Diradylglycerols stimulate phospholipase A_2 and subsequent exocytosis in ram spermatozoa

Evidence that the effect is not mediated via protein kinase C

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We tested the hypothesis that the role of diacylglycerol (DAG) in sperm acrosomal exocytosis is related to the activation of phospholipase A_2, and that this effect is not mediated via protein kinase C. Treatment of [^{14}C]arachidonic acid-labelled ram spermatozoa with Ca^{2+} and the ionophore A23187 stimulated both liberation of arachidonic acid and acrosomal exocytosis. No changes in [^{14}C]DAG or [^{14}C]monoacylglycerol were found after stimulation of spermatozoa, thus suggesting that arachidonic acid may be released exclusively via phospholipase A_2. An increase in the endogenous levels of diradylglycerols (DRGs), resulting from exposure either to the DAG kinase inhibitor R 59022 or to exogenous 1-oleoyl-2-acetyl-*sn*-glycerol or 1,2-dioctanoyl-*sn*-glycerol, led to an increase in both phospholipase A_2 activity and exocytosis when cells were stimulated with A23187 and Ca^{2+}. Addition of DRGs that do not stimulate protein kinase C (1,3-dioctanoylglycerol, 1-*O*-hexadecyl-2-acetyl-*rac*-glycerol) also resulted in an increase in phospholipase A_2 activity and exocytosis. On the other hand, phorbol esters (phorbol 12,13-dibutyrate; phorbol 12-myristate 13-acetate) did not enhance enzyme activity or exocytosis. Finally, exposure to 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol, a compound known to inhibit protein kinase C, did not affect phospholipase A_2 activity or acrosomal exocytosis. We therefore conclude that in spermatozoa the messenger role of DAG is related to the activation of phospholipase A_2, which in turn would generate an array of metabolites directly or indirectly involved in bringing about exocytosis of the acrosome.

INTRODUCTION

Exocytosis of the sperm acrosome (the so-called 'acrosome reaction') is an essential step in fertilization because it exposes or releases enzymes that allow the sperm cell to penetrate the oocyte vestments. The process also primes the sperm head plasma membrane for fusion with the oocyte itself. As a result of recent studies, a series of events underlying signal transduction during exocytosis of the acrosome has been unravelled (for reviews see [1,2]), but little is still known with regard to mechanisms regulating these events.

An early and very rapid event in the sequence activated after stimulation of mammalian and invertebrate spermatozoa (both with cation ionophores and with natural ligands) is the hydrolysis of the polyphosphoinositides [3–5] to generate diacylglycerol (DAG) [6,7]. DAG messenger action in most cells is related to the activation of protein kinase C [8]. In spermatozoa, however, the role of DAG does not appear to be related to the activation of this kinase [4,6,9–11], or to the generation of other active metabolites such as arachidonic acid or phosphatidic acid [7]. The role of DAG during acrosomal exocytosis is as yet unknown, but it has been suggested that it may be related to the activation of phospholipase A_2 and perhaps also the inhibition of lysophosphatide acyltransferase [1,2,6,7].

The interaction between phospholipase A_2 and the acyltransferase regulates the levels of free arachidonic acid (or other fatty acids) and lysophospholipids in intact cells, with the former hydrolysing membrane phospholipids, and the latter reacylating the resulting lyso-compounds to regenerate the phospholipid pools [12,13]. In both somatic and germ cells, activation of phospholipase A_2 (or the net effect resulting from activation of this enzyme and inhibition of the acyltransferase) may play a significant role in exocytosis, because arachidonic or other fatty acids, and lysophospholipids, can either exert direct actions or be further metabolized to a host of molecules with important biological functions. Theories of membrane fusion have traditionally involved lysophospholipids as fusogens in the presence of Ca^{2+} [14,15], but recent evidence also indicates that unsaturated fatty acids can play a role in membrane fusion after annexin-mediated changes [16].

Earlier studies have presented circumstantial evidence favouring the idea that phospholipase A_2 could underlie events leading to membrane fusion during sperm acrosomal exocytosis [17–22]. More recent results have uncovered an association between arachidonic acid release (presumed to represent phospholipase A_2 activity) and exocytosis in spermatozoa [23,24].

The mechanisms regulating phospholipase A_2 activation during cell function are still a matter of debate [25–28]. The possibility that phospholipase A_2 activation may be receptor-regulated (independently from other pathways) has received recent support, although some authors still consider this a speculative idea [29]. On the other hand, the long-standing hypothesis that cytosolic phospholipase A_2 is regulated by a rise in intracellular Ca^{2+} has been strengthened ([29]; and references therein). Nevertheless, it has also been argued that a rise in cytosolic Ca^{2+} induced by physiological agonists may not be sufficient to activate

Abbreviations used: DAG, diacylglycerol; DRG, diradylglycerol ('radyl' refers to acyl, alkyl and alkenyl substituents); MAG, monoacylglycerol; MRG, monoradylglycerol; 1-$C_{18:1}$/2-C_2, 1-oleoyl-2-acetyl-*sn*-glycerol; 1-O-C_{16}/2-C_2, 1-*O*-hexadecyl-2-acetyl-*rac*-glycerol; 1-O-C_{16}/2-O-C_1, 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol; 1-monoC_{18}, 1-mono-oleoyl-*rac*-glycerol; 1-monoC_{16}, 1-monohexadecanoyl-*rac*-glycerol; 2-monoC_{16}, 2-monohexadecanoyl-glycerol; 1-O-monoC_{16}, 1-*O*-hexadecyl-*sn*-glycerol; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate.

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phospholipase A_2 [27]; other messengers such as DAG may be involved in direct or indirect activation of the enzyme. Evidence in favour of an indirect DAG (protein kinase C-mediated) stimulation of phospholipase A_2 has been presented [28,30], but a direct role of DAG in phospholipase A_2 stimulation has also received experimental support [31–35].

In spermatozoa, a rise in intracellular Ca^{2+} seems to be required for the activation of phospholipase A_2 ([23,36]; reviewed in [1]), but it is likely that additional messengers are involved in modulating this enzyme. We have obtained evidence suggesting that various DAGs stimulate sperm phospholipase A_2 in assays *in vitro* [11]; furthermore, various DAGs are capable of stimulating acrosomal exocytosis [6,7]. However, no studies have yet been undertaken to try to understand how is phospholipase A_2 actually regulated during exocytosis in spermatozoa.

