

Effect of Phorbol Diesters, Synthetic Diacylglycerols, and a Protein Kinase C Inhibitor on the Human Sperm Acrosome Reaction

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ABSTRACT: The acrosome reaction of spermatozoa may be analogous to various somatic cell exocytotic events that incorporate cascade reactions. One such cascade system involves the hydrolysis of a membrane-bound phospholipid; generation of the intracellular second messenger, diacylglycerol; and activation of protein kinase C, followed by the phosphorylation of a number of intracellular proteins. Stimulators of protein kinase C, phorbol diesters and synthetic diacylglycerols, were evaluated to determine if this system functions in the human sperm acrosome reaction. Phorbol 12-myristate 13-acetate and 4 β -phorbol 12,13-didecanoate caused a significant ($P < 0.01$) increase in the acrosome reaction of capacitated spermatozoa. Conversely, an inactive phorbol diester had no significant ($P > 0.05$) stimulatory effect on the acrosome reaction. The synthetic diacylglycerols, 1-oleoyl-2-acetyl-sn-glycerol, 1,2-dioctanoyl-sn-glycerol, and 1,2-dioleoyl-sn-glycerol caused a significant ($P <$

0.01) increase in the acrosome reaction of capacitated spermatozoa, and to a similar extent as the phorbol diesters. A nonactivating isomer of 1,2-dioleoyl-sn-glycerol, 1,3-diolein, had no significant ($P > 0.05$) stimulatory effect on the acrosome reaction. Protein kinase C activation is a diacylglycerol-dependent and Ca^{2+} -dependent process, and stimulation of the acrosome reaction by 1,2-dioctanoyl-sn-glycerol required the presence of calcium ions in the capacitation medium. An inhibitor of protein kinase C, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), prevented the diacylglycerol-induced acrosome reaction ($P < 0.01$). These results support the hypothesis that protein kinase C, via activation by the intracellular second messenger diacylglycerol, has a role in the human sperm acrosome reaction.

Key words: Protein kinase C, calcium, diacylglycerol, phorbol diesters, acrosome reaction.

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Successful fertilization by the mammalian spermatozoon depends on two consecutive processes: 1) capacitation, involving biochemical changes and the redistribution of membrane components, and 2) the acrosome reaction, an exocytotic event characterized by the progressive fenestration and disappearance of the outer acrosomal membrane with the overlying plasma membrane, followed by dispersal of the acrosomal matrix. The primary stimulus for the acrosome reaction appears to involve an interaction between extracellular calcium and the sperm plasma membrane.

The signal transduction pathway(s) that culminate in the acrosome reaction of spermatozoa is not well understood. De Jonge et al (1989a) presented evidence demonstrating that the human sperm acrosome reaction can be regulated by using modulators of the adenylate cyclase/cyclic AMP second messenger system, implying a role for that system in the acrosome reaction. Other second messenger systems, however, may be operational.

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The receptor-mediated hydrolysis of membrane-bound inositol phospholipids is known to be a common mechanism for the transduction of extracellular signals across the plasma membrane of somatic cells (for review see Berridge, 1987). The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C generates the second messengers, inositol 1,4,5-trisphosphate and diacylglycerol. Diacylglycerol, in a calcium-dependent and phospholipid-dependent process, activates protein kinase C (Nishizuka, 1984; Nishizuka, 1986; Parker et al, 1986). Protein kinase C, like other kinases, phosphorylates a number of intracellular proteins.

Involvement of the PIP_2 system in sperm prefertilization processes has only recently been investigated. In human spermatozoa, Ribbes et al (1987) demonstrated phospholipase C activity, and the generation of diacylglycerol and inositol phosphates from the corresponding phospholipids. Bennet and co-workers (1987) provided additional support by demonstrating that phospholipid hydrolysis occurs, along with diacylglycerol formation, during the calcium ionophore-induced acrosome reaction. Thomas and Meizel (1989) presented data demonstrating that the hydrolysis of PIP_2 is stimulated by a fraction of human follicular fluid and progesterone in 24-hour incubated human spermatozoa,

with the reaction being dependent on the presence of extracellular calcium. Additionally, Roldan and Harrison (1989) showed that components of the phosphoinositide cycle were labeled when spermatozoa from several species, including humans, were incubated with radioactive phosphate. Subsequent treatment of the spermatozoa with Ca^{2+} and ionophore A23187 resulted in a rapid breakdown of PIP_2 , followed by the acrosome reaction. Nikolopoulou et al (1986) presented evidence that the total lipid content of boar spermatozoa membranes did not change during the acrosome reaction, yet a degradation of phosphoinositides concomitant with an increase in the amount of diacylglycerol and free fatty acids occurred. This change was correlated with Ca^{2+} uptake by the sperm. In addition, for those reports in which the acrosome reaction was the endpoint of the study, it was found that diacylglycerol formation via phospholipase C activity and the acrosome reaction were dependent on calcium. Thus, these reports establish the presence of the precursor for diacylglycerol formation, phospholipase C, and also demonstrate that formation of diacylglycerol occurs as a result of that enzyme's activity.

