphosphoinositides have already been hydrolyzed, but before the peak of DAG mass accumulation). This suggestion is supported by the finding that rabbit and bull sperm PLCs are not inhibited by Mg²⁺ in an in vitro assay (46). Another set of results also supports the idea that PLC is stimulated by phosphoinositide-derived DAG. Exposure of sperm to neomycin, which blocks polyphosphoinositide breakdown induced by A23187/Ca²⁺, also reduced the elevation of DAG mass (10).

The fact that neither Mg²⁺ nor neomycin caused complete inhibition of DAG production suggests that PLC is activated partially in parallel to phosphoinositidase C activation, but that it requires phosphoinositide-derived DAG to maximize its activity. This is supported by the finding that exogenous DRGs stimulated PLC activity and DAG generation. The stimulation of PLC by these DRGs most certainly occurs via mechanisms not involving protein kinase C. This suggestion stems from two major lines of evidence. The first one relates to the fact that both diacyl- and alkylacylglycerol enhanced PLC activity to a similar extent, even though alkylacylglycerol does not appear to activate protein kinase C. It should be mentioned that, although most studies have found that alkylacylglycerol does not activate or actually inhibits protein kinase C (47-52), it appears that this metabolite is capable of activating the kinase under certain circumstances (53, 54). No studies have been carried out to examine whether alkylacylglycerol would activate protein kinase C in spermatozoa. Nevertheless, this is unlikely because although stimulation of spermatozoa with A23187/Ca²⁺ leads to alkylacylglycerol formation (this study), such treatment does not result in changes in the pattern of protein phosphorylation (21), thus suggesting that alkylacylglycerol is not involved in activation of protein kinase C in spermatozoa. This explanation cannot rule out the possibility that during acrosomal exocytosis, protein kinase C might exert effects not related to phosphorylation (55). This, however, is also unlikely because several studies have found that treatment of spermatozoa from various species with phorbol esters, known activators of protein kinase C, does not induce or enhance exocytosis (11, 19, 21). Further studies are needed to confirm this interpretation.

A second line of evidence gives additional support to the idea that DRG effects are not mediated by protein kinase C. It was found that both 1,2- and 1,3-dioctanoylglycerol enhanced PLC activity to the same extent. There is strong evidence demonstrating that 1,3-DAGs, including 1,3-dioctanoylglycerol, do not activate protein kinase C (31, 32). Moreover, the fact that, under our experimental conditions, there is no conversion of 1,3-dioctanoylglycerol to the 1,2-isomer indicates that this possibility cannot account for the stimulation seen with 1,3-diocatroylglycerol. Thus, our results strongly support the view that DRG effects are not mediated by protein kinase C and that DRGs may exert a direct effect on PLC.

There seemed to be a clear relationship between activation of PLC (and generation of DAG) and subsequent exocytosis. Treatment with A23187/Ca²⁺ led to maximal increases in DAG mass, [³H]SUS-DAG, and [³H]alkyl-DG, and such treatment also resulted in acrosomal exocytosis. Conditions that enhanced PLC activity and DAG generation, such as treatment with exogenous DRGs (this study), enhance the number of cells that undergo exocytosis (13, 14). There is only one situation in which DRG rise and exocytosis seem to be uncoupled, and that is when cells are stimulated with A23187 alone (no added Ca²⁺). This, however, can be explained by the fact that although PLC is activated at low Ca²⁺ levels, downstream events such as phospholipase A₂ activation and membrane fusion require higher (millimolar) levels of the cation (12, 13).

In conclusion, based on results presented here and elsewhere, we wish to present our current view of the sequence of events triggered upon Ca²⁺ entry and leading to membrane fusion during exocytosis of the mammalian sperm acrosome (Fig. 5). Four Ca²⁺-requiring steps have been identified after Ca²⁺ entry, the first two requiring micromolar levels of the cation and the other two requiring millimolar concentrations (12). The first event is probably the early and essential breakdown of the polyphosphoinositides by phosphoinositidase C. The second event, as argued in this study, probably corresponds to activation of PLC by Ca²⁺ and by polyphosphoinositide-derived DAG; PLC, by acting on various PC subclasses, generates diacyl- and alkylacylglycerols. These DRGs, along with that derived from the polyphosphoinositides, stimulate a phospholipase A₂ (36), which, being a millimolar Ca²⁺-requiring enzyme, probably represents the third Ca²⁺-requiring step (23). Activation of phospholipase A₂ then leads to the generation of an array of metabolites that directly or indirectly (23) bring about membrane fusion, the final Ca²⁺-requiring event.

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