The role of diacylglycerol in the exocytosis of the sperm acrosome

Studies using diacylglycerol lipase and diacylglycerol kinase inhibitors and exogenous diacylglycerols

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When ram spermatozoa were treated with Ca^{2+} and the ionophore A23187 to induce acrosomal exocytosis, a rise in diacylglycerol (DAG) mass was observed, concomitant with a rapid breakdown of [^{32}P]P$_i$-labelled phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate and a rise in [^{32}P]P$_i$-labelled phosphatidate. Inclusion of the DAG lipase inhibitor RHC 80267 resulted in further but biphasic increases in DAG; there was an increasing accumulation of DAG with concentrations of RHC 80267 up to 10 μM, whereas higher concentrations produced lessening accumulation. Inclusion of RHC 80267 in the ionophore induction system also resulted in significant accelerations of the onset of exocytosis. In spermatozoa stimulated with Ca^{2+}/A23187 and the DAG kinase inhibitor R59022, a similar increase in DAG levels together with stimulation of acrosomal exocytosis were observed. Preincubation of spermatozoa with sn-1-oleoyl-2-acetylglycerol, rac-1-oleoyl-2-acetylglycerol, sn-1,2-dioctanoylglycerol and sn-1,3-dioctanoylglycerol before treatment with Ca^{2+}/A23187 resulted in a dose-dependent stimulation of exocytosis by all these isomers. Neomycin inhibited Ca^{2+}/A23187-induced generation of DAG together with polyphosphoinositide breakdown, as well as acrosomal exocytosis. Inclusion of exogenous DAG, however, overcame the inhibitory effect of neomycin on exocytosis. Our results suggest that DAG has a key role in acrosomal exocytosis and that it acts as a messenger rather than as a substrate from which other active metabolites are generated. The lack of stereospecificity shown by the exogenous DAGs implies that DAG does not act by stimulating protein kinase C, but the metabolite's actual target in the sperm cell is as yet unclear.

INTRODUCTION

Activation of many secretory and non-secretory cell types results in the hydrolysis of membrane polyphosphoinositides (PPIs) and the concomitant generation of the PPI-derived second messengers InsP_3 and 1,2-diacylglycerol (DAG) (Berridge, 1987). Whereas InsP_3 participates specifically in the modulation of intracellular Ca^{2+} levels (Berridge & Irvine, 1989), DAG may have a direct effect on several cellular processes. Thus, although in many cells the target of DAG messenger action is a Ca^{2+}- and phospholipid-dependent protein kinase C (Nishizuka, 1984), this metabolite has been shown to exert other molecular functions (Dawson et al., 1984; Kramer et al., 1987; Das & Rand, 1984).

Moreover, sequential deacylation of DAG, first by DAG lipase and then by monoacylglycerol (MAG) lipase, results in the release of arachidonic or other fatty acids, which might serve to generate eicosanoids (Hokin, 1985). On the other hand, phosphorylation of DAG by DAG kinase results in the generation of phosphatidate (PtdOH), which might in turn serve to modulate other cellular processes (Billah et al., 1981; Sundler & Papahadjopoulos, 1981; Jackowski & Rock, 1989).

At the time of fertilization, the spermatozoon undergoes an exocytotic process (the so-called 'acrosome reaction'), which exposes or releases enzymes to allow the sperm cell to penetrate the egg vestments and also primes the sperm for fusion with the egg itself. Our previous findings have indicated the importance of PPI breakdown as an early event during the exocytosis of the sperm acrosome (Roldan & Harrison, 1989, 1990a; Harrison & Roldan, 1990). In addition, we have suggested that inositol phosphates produced as a result of this breakdown do not seem to be involved as second messengers in this exocytotic process (Harrison et al., 1990). Nothing is known, however, about the

role of the other metabolite, DAG. Although DAG could have a messenger action by activating protein kinase C (as in many other cell types), this is unlikely, since we have been unable to detect protein kinase C in spermatozoa (Roldan & Harrison, 1988, 1990a). It is thus possible that during acrosomal exocytosis DAG may serve either as a substrate to generate other active compounds or as a direct modulator of other events.

Studies on DAG and PtdOH generation after sperm stimulation have led us to conclude that PtdOH, produced by phosphorylation of DAG by DAG kinase, does not seem to participate in events underlying exocytosis in spermatozoa (Roldan & Harrison, 1990b). However, that investigation suggested that DAG may have a key role in this process. Therefore we have carried out studies on the catabolism of DAG via DAG lipase and DAG kinase in order to define further the role of DAG in the exocytosis of the sperm acrosome.

MATERIALS AND METHODS

Reagents

Abbreviations used: PPI, polyphosphoinositide; PtdOH, phosphatidate (= phosphatidic acid); DAG, 1,2-diacylglycerol; DiC$_8$, dioctanoylglycerol; OAG, 1-oleoyl-2-acetylglycerol; DOG, dioleoylglycerol; MAG, monoacylglycerol; PIC, phosphoinositidase C; PLC, (phosphoglyceride-specific) phospholipase C; N.S., not significant.

and was dissolved in dimethyl sulphoxide. PPI standards were kindly provided by Dr. R. F. Irvine of this Institute.