The objective of the work presented here has been to test the hypothesis that diradylglycerols (DRGs) may regulate the activation of phospholipase A_2 in the sequence leading to sperm acrosomal exocytosis, and to explore whether this effect is mediated via protein kinase C. We report that an increase in intracellular DRG levels, brought about either by inhibition of DRG catabolism or by addition of exogenous DRGs, resulted in an increase in phospholipase A_2 activity, and a parallel rise in the proportion of cells undergoing exocytosis, when spermatozoa were stimulated with the ionophore A23187 and Ca^{2+}. The fact that DRGs that do (1,2-DAG) and do not (1,3-DAG, alkyl-acylglycerol) activate protein kinase C stimulated both phospholipase A_2 activity and exocytosis to similar extents allows us to conclude that these effects are unlikely to be mediated via protein kinase C.

MATERIALS AND METHODS

Reagents

[1-^{14}C]Arachidonic acid (toluene solution; sp. radioactivity 54 mCi/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.). Ionophore A23187 was purchased from Calbiochem (Nottingham, U.K.). Poly(vinyl alcohol) (type II), poly(ethylene glycol) compound, EGTA and EDTA were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). The compound R 59022 was obtained from Janssen Pharmaceuticals (Wantage, Oxon, U.K.) and dissolved as described [37]. Hepes was from BDH (Poole, Dorset, U.K.). 1-Oleoyl-2-acetyl-*sn*-glycerol (1-$C_{18:1}$/2-C_2), 1,2-dioctanoyl-*sn*-glycerol, 1,3-dioctanoylglycerol, 1-*O*-hexadecyl-2-acetyl-*rac*-glycerol (1-O-C_{16}/2C_2), 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol (1-O-C_{16}/2-O-C_1), 1-monohexadecanoyl-*rac*-glycerol (1-monoC_{16}), 2-monohexadecanoylglycerol (2-monoC_{16}), 1-mono-oleoyl-*rac*-glycerol (2-monoC_{18}) and 1-*O*-hexadecyl-*sn*-glycerol (1-O-monoC_{16}) were purchased from Sigma. Lipids used as standards (arachidonic acid, 1,2-dioleoyl-*sn*-glycerol, and 1,3-dioleoylglycerol) were also from Sigma. Phorbol 12,13-dibutyrate (PDBu) and phorbol 12-myristate 13-acetate (PMA) were from Calbiochem. Organic solvents were from BDH or Fisons (Loughborough, Leics., U.K.). All other chemicals were of reagent grade and were purchased from BDH or Fluka (Buchs, Switzerland).

Preparation, labelling and treatment of spermatozoa

The standard saline incubation medium used throughout consisted of 142 mM NaCl, 2.5 mM KOH, 10 mM glucose and 20 mM Hepes, adjusted to pH 7.5 at 20 °C with NaOH [9]; 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 poly(ethylene glycol)/ml, and had an osmolality of 305 mosmol/kg.

Ejaculated spermatozoa from Clun Forest or Suffolk rams were separated from seminal plasma by dilution and washing through sucrose medium as described [38]. Labelling was carried out by incubating washed spermatozoa (about 1×10^8/ml) in about 5 ml of saline medium containing 0.3 or 0.5 μCi of [^{14}C]arachidonic acid/ml for 60 min at 37 °C. Before stimulation, spermatozoa were washed through sucrose medium (400 $g_{max.}$ for 5 min and 1000 $g_{max.}$ for 10 min) and resuspended in the saline medium.

Exocytosis of the sperm acrosome was induced by treating cells with Ca^{2+} (3 mM) and the bivalent-cation ionophore A23187 (1 μM) in saline medium at 37 °C, and was monitored by phase-contrast microscopy of glutaraldehyde-fixed samples [39].

The effects of the DAG kinase inhibitor R 59022 [6,7] were examined by adding the compound to the sperm suspensions together with the ionophore, whereas the effects of di- and mono-radylglycerols, or phorbol esters, were examined by adding the compounds to the sperm suspensions either 10 min before A23187 or at the same time as the ionophore (see the Results section for details).

Lipid analyses

At various intervals after the beginning of treatments, incubations were stopped by the addition of chloroform/methanol (1:2, v/v) and lipids were then extracted as described [40]. Neutral lipids were separated by t.l.c. on silica-gel-60-coated glass plates (0.25 mm thickness) (E. Merck, Darmstadt, Germany) by using the solvent n-hexane/diethyl ether/acetic acid (70:30:1, by vol.), and detected by staining in an iodine tank. Lipid spots were identified by comparison with arachidonic acid, 1,2-dioleoyl-*sn*-glycerol and 1,3-dioleoylglycerol standards run on the same plate, scraped off, and the radioactivity in each was determined by liquid-scintillation counting.

For the quantification of DAG changes, lipids were separated in the solvent toluene/diethyl ether/ethanol/conc. NH_3 (250:200:10:1, by vol.) [6,7,41]. DAG was quantified by Coomassie Blue staining [42] and densitometry, by using 1,2-dioleoylglycerol to construct standard curves for each plate as previously described [6,7,41]. 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.).

Statistics

Results are means ± S.E.M. Significance of results was examined by using the Mann–Whitney *U* test or analysis of variance (ANOVA). Regression analyses were done after data transformation [arcsin $\sqrt{(x/100)}$ for percentages of acrosome reactions, and $\log_{10}$ for all other variables]. Values of $P < 0.05$ were regarded as statistically significant.

RESULTS

Generation of arachidonic acid and exocytosis after cell stimulation with A23187/Ca^{2+}

Treatment of labelled ram spermatozoa with Ca^{2+} (3 mM) and the ionophore A23187 (1 μM) resulted in a considerable time-dependent increase in arachidonic acid release (Figure 1a); in the