Even though data may suggest that an enzymatic process becomes activated during a particular event, it does not necessarily mean that the event is regulated by that process. This is particularly true for spermatozoa in which a hyperactivation of motility occurs virtually concurrently with the acrosome reaction. Thus, the data showing activation of phospholipase C, followed by diacylglycerol formation, during capacitation and the acrosome reaction may have occurred in the sperm tail rather than in the sperm head. To more firmly establish such a role, it must be shown that inhibition of the enzymatic process results in the inhibition of the event. Conversely, stimulation of the process should enhance the event. Therefore, if the PIP_2 system is indeed involved in the human sperm acrosome reaction, inhibitors of protein kinase C should prevent the reaction and stimulators, such as synthetic diacylglycerols or phorbol diesters, should induce the reaction.

Because these studies have not been performed with human spermatozoa, a more definitive role for the PIP_2 system in the acrosome reaction is yet to be established. This investigation determined whether stimulatory and inhibitory modulators of protein kinase C have an effect on the human sperm acrosome reaction. The synchronous acrosome reaction assay (De Jonge et al, 1989b) was used for these determinations. Short communications on this topic have been presented (De Jonge et al, 1989c; De Jonge et al, 1990).

Materials and Methods

Chemicals

Forskolin, 4 α -phorbol 12,13-didecanoate, 4 β -phorbol 12,13-didecanoate, phorbol 12-myristate 13-acetate, 1,2-dioctanoyl-sn-glycerol, 1-oleoyl-2-acetyl-sn-glycerol, 1,2-

dioleoyl-sn-glycerol, 1,3-diolein, 1-(5-isoquinolylsulfonyl)-2-methylpiperazine (H-7), ficoll (Type 400), human serum albumin (HSA, fraction V), Hepes, bismark brown, rose bengal, dimethyl sulfoxide (DMSO), and all salts ("cell culture tested") were obtained from Sigma Chemical Company (St. Louis, MO). All modulators tested were of the highest quality and purity that is commercially available. Forskolin, synthetic diacylglycerols, H-7, and phorbol esters were dissolved in DMSO before dilution with capacitation medium (see below). The final concentration of DMSO was less than 2% in the acrosome reaction system. This concentration of DMSO had no effect on sperm motility or the acrosome reaction under the current conditions.

Capacitation Medium

Modified Biggers, Whitten, and Whittingham medium (BWW, pH 7.4; Biggers et al, 1971), lacking glucose, sodium pyruvate, and sodium lactate (approximately 240 mOsm) and containing 35 mg/ml human serum albumin (HSA) and calcium chloride substituted for calcium lactate, was used as capacitation medium for spermatozoa (De Jonge et al, 1989b). The capacitation medium used for Ca^{2+} -deficient experiments contained all components listed previously with the exception of calcium chloride. A calcium chelator was not used.

Sperm Preparation and Treatment

Semen from apparently normal, healthy volunteers was obtained by masturbation. After complete liquefaction, ejaculates were analyzed for standard semen parameters (Zaneveld and Jeyendran, 1988); only those samples demonstrating a minimum of 70% motile spermatozoa ("motility") were used for experimentation. Seminal plasma was removed by layering semen over 11% Ficoll and centrifuging at 500g for 30 minutes. The sperm pellet was resuspended in 1 ml capacitation medium, recentrifuged at 500g for 2 minutes, and resuspended in capacitation medium to 5.0×10^6 spermatozoa/ml (De Jonge et al, 1989b).

The procedure for testing modulators, the synchronous acrosome reaction system, has been described (De Jonge et al, 1989b). In brief, washed sperm samples (0.5 ml) were pipetted into 5-ml plastic centrifuge tubes and incubated at 37°C for 0 and 3 hours. At each time period, stimulators were added to one of the tubes, the other tube served as a nontreatment control (if treatment volume exceeded 5 μl , >0.1% v:v, then the appropriate vehicle volume was added to the control tube), and the tubes were incubated for an additional 15 minutes at 37°C. A sample of medium-containing sperm was removed from each tube for motility assessment (100 sperm with tail motion counted per tube), and then the reaction was stopped by addition of 3% glutaraldehyde.

The protein kinase C inhibitor, H-7, was added either at the onset of incubation or at the end of the incubation period

(5 minutes before the addition of inducer) to assay for its effect on the human sperm acrosome reaction.

For experiments involving either a total or initial exclusion of calcium, sperm were processed and incubated in medium lacking calcium chloride. When calcium content was manipulated during the time course of experimentation, the medium of all the test tubes, including the controls, was changed by centrifugation (2 minutes, 200g) and aspiration of the medium. The sperm pellet was then resuspended in the desired medium. No significant ($P > 0.05$) change in sperm motility was observed when using this method.

