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DIACYLGLYCEROL AND PHOSPHATIDATE PRODUCTION AND THE EXOCYTOSIS OF THE SPERM ACROSOME

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We have investigated the production of diacylglycerol (DAG) and phosphatidate (PtdOH) during the exocytosis of the sperm acrosome. Ram spermatozoa treated with Ca^{2+} and the ionophore A23187 experienced a rapid breakdown of the polyphosphoinositides (PPIs), and a rise in [^{32}P]P_i-labelled PtdOH and DAG mass; PtdOH mass, however, was unaffected. Treatment with Ca^{2+}/A23187 and the DAG kinase inhibitor R59022 resulted in a dose-dependent increase in DAG mass and a concomitant decrease in [^{32}P]PtdOH; such treatment showed a dose-dependent stimulation of acrosomal exocytosis. Pre-incubation with exogenous PtdOHs before stimulation with Ca^{2+}/A23187 did not affect the time-course of exocytosis, whereas treatment with Ca^{2+}/A23187 and exogenous DAGs (dioctanoylglycerol, oleoyl-acetyl-glycerol, or dioleoylglycerol) resulted in a dose-dependent stimulation of acrosomal exocytosis. Our results suggest that DAG, rather than PtdOH, is the important metabolite generated upon PPI hydrolysis; however, since spermatozoa lack protein kinase C, the target of DAG in most cells, a role for DAG in acrosomal exocytosis is as yet unclear.

At fertilization, the spermatozoon undergoes an exocytotic process known as the acrosome reaction, which involves the exposure/release of enzymes that enables the sperm cell to penetrate the egg vestments (1). This exocytotic process is thought to be triggered *in vivo* by egg-associated factors but it can also be induced *in vitro* by divalent cation ionophores in the presence of extracellular Ca^{2+} (2,3). We have recently shown (4) that following such Ca^{2+}/ionophore stimulation, spermatozoa experience an early and rapid breakdown of the polyphosphoinositides (PPIs) which appears to be essential for subsequent exocytosis (5). In many cells, PPI breakdown is a common feature of receptor-mediated activation resulting in the generation of inositol phosphates, diacylglycerol (DAG) and phosphatidate (PtdOH)(6). Our previous work suggests that inositol phosphates generated from PPI breakdown are not involved as second messengers during exocytosis of the acrosome (7). However, although a rise in labelled PtdOH, parallel to PPI breakdown (4), and a rise in DAG after treatment with Ca^{2+}/ionophore (8) have been detected, nothing is known about the metabolism or potential roles of DAG and PtdOH during sperm acrosomal exocytosis (5).

Although DAG can be catabolized via two different pathways, i.e. DAG lipase and DAG kinase (6), it seems that the latter is preferentially used to remove DAG in most cells (9). The phosphorylation of DAG to PtdOH by the kinase has been recognized as an important way of modulating the messenger activity of DAG (10), and inhibition of such enzyme by the compound R59022 (11) has proved to be a useful tool in the study of DAG-modulated cellular responses including exocytosis (12-16). In many cells, the target of

Abbreviations: PPI, polyphosphoinositide; PtdIns, phosphatidylinositol; PtdIns(4)*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdOH, phosphatidate (=phosphatidic acid); DAG, diacylglycerol; DiC8, 1,2-dioctanoyl-*sn*-glycerol; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; DOG, 1,2-dioleoyl-*sn*-glycerol.

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DAG messenger action is a Ca^{2+}- and phospholipid-dependent protein kinase C (17). In spermatozoa, however, this is unlikely since we have recently reported the absence of active protein kinase C from these cells (5,18). Thus, a target for DAG has not yet been found in spermatozoa, although alternative roles may certainly exist (cf. 19,20). On the other hand, since various roles have also been proposed for PtdOH (6,21,22), it is possible that phosphorylation of DAG by DAG kinase actually serves to increase a pool of active PtdOH thus leading to subsequent cellular processes.