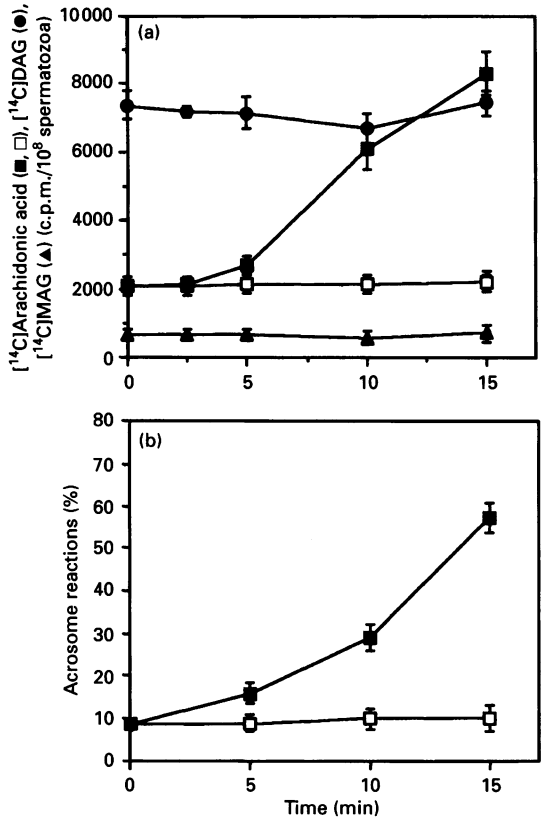

Figure 1 Changes in [^{14}C]arachidonic acid, [^{14}C]DAG and [^{14}C]MAG, and exocytosis in ram spermatozoa treated with Ca^{2+} and A23187

Spermatozoa in saline medium were labelled with 0.5 μCi of [^{14}C]arachidonic acid/ml for 60 min at 37 °C, washed and resuspended in saline medium. (**a**) Cells were exposed to Ca^{2+} (3 mM) alone (□) or to both Ca^{2+} and A23187 (1 μM) (■, ●, ▲) for different times. At the end of treatment, arachidonic acid (■), DAG (●) and MAG (▲) were resolved and quantified as described in the Materials and methods section. Results (means ± S.E.M.) are averages of duplicate assays carried out on three occasions. (**b**) Parallel unlabelled samples were exposed to Ca^{2+} alone (□) or to Ca^{2+}/A23187 (■), and spermatozoa were fixed after different times and examined by phase-contrast microscopy. Means (± S.E.M.) of three experiments are shown.

absence of ionophore no increase in arachidonic acid levels was observed. It is unlikely that the increase in arachidonic acid observed is due to hydrolysis of diacylglycerol by DAG and monoacylglycerol (MAG) lipases, because levels of [^{14}C]DAG and [^{14}C]MAG did not change during this period of time (Figure 1a). Conversely, breakdown of phosphatidylcholine and phosphatidylserine has been found to parallel the increase in arachidonic acid levels (results not shown) (cf. [24]), therefore indicating that generation of arachidonic acid is due to phospholipase A_2 activity.

Treatment of spermatozoa with Ca^{2+} and A23187 caused a time-dependent increase in the number of cells undergoing exocytosis ('acrosome reactions') (Figure 1b). Such changes were not observed if cells were not exposed to the ionophore.

Inhibition of DAG catabolism enhances phospholipase A_2 activity and exocytosis

In order to understand if DAG affects phospholipase A_2 activity and exocytosis, the endogenous levels of DAG were raised by inhibiting its catabolism via DAG kinase; this was done by

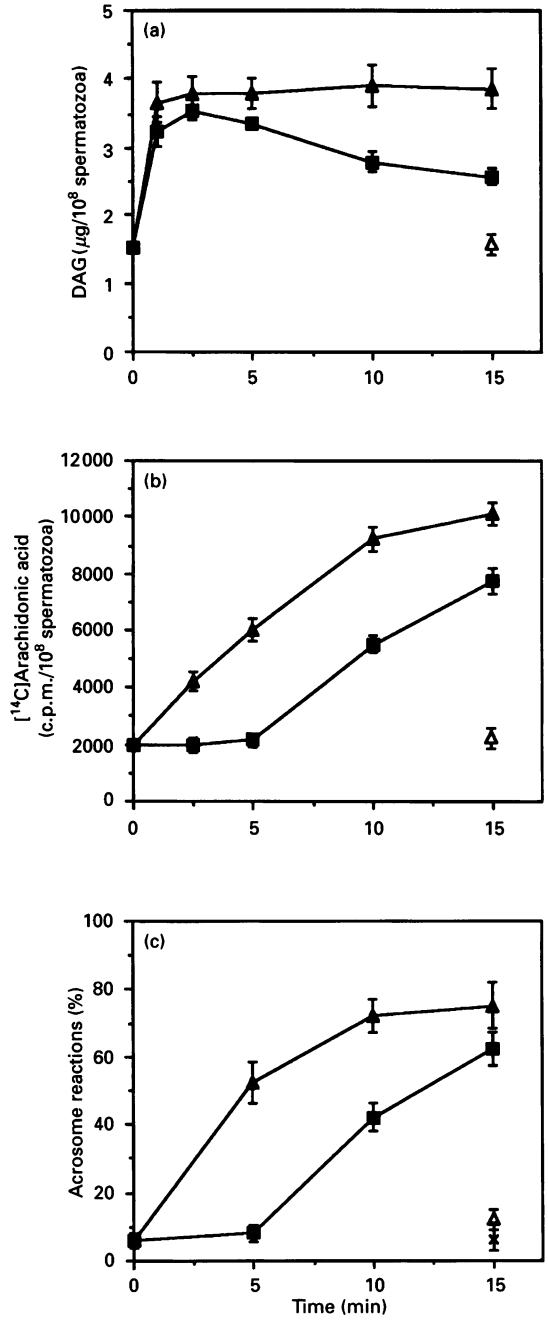

Figure 2 Effect of R 59022 on DAG mass accumulation, arachidonic acid release and acrosomal exocytosis in ram spermatozoa

(**a**) Spermatozoa in saline were treated with Ca^{2+} (3 mM) and A23187 (1 μM) for various times in the absence (■) or presence (▲) of 100 μM R 59022. Parallel control sperm samples were incubated in the absence of ionophore and with 100 μM R 59022 (△). Lipids were extracted with chloroform/methanol (1:2, v/v), 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 ± S.E.M. from three experiments. (**b**) Spermatozoa in saline were labelled with 0.5 μCi of [^{14}C]arachidonic acid/ml for 60 min at 37 °C, washed and resuspended in saline medium. Spermatozoa were treated with Ca^{2+} and A23187 in the absence (■) or presence (▲) of 100 μM R59022, or they were incubated with 100 μM R 59022 alone, in the absence of ionophore (△), for various times. Lipids were extracted and resolved as described in the Materials and methods section. Means (± S.E.M.) of duplicate assays from four experiments are shown. (**c**) Spermatozoa in saline were treated with Ca^{2+}/A23187 in the absence (■) or presence (▲) of 100 μM R 59022. Sub-samples were analysed at various times for the occurrence of the acrosome reaction. Results are means ± S.E.M. from three separate experiments. As controls, parallel samples were incubated in the absence of ionophore either with (△) or without (×) 100 μM R 59022.

