Role of diacylglycerols and calcium in the marsupial acrosome reaction

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Acrosomal loss was induced in marsupial spermatozoa by an intermediate of the phosphoinositide pathway. The diacylglycerol, 1,2-dioctanoyl-*sn*-glycerol (DiC_8 ; $100 \mu\text{mol l}^{-1}$) induced acrosomal loss in 70% of brushtail possum (*Trichosurus vulpecula*) spermatozoa and in 80% of tammar wallaby (*Macropus eugenii*) spermatozoa. The DiC_8 -induced acrosomal loss was not enhanced by co-incubation with calcium ionophore A23187 and occurred in Ca^{2+} -free medium and in the presence of the calcium chelator EGTA (3 mmol l^{-1}). There was no evidence of uptake of $^{45}\text{Ca}^{2+}$ during the DiC_8 -induced acrosomal loss. Inhibitors of protein kinase C [1-(5-isoquinoliny)-2-methylpiperazine] and phospholipase A_2 [dexamethasone] did not effect DiC_8 -induced acrosomal loss in wallaby spermatozoa. The phorbol ester, phorbol 12-myristate 13-acetate, at a concentration of $10 \mu\text{mol l}^{-1}$ had no effect on possum spermatozoa and induced acrosomal loss in only 6% of wallaby spermatozoa. It appears that the DiC_8 -induced acrosome reaction is not mediated by activation of the phosphoinositide pathway and that extracellular calcium is not required for the membrane fusion event. As acrosomal loss was seen only at relatively high concentrations of diacylglycerol ($> 50 \mu\text{mol l}^{-1}$) and there is no evidence of involvement of other phosphoinositide intermediates or analogues, it is likely that its role is as a direct membrane fusogen.

Introduction

The marsupial acrosome is an unusually stable structure, which remains intact in the presence of high concentrations of divalent cations, calcium ionophore A23187 and cyclic nucleotides (Mate and Rodger, 1991). All of these treatments can induce the acrosome reaction in eutherian spermatozoa, although their effectiveness varies among species (Santos-Sacchi and Gordon, 1980; Shams-Borhan and Harrison, 1981; Fraser, 1984; Stock and Fraser, 1989; De Jonge *et al.*, 1991). The stability of the marsupial acrosome appears to lie in the properties of the acrosomal membranes which resist damage by freeze-thawing without cryoprotectant and detergent treatment (Sistina *et al.*, 1993a). Induced acrosomal loss was recently described in marsupial spermatozoa following exposure to high concentrations of the phosphoinositide pathway intermediates, the diacylglycerols, 1,2-dioctanoyl-*sn*-glycerol (DiC_8) and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) (Sistina *et al.*, 1993c). The manner of acrosome loss involved multiple point fusions of the plasma membrane and outer acrosomal membrane (Sistina *et al.*, 1993c) and thus appeared similar to the physiological acrosome reaction of the spermatozoa of placental mammals (Barros *et al.*, 1967; Yanagimachi and Noda 1970; Moore and Bedford, 1978).

The aim of this study was to examine the involvement of the phosphoinositide pathway in the regulation of the marsupial acrosome reaction. In general, activation of the phosphoinositide

messenger system involves hydrolysis of polyphosphoinositides to generate the second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. Diacylglycerol operates as a messenger within the plane of the membrane to activate a Ca^{2+} - and phospholipid-dependent protein kinase C (PKC), whereas IP_3 is released into the cytoplasm to function as a second messenger by mobilizing intracellular calcium.