Sperm Acrosomal Evaluation

Acrosomal status was evaluated using a modification (De Jonge et al, 1989b) of the technique by Talbot and Chacon (1981). In brief, spermatozoa were fixed in glutaraldehyde (3%) for 30 minutes and washed twice by centrifugation with distilled water (1000g for 3 minutes). The pellet was resuspended in ~50 μ l of distilled water and transferred to microscope slides, and smears of the samples were prepared. Slides were then incubated in 0.8% bismark brown (pH 1.8) at 37°C for 10 minutes and then 0.8% rose bengal (pH 5.3) for 20 to 23 minutes at room temperature. After incubation, slides were passed through an alcohol dehydration series (50%, 95%, and 100% alcohol) and cleared in Histo-Clear (National Diagnostics, Manville, NJ). After drying, the slides were examined under oil immersion (1000 \times), and acrosomal status of spermatozoa was evaluated (2 slides prepared per tube, 100 sperm cells counted per slide).

Statistical Analysis

Mean, standard deviations (SD) of the mean, and 90% confidence limits were calculated. The *n* values represent essentially pooled data. Each experiment represents one donor chosen at random from a donor pool of 33. Data that comprise one table represent at least three experiments using a minimum of two donors. Occasionally, two experiments were conducted simultaneously; in those instances, two different donors were used. Frequency data were subjected to arcsine transformation and statistically compared using Bartlett's test for homogeneity, the *F*-test, and then the paired *t* test or Dunnett's multiple comparison test.

Results

Phorbol Diesters

Phorbol diesters are compounds that stimulate protein kinase C *in vivo* and *in vitro* (Castagna et al, 1982). To

determine if these types of compounds have an effect on the human sperm acrosome reaction (AR), initial experiments were performed using phorbol 12-myristate 13-acetate (PMA). Forskolin (10 μ mol/L final concentration), an adenylate cyclase stimulator shown to induce the acrosome reaction in capacitated human spermatozoa (De Jonge et al, 1989a), was used as a positive control. In preliminary experiments (*n* = 2), when PMA (100 μ mol/L final concentration, a concentration based on data from somatic cells; see Castagna et al, 1982) was added to spermatozoa at the onset of the incubation period for 15 minutes (ie, to "noncapacitated" spermatozoa), no significant ($P > 0.05$) stimulatory effect on the acrosome reaction was observed. Similar data were obtained with forskolin. A slight inhibitory effect on sperm motility by PMA ($62 \pm 18\%$) occurred after incubation as compared with the control ($78 \pm 11\%$).

When PMA (100 μ mol/L) and forskolin (10 μ mol/L) were added for 15 minutes to spermatozoa that had been incubated for 3 hours (ie, to "capacitated" spermatozoa), both PMA ($55 \pm 18\%$ AR) and forskolin ($33 \pm 5\%$ AR) induced a significant ($P < 0.01$) increase in the percent acrosome reaction as compared with nontreatment controls ($11 \pm 2\%$ AR). However, in contrast to forskolin-treated spermatozoa ($80 \pm 5\%$ motile spermatozoa) and to untreated spermatozoa ($76 \pm 5\%$ motile spermatozoa), PMA caused a reduction in sperm motility ($39 \pm 13\%$).

Since the synchronous acrosome reaction assay requires maintenance of sperm motility during incubation to induce capacitation after treatment with modulators, lower concentrations of PMA were tested. Results in Table 1 show that neither PMA nor forskolin had a stimulatory effect on the acrosome reaction of noncapacitated spermatozoa in comparison with untreated controls. However, when the stimulators were added for 15 minutes to capacitated spermatozoa, a significant ($P < 0.01$) increase in the percent acrosome reaction occurred in comparison with untreated controls, and no inhibitory effect on sperm motility was detected. The percent acrosome reaction obtained by both stimulators was approximately the same. As the dose of PMA was decreased, the percentage of acrosome-reacted sperm also decreased, suggesting a dose-dependent effect on acrosome reaction induction. The lowest concentration of PMA (0.001 μ mol/L) had no significant ($P > 0.05$) stimulatory effect on the acrosome reaction.

To confirm the acrosome reaction-inducing effect of PMA, an additional phorbol diester, 4 β -phorbol 12,13-didecanoate (β -PDD), was tested for its effect on the acrosome reaction (Table 2). The concentrations of β -PDD selected were based on the previous results using PMA. No significant ($P > 0.05$) difference in the percent acrosome reaction was seen when noncapacitated spermatozoa were treated with β -PDD as compared with untreated controls. However, the addition of β -PDD to capacitated spermatozoa caused a significantly ($P < 0.01$) higher percent acrosome

Table 1. Stimulatory effect of forskolin and phorbol 12-myristate 13-acetate on the human sperm acrosome reaction*