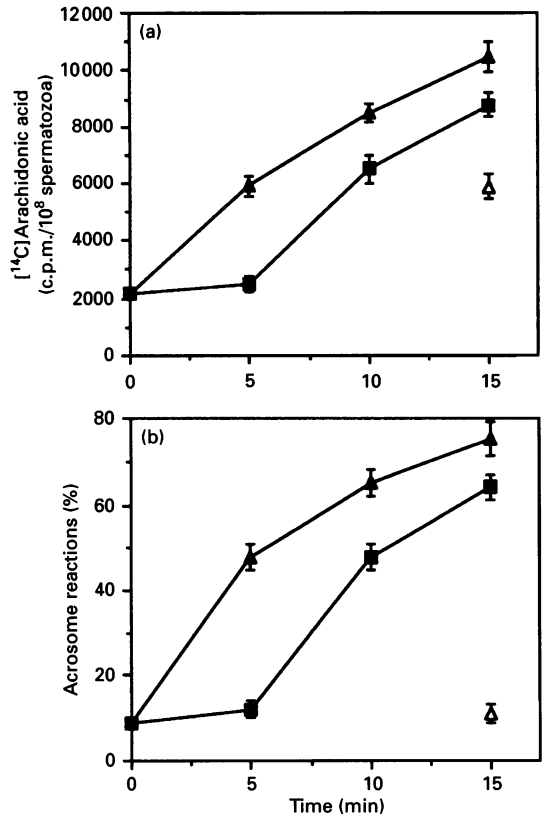

Figure 3 Effect of on 1-$C_{18:1}$/2-C_2 on [^{14}C]arachidonic acid release and exocytosis in ram spermatozoa treated with Ca^{2+}/A23187

Spermatozoa were labelled with 0.5 μCi of [^{14}C]arachidonic acid/ml for 60 min at 37 °C, washed and resuspended in saline medium. (**a**) Labelled cells were exposed to 1 μM A23187 and 3 mM Ca^{2+} (■) or to A23187/Ca^{2+} plus 10 μM 1-$C_{18:1}$/2-C_2 (▲) for various times, and lipids were then extracted and separated by t.l.c. Results (means ± S.E.M.) of duplicate assays from three experiments are shown. (**b**) Parallel unlabelled samples were exposed to A23187/Ca^{2+} (■) or to A23187/Ca^{2+} plus 10 μM 1-$C_{18:1}$/2-C_2 (▲) for various time intervals, and sub-samples were analysed for the occurrence of acrosomal exocytosis. As controls, sperm samples were incubated only with Ca^{2+} and 1-$C_{18:1}$/2-C_2 (△). Means (± S.E.M.) of three experiments are shown.

treating spermatozoa with R 59022 [37], a compound known to increase endogenous levels of DAG in ram spermatozoa [6,7]. The concentration of R 59022 used in this study was previously shown to result in maximal DAG accumulation without any adverse effects on cell viability or integrity.

Spermatozoa treated with R 59022 and A23187/Ca^{2+} showed a time-dependent increase in the mass of DAG that was significantly higher than that seen in spermatozoa exposed only to A23187/Ca^{2+} (2-factor ANOVA; treatment, $F = 4.42$; $P = 0.03$; time, $F = 8.31$, $P = 0.0001$) (Figure 2a). In labelled spermatozoa, treatment with A23187/Ca^{2+} and R 59022 resulted in levels of arachidonic acid that were significantly higher than those seen when cells were stimulated with A23187/Ca^{2+} alone (2-factor ANOVA; treatment, $F = 6.41$, $P = 0.03$; time, $F = 5.62$, $P = 0.02$) (Figure 2b). Spermatozoa in Ca^{2+}-containing saline medium treated with R 59022 alone (i.e. not exposed to A23187) showed levels of DAG and arachidonic acid that were not different from untreated controls (Figures 2a and 2b).

The levels of DAG mass seen after 5 min or 10 min of treatment with A23187/Ca^{2+} and R 59022 were respectively 1.1-fold and 1.3-fold higher than those seen in their A23187/Ca^{2+}-treated counterparts. On the other hand, the increases in arachidonic acid at similar time points were 3-fold (5 min) and 2-fold (10 min) higher when cells were treated with A23187/Ca^{2+} and R 59022 than when spermatozoa were challenged with A23187/Ca^{2+} in the absence of R 59022. Hence, a small rise in DAG appears to be translated into a larger increase in arachidonic acid levels, which supports the idea that DAG is not the source of arachidonic acid, but rather that it is stimulating the release of the fatty acid via a different mechanism.

Parallel unlabelled sperm samples were exposed to A23187/Ca^{2+} in the absence or presence of R 59022, and sub-samples were examined for the occurrence of exocytosis; addition of R 59022 along with the ionophore resulted in an enhancement of acrosomal exocytosis (2-factor ANOVA: treatment, $F = 8.91$, $P = 0.01$; time, $F = 4.51$, $P = 0.03$) (Figure 2c). On the other hand, cells exposed to R 59022 in the absence of A23187 maintained an intact acrosome. Regression analyses comparing arachidonic acid levels and the percentages of cells undergoing acrosomal exocytosis (data transformed as indicated in the Materials and methods section) showed a highly significant relationship between the two variables when cells were exposed to A23187/Ca^{2+} in the absence of R 59022 ($r^2 = 0.91$, $P = 0.001$) and also when cells were exposed to ionophore and Ca^{2+} in the presence of this compound ($r^2 = 0.97$, $P = 0.0003$), thus indicating that higher levels of arachidonic acid resulted in a higher proportion of spermatozoa undergoing exocytosis (and see below).