Phosphoinositides have been detected in the outer acrosomal membrane of boar spermatozoa (Berruti and Franchi, 1986) and a phospholipase C specific for phosphoinositides has been described in human spermatozoa (Ribbes *et al.*, 1987). Studies of the calcium ionophore A23187-induced acrosome reaction indicate that it is accompanied by polyphosphoinositide breakdown and a subsequent rise in phosphatidic acid (PA), IP_3 , diacylglycerol and free arachidonic acid (Bennet *et al.*, 1987; Harrison *et al.*, 1990; Roldan and Harrison, 1990b; Roldan and Mollinedo, 1991). Although it has been reported that diacylglycerols and phorbol esters (modulators of PKC activity) stimulate the acrosome reaction in mouse spermatozoa (Lee *et al.*, 1987), PKC is not detectable in ram, boar or hamster spermatozoa (Roldan and Harrison, 1988, 1990a). It has been suggested that in the absence of PKC in spermatozoa, the second messenger role of diacylglycerol in acrosomal exocytosis may be to activate a phospholipase A_2 (Harrison and Roldan, 1990; Roldan and Harrison, 1990a; Roldan and Mollinedo, 1991). The fusogenic lysophospholipids subsequently produced by the action of phospholipase A_2 may then lead to the fusion of the plasma and outer acrosomal membranes.

This study examined whether the phosphoinositide pathway operates in marsupial spermatozoa during the DiC_8 -induced acrosome reaction.

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Materials and Methods

Animals and semen

Adult male brushtail possums (*Trichosurus vulpecula*) were captured in the Newcastle area and housed as described by Rodger and Mate (1988) in a 14 h light:10 h dark cycle. Tamar wallabies (*Macropus eugenii*) were from the colony of the CSIRO Division of Wildlife and Ecology in Canberra and were housed as described by Mate and Rodger (1991). Work with protected species was licensed by the National Parks and Wildlife Service of New South Wales and animal experimentation had the approval of the University of Newcastle Animal Care and Ethics Committee.

Semen was collected by electroejaculation without dilution as described by Mate and Rodger (1991) from a pool of four possums and seven wallabies. At least seven days separated successive electroejaculations from any one animal. Individual sperm samples were used for each experimental replicate. The mean spermatozoa concentration during the experimental period for the possum ejaculates was 11.5×10^6 spermatozoa ml^{-1} and for the tamar was 135×10^6 spermatozoa ml^{-1} .

Possum and wallaby spermatozoa were washed by a swim-up procedure as described by Mate and Rodger (1991). Wallaby semen was left at room temperature for 15–20 min to coagulate fully before washing (Sistina *et al.*, 1993a). Ejaculates exhibiting poor motility or fewer than 5×10^6 spermatozoa ml^{-1} were discarded.

Test solutions

The agents to be tested were made up at ten times their final concentration in Eagle's minimum essential medium (EMEM; Cytosystems, Australia) containing 75 iu penicillin ml^{-1} and 0.05 mg streptomycin ml^{-1} at pH 7.6. At the beginning of the incubation period the test solution was mixed 1:9 with the washed sperm suspension and incubated at 35°C in 5% CO_2 in air. All agents were obtained from the Sigma Chemical Co. (St Louis, MO). The agents used were: diacylglycerol-1,2-dioctanoyl-*sn* glycerol (DiC_8) (50–100 $\mu\text{mol l}^{-1}$); calcium influx: 10 μmol calcium ionophore A23187 l^{-1} (Ca^{2+} - Mg^{2+} mixed salts) in combination with 100 $\mu\text{mol l}^{-1}$ DiC_8 incubated in the dark; protein kinase C (PKC) activator: phorbol 12-myristate 13-acetate (0.01–10 $\mu\text{mol l}^{-1}$); PKC inhibitor: 1-(5-isoquinolinylnsulfonyl)-2-methylpiperazine (ISMP) (10–100 $\mu\text{mol l}^{-1}$) in combination with 100 $\mu\text{mol l}^{-1}$ DiC_8 ; and phospholipase A_2 (PLA_2) inhibitor: dexamethasone (1 mmol l^{-1}) in combination with 100 $\mu\text{mol DiC}_8 \text{l}^{-1}$.

Effects of calcium concentration

Wallaby and possum spermatozoa were washed by swim-up into either (i) EMEM; (ii) Ca^{2+} -free EMEM, (iii) Ca^{2+} - and Mg^{2+} -free Dulbecco's phosphate-buffered saline (PBS). The agents to be tested were made up at ten times their final concentration in the appropriate medium. At the beginning of the incubation period the test solution was mixed 1:9 with the washed sperm suspension and incubated at 35°C in 5% CO_2 in air. The

agents tested were: 100 $\mu\text{mol DiC}_8 \text{l}^{-1}$; 100 $\mu\text{mol DiC}_8 \text{l}^{-1}$ + 3 mmol EGTA l^{-1} ; 100 $\mu\text{mol DiC}_8 \text{l}^{-1}$ + 10 $\mu\text{mol A23187 l}^{-1}$; and 100 $\mu\text{mol DiC}_8 \text{l}^{-1}$ + 10 $\mu\text{mol A23187 l}^{-1}$ + 3 mmol EGTA l^{-1} .