Capacitation time (h)	n	Inducer	μmol/L	% Motility†	% AR
0	8	control	—	74 ± 9 (68–80)	9 ± 4 (5–11)
0	8	forskolin	10	75 ± 6 (71–79)	7 ± 3 (5–9)
0	3	PMA	10	70 ± 7 (58–82)	15 ± 4 (8–22)
0	3	PMA	1	67 ± 12 (38–96)	14 ± 2 (11–17)
0	4	PMA	0.1	75 ± 5 (69–81)	8 ± 2 (6–10)
0	5	PMA	0.01	74 ± 8 (66–82)	7 ± 1 (6–8)
0	3	PMA	0.001	78 ± 7 (66–90)	8 ± 3 (3–13)
3	8	control	—	76 ± 5 (73–79)	11 ± 2 (9–13)
3	8	forskolin	10	80 ± 5 (77–83)	33 ± 5 (30–36)‡
3	3	PMA	10	74 ± 2 (71–77)	42 ± 8 (29–55)‡
3	3	PMA	1	70 ± 8 (57–83)	41 ± 5 (27–55)‡
3	4	PMA	0.1	78 ± 6 (71–85)	30 ± 5 (24–36)‡
3	5	PMA	0.01	79 ± 8 (71–87)	27 ± 5 (22–32)‡
3	3	PMA	0.001	78 ± 7 (66–90)	17 ± 6 (7–27)

* Values represent the mean ± SD (90% confidence limits in parentheses). Values shown are for 0- and 3-h untreated and forskolin-treated spermatozoa and represent pooled data from experiments under which the different concentrations of PMA were tested. See text for experimental details.

† Motility did not change significantly ($P > 0.05$) in any of the test samples as compared to untreated controls.

‡ Significantly ($P < 0.01$) different from capacitated (3 hours) spermatozoa not treated with inducer.

reaction, at both concentrations tested, than that seen in the nontreatment controls. The percent acrosome reaction induced by the higher concentration of β -PDD was similar to that induced by forskolin. The lower concentration of α -PDD had less of a stimulatory effect than the higher concentration.

To serve as a control for β -PDD (0.1 μmol/L), the inactive isomer 4 α -phorbol 12,13-didecanoate (α -PDD) was tested (0.1 μmol/L final concentration) for its effect on the acrosome reaction of capacitated spermatozoa ($n = 3$). The 4 α -isomer had no significant ($P > 0.05$) stimulatory effect on the acrosome reaction ($11 \pm 2\%$ AR) compared with untreated controls ($12 \pm 2\%$ AR). β -PDD, which was tested at the same time, had a significant ($P < 0.01$) stimulatory effect on the acrosome reaction ($26 \pm 1\%$ AR), confirming the results in Table 2.

Synthetic Diacylglycerols

One of the products of the hydrolysis of phosphatidylinositol 4,5-bisphosphate is diacylglycerol, which serves as

an intracellular second messenger to activate protein kinase C. The addition of exogenous synthetic diacylglycerols, containing a 1,2-sn configuration and various fatty acids of different chain length, to somatic cells *in vitro* activates protein kinase C (for review see Nishizuka, 1986). Having established that phorbol diesters are capable of stimulating the acrosome reaction, the effect of synthetic diacylglycerols on the acrosome reaction was evaluated using β -PDD as a positive control (Tables 3 and 4).

When either 1-oleoyl-2-acetyl-sn-glycerol (OAG), at concentrations based on data from somatic cells (Lapetina et al, 1985), or β -PDD were added to noncapacitated spermatozoa, no significant ($P < 0.05$) stimulatory effect on the percent acrosome reaction was detected as compared with untreated controls (Table 3). However, when OAG was added to capacitated spermatozoa, a significant ($P < 0.01$) stimulation of the acrosome reaction occurred at both concentrations tested. Additionally, the magnitude of the acrosome reaction-inducing effect by OAG was similar to that of β -PDD. Like the phorbol esters, OAG had an apparent dose-dependent effect on acrosome reaction induction.

Table 2. Stimulatory effect of forskolin and 4 β -phorbol 12,13-didecanoate on the human sperm acrosome reaction*

Capacitation time (h)	Inducer	μmol/L	% Motility†	% AR
0	control	—	79 ± 6 (69–89)	7 ± 1 (5–9)
0	forskolin	10	84 ± 6 (74–94)	7 ± 2 (4–10)
0	β -PDD	0.1	79 ± 9 (64–94)	5 ± 2 (2–8)
0	β -PDD	0.01	76 ± 8 (63–89)	8 ± 3 (3–13)
3	control	—	79 ± 1 (77–81)	7 ± 1 (5–9)
3	forskolin	10	84 ± 3 (79–89)	29 ± 3 (24–34)‡
3	β -PDD	0.1	83 ± 6 (73–93)	34 ± 4 (27–41)‡
3	β -PDD	0.01	79 ± 2 (76–82)	15 ± 5 (7–23)‡

* Values represent the mean ± SD (90% confidence limits in parentheses), $n = 3$. See text for experimental details.

† Motility did not change significantly ($P > 0.05$) in any of the test samples as compared to untreated controls.

‡ Significantly ($P < 0.01$) different from capacitated (3 hours) spermatozoa not treated with inducer.