Addition of exogenous DAG enhances phospholipase A_2 activity and exocytosis

The effects of DAG on the release of arachidonic acid (i.e. phospholipase A_2 activity; see above) were also examined by adding exogenous 1-$C_{18:1}$/2-C_2. When spermatozoa were labelled, washed and resuspended in saline medium and exposed to A23187/Ca^{2+} along with 1-$C_{18:1}$/2-C_2, an increase in the amount of arachidonic acid was observed compared with that seen in cells only exposed to Ca^{2+} and the ionophore (2-factor ANOVA: treatment, $F = 18.4$, $P = 0.01$; time, $F = 21.5$, $P = 0.001$) (Figure 3a). Similarly, spermatozoa treated with A23187/Ca^{2+} and 1-$C_{18:1}$/2-C_2 underwent acrosomal exocytosis much more rapidly than cells treated with A23187 and Ca^{2+} (2-factor ANOVA: treatment, $F = 26.74$, $P = 0.001$; time, $F = 84.31$, $P = 0.001$) (Figure 3b). Regression analysis showed that there was a very significant relationship between the levels of arachidonic acid and exocytosis seen after cells were treated with Ca^{2+}/ionophore and the DAG ($r^2 = 0.96$; $P = 0.0006$). Labelled spermatozoa incubated in Ca^{2+}-containing saline medium and exposed only to 1-$C_{18:1}$/2-C_2 showed levels of arachidonic acid that were higher than those found in untreated controls (zero time). However, in parallel samples similarly treated, acrosomal exocytosis did not occur when cells were exposed only to 1-$C_{18:1}$/2-C_2. These results suggest that the sole addition of exogenous DAG may either partially activate phospholipase A_2 or inhibit lysophosphatide acyltransferase (resulting in a net rise in arachidonic acid), but that this is not enough to activate/inhibit these enzymes fully and complete exocytosis.

Specificity of the DAG effect on phospholipase A_2 activity and exocytosis

The ability of 1,2-DAG to stimulate enzymes such as protein kinase C is specific: neither 1,3-DAG nor alkyl-acyl-glycerol would activate this enzyme [8,43–48]. Therefore, in order to test whether there was any specificity in the capacity of DAG to

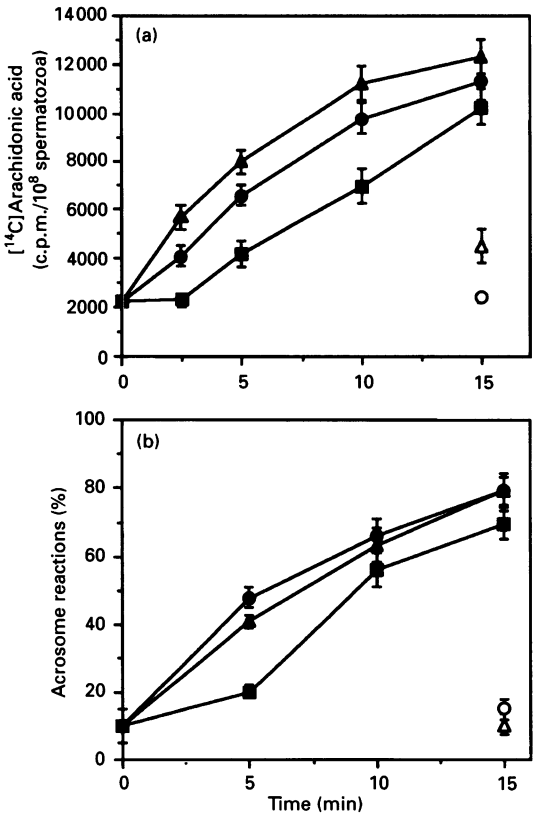

Figure 4 Effect of 1,2-DAG and 1,3-DAG on [^{14}C]arachidonic acid release and exocytosis in ram spermatozoa treated with Ca^{2+}/A23187

(**a**) Spermatozoa were labelled with 0.5 μCi of [^{14}C]arachidonic acid/ml for 60 min at 37 °C, washed and resuspended in Ca^{2+}-containing saline medium. Labelled cells were preincubated for 10 min in the absence (■) or the presence of 25 μM 1,2-dioctanoyl-*sn*-glycerol (▲) or 25 μM 1,3-dioctanoylglycerol (●), before stimulation with 1 μM A23187 for various times. Spermatozoa exposed only to Ca^{2+} and 25 μM 1,2-dioctanoyl-*sn*-glycerol (△), or to Ca^{2+} and 25 μM 1,3-dioctanoylglycerol (○), served as controls. After incubating spermatozoa for different times, lipids were extracted and separated by t.l.c., and radioactivity in each was determined by liquid-scintillation counting. Results (means ± S.E.M.) of duplicate assays from three experiments are shown. (**b**) Parallel unlabelled samples were similarly treated, and at various time intervals sub-samples were analysed for the occurrence of acrosomal exocytosis. Means (± S.E.M.) of three experiments are shown.

Table 1 Effect of DRGs and phorbol esters on arachidonic acid release and acrosomal exocytosis

Ram spermatozoa were labelled with 0.3 μCi of [^{14}C]arachidonic acid/ml for 60 min at 37 °C, washed and resuspended in saline medium containing 3 mM Ca^{2+}. Labelled cells were incubated in the absence or the presence of the indicated compounds for 10 min and were then exposed to either 1 μM A23187 or dimethyl sulphoxide. After 10 min, incubations were stopped and lipids were extracted and resolved by t.l.c.; radioactivity in each lipid spot was quantified by liquid-scintillation counting. Parallel unlabelled samples were similarly treated, and incubations were stopped after 15 min and examined for the occurrence of acrosomal exocytosis. Results are means (± S.E.M.) of duplicate assays carried out on four occasions (for arachidonic acid release) or three different experiments (acrosome reactions): *different from control ($P < 0.01$); †different from A23187/Ca^{2+} ($P < 0.05$).

Treatment	[^{14}C]Arachidonic acid released after 10 min (c.p.m./10^8 spermatozoa)	Acrosome reactions after 15 min (%)
Control	457 ± 54	9 ± 1
A23187/Ca^{2+}	1675 ± 111*	51 ± 2*
+ 1-$C_{18:1}$/2-C_2 (25 μM)	4311 ± 422*†	67 ± 3*†
+ 1-O-C_{16}/2-C_2 (25 μM)	2551 ± 293*†	73 ± 4*†
+ PDBu (1 μM)	1977 ± 570*	52 ± 2*
+ PMA (0.2 μM)	1726 ± 323*	55 ± 3*
+ 1-O-C_{16}/2-O-C_1 (25 μM)	1945 ± 340*	56 ± 2*
1-$C_{18:1}$/2-C_2 (25 μM) alone	3327 ± 658*	8 ± 1
1-O-C_{16}/2-C_2 (25 μM) alone	1587 ± 360*	8 ± 2
PDBu (1 μM) alone	365 ± 57	8 ± 2
PMA (0.2 μM) alone	475 ± 78	10 ± 3
1-O-C_{16}/2-O-C_1 (25 μM) alone	1295 ± 262*	9 ± 2