Determination of calcium ion concentrations

The concentration of calcium present in EMEM and Ca^{2+} -free EMEM before and after washing of wallaby spermatozoa using the swim-up method was measured by atomic absorption spectroscopy. Samples of EMEM and Ca^{2+} -free EMEM were used without modification for measurement before swim-up. To measure the calcium content of EMEM and Ca^{2+} -free EMEM after swim-up, the sperm suspension was centrifuged at 1500 *g* for 15 min to pellet the spermatozoa, and the supernatant was removed for calcium determination. The calcium concentration of seminal plasma was also measured. Wallaby semen was collected into a graduated 50 ml centrifuge tube and subsequently diluted 1:9 with Ca^{2+} -free EMEM. The diluted semen was then centrifuged at 1500 *g* for 15 min to pellet the spermatozoa and the supernatant was used for calcium determination.

Standard solutions of calcium were prepared in Ca^{2+} -free EMEM and ranged from 0 to 70 mg l^{-1} . Calcium concentrations were determined using a Varian AA-175 series atomic absorption spectrophotometer at the following settings: lamp current 3 mA; acetylene nitrous oxide flame with a reducing red cone approximately 1.0–1.5 cm high; wavelength 422.7 nm; and spectral band pass 0.1 nm.

Calcium uptake

The uptake of $^{45}\text{Ca}^{2+}$ by wallaby spermatozoa was determined by a radiochemical procedure essentially as described by Simpson *et al.* (1987). The spermatozoa were adjusted to a concentration 1×10^7 spermatozoa ml^{-1} in Ca^{2+} -free EMEM containing 75 iu penicillin ml^{-1} and 0.05 mg streptomycin ml^{-1} . $^{45}\text{CaCl}_2$ in EMEM was added to the sperm suspension to give a final concentration of 1 mmol l^{-1} . The uptake of $^{45}\text{Ca}^{2+}$ by wallaby spermatozoa was measured in control EMEM, EMEM containing 100 $\mu\text{mol DiC}_8 \text{l}^{-1}$ and high ionic strength (380 mOsmol) EMEM (HIS). Aliquots (0.1 ml) of sperm suspension were taken with an automatic pipette at the times indicated in the results. The sperm samples were immediately filtered at a low pressure vacuum (2 psi) through glass fibre discs (Whatman/GFC, Clifton, NJ). The filters were washed five times with 3 ml aliquots of EMEM that had been supplemented with 10 mmol nonradioactive $\text{CaCl}_2 \text{l}^{-1}$. The filters were then dried and counted in 3 ml Liquiscint (National Diagnostics, USA) using a Hewlett-Packard Tri-Carb 1500 liquid scintillation analyser.

Acrosome assessment

Sperm samples were fixed for acrosomal assessment at the times indicated. Acrosomal status was scored for 100–200 spermatozoa per sample after Bryan's staining as described by Mate and Rodger (1991). Statistical analysis was by Student's *t* test after square root transformation.

Table 1. The concentration of calcium ions in the seminal plasma of tammar wallaby (*Macropus eugenii*) semen and culture media before and after swim-up

Fluid	Calcium concentration (mmol l ⁻¹) (mean $\pm$ SEM; n = 3)	
	Before swim-up	After swim-up
Seminal plasma	1.41 $\pm$ 0.21	nd
EMEM	1.50 $\pm$ 0.09	1.50 $\pm$ 0.30
Ca ²⁺ -free EMEM	0 ^a	0.23 $\pm$ 0.02

^aNot detectable; nd: not determined; EMEM: Eagle's minimum essential medium.