Table 3. Stimulatory effect of 4 β -phorbol 12,13-didecanoate and 1-oleoyl-2-acetyl-sn-glycerol on the human sperm acrosome reaction*

Capacitation time (h)	Inducer	$\mu\text{mol/L}$	% Motility†	% AR
0	control	—	77 \pm 1 (75–79)	6 \pm 2 (3–9)
0	β -PDD	0.1	75 \pm 4 (68–82)	7 \pm 3 (2–12)
0	OAG	50	81 \pm 2 (78–84)	6 \pm 3 (1–11)
0	OAG	5	79 \pm 5 (71–87)	6 \pm 2 (3–9)
3	control	—	82 \pm 5 (74–90)	9 \pm 2 (6–12)
3	β -PDD	0.1	83 \pm 2 (80–86)	28 \pm 3 (22–34)‡
3	OAG	50	83 \pm 7 (71–95)	27 \pm 3 (22–32)‡
3	OAG	5	78 \pm 7 (66–90)	17 \pm 3 (12–22)‡

* Values represent the mean \pm SD (90% confidence limits in parentheses), n = 3. See text for experimental details.

† Motility did not change significantly ($P > 0.05$) in any of the test samples as compared to untreated controls.

‡ Significantly ($P < 0.01$) different from capacitated (3 hours) spermatozoa not treated with inducer.

The synthetic diacylglycerol 1,2-dioctanoyl-sn-glycerol (DOG) was tested to confirm the acrosome reaction-inducing effect of these types of compounds (Table 4). No significant ($P > 0.05$) effect on the acrosome reaction occurred when noncapacitated spermatozoa were treated with either DOG or β -PDD. However, a significant ($P < 0.01$) stimulation of the acrosome reaction occurred when either DOG or β -PDD were added to capacitated spermatozoa as compared with untreated controls. The percent acrosome reaction induced by DOG at the higher concentration was similar to that induced by β -PDD. The lower concentration of DAG, although not statistically ($P > 0.05$) different from untreated controls, had a slight stimulatory effect on the percent acrosome reaction.

In contrast to the previously tested diacylglycerols that have a 1,2-sn configuration, diacylglycerols with a 1,3-sn configuration are unable to activate protein kinase C (for review see Nishizuka, 1986). Therefore, the effects of an activator and a nonactivator of protein kinase C were compared for their effect(s) on the acrosome reaction; DOG was used as a positive control (n = 3). Neither 1,2-dioleoyl-sn-glycerol (50 $\mu\text{mol/L}$ final concentration), 1,3-diolein (50 $\mu\text{mol/L}$ final concentration), nor DOG (50 $\mu\text{mol/L}$ final concentration) had any significant ($P > 0.05$) stimulatory effect on the acrosome reaction of noncapaci-

tated spermatozoa. However, when the 1,2-sn isomer was added to capacitated spermatozoa, a significant ($P < 0.01$) stimulation of the acrosome reaction (38 \pm 4% AR) occurred as compared with the untreated control (7 \pm 1% AR). The percent acrosome reaction was similar to that induced by DOG (38 \pm 6% AR). In contrast to the 1,2-sn compounds, the 1,3-sn isomer had no significant ($P > 0.05$) stimulatory effect on the acrosome reaction (9 \pm 1% AR). None of the synthetic diacylglycerols had any significant ($P > 0.05$) effect on sperm motility in comparison with untreated controls.

Role of Calcium in the Diacylglycerol-Induced Acrosome Reaction

In somatic cells, the activation of protein kinase C by diacylglycerol is a calcium-dependent process (Nishizuka, 1984). To determine whether this same condition applies to spermatozoa, extracellular Ca^{2+} was either present or absent from the medium (Table 5). The spermatozoa were incubated to induce capacitation, followed by the addition (50 $\mu\text{mol/L}$ final concentration) of 1,2-dioctanoyl-sn-glycerol (DOG) to stimulate the acrosome reaction. An analogue of adenosine 3',5'-cyclic monophosphate (cAMP), dibutyryl cAMP (dbcAMP, 1 mmol/L final con-

Table 4. Stimulatory effect of 4 β -phorbol 12,13-didecanoate and 1,2-dioctanoyl-sn-glycerol on the human sperm acrosome reaction*

Capacitation time (h)	Inducer	$\mu\text{mol/L}$	% Motility†	% AR
0	control	—	77 \pm 5 (69–85)	7 \pm 2 (4–10)
0	β -PDD	0.1	82 \pm 3 (77–87)	9 \pm 2 (6–12)
0	DOG	50	77 \pm 7 (65–89)	9 \pm 2 (6–12)
0	DOG	5	77 \pm 3 (71–82)	6 \pm 1 (4–8)
3	control	—	78 \pm 7 (66–90)	10 \pm 1 (9–11)
3	β -PDD	0.1	76 \pm 1 (74–78)	34 \pm 4 (28–40)‡
3	DOG	50	80 \pm 1 (78–82)	36 \pm 3 (30–42)‡
3	DOG	5	77 \pm 3 (71–82)	20 \pm 5 (12–28)‡

* Values represent the mean \pm SD (90% confidence limits in parentheses), n = 3. See text for experimental details.