Preparation, labelling 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 (Roldan & Harrison, 1988); a medium containing 222 mM-sucrose in place of the NaCl was used for washing spermatozoa. Both media also contained 1 mg of poly(vinyl alcohol)/ml and 1 mg of polyvinylpyrrolidone/ml and had an osmolality of 305 mosmol/kg.

Ejaculated ram spermatozoa were separated from seminal plasma by dilution and washing through sucrose medium as described by Harrison et al. (1982). For experiments in which labelled cells were required, washed spermatozoa (about 1×10^8/ml) were incubated in about 5 ml of saline medium containing 125–250 μCi of $[^{32}P]P_i$/ml for 60 min at 37 °C (Roldan & Harrison, 1989).

Exocytosis of the sperm acrosome was induced by treating cells with Ca^{2+} and the bivalent-cation ionophore A23187 in saline medium at 37 °C (Shams-Borhan & Harrison, 1981), and was monitored by phase-contrast microscopy of glutaraldehyde-fixed samples.

DAG lipase and DAG kinase inhibitors were added to the sperm suspensions together with the ionophore, whereas exogenous DAGs and neomycin were preincubated with the suspensions for 15 min at 37 °C before initiation of ionophore treatment.

Lipid analyses

For the quantification of DAG changes, reactions were stopped at various intervals after the beginning of Ca^{2+}/ionophore treatment by addition of chloroform/methanol (1:2, v/v), and lipids were then extracted as described by Bligh & Dyer (1959). To measure changes in PPIs and PtdOH, lipids were extracted essentially as described previously (Roldan & Harrison, 1989), except that reactions were stopped with 10% (w/v) $HClO_4$ and the pellet obtained after the first centrifugation (at 1000 g_{max} for 5 min) was resuspended in 5% $HClO_4$. After a second centrifugation, lipids were extracted with chloroform/methanol/conc. HCl (250:500:3, by vol.) and further washed as previously described (Roldan & Harrison, 1989). In some experiments, changes in DAG mass and in labelled PPIs and PtdOH were measured concomitantly in portions of split samples of Ca^{2+}/A23187-treated spermatozoa, when reactions were stopped with 10% $HClO_4$ and lipids extracted with chloroform/methanol/conc. HCl as described above. Preliminary studies showed that the levels of 1,2-DAG detected with this extraction protocol were not significantly different from those obtained by the Bligh & Dyer (1959) method.

Lipids were separated by t.l.c. on silica-gel 60 F_{254}-coated plates (0.25 mm thickness; E. Merck, Darmstadt, Germany). DAG and other neutral lipids were separated in the solvent toluene/diethyl ether/ethanol/conc. NH_3 (250:200:10:1, by vol.) (Bocckino et al., 1987). DAG was quantified by Coomassie Blue staining (Nakamura & Handa, 1984) and densitometry, using 1,2-dioleoylglycerol (DOG) to construct standard curves for each plate, as described by Bocckino et al. (1987). Briefly, developed 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.). By this method, quantities of 0.05 μg were easily detected

on the t.l.c. plates (cf. Nakamura & Handa, 1984), which in our experiments corresponded to as little as 0.1 μg/10^8 spermatozoa.

Labelled PPIs and PtdOH were separated in the solvent system chloroform/methanol/water/conc. NH_3 (38:40:7:5, by vol.) (Mitchell et al., 1986). Lipid spots were made visible by autoradiography, identified by using the autoradiograph as template, scraped off, and the radioactivity in each was determined by liquid-scintillation counting.

RESULTS

DAG production and inhibition of DAG catabolism

Treatment of ram spermatozoa with Ca^{2+} (3 mM) and A23187 (1 μM) to induce acrosomal exocytosis resulted in a rapid and considerable increase in DAG and PtdOH (Table 1; Fig. 1a), and a parallel decrease in both $PtdIns(4,5)P_2$ and PtdIns4P (Table 1). DAG levels reached a maximum at 2.5 min, and then declined slowly (Fig. 1a).