Fig. 1. The percentage of acrosome intact tammar wallaby (*Macropus eugenii*) spermatozoa during incubation in Eagle's minimum essential medium (EMEM) (filled symbols) with 1,2-dioctanoyl-*sn*-glycerol (DiC₈) at concentrations 0 μmol l⁻¹ (●), 100 μmol l⁻¹ (▼), 100 μmol l⁻¹ + 3 mmol EGTA l⁻¹ (■) and in Ca²⁺-free EMEM (hollow symbols) with DiC₈ at concentrations 0 μmol l⁻¹ (○; obscured by filled circle), 100 μmol l⁻¹ (▽), 100 μmol l⁻¹ + 3 mmol EGTA l⁻¹ (□). All points are means $\pm$ SEM; n = 6.

Results

Calcium ion concentration of seminal plasma and test solutions

The calcium concentration of seminal plasma obtained from wallabies by electroejaculation was approximately 57 mg l⁻¹ or 1.41 mmol l⁻¹. The calcium concentration of EMEM was 1.5 mmol l⁻¹, and did not change after the swim-up procedure (Table 1). Calcium-free EMEM did not contain measurable amounts of calcium; however, 0.23 mmol calcium l⁻¹ was detected after the swim-up procedure (Table 1).

DiC₈-induced acrosomal loss in calcium-free medium

Acrosomal loss was induced in 80–90% of wallaby spermatozoa by 100 μmol DiC₈ l⁻¹ (Fig. 1). There was no difference in the effectiveness of DiC₈ in calcium-containing and calcium-free

Fig. 2. The percentage of acrosome intact spermatozoa from the tammar wallaby (*Macropus eugenii*) during incubation with 1,2-dioctanoyl-*sn*-glycerol (DiC₈) at concentrations of (a) 0 μmol l⁻¹ (●), 50 μmol l⁻¹ (▼) or 100 μmol l⁻¹ in combination with 10 μmol calcium ionophore A23187 l⁻¹ (■) and (b) 0 μmol l⁻¹ (●), 100 μmol l⁻¹ (▼), 100 μmol l⁻¹ + 10 μmol calcium ionophore A23187 l⁻¹ (■) and 100 μmol l⁻¹ + 10 μmol calcium ionophore A23187 l⁻¹ + 3 mmol EGTA l⁻¹ (▲). All points are means $\pm$ SEM; (a) n = 6–10 (b) n = 40.

EMEM. Addition of 3 mmol EGTA l⁻¹ inhibited the actions of DiC₈, so that significantly more spermatozoa remained acrosome intact ($P < 0.05$ at 30 and 60 min in media with or without calcium). However, in the presence of EGTA, acrosomal loss occurred in approximately 40% of spermatozoa in both calcium-containing and calcium-free media, which was significantly greater than occurred in the control (60 min EMEM: $P < 0.05$; 30 min Ca²⁺-free EMEM: $P < 0.01$).

Acrosomal loss in the presence of DiC₈ and the calcium ionophore A23187

The presence of 50 μmol DiC₈ l⁻¹ alone or in combination with 10 μmol A23187 l⁻¹ induced acrosomal loss in 20% of wallaby spermatozoa (Fig. 2a), significantly greater than occurred in the control ($P < 0.01$ or 0.001). One hundred μmol DiC₈ l⁻¹ alone induced acrosomal loss in approximately 90% of spermatozoa after 30 min incubation (Fig. 2b), significantly more than occurred in the control ($P < 0.0001$). Around 60% of spermatozoa had lost their acrosome after treatment with either 100 μmol DiC₈ l⁻¹ + 10 μmol A23187 l⁻¹ (DiC₈-A) or 100 μmol DiC₈ l⁻¹ + 10 μmol A23187 l⁻¹ + EGTA (DiC₈-A-E) (Fig. 2b). This was significantly fewer than with DiC₈ alone

Fig. 3. The percentage of acrosome intact brushtail possum (*Trichosurus vulpecula*) spermatozoa during incubation in Eagle's minimum essential medium (EMEM) only (●), 100 μmol 1,2-dioctanoyl-*sn*-glycerol l^{-1} (DiC_8 ; ▲), 100 μmol DiC_8 l^{-1} + 10 μmol calcium ionophore A23187 l^{-1} (▼) or, 100 μmol DiC_8 l^{-1} + 10 μmol calcium ionophore A23187 l^{-1} + 3 mmol EGTA l^{-1} (■). All points are means $\pm$ SEM; $n = 4$.