enhance arachidonic acid release and the ensuing exocytosis (and thus to infer whether the DAG action was mediated via protein kinase C), we compared the effects of 1,2- and 1,3-DAG isomers. We predicted that if the effect was not mediated via protein kinase C both isomers would stimulate arachidonic acid release and acrosomal exocytosis to the same extent. As shown in Figure 4(a), preincubation of spermatozoa with either 1,2- or 1,3-dioctanoylglycerol enhanced the levels of arachidonic acid released when cells were stimulated with A23187/Ca^{2+} (ANOVA: +1,2-DAG, $F = 5.14$, $P = 0.02$; +1,3-DAG, $F = 4.21$, $P = 0.03$). Addition of either the 1,2- or the 1,3-DAG isomer also resulted in an increase in the number of cells undergoing exocytosis (ANOVA: +1,2-DAG, $F = 5.62$, $P = 0.01$; +1,3-DAG, $F = 5.2$, $P = 0.02$) (Figure 4b). Regression analyses, carried out after data were transformed as indicated in the Materials and methods section, showed significant relationships between the levels of arachidonic acid measured after treatment of spermatozoa with Ca^{2+}/ionophore plus each isomer and the percentages of cells undergoing acrosomal exocytosis after similar treatments (+1,2-DAG, $r^2 = 0.95$, $P = 0.008$; +1,3-DAG, $r^2 = 0.91$, $P = 0.005$). Taken together, these results argue against the idea that the DAG effect is mediated via protein kinase C.

The specificity of the DRG effect was examined further by exposing sperm cells to an alkyl-acyl-glycerol before treatment with A23187/Ca^{2+}. Spermatozoa labelled, washed and preincubated in Ca^{2+}-containing saline medium with 1-O-C_{16}/2-C_2 for 10 min showed higher levels of arachidonic acid when challenged with A23187 than did sperm cells that were not exposed to 1-O-C_{16}/2-C_2 (Table 1). The rise in arachidonic acid levels was, however, not as high as that seen in cells preincubated with 1-$C_{18:1}$/2-C_2. Spermatozoa exposed only to 1-O-C_{16}/2-C_2 also showed an increase in the levels of arachidonic acid, but, although these levels were higher than those found in controls, they were lower than those found in cells also challenged with A23187. Acrosomal exocytosis was also enhanced in cells preincubated in 1-O-C_{16}/2-C_2 before ionophore treatment (Table 1), but exposure to this metabolite without ionophore challenge did not result in exocytosis. These results also support the idea that PLA_2 activation, and the ensuing exocytosis, are not mediated via protein kinase C.

To examine further the possible involvement of protein kinase C in these phenomena, we exposed labelled spermatozoa to phorbol esters (PDBu or PMA) before ionophore treatment. None of the phorbol esters enhanced arachidonic acid release, nor exocytosis, above the levels seen when spermatozoa were exposed to A23187 and Ca^{2+} alone (Table 1).

Finally, we also examined the effects of a metabolite structurally related to the DRGs (1-O-C_{16}/2-O-C_1), which has been previously found to inhibit protein kinase C [44,49–51]. Preincubation of spermatozoa with 1-O-C_{16}/2-O-C_1 before Ca^{2+}/ionophore treatment did not enhance arachidonic acid levels above those seen when cells were treated with A23187/Ca^{2+} alone and, more importantly, did not decrease them either (Table

Table 2 Effect of MRGs on arachidonic acid release and acrosomal exocytosis

Ram spermatozoa were labelled with 0.3 μCi of [^{14}C]arachidonic acid/ml for 60 min at 37 °C, washed and resuspended in saline medium containing 3 mM Ca^{2+}. Labelled cells were incubated in the absence or the presence of MRGs for 10 min and then were exposed to either 1 μM A23187 or dimethyl sulphoxide. After 10 min, incubations were stopped and lipids were extracted and resolved by t.l.c.; radioactivity in each lipid spot was quantified by liquid-scintillation counting. Parallel unlabelled samples were similarly treated, and incubations were stopped after 15 min and examined for the occurrence of acrosomal exocytosis. Results are means (±S.E.M.) of duplicate assays carried out on four occasions (for arachidonic acid release) or five different experiments (acrosome reactions): *different from control ($P < 0.01$); †different from A23187/Ca^{2+} ($P < 0.05$).

Treatment	[^{14}C]Arachidonic acid released after 10 min (c.p.m./10^8 spermatozoa)	Acrosome reactions after 15 min (%)
Control	457±54	9±1
A23187/Ca^{2+}	1675±111*	55±3*
+1-monoC_{18} (25 μM)	6508±215*†	76±3*†
+1-monoC_{16} (25 μM)	4220±228*†	71±3*†
+2-monoC_{16} (25 μM)	4608±122*†	70±4*†
+1-O-monoC_{16} (25 μM)	2595±243*†	67±4*†
1-monoC_{18} (25 μM) alone	3944±880*	12±3
1-monoC_{16} (25 μM) alone	4226±643*	9±3
2-monoC_{16} (25 μM) alone	3605±752*	8±1
1-O-monoC_{16} (25 μM) alone	1043±236*	9±1

1). In parallel treated samples, addition of 1-O-C_{16}/2-O-C_1 did not enhance or decrease the number of cells undergoing acrosomal exocytosis when sperm cells were treated with A23187/Ca^{2+}. Exposure of cells to 1-O-C_{16}/2-O-C_1 alone did not affect their acrosome integrity (Table 1) or viability (results not shown). Interestingly, incubation of spermatozoa with this metabolite alone resulted in some increase in the levels of arachidonic acid; exocytosis, however, was not observed under these conditions.

Effect of monoradylglycerols (MRGs) on phospholipase A_2 activity and exocytosis

Spermatozoa labelled, washed and resuspended in saline with Ca^{2+} (3 mM) were exposed to monoacyl- or monoalkyl-glycerols for 10 min and then treated with 1 μM-A23187. Exposure to the various MRGs before treatment with A23187 significantly enhanced the release of arachidonic acid above the levels seen when cells were treated with ionophore without prior exposure to these metabolites (Table 2). Both 1-monoC_{16} and 2-monoC_{16} enhanced arachidonic acid release to the same extent. A longer acyl chain in position 1 appeared to confer greater activity, because 1-monoC_{18} was more effective at enhancing arachidonic acid release than 1-monoC_{16}. The monoalkylglycerol 1-O-monoC_{16}, on the other hand, was less effective than the monoacylglycerols. Exposure to the various MRGs (without A23187 stimulation) enhanced the accumulation of arachidonic acid, but usually to a lesser extent than that seen when cells were exposed to the MRGs and A23187.

