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DIACYLGLYCEROL STIMULATES THE Ca^{2+}-DEPENDENT PHOSPHOLIPASE A_2 OF RAM SPERMATOZOA

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We have investigated phospholipase A_2 (PLA_2) activity in sonicates from ram spermatozoa and have analyzed the enzyme's susceptibility both to various inhibitors and to stimulation by diacylglycerols (DAGs). Ram sperm PLA_2 activity was Ca^{2+}-dependent and was inhibited by dexamethasone, chloracysine and compounds Ro 31-4493 and Ro 31-4639; mepacrine, however, did not inhibit PLA_2 activity. Addition of three different 1,2-DAGs (dioctanoyl-*sn*-glycerol, oleoyl-acetyl-*sn*-glycerol and dioleoyl-*sn*-glycerol) markedly increased PLA_2 activity; moreover, both 1,2- and 1,3-isomers enhanced enzyme activity. Since ram spermatozoa lack active protein kinase C, the target of DAG in most cells, our results suggest that stimulation of PLA_2 activity by DAG may play an important role in intracellular signalling in the sperm cell. © 1991 Academic Press, Inc.

Phospholipase A_2 (PLA_2) plays a fundamental role in several cellular processes by generating a variety of metabolites with different biological functions: PLA_2-mediated hydrolysis of phosphoglycerides results in the release of arachidonic acid and lysophospholipids. While arachidonic acid serves as substrate for the generation of eicosanoids (1), lysophospholipids may either be involved directly in membrane perturbation and membrane fusion (2) or act as substrates for the generation of platelet activating factor (3). Despite widespread interest in the biosynthesis of these metabolites, there is still controversy regarding the mechanisms involved in PLA_2 modulation. Both activation of G-proteins as well as a rise in intracellular Ca^{2+} have been reported to activate PLA_2 (4). Recently, diglycerides generated upon cell activation have also been invoked as stimulators of PLA_2 acting either directly or indirectly, via protein kinase C (5-10).

Work by others (e.g. 11,12) has recognized the potential role of PLA_2 in the exocytosis of the sperm acrosome, an essential process in the series of phenomena leading to fertilization. The mechanisms which modulate PLA_2 activity in the sperm cell are, nevertheless, virtually unknown. Recent work has shown that following Ca^{2+} entry (triggered either by treatment with Ca^{2+}/ionophore or natural ligands) there is a rapid breakdown of sperm polyphosphoinositides (13-15) and a concomitant generation of diacylglycerol (DAG)(16). It has been proposed that such DAG could modulate PLA_2 activity directly during this exocytotic process (17,18), especially since active protein kinase C, an important target of DAG action in other cells, is absent from spermatozoa (19).

Abbreviations: PLA_2, phospholipase A_2; DAG, diacylglycerol; PMSF, phenylmethyl sulfonyl fluoride; pAB, p-aminobenzamidine.

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MATERIALS AND METHODS

Chemicals and reagents. Snake venom phosphodiesterase 1, Histone type VII S and other chemicals were obtained from Sigma Chemical Company, St.Louis, MO. [α-^{32}P]adenylate labeled NAD (800 Ci/mmol= 29.6 TBq/mmol) was obtained from New England Nuclear, MA. [p-(Nitrobenzylidine)amino]guanidine (NBAG) was synthesized as described earlier (12). The culture media used were DMEM (Flow laboratories, Inc., McLean, VA) supplemented with 1 mM glutamine, 0.1 mM sodium pyruvate (Gibco, Grand Island, NY) or RPMI 1640. Cells were grown in the media supplemented with 10% heat inactivated (56°C, 30 minutes) bovine calf serum (BCS) (Cell culture laboratories, Cleveland OH) 100 U/ml penicillin and 100 µg/ml streptomycin.

Cell lines. The cell lines used in this study are from ATCC. The T cell hybridomas used were constructed in our laboratory by using standard fusion techniques using BW5147 line as the tumor partner (20). The T cell partner for the herpes simplex virus specific hybridoma G was obtained from Balb/c mice immunized with herpes simplex virus type 1 (mP strain) (21). Similarly the T cell partner for the vesicular stomatitis virus specific T cell hybridoma E15 was obtained from Balb/c mice immunized with vesicular stomatitis virus (Indiana strain) (22). The murine skeletal muscle derived mononuclear cell or myoblast line SP25 was derived in our laboratory from adult C57BL/6 mice by a modification of the method described by Koningsberg (23). T lymphocytes were purified from 5-8 weeks old Balb/c mouse splenocytes by passing through Nylon wool column. The nonadherent T cells were eluted using warm medium (37^0) and the adherent cells (B cells and macrophages) were eluted by pressing the column with ice cold PBS. The red cells were removed by centrifugation over Ficoll-hypaque gradient followed by ammonium chloride lysis.