In order to delineate a possible role for DAG in acrosomal exocytosis, inhibitors of DAG catabolism were included in the induction system to determine their effect both on DAG levels and on rates of exocytosis. When spermatozoa were treated for 5 min with Ca^{2+}/A23187 and increasing concentrations of the DAG lipase inhibitor RHC 80267 (Sutherland & Amin, 1982), there was a biphasic accumulation of DAG (Table 1): inclusion of up to 10 μM-RHC 80267 caused a dose-dependent increase in the amount of DAG accumulated, whereas greater concentrations of the inhibitor resulted in lesser increases. Concentrations of RHC 80267 of up to 100 μM did not affect either PPI breakdown or PtdOH accumulation induced by Ca^{2+}/A23187 (Table 1). RHC 80267 alone did not affect the resting levels of PPIs, and did not lead to the generation of either DAG or PtdOH (Table 1).
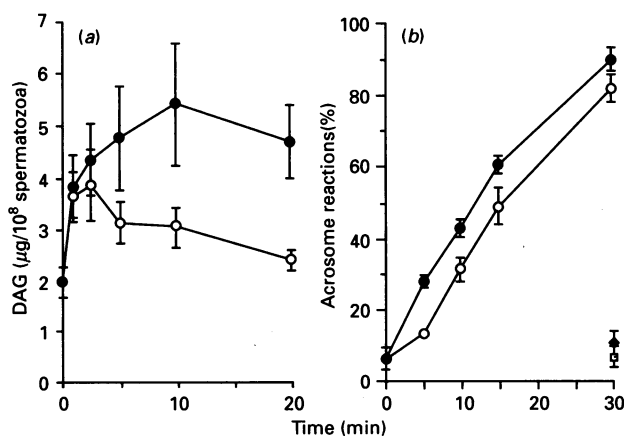
Fig. 1 shows the effect of RHC 80267 on the time course of DAG accumulation and acrosomal exocytosis in Ca^{2+}/A23187-treated spermatozoa. Treatment with Ca^{2+}/A23187 and 10 μM-RHC 80267 resulted in a greater accumulation of DAG as compared with cells treated with Ca^{2+}/A23187 alone (2-factor ANOVA: treatment, $F = 9.25$, $P = 0.004$; time, $F = 3.08$, $P = 0.02$; Fig. 1a). In parallel sperm samples treated with Ca^{2+}/A23187 and different concentrations of RHC 80267, 10 μM-RHC 80267 induced a significant stimulation of acrosomal exocytosis (2-factor ANOVA: treatment, $F = 26.06$, $P = 0.0001$; time, $F = 63.36$, $P = 0.0001$; Fig. 1b). Higher concentrations of RHC 80267 (30 and 100 μM), which were unable to raise the concentration of DAG beyond those obtained with 10 μM (cf. Table 1), also induced a significant stimulation of acrosomal exocytosis (results not shown; 2-factor ANOVA: Ca^{2+}/A23187 versus + 30 μM-RHC 80267, $F = 11.12$, $P = 0.004$; time, $F = 38.52$, $P = 0.0001$; Ca^{2+}/A23187 versus + 100 μM-RHC 80267, $F = 18.44$, $P = 0.0004$; time, $F = 52.12$, $P = 0.0001$). However, the degree of stimulation was not greater than that obtained when cells were treated with Ca^{2+}/A23187 and 10 μM-RHC 80267 [2-factor ANOVA: + 10 μM- versus + 30 μM-RHC 80267, $F = 1.11$, not significant (N.S.); + 10 μM- versus + 100 μM-RHC 80267, $F = 0.001$, N.S.]. Spermatozoa treated with 100 μM-RHC 80267 alone remained intact (Fig. 1b) and motile (result not shown) during the period examined.

These experiments showed that increases in sperm DAG levels could be induced by inhibition of DAG lipase and that such increases were associated with acceleration of the onset of exocytosis; because inhibition of the DAG lipase pathway did not result in rates of acrosomal exocytosis lower than controls, it was concluded that DAG's role in acrosomal exocytosis was not as a source of MAG or fatty acids.

Table 1. Changes in PPIs, PtdOH and DAG after treatment of ram spermatozoa with Ca^{2+}/A23187 in the absence or presence of compound RHC 80267

Spermatozoa were labelled for 60 min with 250 μCi of [^{32}P]P$_i$/ml and treated with Ca^{2+} (3 mM) and A23187 (1 μM) for 5 min in the absence or presence of different concentrations of compound RHC 80267. For quantification of ^{32}P labelling, lipids were extracted with chloroform/methanol/conc. HCl, separated by t.l.c., and radioactivity in each spot was counted. For quantification of DAG changes, lipids were extracted with chloroform/methanol, separated by t.l.c., and mass was quantified by Coomassie Blue staining and densitometry using sn-1,2-DOG as standard (for details see the Materials and methods section). Results are means $\pm$ s.e.m. from three separate experiments: [a]different from control ($P < 0.0001$); [b]different from Ca^{2+}/A23187 ($P < 0.05$); [c]different from $+3$ μM-RHC 80267 ($P = 0.05$); [d]different from $+10$ μM-RHC 80267 ($P = 0.002$).

| | Lipids 5 min after treatment | | | |
| | [^{32}P]PtdIns(4,5)P_2 (c.p.m./10^8 spermatozoa) | [^{32}P]PtdIns4P (c.p.m./10^8 spermatozoa) | [^{32}P]PtdOH (c.p.m./10^8 spermatozoa) | DAG (μg/10^8 spermatozoa) |
Treatment				
None (control)	25291 ± 2156	65027 ± 5840	39486 ± 4258	1.26 ± 0.04
Ca^{2+}/A23187	$3456\pm658^{(a)}$	$9970\pm1146^{(a)}$	$139648\pm3983^{(a)}$	$3.08\pm0.12^{(a)}$
$+1$ μM-RHC 80267	$3035\pm876^{(a)}$	$6502\pm1720^{(a)}$	$127359\pm7295^{(a)}$	$3.60\pm0.50^{(a)}$
$+3$ μM-RHC 80267	$2866\pm607^{(a)}$	$6068\pm1692^{(a)}$	$127671\pm12294^{(a)}$	$3.68\pm0.04^{(a,b)}$
$+10$ μM-RHC 80267	$2697\pm938^{(a)}$	$6079\pm1926^{(a)}$	$138727\pm8915^{(a)}$	$4.28\pm0.09^{(a,b,c)}$
$+30$ μM-RHC 80267	$2444\pm989^{(a)}$	$6097\pm1299^{(a)}$	$148203\pm14053^{(a)}$	$3.95\pm0.06^{(a,b,d)}$
$+100$ μM-RHC 80267	$2454\pm968^{(a)}$	$5635\pm1599^{(a)}$	$148072\pm10269^{(a)}$	$3.51\pm0.40^{(a)}$
RHC 80267 (100 μM) alone	30855 ± 2309	74130 ± 3754	44882 ± 2500	1.15 ± 0.30