† Motility did not change significantly ($P > 0.05$) in any of the test samples as compared to untreated controls.

‡ Significantly ($P < 0.01$) different from capacitated (3 hours) spermatozoa not treated with inducer.

Table 5. Effect of 1,2-dioctanoyl-sn-glycerol and dibutyryl cAMP on the acrosome reaction of spermatozoa capacitated in the presence or absence of extracellular calcium*

Inducer	Conc.	Ca ²⁺ †	% Motility‡	% AR
0	0	+	81 ± 3 (77–84)	7 ± 2 (4–9)
0	0	–	78 ± 5 (71–84)	6 ± 2 (3–8)
DOG	50 µmol/L	+	79 ± 10 (68–90)	29 ± 4 (25–33)§
DOG	50 µmol/L	–	78 ± 3 (74–81)	9 ± 2 (7–11)
dbcAMP	1 mmol/L	+	82 ± 3 (79–85)	28 ± 4 (24–32)§
dbcAMP	1 mmol/L	–	80 ± 3 (77–83)	29 ± 5 (23–35)§

* Values represent the mean ± SD (90% confidence limits in parentheses), n = 4. See text for experimental details.

† Presence (+) or absence (–) of 1.71 mmol/L CaCl₂ in incubation medium.

‡ Motility did not change significantly (P > 0.05) in any of the test samples as compared to untreated controls incubated in medium containing Ca²⁺.

§ Significantly (P < 0.01) different from capacitated (3 hours) spermatozoa not treated with inducer.

centration), which has previously been shown (De Jonge et al, 1989a) to bypass a requirement of extracellular Ca²⁺ for stimulation of the acrosome reaction, was used as a positive control. Neither DOG nor dbcAMP had an effect on the acrosome reaction of noncapacitated spermatozoa incubated either in the presence or absence of Ca²⁺ (data not shown). When Ca²⁺ was included in the capacitation medium, DOG and dbcAMP caused a significant (P < 0.01) increase in the acrosome reaction. However, when Ca²⁺ was excluded from the medium, only dbcAMP stimulated the acrosome reaction significantly (P < 0.01). No significant (P > 0.05) decrease in sperm motility was observed after incubation to induce capacitation or after treatment with modulators as compared with nonincubated, untreated controls.

The inability of diacylglycerol to stimulate the acrosome reaction of human spermatozoa capacitated in the absence of Ca²⁺ implies that Ca²⁺ is required for either the capacitation period or the acrosome reaction process. To determine whether Ca²⁺ plays a role in capacitation or the acrosome reaction, sperm were incubated in medium containing or lacking Ca²⁺ (Table 6). At the end of the capacitation period and 5 minutes before stimulation of the

acrosome reaction by DOG, the incubation medium was changed so that the solution contained the opposite Ca²⁺ content than that present in the medium during the capacitation period (see Materials and Methods). The results (Table 6) indicate that for a significant (minimally, P < 0.05) stimulation of the acrosome reaction to occur via DOG, extracellular Ca²⁺ is required during the incubation period to induce capacitation. For spermatozoa incubated for 3 hours in the absence of Ca²⁺, the addition of calcium immediately before stimulation of the acrosome reaction by DOG is insufficient to allow its occurrence.

Protein Kinase C Inhibitor

To further establish a role for protein kinase C and diacylglycerol in the human sperm acrosome reaction, an inhibitor of the kinase, 1-(5-isoquinolinylnsulfonyl)-2-methylpiperazine (H-7), was tested for its ability to prevent the DOG-induced acrosome reaction (Table 7). Induction of the acrosome reaction by DOG was prevented by H-7 at both concentrations tested, regardless of whether the spermatozoa were capacitated in the presence of inhib-

Table 6. Extracellular calcium requirement during the capacitation period for the 1,2-dioctanoyl-sn-glycerol-induced acrosome reaction*

DOG†	Incubation with Ca ²⁺ ‡	% Motility§	% AR
–	B	76 ± 10 (59–93)	8 ± 2 (5–11)
+	B	72 ± 2 (69–75)	30 ± 3 (24–36) [¶]
–	N	75 ± 7 (63–87)	8 ± 2 (5–11)
+	N	74 ± 4 (67–81)	8 ± 2 (5–11)
–	C	74 ± 4 (67–81)	8 ± 3 (4–12)
+	C	76 ± 4 (69–83)	31 ± 2 (27–35) [¶]
–	A	74 ± 7 (62–86)	8 ± 1 (6–10)
+	A	73 ± 4 (67–79)	10 ± 2 (7–13)

* Values represent the mean ± SD (90% confidence limits, in parentheses), n = 3. See text for experimental details.