Preparation of cell membranes and cell lysates. Membranes were prepared from cells in the exponential growth phase, by a procedure similar to that reported by Koski and Klee (24). Briefly, cells were collected by centrifugation at 900 x *g*, washed three times with PBS, resuspended in 10 mM Tris-HCl, 0.1mM EDTA (pH 7.4) buffer, and disrupted by one cycle of quick freezing and thawing, and then homogenized in glass homogenizer by using 25 strokes. The homogenate was centrifuged at 900 x *g* to remove cell nuclei and debris. The supernatant was centrifuged at 100,000 x *g* in a Beckman 70.1 Ti rotor in Beckman model L8-M ultracentrifuge. The residue was suspended in 10 mM Tris-0.1 mM EDTA (pH 7.4) buffer and centrifuged at 100,000 x *g* for one more time. The resulting pellet was suspended in 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.4), 10% sucrose at a concentration of 4-5 mg protein/ ml and quick frozen in liquid nitrogen and stored at -70°C.

Cell lysates for transferase assay were prepared from cells in the exponential growth phase. Cells were collected by centrifugation at 900 x *g* for 10 min, washed three times in PBS and lysed by incubating in 1 ml of 2% polyoxyethylene-9-lauryl ether in 10 mM Tris-HCl, 0.1mM EDTA (pH 7.4) per 2x10^7 cells for 1 h at 4 °C. The lysate is then centrifuged at 900 x *g* for 10 min and the supernatant was used for transferase assay.

ADP-ribosyltransferase assay. Guanidine group specific mono ADP-ribosyl transferase activity was measured using the synthetic guanylhydrazone, NBAG, as the substrate by the spectrophotometric method (19). Briefly, reaction systems were set up with 1 mM NBAG, 10 mM dithiothreitol, 5 mM NAD, 50 mM Tris-HCl (pH 7.4) and 50 µl of the cell lysate or 200 µg/ml of membrane proteins in a total volume of 100 µl. Control samples lacking either NAD or NBAG or enzyme source were also set up simultaneously. After 1-2 h incubation at 37°C, the reaction was terminated by adding 400 µl of a 1:1 slurry of Dowex 50W-X4 resin suspended in 10 mM phosphate (pH 7.4) and 1.6 ml of 10 mM phosphate (pH 7.4). Samples were mixed well, the resin was allowed to settle and 900 µl of supernatant mixed with 100 µl of 1 N NaOH, and the optical density at 375 nm was recorded. The optical density was converted to amount of product formed by determining the molar absorption coefficient of NBAG under identical conditions. The identity of the ADP-ribosylated product was confirmed by HPLC analysis of the reaction system as described by Soman *et al* (5) followed by measuring absorption characteristics of the product peaks. A Waters model 600E pump with a Waters model 464 detector and microsorb C18 column (4 mm x 15 cm) from Rainin Instrument Co., MA, were used for analysis.

Endogenous ADP-ribosylation of membrane proteins. Membranes (100-150 µg/ml) were incubated with 10 µM [^{32}P]NAD (4-5 Ci/mole) in the presence of the indicated ligands for 30 min at 37°C . At the end of reaction the proteins were precipitated with 10% ice cold trichloroacetic acid. Precipitated proteins were collected by centrifugation, washed once with 5% trichloroacetic acid followed by a wash in water saturated ice cold ether. The pellet was dissolved in SDS- PAGE sample buffer. SDS -PAGE was performed by the procedure of Laemmli (25). Gels were stained in Coomassie blue, destained, dried and exposed to Kodak XAR-5 film.