Fig. 1. Effect of RHC 80267 on the time course of DAG accumulation and acrosomal exocytosis induced by Ca^{2+} and A23187 in ram spermatozoa

(a) Spermatozoa were treated with 3 mM-Ca^{2+} and 1 μM-A23187 for various times in the absence (O) or presence (●) of 10 μM-RHC 80267. Lipids were extracted with chloroform/methanol, separated by t.l.c., and DAG mass was quantified by Coomassie Blue staining and densitometry (see the Materials and methods section). Results are means $\pm$ s.e.m. from two separate experiments. (b) Spermatozoa were treated with similar concentrations of Ca^{2+} and A23187 in the presence (●) or absence (O) of 10 μM-RHC 80267. As controls, parallel samples were incubated in the absence of ionophore with (▣) or without (◆) 100 μM-RHC 80267. At various times, subsamples were analysed for the occurrence of the acrosome reaction. Results are means $\pm$ s.e.m. from four separate experiments.
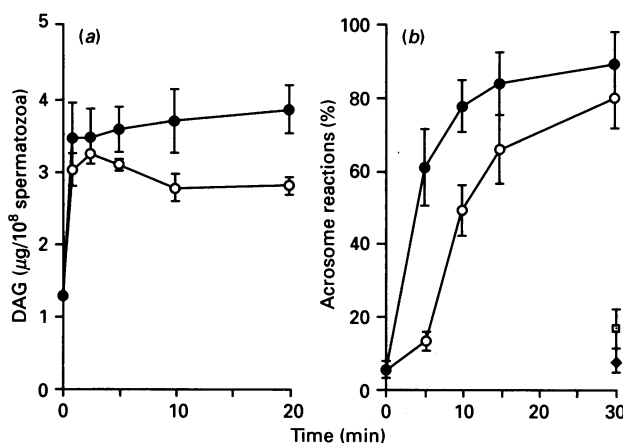
Fig. 2. Effect of R59022 on the time course of DAG accumulation and acrosomal exocytosis induced by Ca^{2+} and A23187 in ram spermatozoa

(a) Spermatozoa were treated with 3 mM-Ca^{2+} and 1 μM-A23187 for various times in the absence (O) or presence (●) of 100 μM-R59022. Lipids were extracted with chloroform/methanol, separated by t.l.c., and DAG mass was quantified by Coomassie Blue staining and densitometry (see the Materials and methods section). Results are means $\pm$ s.e.m. from three separate experiments. (b) Spermatozoa were treated with Ca^{2+} and A23187 in the absence (O) or presence (●) of 100 μM-R59022. As controls, parallel samples were incubated in the absence of ionophore with (▣) or without (◆) 100 μM-R59022. Sub-samples were analysed at various times for the occurrence of the acrosome reaction. Results are means $\pm$ s.e.m. from four separate experiments.

The other pathway through which DAG can be catabolized is that involving DAG kinase, which phosphorylates DAG to PtdOH. We have previously found that in ram spermatozoa the DAG kinase inhibitor R59022 (de Chaffoy de Courcelles et al., 1985) stimulates the accumulation of DAG in a dose-dependent fashion, and that this is accompanied by a parallel decrease in PtdOH (Roldan & Harrison, 1990b). To test the possible importance of this pathway in events leading to exocytosis of the acrosome, spermatozoa were treated with Ca^{2+}/A23187 in the absence or presence of R59022. As shown in Fig. 2(a), treatment