Spermatozoa preincubated with the various MRGs and then treated with ionophore showed higher percentages of cells undergoing exocytosis. Exposure to MRGs alone, in the presence of Ca^{2+}, did not induce exocytosis.

Regression analysis showed that there was a significant positive relationship between the levels of arachidonic acid generated after treatment with A23187/Ca^{2+} in the absence or the presence of the various MRGs, and the percentages of cells undergoing exocytosis after similar treatments ($r^2 = 0.94$; $P = 0.005$).

DISCUSSION

The results of the present study constitute the first direct evidence in support of the hypothesis that DRGs generated after sperm activation exert their messenger role through stimulation of phospholipase A_2 and thus lead to exocytosis. The DRGs' effect, however, does not appear to be mediated by protein kinase C.

Phospholipase A_2 involvement in sperm function has attracted considerable attention because of its potential role in the generation of metabolites (fatty acids and lysophospholipids) that would directly or indirectly promote membrane fusion during exocytosis. However, despite various studies dealing with the characterization and partial purification of this enzyme (e.g. [11,19,52–55]) there is only one previous study [23] in which a metabolite generated through phospholipase A_2 activity (i.e. arachidonic acid) has actually been quantified after sperm stimulation (even though the sources of the fatty acid were not unequivocally established). That work also constituted the first attempt to establish whether arachidonic acid release was linked with acrosomal exocytosis. Other studies designed to uncover an association between phospholipase A_2 and exocytosis have contributed only circumstantial evidence [17,18,20–22]. Thus it should be pointed out that the results presented here indicate clearly that arachidonic acid release occurs mainly, if not exclusively, through activation of phospholipase A_2 (rather than via the sequential deacylation of DAG). The accumulation of [^{14}C]arachidonic acid was paralleled by a decrease in labelled choline- and serine-containing glycerophospholipids, in agreement with previous preliminary observations [24], whereas no decrease in either [^{14}C]DAG or [^{14}C]MAG took place in parallel to the rise in [^{14}C]arachidonic acid after stimulation with Ca^{2+} and A23187. In addition, the results of this study represent very strong support in favour of the idea that phospholipase A_2 activation is closely (and significantly) related to acrosomal exocytosis.

There is still controversy regarding the mechanisms that modulate phospholipase A_2 after cell activation. Studies in various cellular systems have revealed that activation of the enzyme may occur as a result of either receptor-mediated activation of G-proteins (independent of the inositol-lipid pathway) or a rise in intracellular Ca^{2+} [25–29]. Nonetheless, DRGs seem capable of affecting phospholipase A_2 activity either directly [31–35] or through the activation of protein kinase C [28,30]. In spermatozoa, Ca^{2+} entry may lead to phospholipase A_2 activation, because enzyme activity measured *in vitro* in sperm homogenates requires Ca^{2+} [11,20,52–54], and because maximal phospholipase A_2 activation in labelled cells is only seen after treatment with the ionophore A23187 and millimolar extracellular Ca^{2+} [23,24]. However, it is worth noting that Ca^{2+} entry into mammalian spermatozoa also leads to the rapid activation of phosphoinositidase C [3,4], and perhaps also phospholipases C [23] and D [10], and the generation of DRGs [7]. It is therefore possible that the activation of sperm phospholipase A_2 observed after Ca^{2+} entry is in fact triggered by a metabolite generated through the activation of an upstream event. It is also possible (and perhaps more likely) that sperm phospholipase A_2 activation is brought about as a result of both a Ca^{2+} rise and targeting by a messenger. We think that DAG may display this messenger role in events underlying acrosomal exocytosis [1,2]. The work which has led to this proposal has been summarized elsewhere [7].

The hypothesis that DAG plays a central role in events leading

to membrane fusion during exocytosis via activation of phospholipase A_2 [1,2] has allowed us to make the following predictions: first, that enhancement of endogenous DAG through inhibition of its catabolism should stimulate phospholipase A_2 and exocytosis; second, that addition of exogenous DAGs should also enhance phospholipase A_2 activity and exocytosis; third, that only DAGs should enhance phospholipase A_2 activity and exocytosis (i.e. related compounds such as MRGs should have no effect). Predictions were also made to test whether DAG action is mediated via protein kinase C. If the latter is true, then reagents known to stimulate the kinase should enhance phospholipase A_2 activity and exocytosis, whereas those that do not stimulate protein kinase C should not have such an effect. Moreover, protein kinase C inhibitors should block phospholipase A_2 activity and exocytosis. Evidence obtained when testing these predictions is discussed below.

An increment in the endogenous levels of diacylglycerols, resulting from the exposure of spermatozoa to the DAG kinase inhibitor R 59022 when cells were stimulated with A23187 and Ca^{2+} [6,7], led to an increase in phospholipase A_2 activity, and this was paralleled by an enhancement in the number of cells undergoing exocytosis; a significant relationship was demonstrated between the two phenomena. Likewise, exposure of spermatozoa to exogenous DAGs (either 1-oleoyl-2-acetylglycerol or 1,2-dioctanoylglycerol) resulted in an enhancement of phospholipase A_2 activity when sperm cells were stimulated with A23187/Ca^{2+}. A significant relationship was again found between rises in arachidonic acid and exocytosis. These results are therefore consistent with the first two predictions.