($P < 0.01$ or 0.001). There was no difference between DiC_8 -A and DiC_8 -A-E in the induction of acrosomal loss.

In possum spermatozoa, 70–90% acrosomal loss occurred in response to DiC_8 alone and to DiC_8 in combination with A23187 and EGTA (Fig. 3). There were significantly fewer acrosome intact possum spermatozoa in the treated samples compared with the control ($P < 0.01$ or 0.0001). There was no difference in acrosomal loss between spermatozoa treated with DiC_8 , DiC_8 + A23187 (DiC_8 -A) or DiC_8 + A23187 + EGTA (DiC_8 -A-E).

In wallaby spermatozoa, the effects of DiC_8 + A23187 (DiC_8 -A) and DiC_8 + A23187 + EGTA (DiC_8 -A-E) on acrosomal loss were similar during incubation in EMEM (Fig. 4a), Ca^{2+} -free EMEM (Fig. 4b) and PBS (Fig. 4c). Both DiC_8 -A and DiC_8 -A-E induced significant acrosomal loss in wallaby spermatozoa in each of the media tested. Acrosomal loss (30–40%) was statistically significant after 5 min incubation in DiC_8 -A in both EMEM and after 10 min in PBS ($P < 0.05$). Results with DiC_8 -A-E were more variable but achieved statistical significance after 30 min in EMEM, 20 min in Ca^{2+} -free EMEM and 10 min in PBS ($P < 0.05$).

Uptake of radiolabelled calcium during DiC_8 -induced acrosomal loss

The presence of 100 μmol DiC_8 l^{-1} + 10 μmol calcium ionophore A23187 l^{-1} did not increase the uptake of $^{45}\text{Ca}^{2+}$ by wallaby spermatozoa (Fig. 5). The uptake of $^{45}\text{Ca}^{2+}$ was highest immediately after the addition of DiC_8 + calcium ionophore A23187 but did not differ significantly from the control and had fallen to control values by 5 min of incubation. Less than 4 nmol $^{45}\text{Ca}^{2+}$ were accumulated per 10^8 spermatozoa during the incubation period.

Effect of PKC and PLA_2 inhibitors on DiC_8 -induced acrosomal loss

The PKC inhibitor, 1-(5-isoquinolyl-sulfonyl)-2-methylpiperazine (ISMP), did not inhibit the DiC_8 -induced acrosome

Fig. 4. The percentage of acrosome intact tammar wallaby (*Macropus eugenii*) spermatozoa during incubation in (a) Eagle's minimum essential medium (EMEM), (b) Ca^{2+} -free EMEM or, (c) Dulbecco's phosphate-buffered saline (PBS) only (●) or with the addition of 100 μmol 1,2-dioctanoyl-*sn*-glycerol l^{-1} (DiC_8) + 10 μmol calcium ionophore A23187 l^{-1} (▼) or 100 μmol DiC_8 l^{-1} + 10 μmol A23187 l^{-1} + 3 mmol EGTA l^{-1} (■). All points are means $\pm$ SEM; $n = 8$ –18.

Fig. 5. The uptake of $^{45}\text{Ca}^{2+}$ by tammar wallaby (*Macropus eugenii*) spermatozoa during incubation in Eagle's minimum essential medium (EMEM) (●) or EMEM containing 100 μmol 1,2-dioctanoyl-*sn*-glycerol l^{-1} (DiC_8) and 10 μmol A23187 l^{-1} (▼). All points are means $\pm$ SEM; $n = 5$.

reaction in wallaby spermatozoa. The addition of $10\text{ }\mu\text{mol}$ or $100\text{ }\mu\text{mol}$ ISMP l^{-1} resulted in 97% ($n = 4$) or 102% ($n = 4$) of the acrosomal loss induced by DiC_8 alone.