The aim of this paper is to examine the metabolism of DAG via DAG kinase and to explore the possible participation of DAG and PtdOH in events leading to the exocytosis of the sperm acrosome.

MATERIALS AND METHODS

Reagents. $[^{32}P]P_i$ (carrier-free; 10 mCi/ml) was purchased from Amersham International (Amersham, Bucks., U.K.). Ionophore A23187 was a gift from Eli Lilly, Indianapolis, IN, U.S.A. Poly(vinyl)alcohol (PVA; type II, average Mr 10,000), diacylglycerols, phosphatidates, and phospholipid standards were from Sigma Chemical Co. (Poole, Dorset, U.K.). Polyvinylpyrrolidone (PVP; average Mr 44,000) and Hepes were from BDH (Poole, Dorset, U.K.). R59022 was purchased from Janssen Pharmaceuticals (Wantage, Oxon, U.K.) and dissolved as described (11). Polyphosphoinositide standards were kindly provided by Dr R.F. Irvine of this Institute.

Preparation and treatment of spermatozoa. Throughout the experiments the standard saline incubation medium used consisted of 142 mM-NaCl; 2.5 mM-KOH, 10 mM-glucose and 20 mM-Hepes, adjusted to 7.55 at 20°C with NaOH (18); a medium containing 222 mM-sucrose in place of the NaCl was used for washing spermatozoa. Both media also contained 1 mg PVA/ml, 1 mg PVP/ml and had an osmolality of 305 mOsm/kg.

Ejaculated ram spermatozoa were separated from seminal plasma by dilution and washing through sucrose medium as described (23). For experiments in which labelled cells were required, washed spermatozoa (about 1.0×10^8/ml) were incubated in about 5 ml of saline medium containing 125-250 μCi $[^{32}P]P_i$/ml for 60 min at 37°C (4).

Exocytosis of the sperm acrosome was induced by treating cells with Ca^{2+} and the divalent cation ionophore A23187 in saline medium at 37°C (24), and was monitored by phase-contrast microscopy of glutaraldehyde-fixed samples.

Lipid analysis. At various intervals after the beginning of Ca^{2+}/ionophore treatment, lipids were extracted essentially as previously described (4) except that reactions were stopped with 10% perchloric acid (v/v) and the pellet obtained after the first centrifugation (at 1000 g_{max} for 5 min) was resuspended in 5% perchloric acid (v/v). After a second centrifugation, lipids were extracted with chloroform/methanol/conc. HCl (500:1000:6 by vol.) and further washed as reported (4). In some experiments, neutral lipids were extracted according to Bligh & Dyer (25).

Lipids were separated by t.l.c. on silica-gel 60 F_{254}-coated plates (0.25 mm thickness; E. Merck, Darmstadt, W. Germany). Labeled PPIs and PtdOH were separated in a solvent system consisting of chloroform/methanol/water/conc. NH_3 (38:40:7:5 by vol.)(26). Lipid spots were detected by autoradiography, identified using the autoradiograph as template, scraped off and the radioactivity in each determined by liquid scintillantion counting. DAG/neutral lipids were separated using the solvent toluene/diethylether/ethanol/conc. NH_3 (50:40:2:0.2 by vol.)(27). 1,2-Diacylglycerol was quantitated by Coomassie Blue staining (28) and densitometry, using 1,2-dioleoylglycerol to construct standard curves for each plate as previously described (27). Briefly, after development, plates were air-dried, stained with Coomassie Brilliant Blue R250 (0.03% w/v in 30% v/v methanol 100 mM-NaCl) for 30 min and destained for 5 min in 30% methanol 100 mM-NaCl. The plates were air-dried and scanned with a Chromoscan-3 UV densitometer (Joyce-Loebl, Gateshead, Tyne and Wear, U.K.). Phosphatidate was separated from other lipids by a multidevelopment system (27). Samples were first developed to the top of the plate in a solvent consisting of butanol/acetic acid/water (6:1:1 by vol.). After removing the top 1 cm of the plate contanining the neutral lipids and turning the plate 180°C, the plates were finally developed in a solvent of chloroform/methanol/conc. NH_3 (10:3:0.6 by vol.). Phosphatidate was quantitated by Coomassie Blue staining and densitometry using dipalmitoyl-phosphatidate as standard.

