Induction of the marsupial sperm acrosome reaction *in vitro* by treatment with diacylglycerols

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The diacylglycerol DiC₈ (1,2-diocanoyl-*sn*-glycerol; 100 $\mu\text{mol l}^{-1}$) was found to induce acrosomal loss in 70% of wallaby spermatozoa and 40% of possum spermatozoa after incubation for 120 min. If 10 μmol calcium ionophore l^{-1} was present, the time required to reach these end points was reduced to 30 and 60 min, respectively. The diacylglycerol OAG (1-oleoyl-2-acetyl-*sn*-glycerol; 100 $\mu\text{mol l}^{-1}$) produced some acrosomal loss, particularly when 10 μmol calcium ionophore l^{-1} was present, but when the ionophore was absent, results were equivocal. The other diacylglycerol tested DOG (1,2-dioleoyl-*sn*-glycerol; 100 $\mu\text{mol l}^{-1}$) did not induce significant acrosomal loss in possum or wallaby spermatozoa. The concentration of DiC₈ required to induce acrosomal loss in marsupial spermatozoa (50–100 $\mu\text{mol l}^{-1}$) was relatively high compared with that for placental mammals, suggesting a less specific mode of action than enzyme activation. Of the diacylglycerols tested alone, only DiC₈ led to significant loss of motility. In wallabies, this was detectable after incubation for 60 min and in possums after incubation for 120 min. The ultrastructure of the DiC₈-induced acrosomal loss was essentially identical to the acrosome reaction described for a broad range of placental mammals. A membrane vesicle shroud covered the acrosomal surface of the sperm head. The vesicles appeared to be formed by multiple point fusions between the plasma membrane and the outer acrosomal membrane. The mechanism of DiC₈-induced acrosomal loss remains to be established. However, fusion of plasma membrane with outer acrosomal membrane was evident after incubation for 5 min in the diacylglycerol, long before a significant decline in motility was detected.

Introduction

During fertilization the spermatozoa of placental mammals undergo the acrosome reaction, an exocytotic event required for penetration of the oocyte vestments and fusion with the oocyte plasma membrane (Barros *et al.*, 1967; Yanagimachi and Noda, 1970; Moore and Bedford, 1978). *In vivo*, the acrosome reaction is almost certainly triggered by the zona pellucida, or a cumulus-derived agonist, but the underlying mechanism remains to be unequivocally established (reviewed by Yanagimachi, 1988; Meizel *et al.*, 1990; Saling *et al.*, 1990; Kopf and Gerton, 1991). The acrosome reaction in mammals involves multiple point fusions between the plasma membrane and the adjacent outer acrosomal membrane (Barros *et al.*, 1967; Bedford and Cooper, 1978). Hybrid membrane vesicles are formed, composed of equal proportions of plasma membrane and outer acrosomal membrane (Russell *et al.*, 1979). The equatorial segment of the acrosome does not participate in the acrosome reaction (Barros *et al.*, 1967; Bedford and Cooper, 1978) and the small segment of persisting plasma membrane overlying it is

the site of sperm–oocyte membrane fusion (Bedford *et al.*, 1979; Moore and Bedford, 1983).

The acrosome reaction has not been described for marsupial spermatozoa. The marsupial acrosome is unusually stable and 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 spermatozoa of placental mammals to undergo an acrosome reaction, although their effectiveness varies between species (Santos-Sacchi and Gordon, 1980; Shams-Borhan and Harrison, 1981; Fraser, 1984; Stock and Fraser, 1989; DeJonge *et al.*, 1991). The marsupial acrosome also remains intact after freeze–thawing in the absence of cryoprotectant, and exposure to the detergent Triton X-100 at concentrations that disturb the sperm plasma membrane (Sistina *et al.*, 1993a). Acrosomal damage, in the presence of cryoprotectants, is a common occurrence when the spermatozoa of placental mammals are frozen (reviewed by Watson, 1979), and detergent treatment has been used to induce an acrosome reaction (Yanagimachi, 1975; Wooding, 1975; Trejo and Mujica, 1990).