of spermatozoa with Ca^{2+}/A23187 and 100 μM-R59022 resulted in DAG values higher than those corresponding to cells treated with Ca^{2+}/A23187 alone (2-factor ANOVA: treatment, $F = 4.49$, $P = 0.03$; time, $F = 7.51$, $P = 0.0001$). Although such a concentration of R59022 may seem high in comparison with that used in other cell systems, it did not affect cell integrity, nor did it induce DAG formation in the absence of ionophore treatment (results not shown). Parallel sperm samples treated with Ca^{2+}/A23187 and 100 μM-R59022 showed a considerable stimulation of acrosomal exocytosis (2-factor ANOVA: treatment,
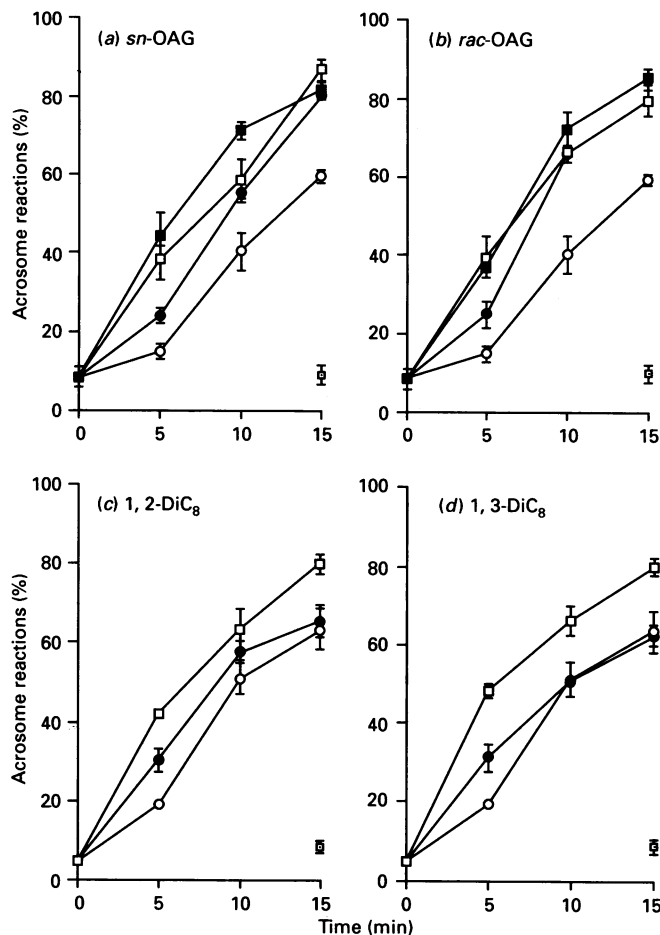
Fig. 3. Effect of exogenous DAGs on the $Ca^{2+}/A23187$-induced acrosomal exocytosis of ram spermatozoa

Spermatozoa were preincubated for 15 min with various concentrations of the indicated DAG and then treated (at 'zero time') with $3\ mM$-Ca^{2+} and $1\ \mu M$-A23187. Control samples were incubated without addition of ionophore. At intervals, sub-samples were analysed for the occurrence of the acrosome reaction. Results are means ± S.E.M. from three separate experiments. Key: (a), (b); $\boxdot$, Ca^{2+}/OAG (10 μM); $\bigcirc$, $Ca^{2+}/A23187$; $\bullet$, $+0.01\ \mu M$-OAG; $\square$, $+0.1\ \mu M$-OAG; $\bullet$, $+1\ \mu M$-OAG; (c), (d): $\boxdot$, Ca^{2+}/DiC_8 (25 μM); $\bigcirc$, $Ca^{2+}/A23187$; $\bullet$, $+10\ \mu M$-DiC$_8$; $\square$, $+25\ \mu M$-DiC$_8$.

$F = 9.09$, $P = 0.01$; time, $F = 4.34$, $P = 0.03$; Fig. 2b). Thus blockade of PtdOH generation with concomitant DAG accumulation increased rather than diminished ionophore-induced exocytosis, suggesting again a direct role for DAG in this process.

Effect of exogenous DAGs

We have already shown that fatty acid chain length seems to be without effect on the ability of exogenous DAGs to stimulate exocytosis in spermatozoa (Roldan & Harrison, 1990b). In order to test whether there was any stereospecificity in the DAG effect, various isomers were examined for their ability to stimulate acrosomal exocytosis. The purity of the DAG isomers tested was confirmed by t.l.c. (cf. Rando & Young, 1984).

Both sn-1-oleoyl-2-acetylglycerol (sn-OAG) and rac-1-oleoyl-2-acetylglycerol (rac-OAG) stimulated acrosomal exocytosis. (2-factor ANOVA: $Ca^{2+}/A23187$ versus +sn-OAG, $F = 28.83$, $P = 0.0001$; time, $F = 91.83$, $P = 0.0001$; $Ca^{2+}/A23187$ versus

+rac-OAG, $F = 54.38$, $P = 0.0001$; time, $F = 208.43$, $P = 0.0001$; Figs. 3a and 3b). Since the racemic mixture contains about 50 % of sn-2,3-enantiomer (i.e. sn-2-acetyl-3-oleoyl-glycerol), a compound which is incapable of activating protein kinase C (Rando & Young, 1984; Nomura et al., 1986), a 50 % decrease in the stimulatory ability of the rac-OAG should have been observed if DAG effects on acrosomal exocytosis were stereospecific. However, sn-OAG and rac-OAG stimulated exocytosis to the same extent (e.g. 2-factor ANOVA: $+0.1\ \mu M$-sn-OAG versus $+0.1\ \mu M$-rac-OAG, $F = 0.009$, N.S.; time, $F = 55.35$, $P = 0.0001$; Figs. 3a and 3b). Likewise, both sn-1,2- and sn-1,3-dioctanoylglycerol (DiC$_8$) were able to stimulate acrosomal exocytosis (2-factor ANOVA: $Ca^{2+}/A23187$ versus +sn-1,2-DiC$_8$, $F = 5.43$, $P = 0.01$; time, $F = 32.5$, $P = 0.0001$; $Ca^{2+}/A23187$ versus +sn-1,3-DiC$_8$, $F = 5.2$, $P = 0.02$; time, $F = 16.53$, $P = 0.0002$; Figs. 3c and 3d), but both isomers stimulated exocytosis to the same extent (e.g. 2-factor ANOVA: $+25\ \mu M$-sn-1,2-DiC$_8$ versus $+25\ \mu M$-sn-1,3-DiC$_8$, $F = 0.36$, N.S.; time, $F = 15.85$, $P = 0.004$; Figs. 3c and 3d), a similarly significant finding because 1,3-DAGs are also incapable of activating protein kinase C (Boni & Rando, 1985; Nomura et al., 1986). The lack of stereospecificity in the DAG effect argues against a role of protein kinase C in acrosomal exocytosis, in agreement with our previous conclusion (Roldan & Harrison, 1988).