RESULTS

Ram spermatozoa treated with Ca^{2+} (3 mM) and A23187 (1 μM) experienced a rapid and considerable loss of ^{32}P label from the region of the t.l.c. plates corresponding to both PtdIns(4,5)P_2 and PtdIns(4)P and a concomitant rise in the region corresponding to PtdOH ; no changes were seen in the labelling of

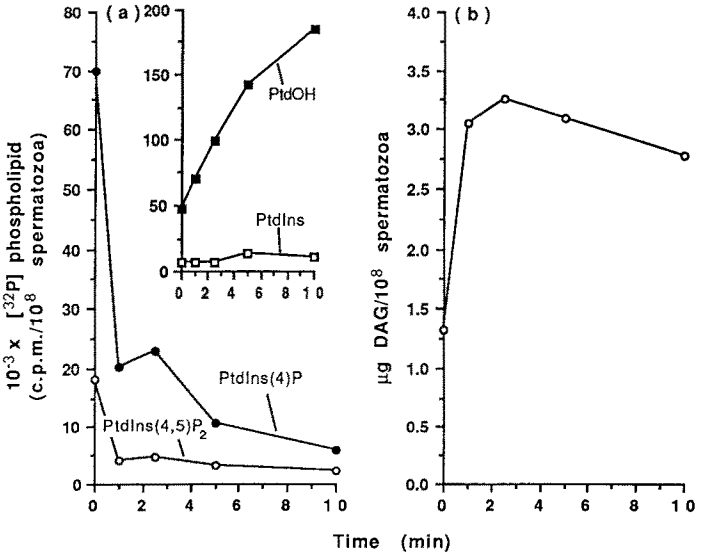

Fig. 1. Changes in (a)[^{32}P]P_i-labelled phosphoinositides and PtdOH, and (b) DAG mass following Ca^{2+}/A23187 treatment of ram spermatozoa. Spermatozoa were labelled for 60 min with 125 µCi [^{32}P]P_i/ml and then treated with Ca^{2+} (3 mM) and A23187 (1 µM) for different times. Lipids were extracted, separated by t.l.c., and radioactivity in each spot counted. Neutral lipids were separated by t.l.c. and DAG mass was quantitated by Coomassie Blue staining and densitometry using 1,2-dioleoylglycerol as standard (see "Materials and Methods" for details). Results are means of 3 separate experiments.

PtdIns (Fig. 1a). Parallel to the decrease in ^{32}P label of the PPIs, a rise in DAG mass was observed (Fig. 1b). However, no apparent changes in the mass of PtdOH could be detected during the time period studied (not shown). Therefore, the remaining studies were done analysing changes in labelled PPIs and PtdOH, and DAG mass.