† 50 µmol/L DOG (final concentration) added (+) or not added (–) after incubation period (3 hours) to induce capacitation.

‡ 1.71 mmol/L CaCl₂ present during the incubation period to induce capacitation and acrosome reaction (B), completely absent from medium (N), present during capacitation period only (C), present during acrosome reaction period only (A).

§ Motility did not change significantly (P > 0.05) in any of the test samples as compared to untreated controls (spermatozoa incubated for capacitation/acrosome reaction period in medium containing Ca²⁺).

¶ Significantly (P < 0.01) different from spermatozoa incubated for capacitation/acrosome reaction period in the presence of Ca²⁺ and not treated with inducer.

Table 7. Inhibitory effect of 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine on the 1,2-dioctanoyl-sn-glycerol-induced acrosome reaction*

Capacitation time (h)	DOG ($\mu\text{mol/L}$)	Inhibitor ($\mu\text{mol/L}$)	Time added (h)	% Motility†	% AR
0	0	0	—	79 \pm 3 (74–84)	4 \pm 1 (2–6)
0	50	0	—	79 \pm 5 (71–87)	6 \pm 1 (4–8)
3	0	0	—	83 \pm 3 (78–88)	9 \pm 1 (7–11)
3	50	0	—	82 \pm 6 (72–92)	31 \pm 3 (26–36)‡
3	50	10	0	81 \pm 2 (78–84)	8 \pm 2 (5–12)
3	50	1	0	80 \pm 6 (70–90)	9 \pm 2 (5–13)
3	50	10	2:55	80 \pm 2 (77–83)	12 \pm 1 (10–14)
3	50	1	2:55	81 \pm 4 (74–88)	11 \pm 1 (10–12)

* Values represent the mean \pm SD (90% confidence limits in parentheses), n = 3. See text for experimental details.

† Motility did not change significantly ($P > 0.05$) in any of the test samples as compared to untreated controls.

‡ Significantly ($P < 0.01$) different from capacitated (3 hours) spermatozoa not treated with inducer.

itor before addition of DOG or in the absence of inhibitor and then incubated with inhibitor 5 minutes before the addition of DOG. No significant ($P > 0.05$) effect on sperm motility was detected under the various incubation conditions.

Discussion

These results provide strong support for a role of diacylglycerol and protein kinase C in the human sperm acrosome reaction. All active isomers of synthetic diacylglycerols and phorbol diesters (ie, protein kinase C activators) that were tested stimulated the acrosome reaction, but only after the spermatozoa had been incubated under capacitating conditions. Inactive isomers had no stimulatory effect on the acrosome reaction of capacitated spermatozoa. In addition, the acrosome reaction response stimulated by the active compounds was equivalent to that induced by calcium ionophore A23187, cAMP analogues, or the adenylate cyclase stimulator, forskolin (De Jonge et al, 1989a; De Jonge et al, 1989b). The percent acrosome reaction induced by the active compounds was also equivalent to that achieved when spermatozoa were exposed to oocytes or oocyte-associated factors, such as follicular fluid, cumulus oophorus, or zona pellucidae (Tesarik, 1985; Suarez et al, 1986; Cross et al, 1988; De Jonge et al, 1988). Stimulation of the acrosome reaction by diacylglycerol was dependent on the presence of calcium in the medium during incubation to induce capacitation. These findings are commensurate with somatic cell data demonstrating that Ca^{2+} is required for protein kinase C activation (Nishizuka, 1984). Finally, an inhibitor of protein kinase C prevented the induction of the acrosome reaction by diacylglycerol.

Since few compounds are entirely specific in their mechanism or action, it is difficult to conclude that a modulator was acting specifically on its proposed target. For this investigation, questions concerning modulator specificity were diminished by testing the following: 1) several com-

pounds of each type of stimulator, 2) inactive isomers of each stimulator type, and 3) an inhibitor of protein kinase C. More conclusive determinations of whether the compounds act in the manner proposed will require the isolation and assay of the various pathway components. However, since all the stimulatory modulators of protein kinase C induced the acrosome reaction and an inhibitor of the kinase prevented acrosome reaction induction, sufficient evidence is provided to suggest a role for the diacylglycerol and protein kinase C branch of the phosphatidylinositol 4,5-bisphosphate system in the mechanism of the human sperm acrosome reaction.

Phorbol diesters, membrane-permeable activators of protein kinase C, are not readily metabolized in somatic cells. As a consequence, very low concentrations of phorbol diester are able to stimulate protein kinase C and thus elicit cellular responses (for review see Castagna et al, 1982; Blumberg, 1988). In contrast, the active permeable synthetic diacylglycerols, which closely mimic the actions of naturally occurring diacylglycerols, are metabolized rapidly. As a result, the concentrations of diacylglycerol that can stimulate cellular responses are several orders of magnitude higher than those of phorbol diesters. The compounds have been shown, however, to interact at the same sites to activate protein kinase C (for review see Nishizuka, 1986). In this investigation, the concentrations of phorbol diesters and diacylglycerols that stimulated the acrosome reaction are similar to concentrations used to elicit responses in somatic cells. In addition, the magnitude of difference in effective concentration between the phorbol diesters and diacylglycerols for acrosome reaction stimulation is comparable to the effective dose difference for somatic cells, roughly a 1000- to 5000-fold difference (Castagna et al, 1982).