## DAG overcomes the neomycin-induced inhibition of acrosomal exocytosis

Neomycin, an aminoglycoside antibiotic that binds to PPIs (Schacht, 1978) and inhibits PPI metabolism (Cockcroft & Gomperts, 1985; Whitaker & Aitchison, 1985; Carney et al., 1985), has been found to prevent the breakdown of PPIs and the rise in PtdOH induced by $Ca^{2+}/A23187$ treatment in spermatozoa of several mammalian species (Roldan & Harrison, 1989). The exocytosis of the sperm acrosome is greatly delayed under these conditions. Experiments were carried out to see if DAG production was similarly affected by such treatment and whether exogenous DAG could overcome the inhibition exerted by neomycin on acrosomal exocytosis. As shown in Table 2, neomycin caused a considerable decrease in the accumulation of DAG in spermatozoa treated with $Ca^{2+}/A23187$, concomitant with the decreased PPI breakdown and PtdOH accumulation. Fig. 4 shows that spermatozoa that were treated with $Ca^{2+}/A23187$ and neomycin also experienced a considerable inhibition of the acrosome reaction as compared with cells treated with $Ca^{2+}/A23187$ alone (2-factor ANOVA: treatment, $F = 97.42$, $P = 0.0001$; time, $F = 29.12$, $P = 0.001$). However, if sn-1,2-DOG [a DAG that is able to stimulate the $Ca^{2+}/$ionophore-induced exocytosis in spermatozoa (2-factor ANOVA: treatment, $F = 9.02$, $P = 0.007$; time, $F = 46.94$, $P = 0.0001$; Fig. 4)] was also included, the inhibitory effect of neomycin was overcome (2-factor ANOVA: $Ca^{2+}/A23187$ versus $Ca^{2+}/A23187$+neomycin+DOG, $F = 0.69$, N.S.; time, $F = 45.91$, $P = 0.0001$; $Ca^{2+}/A23187$+neomycin versus $Ca^{2+}/A23187$+neomycin+DOG, $F = 154.2$, $P = 0.0001$; time, $F = 27.95$, $P = 0.0001$). Similar results were obtained if sn-OAG was used instead of 1,2-DOG (results not shown). As exogenous DAG did not stimulate exocytosis in the absence of ionophore treatment (Fig. 4), these results suggest that DAG is acting downstream from neomycin. They also suggest that neomycin does not act by blocking pathways other than those involving the PPIs, because cells treated with neomycin and DAG were still able to exocytose the acrosome.

Because of its highly charged nature, the ability of neomycin to enter intact cells has been questioned, and it has been argued that neomycin might exert its effects by restricting Ca^{2+} entry

Table 2. Changes in PPIs, PtdOH and DAG after treatment of ram spermatozoa with Ca²⁺/A23187 in the absence or presence of neomycin

Spermatozoa were labelled for 60 min with 125 μCi of [^{32}P]P$_i$/ml and treated with Ca^{2+} (3 mM) and A23187 (1 μM) for 5 min in the absence or presence of 10 mM-neomycin. Lipids were extracted with chloroform/methanol/conc. HCl, separated by t.l.c., and radioactivity in each spot was counted. DAG mass was quantified by Coomassie Blue staining and densitometry with sn-1,2-DOG as standard (for details see the Materials and methods section). Results are means±S.E.M. from two separate experiments; [a]different from control ($P < 0.0001$); [b]different from Ca^{2+}/A23187 ($P < 0.0001$).

Treatment	Lipids 5 min after treatment			
	[^{32}P]PtdIns(4,5)P_2 (c.p.m./10^8 spermatozoa)	[^{32}P]PtdIns4P (c.p.m./10^8 spermatozoa)	[^{32}P]PtdOH (c.p.m./10^8 spermatozoa)	DAG (μg/10^8 spermatozoa)
None (control)	7239±203	23966±359	13260±278	1.12±0.08
Ca^{2+}/A23187	2337±107[a]	5968±185[a]	53042±530[a]	3.19±0.15[a]
Ca^{2+}/A23187+neomycin (10 mM)	3155±129[b]	10302±237[b]	32729±425[b]	1.59±0.06[b]
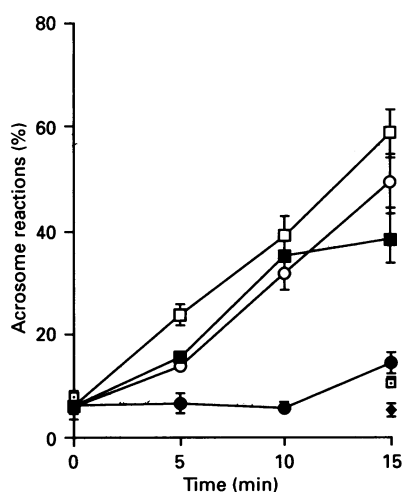
Fig. 4. Effect of neomycin and a DAG on the Ca²⁺/A23187-induced acrosomal exocytosis of ram spermatozoa