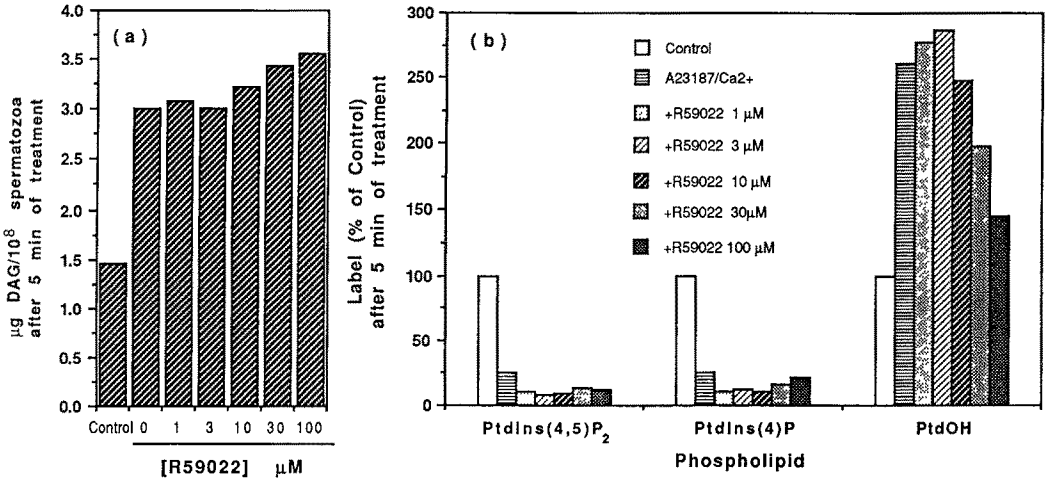

Fig. 2. Effect of different concentrations of R59022 on changes in (a) DAG mass and (b) [^{32}P]P_i-labelled PPIs and PtdOH after treatment of ram spermatozoa with Ca^{2+}/A23187. Spermatozoa were labelled for 60 min with 250 µCi [^{32}P]P_i/ml and then treated with Ca^{2+} (3 mM) and A23187 (1 µM) for 5 min in the presence of different concentrations of R59022. Lipids were extracted, separated by t.l.c., and radioactivity in each spot measured as described in "Materials and Methods". DAG mass was quantitated by Coomassie Blue staining and densitometry using 1,2-dioleoylglycerol as standard (see "Materials and Methods" for details). Results are means of 2 separate experiments.