Addition of dexamethasone, an inhibitor of phospholipase A_2 , at a concentration of 1 mmol l^{-1} , resulted in 111% ($n = 4$) of the acrosomal loss induced by DiC_8 alone.

Effect of phorbol ester on the acrosomes of possum and wallaby spermatozoa

The motility of possum and wallaby spermatozoa incubated in the presence of the phorbol ester, phorbol 12-myristate 13-acetate (PMA), did not differ from that of control spermatozoa. PMA did not induce acrosomal loss in possum spermatozoa at the concentrations tested: $100 \pm 0\%$ and $97.7 \pm 1.5\%$ of possum spermatozoa remained acrosome intact after 3 h exposure to $0.01\text{ }\mu\text{mol l}^{-1}$ or $1.0\text{ }\mu\text{mol l}^{-1}$ PMA, respectively ($n = 4$). The percentage of acrosome-intact wallaby spermatozoa decreased to $94.2 \pm 1.3\%$ after exposure to $10\text{ }\mu\text{mol PMA l}^{-1}$ for 3 h, which was significantly lower than the control ($98.8 \pm 0.7\%$; $P < 0.01$). PMA did not induce acrosomal loss in wallaby spermatozoa at a concentration of $0.1\text{ }\mu\text{mol l}^{-1}$.

Discussion

Millimolar amounts of calcium are required for the acrosome reaction to occur in eutherian mammals; 1.8 mmol l^{-1} is the minimal requirement in mouse and human spermatozoa (Fraser, 1987; Stock and Fraser, 1989). Marsupial spermatozoa, however, appear able to undergo the acrosome reaction in the absence of extracellular calcium. The calcium ionophore A23187 does not induce the acrosome reaction (Mate and Rodger, 1991) and, in the present study, did not accelerate the rate of the DiC_8 -induced acrosome reaction in possum or wallaby spermatozoa. Indeed, A23187 inhibited the DiC_8 -induced acrosome reaction of wallaby spermatozoa, reducing the number of acrosome-reacted spermatozoa by approximately 50%. The DiC_8 -induced acrosome reaction occurred in the absence of added calcium and in the presence of EGTA, and did not involve uptake of radiolabelled Ca^{2+} . However, EGTA did reduce the number of wallaby spermatozoa undergoing the acrosome reaction in response to DiC_8 by 50%. These results indicate that if calcium is involved in the DiC_8 -induced marsupial acrosome reaction, that (i) only a very small amount of extracellular calcium is required or (ii) intracellular stores of calcium are sufficient.

The percentage of marsupial spermatozoa undergoing acrosomal loss in response to DiC_8 was slightly higher in this study than was described by Sistina *et al.* (1993c). These differences may be attributable to a number of factors: the studies were carried out using different animals and batches of reagents, and at different times of the year, perhaps introducing seasonal effects in the semen samples. The Ca^{2+} -independent DiC_8 -induced acrosome reaction of marsupial spermatozoa appears ultrastructurally as a true acrosome reaction involving point fusion between the plasma membrane and the outer acrosomal membrane (Sistina *et al.*, 1993c). By contrast, other agents such as Triton X-100 and lysophosphatidylcholine have been

demonstrated to result in degenerative acrosomal loss in marsupial spermatozoa (Sistina *et al.*, 1993a,b). Spontaneous acrosomal loss in the absence of calcium has been reported in human spermatozoa, but was considered degenerative rather than due to a true acrosome reaction, although the ultrastructure of acrosome loss was not examined (White *et al.*, 1990). Although calcium is required for membrane fusion in many types of cell and is of major importance in many processes of secretion that involve exocytosis, a requirement for calcium is not universal (Lucy, 1978). The fusion of erythrocytes induced by lysophosphatidylcholine or Sendai virus can occur in the absence of calcium (Howell and Lucy, 1969; Peretz *et al.*, 1974; Hart *et al.*, 1976) and a lysin obtained from the acrosome of abalone spermatozoa can induce liposome fusion in the absence of calcium (Hong and Vacquier, 1986).