Spermatozoa were preincubated for 15 min in the absence or the presence of 10 mM-neomycin, or 100 μM-sn-1,2-DOG, or both, and then treated (at 'zero time') with 3 mM-Ca^{2+} and 1 μM-A23187; control samples received no ionophore. At intervals, sub-samples were analysed for the occurrence of the acrosome reaction. Results are means±S.E.M. from four separate experiments. Key: ◆, control (Ca^{2+} alone); ◙, Ca^{2+}/DOG (100 μM); ○, Ca^{2+}/A23187; ●, +neomycin (10 mM); □, +DOG; ■, +neomycin+DOG.

(Griffin et al., 1980) or by increasing the Ca^{2+} requirement for exocytosis (Whitaker, 1987). If this were the case in our sperm system, cells treated with exogenous DAG would not have been able to overcome the inhibitory effect of neomycin, because extracellular Ca^{2+} is needed for several steps during exocytosis in spermatozoa (Roldan & Harrison, 1989).

DISCUSSION

Exocytosis in many cells encompasses the generation of an array of messengers, and involves several enzymic processes and metabolites. Moreover, cells may exocytose more than one type of secretory granule, and different sets of processes underlying each secretory event may be triggered simultaneously in response to particular agonists. In such cells, the overlapping generation of messengers and metabolites that takes place after cell stimulation confounds the investigation of the molecular basis of signal transduction. From this viewpoint, spermatozoa are very useful models for studying the molecular mechanisms of exo-

cytosis because, being highly differentiated cells, they have a number of distinctive attributes and lack processes common to other cells. For instance, exocytosis relies entirely on the presence of extracellular Ca^{2+} (reviewed by Roldan & Harrison, 1990a), and it is only after Ca^{2+} entry that phosphoinositidase C (PIC)-mediated hydrolysis of PPIs takes place in both invertebrate (Domino & Garbers, 1988) and mammalian spermatozoa (Roldan & Harrison, 1989; Thomas & Meizel, 1989). Our earlier studies (Roldan & Harrison, 1989) have indicated that PPI breakdown is an essential early event in the exocytotic process in spermatozoa, and we have therefore attempted subsequently to establish the reason for this.

Of the two products generated from PPI breakdown, our previous studies have suggested that there is no apparent messenger role for Ins(1,4,5)P_3 (Harrison et al., 1990), especially since there are no obvious Ca^{2+} stores in these cells (see discussion in Harrison & Roldan, 1990). We have recently demonstrated that the other product generated during PPI hydrolysis, i.e. DAG, accumulates in spermatozoa in parallel to Ins(1,4,5)P_3 and appears to be a key metabolite (Roldan & Harrison, 1990b). However, DAG's role is not yet clear, because protein kinase C, the target of DAG action in most cells, is not detectable in spermatozoa (Roldan & Harrison, 1988). The results of our studies using inhibitors of DAG catabolism presented above reinforce the importance of DAG and strongly suggest that it is DAG itself, rather than a derived metabolite, that is the key messenger generated by PPI hydrolysis in spermatozoa.

Spermatozoa stimulated with Ca^{2+} and A23187 experience a rapid and considerable accumulation of DAG concomitant with the breakdown of both PtdIns(4,5)P_2 and PtdIns4P. However, the hydrolysis of the PPIs is insufficient to account for all the DAG generated (Roldan & Harrison, 1990b). Work by others has shown that in various cell types the DAG generated after stimulation may originate from several sources (Pelech & Vance, 1989; Exton, 1990). For example, cell stimulation may result in parallel activation of both a PIC and a phosphatidylcholine-specific phospholipase C, the latter producing a greater and prolonged accumulation of DAGs with a composition different from the PPI-derived DAG (Rodriguez de Turco & Spitzer, 1988; Pessin & Raben, 1989). In addition, the PPI-derived DAG may exert a positive feedback effect by stimulating PLC to generate additional DAG from several phosphoglycerides (Exton, 1990). At present, it is not known whether such interaction between phospholipid pools exists in spermatozoa, though a PLC has been described in bull spermatozoa (Sheikh-nejad & Srivastava, 1986). It should also be noted that DAGs are by no means the only diradylglycerides generated upon cell activation (see Rider et al., 1988), and an unusually high

proportion of choline and ethanolamine phosphoglycerides present in spermatozoa of various mammalian species have side chains consisting mainly of alkyl or alkenyl groups (Neill & Masters, 1973; Selivonchick et al., 1980; Nikopoulou et al., 1985). It would therefore be of great interest to study the molecular species of DAG generated after sperm stimulation to determine the origin and nature of such a considerable mass of DAG.