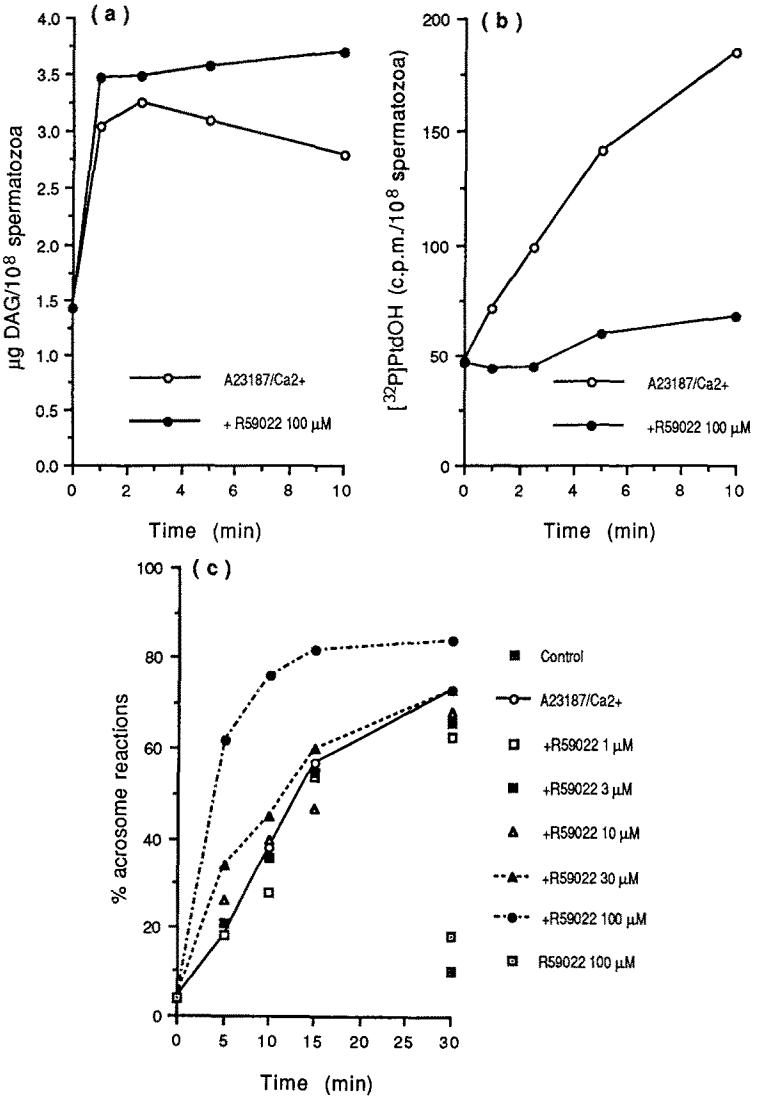

Fig. 3. Effect of R59022 on the time-course of (a) DAG mass accumulation, (b) [^{32}P]PtdOH production, and (c) acrosomal exocytosis induced by Ca^{2+} and A23187 in ram spermatozoa. Spermatozoa were labelled for 60 min with 250 µCi [^{32}P]P_i/ml and then treated with Ca^{2+} (3 mM) and A23187 (1 µM) for various times in the absence or the presence of 100 µM-R59022. Lipids were extracted, separated by t.l.c., and radioactivity in each spot counted (see "Materials and Methods"). DAG mass was quantitated by Coomassie Blue staining and densitometry using 1,2-dioleoylglycerol as standard as described in "Materials and Methods". Parallel unlabelled samples were also treated with Ca^{2+} and A23187, in the presence of different concentrations of R59022 and, at various intervals, subsamples were analyzed for the occurrence of the acrosome reaction. Results are means of 3 separate experiments.

Spermatozoa treated with Ca^{2+}/A23187 and increasing concentrations of R59022 (a well-known inhibitor of DAG kinase) for 5 min showed a dose-dependent increase in the amount of DAG accumulated (Fig. 2a). In addition, increasing concentrations of R59022 inhibited in a dose-dependent manner the formation of [^{32}P]PtdOH following Ca^{2+}/A23187 treatment without affecting [^{32}P]PtdIns(4,5)P_2 or [^{32}P]PtdIns(4)P breakdown (Fig. 2b). Treatment of spermatozoa with R59022 (100 µM) alone did not induce the breakdown of [^{32}P]PtdIns(4,5)P_2 or [^{32}P]PtdIns(4)P, or the generation of DAG, and it did not affect the resting levels of [^{32}P]PtdOH (not shown).

Figure 3 shows the effects of R59022 (100 μM) on the time-course of DAG and [^{32}P]PtdOH accumulation induced by Ca^{2+}/A23187 treatment of spermatozoa. Treatment with R59022 resulted in a faster and greater accumulation of DAG (Fig. 3a) and completely prevented the rise in [^{32}P]PtdOH (Fig. 3b). Parallel sperm samples treated with Ca^{2+}/A23187 and increasing concentrations of R59022 showed a dose-dependent stimulation of acrosomal exocytosis (Fig. 3c). Spermatozoa that received no treatment or were treated with R59022 (100 μM) alone remained intact during the period examined.

Exogenous DAG and PtdOH were used to test whether these metabolites would affect the occurrence of acrosomal exocytosis. To examine the effect of DAG, spermatozoa were treated with Ca^{2+}/A23187 and