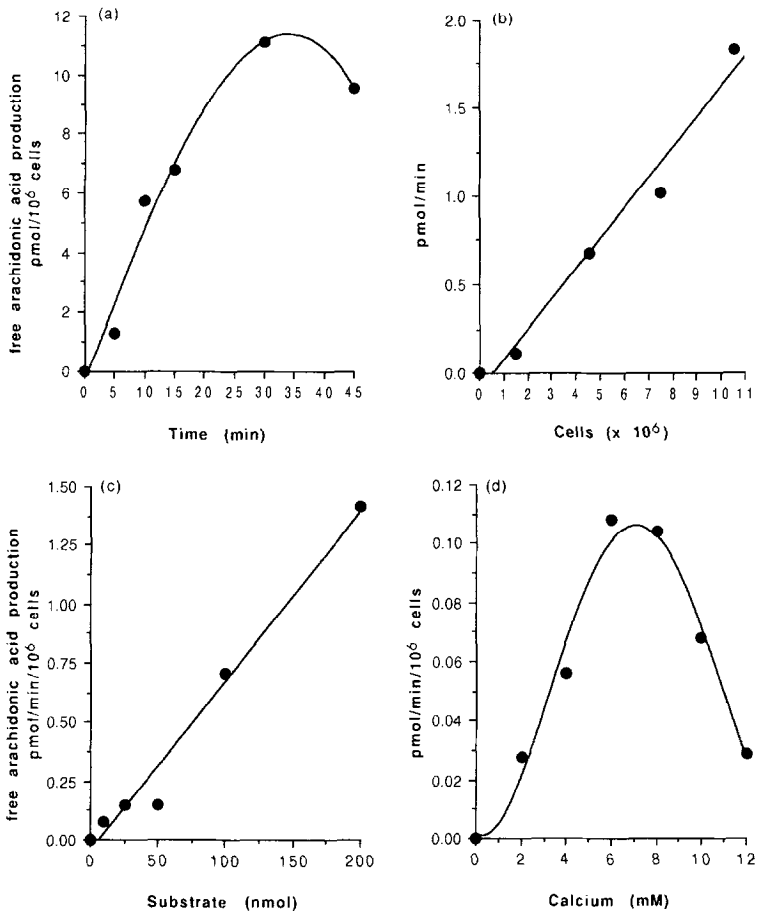

Fig. 1. Properties of PLA_2 activity in sonicates from ram spermatozoa. (a) Time course of enzyme action (3.5 x 10^6 cells, 6 mM Ca^{2+}). (b) Effect of cell concentration. (c) Effect of substrate concentration. (d) Effect of Ca^{2+} concentration. Unless otherwise stated, incubations were for 30 min at 37°C in a total volume of 250 µl (see Materials and methods for details). Results are averages of duplicate determinations of at least three separate experiments.

The effects of reagents known to inhibit PLA_2 activity were examined. As seen in Table 1, mepacrine did not inhibit ram sperm PLA_2; on the contrary, at 1 mM it appeared stimulatory. On the other hand, 1 mM dexamethasone totally abolished PLA_2 activity. Chloracysine, Ro 31-4493 and Ro-31-4639 showed a dose-dependent inhibition of PLA_2 activity.

Addition of various DAGs markedly increased PLA_2 activity (Fig. 2), especially in the cases of 1-oleoyl-2-acetyl-*sn*-glycerol and 1,2-dioleoyl-*sn*-glycerol (Fig. 2a,b). Concentrations of 25 µM 1-oleoyl-2-acetyl-*sn*-glycerol and 50 µM 1,2-dioleoyl-*sn*-glycerol caused maximal stimulation of PLA_2, with 5.5- and 7.7-fold increases in enzyme activity, respectively. That both 1,2- and 1,3-DAG isomers are capable of stimulating the enzyme is shown by the fact that 1,2- and 1-3-dioctanoyl-*sn*-glycerol enhanced PLA_2 activity (Fig. 2c,d).

Finally, when reagents previously shown to block PLA_2 activity, (i.e. dexamethasone, Ro-31-4493) were added together with DAGs, no PLA_2 activity was detected thus suggesting that DAGs cannot overcome the effect of the inhibitors (Table 2).

Table 1. Effect of inhibitors on the activity of ram sperm PLA_2

Reagent	Concentration	% Control
Control	-	100
Mepacrine	0.1 mM	95
	1.0 mM	152
Dexamethasone	1.0 mM	0
Ro 31-4493	12.5 μM	87
	25 μM	0
Ro 31-4639	25 μM	126
	50 μM	31
	100 μM	0
Chloracysine	0.5 mM	95
	1.0 mM	68
	2.0 mM	0

Reagents were added after the radiolabelled substrate was sonicated in the reaction mixture. Assays were carried out by incubating 8 μl of sperm sonicate (3.5×10^6 cells) for 30 min at 37°C as described in the Materials and methods section. Results are averages of duplicate assays carried out on at least two different occassions. Specific activity of control was 0.104 pmol/min/10^6 cells.