Conventionally, the action of DiC_8 is mediated through the activation of PKC. However, failure of the PKC inhibitor, ISMP, to prevent the DiC_8 -induced acrosome reaction in wallaby spermatozoa indicates that the activation of PKC is not involved in this case. Phorbol esters are more potent activators of PKC than are diacylglycerols (Bell, 1986; Leach and Blumberg, 1989), but in this study the phorbol ester, PMA, induced acrosomal loss in a very small number of spermatozoa, more suggestive of nonspecific effects on membrane structure. PKC has been detected in mouse spermatozoa (Kopf and Gerton, 1991) and induction of the acrosome reaction in mouse and human spermatozoa with phorbol esters and diacylglycerol is consistent with the involvement of PKC (Lee *et al.*, 1987; De Jonge *et al.*, 1989). However, potentially active PKC appears not to be present in ram or boar spermatozoa (Roldan and Harrison, 1988, 1990a). One of the difficulties in establishing a role for PKC is the existence of different types of PKC, each with different requirements for activation, kinase activities and binding affinities. Heterogeneity in the binding activity of PKC has been described in several types of cell (Konig *et al.*, 1985; Dougherty and Niedel, 1986) and may occur in spermatozoa as evidenced by the markedly different effects of phorbol ester and DAG on capacitated and non-capacitated human and mouse spermatozoa (Lee *et al.*, 1987; De Jonge *et al.*, 1989). In addition, Roldan and Harrison (1990b) found that diacylglycerols alone could not induce acrosomal loss in ram spermatozoa but did accelerate the onset of the A23187-induced acrosome reaction. Leach and Blumberg (1989) suggested that the differences in the binding affinity of PKC were the result of modulation by changes in calcium concentration or phospholipid composition.

In ram spermatozoa the acrosome reaction inducing activity of diacylglycerols is mediated via the activation of Ca^{2+} -dependent phospholipase A rather than PKC (Roldan and Harrison, 1988; Roldan and Mollinedo, 1991). Although calcium-independent PLA_2 has been described (Kent and Vagelos, 1975; Pierik *et al.*, 1988), the failure of the PLA_2 inhibitor, dexamethasone, to alter DiC_8 -induced acrosomal loss indicates that DiC_8 -induced acrosomal loss in marsupial spermatozoa was probably not due to stimulation of PLA_2 .

In addition to their role in enzyme activation, diacylglycerols can play a more direct role in membrane fusion (Das and Rand, 1984; Dawson *et al.*, 1984; Siegel, 1989). Diacylglycerols with long acyl chains effectively induce biomembrane fusion by reducing lamellar/inverted hexagonal phase transition temperature (Dawson *et al.*, 1984; Siegel *et al.*, 1989). Das and Rand

(1984) suggested that when diacylglycerol is incorporated into phospholipid bilayers its highly conical shape induces distortions and spreading apart of polar groups creating non-lamellar, hexagonal phases. Although such structural changes alone may not be sufficient for membrane fusion, they may enhance the access of proteins to the membrane hydrocarbon core and facilitate the interaction of enzymes with their phospholipid core (Dawson *et al.*, 1984). The membrane transitions induced by DAG depend on the phospholipid species: they are most effective in phosphatidylethanolamine and phosphatidylcholine and least effective in phosphatidylserine (Das and Rand, 1984). The relatively high concentrations ($>50 \mu\text{mol l}^{-1}$) of diacylglycerol required to induce the acrosome reaction in marsupial spermatozoa are consistent with its action as a direct membrane fusogen.

The mechanism of DAG-induced acrosome reaction in marsupial spermatozoa differs markedly from the eutherian acrosome reaction. Perhaps there is no absolute requirement for extracellular calcium. Inhibitor studies indicate that activation of protein kinase C or phospholipase A_2 is probably not involved. It is suggested that the membrane fusion events observed in this study may be modulated by physical interaction of DiC_8 with the sperm membranes and are probably not directly related to events in the phosphoinositide pathway.

Although DiC_8 -induced acrosome loss by marsupial spermatozoa mimicked the placental sperm acrosome reaction, it is not known whether the DAG-induced acrosome reaction in marsupials bears any relationship to the regulatory processes of the naturally occurring acrosome reaction.

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