Marsupial spermatozoa are essentially similar to mammalian spermatozoa in character but differ in several features due to the 90° difference in the plane of nuclear flattening during spermiogenesis (marsupials – dorso-ventral, placentals – lateral; reviewed by Rodger, 1991). The marsupial acrosome lies on the dorsal surface of the sperm head and does not form a cap over

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the leading edge. In the species examined here, the acrosome is a compact 'button' adjacent to the leading edge of the sperm head. However, in other Australian marsupial species, and the American opossums, it extends over a large portion of the dorsal surface of the sperm head (e.g. Harding *et al.*, 1979, 1984; Temple-Smith, 1987). There is no evidence that the marsupial acrosome has a morphologically distinct equatorial segment (Harding *et al.*, 1976; Temple-Smith and Bedford, 1976). However, it is unwise to ignore the possibility of functional subregions since the acrosome reaction has yet to be described for any marsupial spermatozoa.

The present study began by examining the possibility that, although calcium ionophore does not induce marsupial spermatozoa to undergo an acrosome reaction, this might be achieved if the ionophore treatment was accompanied by an appropriate diacylglycerol. This followed on from work (Roldan and Harrison, 1989, 1990a; Harrison and Roldan, 1990; Harrison *et al.*, 1990) which demonstrated a role for the phosphoinositide pathway in the regulation of ionophore-induced acrosome reaction in the spermatozoa of placental mammals.

Materials and Methods

Animals and semen

Adult, common brushtail possums (*Trichosurus vulpecula*) were captured in the Newcastle area and housed as described earlier (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 in grassed yards as described by Mate and Rodger (1991). Animal experimentation was approved by the University of Newcastle Animal Care and Ethics Committee, and the use of protected animals licensed by the New South Wales National Parks and Wildlife Service.

Semen was collected by electroejaculation (Mate and Rodger, 1991) from a pool of four possums and seven wallabies. At least seven days separated successive electroejaculations from any one animal. Spermatozoa were scored for percentage motility (0–100%) and progressive motility (0–5) (Rodger *et al.*, 1991). Ejaculates exhibiting poor sperm motility (% motile < 90%; progressive motility < 4) were discarded. In general, individual sperm samples were used for each experimental replicate, although pooled semen was used on occasions when sperm numbers were low. During the experimental period, possum ejaculates had a sperm concentration of $1.5 \pm 0.4 \times 10^7$ spermatozoa ml^{-1} (mean $\pm$ SEM) and volume of 3.8 ± 0.3 ml ($n = 26$). Possum spermatozoa were washed by the 'swim-up' method as described by Mate and Rodger (1991).

Wallaby semen was collected and washed by the 'swim-up' method without dilution. Briefly, 10–20 ml of wallaby semen was collected into a graduated 50 ml centrifuge tube. A small sample of semen was examined immediately for the presence of spermatozoa. The remainder of the semen was left at room temperature for approximately 30 min to coagulate. Twenty to thirty millilitres of Eagle's minimum essential medium (EMEM) containing 100 iu penicillin ml^{-1} and 0.01 mg streptomycin ml^{-1} at pH 7.4 (Cytosystems Sydney) was placed over the

coagulated semen and incubated at 35°C for 30 min to allow spermatozoa to 'swim-up'. Sperm concentration in the 'swim-up' was $1.3 \pm 0.4 \times 10^6$ wallaby sperm ml^{-1} (mean $\pm$ SEM) in 29.0 $\pm$ 2.9 ml of EMEM ($n = 17$).

Assessment of acrosomal status by light microscopy

Paraformaldehyde-fixed spermatozoa were attached to microscope slides by centrifugation ('Cytospin', Shandon Inc., Pittsburgh) at 210 g for 3 min (Mate and Rodger, 1991) and then stained by the Bryan's technique (Bryan, 1970). Briefly, spermatozoa were fixed for 10 min by adding 0.1–0.5 ml sperm suspension (depending on sperm concentration) to 2 ml 2% paraformaldehyde in PBS (pH 7.4). Acrosomes were visualized by staining for 4 min in Bryan's stain (0.1% eosin Y, Sigma No. E4382; 0.1% fast green FCF, Sigma No. F7258; 0.1% flavianic acid, Sigma No. F6500 in 1% acetic acid: all from Sigma, St Louis, MO). Data were obtained from 600 possum or wallaby spermatozoa per treatment (three slides: 200 spermatozoa per slide). Statistical analysis of acrosome and motility data was by Student's *t* test after square root transformation.