Previous reports in which synthetic diacylglycerols and phorbol diesters were tested have yielded apparently conflicting results. Visconti and Tezon (1989) demonstrated that the addition of either a phorbol diester or a synthetic diacylglycerol to hamster epididymal spermatozoa incu-

bated in capacitation medium induced a 20- to 50-fold increase in cAMP levels. However, no stimulatory effect on the acrosome reaction was detected. These same types of compounds were shown to have a stimulatory effect on mouse spermatozoa incubated in combination with mouse zona pellucida, apparently accelerating an initial step in the acrosome reaction (Lee et al, 1987). In contrast, Roldan and Harrison (1988) found that phorbol dibutyrate had no stimulatory effect on the acrosome reaction of ram spermatozoa. We tested several forms of each stimulator type and each was found to activate the acrosome reaction process.

To further establish that the effect of the diacylglycerols was mediated via protein kinase C, an inhibitor of the enzyme (Hidaka et al, 1984) was evaluated for its ability to prevent the diacylglycerol-induced acrosome reaction. Previously, Roldan and Harrison (1988) presented data, albeit in the ram, implying that spermatozoa lack any detectable protein kinase C. The current data show that the acrosome reaction was inhibited by 1-(5-isoquinolinylnsulfonyl)-2-methylpiperazine (H-7, $K_i = 6.0 \mu\text{mol/L}$ for protein kinase C), regardless of whether the compound was present throughout the incubation period (ie, capacitation period) before addition of the diacylglycerol or added for only 5 minutes at the end of the incubation period. Inhibition of the acrosome reaction by this compound may have resulted from a mechanism other than that proposed, since H-7 inhibits not only protein kinase C but cAMP-dependent protein kinase ($K_i = 3.0 \mu\text{mol/L}$; Hidaka et al, 1984). However, the fact that an inhibitor of the enzyme in question could prevent induction of the acrosome reaction by an effector of the enzyme implies that the enzyme may have a pivotal role in the acrosome reaction sequence.

In somatic cells, Ca^{2+} is required for protein kinase C activation (Nishizuka, 1984). Similarly, in sperm cells, the hydrolysis of PIP_2 , the generation of the second messenger diacylglycerol, and finally, the acrosome reaction, have been shown to be activated in a calcium-dependent fashion during the capacitation period (Nikolopoulou et al, 1986; Roldan and Harrison, 1989; Thomas and Meizel, 1989). The current data support these findings. Additionally, the preliminary data suggest that calcium is required only for the capacitation period and not, apparently, for the acrosome reaction. These findings, however, require further investigation.

Visconti and Tezon (1989) showed that the addition of phorbol 12-myristate 13-acetate (PMA) or 1-oleoyl-2-acetyl-sn-glycerol to hamster spermatozoa induced a 20- to 50-fold increase in cAMP levels. These authors speculated that the increase in cAMP levels may have resulted from adenylate cyclase activation, and that this activation might be linked to protein kinase C activity. Data were presented recently (De Jonge et al, 1989a; De Jonge et al, 1991) demonstrating the stimulation of the acrosome reaction using the adenylate cyclase stimulator forskolin, cAMP ana-

logues, and phosphodiesterase inhibitors. The activation of the adenylate cyclase pathway in the human sperm acrosome reaction has been open to criticism because no evidence for the required guanine nucleotide-stimulatory (Gs) protein has been shown. However, Kopf (1988; and personal communication) has presented strong evidence for a guanine nucleotide-inhibitory (Gi) protein in human spermatozoa. Furthermore, in somatic cells, adenylate cyclase has been shown to be activated by protein kinase C inactivation of the Gi-protein (Bell and Brunton, 1986; Johnson et al, 1986; Katada et al, 1987; Levitski, 1987). It is possible that protein kinase C activates adenylate cyclase; thus, an explanation arises for how the adenylate cyclase pathway could become activated to stimulate the acrosome reaction.

In conclusion, our data provide evidence for the involvement of the diacylglycerol/protein kinase C pathway in the human sperm acrosome reaction, which is directly analogous to the second messenger cascade system that operates in somatic cells. It is likely that the mediation of one or more external signals by the sperm plasma membrane results in PIP_2 hydrolysis, diacylglycerol formation, and protein kinase C activation. Based on results obtained with somatic cells (Anderson et al, 1985; Nishizuka, 1986), it is possible that this messenger system interacts with the adenylate cyclase/cAMP pathway in modulating the human sperm acrosome reaction. These pathways may act in sequence, bidirectionally or alternatively, in an antagonistic fashion, possibilities that are currently under investigation.

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