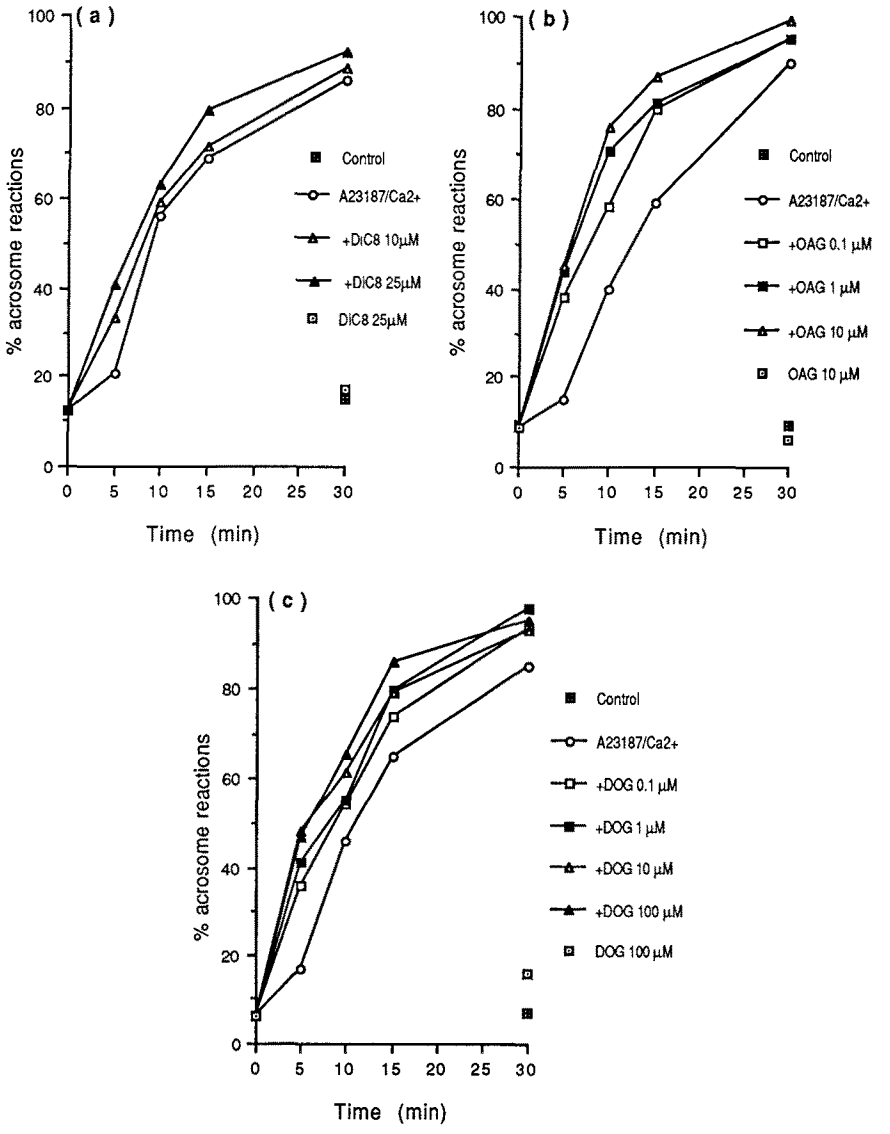

Fig. 4. Effect of exogenous DAGs on acrosomal exocytosis. Spermatozoa in saline medium were pre-incubated for 15 min with various concentrations of the indicated DAG and then treated with 3mM-Ca^{2+} and 1 μM-A23187 (time zero). At various intervals, subsamples were analyzed for the occurrence of the acrosome reaction. (a) 1,2-dioctanoyl-*sn*-glycerol (DiC8); (b) 1-oleoyl-2-acetyl-*sn*-glycerol (OAG); (c) 1,2-dioleoyl-*sn*-glycerol (DOG). Results are averages of 3 experiments.