Assessment of acrosomal status by transmission electron microscopy

Treated spermatozoa were fixed in 2/3 Superfix or Karnovsky's fixative for 10 min and then centrifuged at 750 g for 10 min to form sperm pellets. The fixation and processing of sperm pellets for electron microscopy has been described (Mate and Rodger, 1991). Thin sections were cut using an Ultracut E (Reichert-Jung, Vienna) ultramicrotome and stained in uranyl acetate (10 min), lead citrate (10 min) and then examined in a Jeol 100-CX electron microscope.

Diacylglycerol treatments

The diacylglycerols (1,2-dioctanoyl-*sn* glycerol (DiC₈); 1,2-dioleoyl-*sn*-glycerol (DOG); and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG)) tested were obtained from the Sigma. Where possible diacylglycerols were transported frozen to minimize breakdown. Stock solutions of diacylglycerol were made up in dimethylsulfoxide (DMSO) at 10 mmol l^{-1} and stored frozen in individual aliquots (containers wrapped in aluminium foil) until required. Calcium ionophore (A23187) was dissolved in DMSO at 1 mg ml^{-1} and stored frozen until required. The reagents were made up at twice the final concentration to be tested in EMEM. Each test solution was mixed 1:1 with the washed sperm suspension and incubated at 35°C in 5% CO₂ in air. Control spermatozoa were treated with EMEM containing DMSO at the same concentration as experimental treatments.

Results

Acrosomal status of marsupial spermatozoa in the presence of diacylglycerols plus calcium ionophore

Acrosomal loss was evident when possum and wallaby spermatozoa were incubated in the presence of calcium ionophore plus DiC₈ or OAG (Fig. 1). DiC₈ was the more potent agent and

Fig. 1. The percentage of spermatozoa undergoing acrosome loss, as assessed by Bryan's staining, of (a, c and e) common brushtail possums (*Trichosurus vulpecula*) and (b, d and f) tammar wallabies (*Macropus eugenii*) after incubation for 30 or 60 min. The incubation medium contained 10 $\mu\text{mol l}^{-1}$ calcium ionophore (A23187) plus 50 $\mu\text{mol l}^{-1}$ (●) or 100 $\mu\text{mol l}^{-1}$ (■) of the diacylglycerols: (a, b) DOG (1,2-dioleoyl-*sn*-glycerol); (c, d) OAG (1-oleoyl-2-acetyl-*sn*-glycerol) and (e, f) DiC₈ (1,2-dioctanoyl-*sn*-glycerol). (○) DMSO controls. Significantly different from control: * $P < 0.01$, ** $P < 0.001$.

wallaby spermatozoa were the more sensitive. Acrosomal loss did not occur when spermatozoa were incubated with DOG plus ionophore (Fig. 1).

Ten and thirty per cent acrosomal loss was seen after incubation of possum spermatozoa for 30 min with 50 $\mu\text{mol l}^{-1}$ ($P = 0.05$) and 100 $\mu\text{mol l}^{-1}$ ($P < 0.01$) DiC₈, respectively. After 60 min incubation, acrosomal loss increased to 20% ($P < 0.01$) and 45% ($P < 0.001$), respectively. Possum spermatozoa incubated with 50 $\mu\text{mol l}^{-1}$ OAG plus ionophore showed no acrosomal loss after incubation for 30 min, but after 60 min exhibited similar acrosomal loss (15% and 25%) to that found with DiC₈ plus ionophore after 30 min ($P < 0.01$). Twenty-five per cent ($P < 0.01$) and 70% ($P < 0.001$) of wallaby spermatozoa had lost their acrosome after 30 min incubation with 50 or 100 $\mu\text{mol l}^{-1}$ DiC₈, respectively, and there was little change after incubation for a further 30 min. Wallaby spermatozoa incubated with OAG plus ionophore showed no significant acrosomal loss after 30 min, but significant (30 to 40%) acrosomal loss was found after 60 min (50 $\mu\text{mol l}^{-1}$, $P = 0.01$; 100 $\mu\text{mol l}^{-1}$ $P < 0.001$).