Treatment with inhibitors of both DAG kinase and DAG lipase led to increased DAG levels in stimulated spermatozoa. However, comparison of the effects of the two inhibitors revealed considerable differences. Whereas treatment with the DAG kinase inhibitor R59022 caused a dose-dependent rise in DAG at all concentrations of the inhibitor tested (Roldan & Harrison, 1990b), treatment with the DAG lipase inhibitor RHC 80267 resulted in a biphasic effect (the present study). Concentrations of RHC 80267 above 10 μM showed lower stimulatory effects on the amount of DAG accumulated. Since some of the DAG might be the product of PLC activity, inhibition of this PLC by high doses of RHC 80267 (see Sutherland & Amin, 1982; Chang et al., 1988) could account for the lower values of DAG mass, and thus explain the biphasic effect of the reagent.

Surprisingly, when spermatozoa were stimulated with Ca^{2+}/A23187 and RHC 80267, the resulting rise in DAG did not result in any increase in PtdOH labelling, despite the apparently greater availability of substrate for a DAG kinase. It would therefore appear that the DAG arising from inhibition of DAG lipase is unavailable to the kinase. Obvious possible explanations could be that lipase activity is located in a different cellular compartment from the kinase activity, or that the kinase is saturated at low levels of DAG. On the other hand, the bulk of the DAG may arise from PLC action (see above) and may either be generated in a different compartment or constitute a poor substrate for the kinase; recent studies have shown that DAG kinase isoenzymes vary in their 'preference' for DAG substrates (Lemaitre et al., 1990).

It is also interesting that RHC 80267 and R59022 differed in their abilities to stimulate ionophore-induced exocytosis. Although both compounds raised endogenous DAG levels above those observed after Ca^{2+}/A23187 treatment, the enhancement of exocytosis seemed to be inversely related to the degree of DAG accumulation provoked by each of the two inhibitors. No clear explanation can be offered to account for these effects, but it is possible that they result from differences in location, source or type of DAG generated, as discussed above.

Taken together, our findings suggest that it is DAG, and not a derived metabolite, that is the important molecule in events leading to exocytosis in spermatozoa. This is indicated by the following observations: (a) increases in endogenous DAG take place during cell stimulation leading to exocytosis, and inhibitors of DAG catabolism enhance exocytosis at the same time as causing enhanced accumulation of DAG; (b) addition of exogenous DAGs also enhances exocytosis in stimulated cells; (c) a decrease in endogenous DAG generation, resulting from inhibition of PPI hydrolysis with neomycin, is accompanied by an inhibition of exocytosis which can be overcome by addition of exogenous DAG; (d) since inhibition of DAG kinase results in a decrease in PtdOH production (cf. Roldan & Harrison, 1990b) but is accompanied by an enhancement of exocytosis, a role for PtdOH seems unlikely; (e) since inhibition of DAG lipase does not inhibit exocytosis, but actually enhances it slightly, it is unlikely that DAG is serving as a source for the generation of MAG or the release of arachidonic or other fatty acids.

At first glance, the absence of a role for MAG seems to be in conflict with results presented by Fleming & Yanagimachi (1981), who found a stimulatory effect of MAG on exocytosis in guinea-

pig spermatozoa. In fact, addition of MAG also stimulates exocytosis in ram spermatozoa (E. R. S. Roldan, unpublished work); however, high concentrations are needed, and they show an effect regardless of whether or not the cells are simultaneously treated with Ca^{2+}/A23187, thus suggesting an unspecific action. In any case, added MAG that is incorporated into the cell can always be acylated to DAG by a MAG acyltransferase (Bishop & Bell, 1988) and may thereby set in train DAG-mediated events.

Evidence for a role for arachidonic acid in acrosomal exocytosis has also been presented by others (Meizel & Turner, 1983; Fleming & Yanagimachi, 1984). Although our results imply that arachidonic acid derived from DAG is not important for this process, other sources of this metabolite clearly exist in spermatozoa, from which it may be released to play a later role. In many cells, arachidonic acid is released from phosphoglycerides by the action of a phospholipase A_2, and a considerable amount of circumstantial evidence has been presented to suggest that products derived from phospholipase A_2 action may have fundamental roles in sperm exocytosis (reviewed in Yanagimachi, 1988; Harrison & Roldan, 1990).

In conclusion, it is clear that the role and site of action of DAG during exocytosis of the sperm acrosome remain to be elucidated. However, our findings presented above allow us to conclude that, after Ca^{2+} entry, PPI breakdown mainly serves to generate DAG, which exerts a messenger action by stimulating additional cellular processes leading to membrane fusion. Although fusogenic properties have been recognized for DAG (Das & Rand, 1984; Siegel et al., 1989), we have argued that such a role is unlikely in sperm acrosomal exocytosis (Roldan & Harrison, 1990a; Harrison & Roldan, 1990). The lack of stereospecificity of DAG stimulation of exocytosis described above supports the idea that DAG is not acting to stimulate protein kinase C (Roldan & Harrison, 1988). Instead, we suspect that, as reported for other cellular systems (Kramer et al., 1987; Bauldry et al., 1988), DAG messenger action in spermatozoa may be related to the activation of phospholipase A_2.

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