To test whether the DAG effect was mediated via protein kinase C, we compared the effects of DAGs that activate this enzyme (e.g. 1,2-DAGs) with DRGs that cannot (1,3-DAG or alkyl-acyl-glycerol) [8,43–48]. We found that both 1,2- and 1,3-DAG added exogenously enhanced the levels of arachidonic acid and exocytosis upon stimulation of spermatozoa with A23187 and Ca^{2+} and, similarly, that both DAG and alkyl-acyl-glycerol were able to enhance enzyme activity and the ensuing acrosomal exocytosis. This agrees with the finding that stimulation of ram sperm phospholipase A_2 activity by DAGs in assays *in vitro* lacked specificity [11] and, moreover, that DRGs containing either a fatty acid or a fatty alcohol in position 1 of the glycerol backbone were capable of enhancing enzyme activity *in vitro* (E. R. S. Roldan, unpublished work). The fact that both 1,2- and 1,3-DAG, and both DAG and alkyl-acyl-glycerol, enhanced arachidonic acid release and exocytosis in Ca^{2+}/ionophore-stimulated spermatozoa indicates very clearly that the effect is not mediated via protein kinase C. That both 1,2- and 1,3-DAGs can enhance phospholipase A_2 activity in assays *in vitro* has also been recorded in other cellular systems [31,56]. Moreover, pretreatment with either DAG or alkyl-acyl-glycerol has also been found to result in an enhanced phospholipase A_2 activity when human neutrophils were stimulated with either A23187 or *N*-formyl-Met-Leu-Phe [35,57].

To test further whether protein kinase C is involved in phospholipase A_2 activation, we examined the effects of two phorbol esters, compounds which are extensively used to stimulate kinase C activity in many cells [8]. We found that addition of either PMA or PDBu did not induce or enhance liberation of arachidonic acid, or exocytosis, when added alone or at the same time as ionophore and Ca^{2+}. Lastly, we studied the effects of 1-O-C_{16}/2-O-C_1, a compound which has been found to inhibit protein kinase C [44,49–51]. Exposure of spermatozoa to this compound before and during A23187/Ca^{2+} stimulation did not inhibit phospholipase A_2 activity or exocytosis. We therefore conclude from this evidence that protein kinase C is not mediating the effect of DRGs on phospholipase A_2.

It is not clear how DRGs may stimulate phospholipase A_2 activity if protein kinase C is not involved. It has been suggested that DRGs may translocate phospholipase A_2 from the cytosol to the membrane [58] in a manner similar to the effect of DAG on protein kinase C [8,28], or that DRGs may perturb the structure of the cell membranes so that phospholipase A_2 substrate(s) may become available to the enzyme. At present, we do not have any indication as to how the DRGs enhance phospholipase A_2 activity in spermatozoa, and further studies are under way to clarify this point.

It is also possible that, in addition to phospholipase A_2, DRGs may also affect enzymes involved in the metabolism of the products of phospholipase A_2 activity (e.g. lipo- or cyclo-oxygenase on fatty acids, or acyltransferases on lysophospholipids). This could explain the observation that some DRGs stimulated higher rises in arachidonic acid levels than did others, regardless of whether they were added alone or with A23187/Ca^{2+}. For example, exposure to 1-oleoyl-2-acetylglycerol resulted in a higher release of arachidonic acid than did treatment with a similar alkyl-containing DRG (1-*O*-hexadecyl-2-acetyl-glycerol). In human neutrophils, both DAGs and alkyl-acyl-glycerols stimulated phospholipase A_2, but they varied in their ability to affect other enzymes such as 5-lipoxygenase and acetyltransferase, and this was reflected in the net amount of arachidonic acid quantified after cell stimulation [35,57]. It is therefore possible that, in spermatozoa, 1,2-DAGs (which induced a higher accumulation of arachidonic acid) are also inhibiting an acyltransferase, thus preventing reacylation of arachidonic acid into lysophospholipids (cf. [59]). Further support for these ideas comes from experiments in which it was found that sperm phospholipase A_2 activity measured in assays *in vitro* was stimulated to the same extent by both 1,2- and 1,3-DAGs [11] and by DAGs, alkyl-acyl- and di-alkyl-glycerols of similar structure (E. R. S. Roldan, unpublished work). This indicates that the direct effects of the DRGs on phospholipase A_2 itself may not vary greatly. Finally, the experiments in which spermatozoa were exposed to DRGs alone (i.e. not challenged with A23187) also revealed diverse effects on 'basal' arachidonic acid levels: 1,2-DAGs enhanced 'basal' levels of arachidonic acid considerably, whereas 1,3-DAG and alkyl-acyl-glycerol did so to a much lesser extent. This again could be explained by a differential role of DRGs on enzymes such as acyltransferase: increases in arachidonic acid in unstimulated cells could simply occur by prevention of reacylation [12,60].

Contrary to one of our predictions, incubation of labelled spermatozoa with MRGs before treatment with A23187/Ca^{2+} led to an enhancement of arachidonic acid levels and acrosomal exocytosis. However, since MRGs are incapable of directly enhancing phospholipase A_2 activity in assays *in vitro* (E. R. S. Roldan, unpublished work), it is tempting to speculate that the stimulation of arachidonic acid release and exocytosis by MRGs does not represent a direct effect. The MRGs could be acting either through an inhibition of DAG kinase, as reported previously [61], or after being converted into DRGs by the action of a MAG acyltransferase [62]. Both explanations are consistent with the finding that spermatozoa treated with A23187/Ca^{2+} and either the DAG kinase inhibitor R 59022 or exogenous DRGs showed an enhancement of arachidonic acid levels and acrosomal exocytosis. The second hypothesis (i.e. acylation of MRGs to DRGs) seems at first glance more plausible, because of the effects observed when spermatozoa where incubated with MRGs alone (i.e. not challenged with A23187). In this latter case, levels of arachidonic acid after exposure to MRGs were higher than those

seen in untreated spermatozoa, resembling the results found when sperm cells were exposed to DRGs alone, but not those found when spermatozoa were exposed only to the DAG kinase inhibitor. The problem with this explanation is that in most cells MAG acyltransferase only uses *sn*-2-monoacylglycerol as a substrate [63–66], although it should be mentioned that a MAG acyltransferase that can also use *sn*-1-monoacylglycerol or monoalkylglycerol has been discovered in intestinal cells [67,68]. Thus the clarification of the mechanism by which MRGs enhance arachidonic acid release and acrosomal exocytosis will require further investigations involving, in particular, studies on the specificity of the sperm MAG acyltransferase.

In conclusion, this study has shown that DRG activation of Ca^{2+}-dependent phospholipase A_2 may play a central role in the sequence leading to exocytosis. The activation of this enzyme results in the generation of lysophospholipids and fatty acids that can directly affect other cellular processes, or that can serve as substrate for further metabolism to bioactive molecules. Although lysophospholipids have traditionally been considered as important fusogens in hypotheses concerning acrosomal exocytosis, the role of fatty acids and derived metabolites has received less attention. We anticipate that new insight into this problem would allow us to improve our understanding of events leading to membrane fusion and to explore whether the DRGs modulate any of these events.

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