DISCUSSION

Ram sperm PLA_2 was capable of releasing, on average, about 0.102 pmol arachidonic acid/min/10^6 cells (about 0.73 nmol/h/mg protein). Since protein concentration cannot be measured reliably in a sperm homogenate, results are expressed in relation to number of cells per assay; activivity per mg protein is given only for gross comparative purposes. The activity of ram sperm PLA_2 is low in relation either to the enzyme activity measured in other cells or that found in spermatozoa from other mammalian species. A neutral neutrophil PLA_2, for example, has an activity of 42 nmol/h/mg protein when assayed on a similar phosphatidylcholine subtrate (23). On the other hand, sperm sonicates from different species showed PLA_2 activities of 6.0 (rabbit), 62.1 (mouse), and 805.4 (man) nmol/h/mg protein (24); interestingly, spermatozoa from bull, a species closely related to ram, were reported to have low PLA_2 activity (0.2 nmol/h/mg protein)(24).

Ram sperm PLA_2 activity was inhibited in a dose-dependent fashion by chloracysine and two alkylamines, a series of novel agents that block porcine pancreatic PLA_2 (25) as well as PLA_2 activity in human rheumatoid synovial cells (25), gastric mucosal sections (26), and human neutrophil cytoplasts (20). Two other inhibitors examined seem to have disparate effects on PLA_2 from spermatozoa of different species. Mepacrine, a classic although less specific PLA_2 inhibitor (27), was found to inhibit mouse (24,28), guinea pig (11), and golden hamster (12) sperm PLA_2 but showed no effect on PLA_2 from either man (24) or ram (this study) spermatozoa. Dexamethasone, on the other hand, which has been previously found to inhibit PLA_2 activity in other cells (29,30), totally abolished ram sperm enzyme activity (this study) while showing no effect whatsoever on mouse sperm enzyme (28). The reasons and significance of these differences remain obscure.

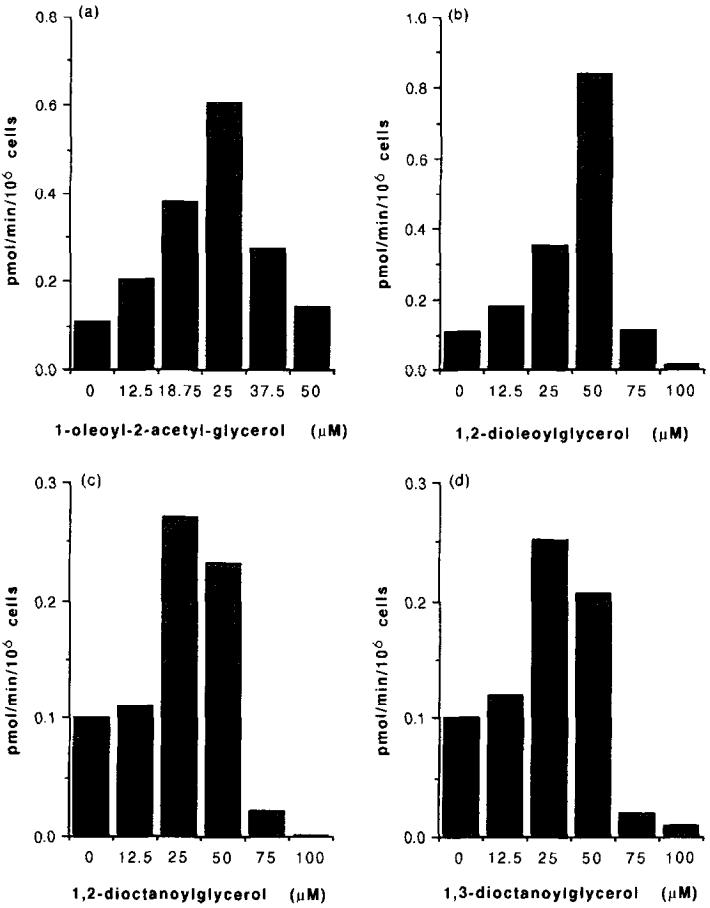

Fig. 2. Effect of diacylglycerols on the production of arachidonic acid by ram sperm PLA_2. Diacylglycerols were added to the reaction mixture and sonicated with the radiolabelled phosphatidylcholine substrate. Assays were carried out by incubating 5 μl of sperm sonicate (5 x 10^6 cells) for 30 min at 37°C in a total volume of 250 μl as described in the Materials and methods section. Results are averages of duplicate assays carried out on at least three different occassions. (a) 1-oleoyl-2-acetyl-*sn*-glycerol; (b) 1,2-dioleoyl-*sn*-glycerol; (c) 1,2-dioctanoyl-*sn*-glycerol; (d) 1,3-dioctanoyl-*sn*-glycerol.