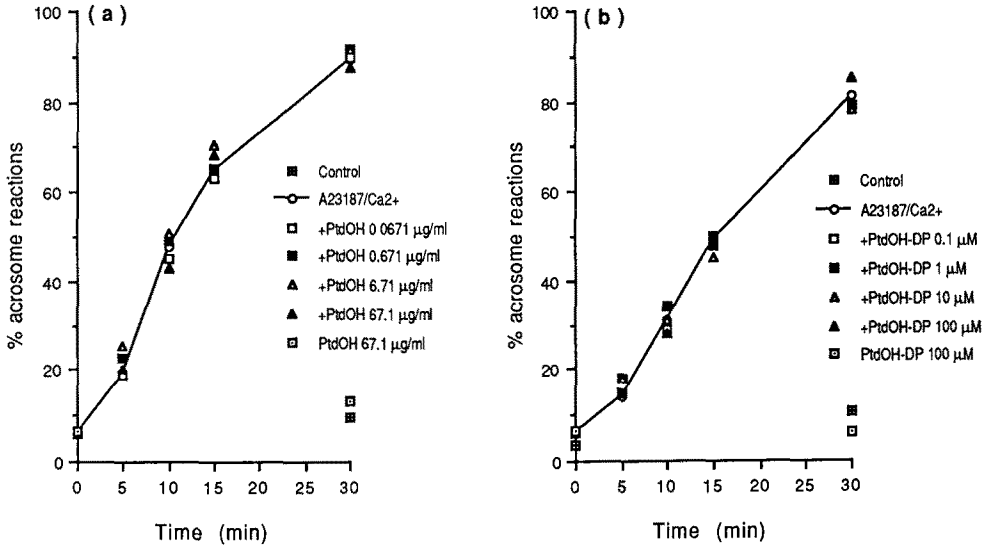

Fig. 5. Effect of exogenous PtdOHs on acrosomal exocytosis. Spermatozoa in saline were pre-incubated for 15 min with various concentrations of the indicated PtdOH and then treated with 3 mM-Ca^{2+} and 1 µM-A23187 (time zero). At different times, subsamples were analyzed for the occurrence of the acrosome reaction. (a) PtdOH from egg yolk lecithin.; (b) dipalmitoyl-PtdOH. Results are averages of 4 separate experiments.

either 1,2-dioctanoyl-*sn*-glycerol (DiC8), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), or 1,2-dioleoyl-*sn*-glycerol (DOG). As shown in Fig. 4 (a-c), all three DAGs showed a dose-dependent stimulation of the acrosome reaction. None of the DAGs on their own had any effect at the concentrations used. On the other hand, pre-incubation of spermatozoa with two different PtdOHs (from egg-yolk and a synthetic dipalmitoyl-PtdOH) before treatment with Ca^{2+}/A23187 had no detectable effect upon the acrosome reaction.(Fig. 5).

DISCUSSION

The results of this study clearly indicate that DAG may be involved in events leading to exocytosis of the sperm acrosome. On the other hand, the DAG-derived PtdOH, generated via a DAG kinase, does not seem to participate in events ending in membrane fusion in this cell.

After stimulation with Ca^{2+}/A23187, ram spermatozoa experienced a rapid disappearance of ^{32}P label from the PPIs, which was accompanied by an increase in DAG levels. This rise in DAG levels paralleled the curve of inositol 1,4,5-trisphosphate production measured after a similar Ca^{2+}/A23187 stimulation (7). Thus, these results lend support to our previous suggestion that the disappearance of ^{32}P label from PPIs actually represents a phosphoinositidase C-mediated hydrolysis of both PtdIns(4,5)P_2 and PtdIns(4)*P* after Ca^{2+} entry (4,7).

Resting levels of DAG seemed high in ram spermatozoa (about 1.5 µg/10^8 spermatozoa) as compared to those seen in other cells (cf. discussion in ref. 29). However, studies of lipid composition of ram, bull and boar spermatozoa (30-32) have clearly shown that basal DAG actually consists of DAG species mainly with 14:0 and 16:0 fatty acids. Such composition is certainly different from what may be expected in a PPI-derived DAG, which usually contains 18:0/20:4 fatty acids (33). Whether the sperm basal DAG is part of a pool that is unavailable as "messenger" or whether it has a poor messenger "quality" because of its fatty

acid composition is unknown. The latter, however, seems unlikely because results presented here show that three different DAGs are able to stimulate the sperm exocytotic process.

It is noteworthy that since PPIs only represent a minor proportion of the sperm membrane phospholipids (32,34) they cannot account for the total amount of DAG mass generated, even if one takes into account that in spermatozoa both PtdIns(4,5)P_2 and PtdIns(4)P are hydrolysed. It is unlikely that DAG derives from PtdIns, since no changes were detected in the levels of this phosphoinositide after spermatozoa were stimulated with Ca^{2+}/A23187. Additional sources of DAG clearly exist in spermatozoa. For instance, the activation of a choline-, serine-, or ethanolamine-phosphoglyceride-specific phospholipase C, either directly or by the PPI-derived DAG generated after cell stimulation (35,36), may contribute to increase the DAG pool. It is not known whether such mechanisms exist in spermatozoa.