Several DAGs were found to greatly enhance PLA_2 activity in a dose-dependent manner. There seemed to be a relation between the length of the acyl chain and the stimulatory ability of the DAGs: 1,2-dioctanoyl-*sn*-glycerol was the compound with the least stimulatory effect, 1-oleoyl-2-acetyl-*sn*-glycerol had an intermediate potency, and 1,2-dioleoyl-*sn*-glycerol showed the maximum effect. The ability of DAGs to stimulate enzyme activity appears to vary according to the type of PLA_2 assayed. For example, 1-oleoyl-2-acetyl-*sn*-glycerol is a poor stimulator of intestinal mucosa (5), and human platelet PLA_2 (7), and moderately enhances Swiss 3T3 fibroblast PLA_2 (10); these results contrast with the high stimulatory ability it showed on ram sperm PLA_2. Moreover, dioleoylglycerol seems to stimulate phospholipases from various sources to different extents (7,10). Since DAGs cause a restructuring of phospholipid vesicles (31-33), this may allow the enzyme a more ready access to the substrate. However, DAG also causes the translocation of PLA_2 from the cytosol to cellular membranes (8), thus resembling the effect of DAG on protein kinase C (34). Interestingly, it has been

Table 2. PLA_2 inhibitors block stimulation by diacylglycerols

Treatment	% Control
Control	100
+Oleoyl-acetyl-glycerol (OAG) 25 μM	560
+1,2-Dioctanoylglycerol (DiC8) 50 μM	220
+Dexamethasone 1 mM	0
+Ro 31-4493 25 μM	0
+Dexamethasone / +OAG	0
+Dexamethasone / +1,2-DiC8	0
+Ro 31-4493 / +OAG	0
+Ro 31-4493 / +1,2-DiC8	0

Diacylglycerols were added to the reaction mixture and sonicated with the radiolabelled phosphatidylcholine substrate. Inhibitors were added immediately before the sperm sonicate. Assays were carried out by incubating 8 μl of sperm sonicate (3.5 x 10^6 cells) for 30 min at 37°C (see Materials and methods for details). Results are averages of duplicate assays carried out on at least two different occasions. Specific activity of control was 0.108 pmol/min/10^6 cells.

suggested that PLA_2 and protein kinase C contain similar regulatory sequences (35) and that, similar to its effect on kinase C, DAG may directly bind to and activate PLA_2 (10). Whether any of these mechanisms participate in the stimulation of sperm PLA_2 is at present a matter of speculation.

In spermatozoa, exocytosis of the acrosomal granule involves at least three Ca^{2+}-requiring events (17), the first one being the generation of DAG through phosphoinositide breakdown (13,16), and the final one probably representing membrane fusion itself. The nature of the second Ca^{2+}-requiring step remains unknown but it has been postulated that, since spermatozoa lack active protein kinase C (19), such second step may actually be the activation of another Ca^{2+}-dependent enzyme; indeed, this enzyme may be the target of the DAG generated during the preceding step (17,18). Previous studies have identified a Ca^{2+}-dependent PLA_2 in mammalian spermatozoa (11,12,24,36) and have provided circumstantial evidence of PLA_2 involvement during exocytosis of the sperm acrosome (11,12). However, nothing is known about the processes that modulate sperm PLA_2 activity. The findings that several DAGs stimulate the Ca^{2+}-dependent sperm PLA_2 (present study), and also stimulate acrosomal exocytosis of Ca^{2+}/ionophore-treated cells (16), lend support to the hypothesis that under physiological conditions this enzyme might be modulated by phosphoinositide-derived DAG, and that this may constitute the second Ca^{2+} requiring event. Phospholipase A_2 action on phosphoglycerides removes arachidonic or other fatty acids from the *sn*-2 position and thus generates lysophopholipids which may either have fusogenic properties or may serve as substrates for the generation of other compounds. Therefore, this enzyme probably plays a central role in the production of metabolites that will be instrumental in events leading to membrane fusion. The study of PLA_2 regulation in whole sperm cells and the role of the resulting metabolites is thus warranted.

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