Unlike studies carried out in other cells (27), we have been unable to detect changes in PtdOH mass after spermatozoa were stimulated with Ca^{2+}/A23187. Such treatment, however, did result in a considerable increase in the amount of labelled [^{32}P]PtdOH. This suggests that the pool of DAG actually being phosphorylated to PtdOH is a very small one. Since initial changes in [^{32}P]PtdOH parallel those taking place in the lipids of the PPI cycle, it is likely that the initial rise in [^{32}P]PtdOH is due to a phosphorylation of the PPI-derived DAG. Because the small mass of either PtdIns(4,5)P_2 or PtdIns(4)P present in spermatozoa (32,34) is not detected by the Coomassie Blue staining method we used, it is not surprising that a rise in PPI-derived PtdOH would not result in a change in PtdOH mass. Further increases in DAG, perhaps due to the hydrolysis of phosphoglycerides other that PPIs (35,36), and the possibility that only a small fraction of this DAG is available to the DAG kinase (cf. ref. 10), could explain, firstly, the continuous rise in [^{32}P]PtdOH seen after PPI breakdown is complete and, secondly, the lack of increase in PtdOH mass that accompanies the rise in DAG mass. Moreover, the limited availability of DAG to the DAG kinase raises the point that a large proportion of the DAG generated might be catabolized via a different pathway, such as that involving a DAG lipase.

Several roles, some of them recently challenged, have been proposed for PtdOH: (a) a substrate for phospholipase A_2 (21), (b) a Ca^{2+} ionophore (6, and see refs. 37,38), and (c) a fusogen (22). In spermatozoa, however, PPI-derived PtdOH does not seem to have any apparent role in events leading to exocytosis. The addition of exogenous PtdOH did not affect the time-course of the acrosome reaction. Moreover, the inhibition of endogenous PtdOH production was not accompanied by an inhibition of acrosomal exocytosis. Although evidence for a phospholipase D-mediated generation of PtdOH has been presented in sea urchin spermatozoa (39), it is not known whether PtdOH itself has any role in exocytosis in these cells. In addition, a similar enzyme has not yet been reported in mammalian sperm cells. Thus, no role for PtdOH is so far apparent in spermatozoa.

In contrast, our results suggest that PPI-derived DAG does have a role in events leading to exocytosis of the sperm acrosome. A rise in endogenous DAG, as a result of either treatment with R59022 (a DAG kinase inhibitor) or incubation with three different exogenous DAGs, accelerated exocytosis. Others have also found that treatment with R59022 or exogenous DAG results in an increase in cellular responses (6,11,13,14), exocytosis (12) or cell proliferation (16). In all these cases, the target of DAG action seemed to be protein kinase C. Spermatozoa, however, lack protein kinase C (18) and, therefore, it is tempting to propose that in these cells DAG might have another role, either as messenger or as a substrate from which other active metabolites might be generated. Various functions have already been recognized: DAG may (a) act as substrate for DAG lipase (from which arachidonic acid may be liberated)(33), (b) be a fusogen (by

perturbing the lipid bilayer)(19), and (c) stimulate phospholipase activity, either rendering substrates accessible to enzyme attack (e.g. 40), or directly activating enzymes such as phospholipase A_2 (20), with the generation of other active metabolites (lysophospholipids and arachidonic or other fatty acids).

In conclusion, since our previous work has found no role for inositol phosphates (7), DAG seems to be *the* important messenger generated upon PPI hydrolysis. Whether it acts by modulating additional cellular processes or by serving as substrate for the release of additional metabolites is presently unknown and is currently the subject of further study.

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