Acrosomal status after incubation with diacylglycerols alone

When possum and wallaby spermatozoa were treated with 100 $\mu\text{mol l}^{-1}$ DOG, OAG and DiC₈ without calcium ionophore

Fig. 2. The percentage of spermatozoa undergoing acrosome loss, as assessed by Bryan's staining, of (a, c and e) common brushtail possums (*Trichosurus vulpecula*) and (b, d and f) tammar wallabies (*Macropus eugenii*) after incubation for 60 or 120 min. The incubation medium contained 100 $\mu\text{mol l}^{-1}$ (●) of the diacylglycerols: (a, b) DOG (1,2-dioleoyl-*sn*-glycerol); (c, d) OAG (1-oleoyl-2-acetyl-*sn*-glycerol) and (e, f) DiC₈ (1,2-dioctanoyl-*sn*-glycerol). (○) DMSO controls. Significantly different from control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Fig. 2), an essentially similar pattern of acrosomal loss was seen to that found when 10 $\mu\text{mol l}^{-1}$ ionophore was present (Fig. 1). However, similar acrosomal loss was only achieved with DiC₈ (40% possum; 70% wallaby) after a longer period of incubation (120 min).

Twenty-five per cent of possum spermatozoa had lost their acrosome after incubation for 60 min with DiC₈ ($P < 0.05$) and 40% after 120 min ($P < 0.05$). Forty per cent of wallaby spermatozoa had lost their acrosome after 60 min ($P < 0.01$) and 65% after 120 min ($P < 0.001$). DOG and OAG did not induce significant acrosomal loss in the absence of ionophore.

Sperm motility in the presence of the diacylglycerols plus calcium ionophore

Spermatozoa treated with each of the three diacylglycerols and calcium ionophore were essentially immotile when examined after incubation for 30 min (data not shown), although only 100 $\mu\text{mol l}^{-1}$ DiC₈ had induced statistically significant acrosomal loss by that time (Fig. 1). In a further experiment, the effect of ionophore on motility loss was examined (Fig. 3). There was no significant loss of motility when wallaby spermatozoa were incubated for 28 min in the presence of 100 $\mu\text{mol l}^{-1}$ DiC₈ and 1 $\mu\text{mol l}^{-1}$ calcium ionophore. However, the motility of possum and wallaby spermatozoa had declined significantly after 7 and 14 min, respectively, of incubation with DiC₈ plus 5 or 10 $\mu\text{mol l}^{-1}$ ionophore.

Fig. 3. The percentage of spermatozoa from (a) common brushtail possums (*Trichosurus vulpecula*) and (b) tammar wallabies (*Macropus eugenii*) that were motile after incubation for 7, 14, 21 or 28 min with 100 μmol l⁻¹ of the diacylglycerol DiC₈ (1,2-dioctanoyl-*sn*-glycerol) plus 1 μmol l⁻¹ (▼; wallabies only), 5 μmol l⁻¹ (●) or 10 μmol l⁻¹ (■) calcium ionophore (A23187). (○, □) DMSO controls. Significantly different from controls: **P* < 0.05, ***P* < 0.01 or ****P* < 0.001.

Acrosomal loss was correlated with a decline in motility (Fig. 4). Possum spermatozoa incubated with 100 μmol DiC₈ l⁻¹ plus 5 μmol calcium ionophore l⁻¹ exhibited statistically significant acrosomal loss and a decline in percentage motility after 7 min. Regression of percentage acrosome loss against percentage motility for possum data (Fig. 4) had a correlation coefficient of 0.99. Wallaby spermatozoa incubated with 100 μmol DiC₈ l⁻¹ plus 5 μmol ionophore l⁻¹ exhibited the same pattern; however, significant acrosomal loss was seen after 7 min (*P* < 0.001), while a significant decline in motility required 14 min of incubation. Regression of percentage acrosome loss against percentage motility for wallaby data (Fig. 4) had a correlation coefficient of 0.83.

Motility of spermatozoa incubated with diacylglycerols alone

The motility of possum spermatozoa treated with 100 μmol DiC₈ l⁻¹ differed from controls after 120 min of incubation (*P* < 0.05; Fig. 5). Although motility of possum spermatozoa declined to about 40% after incubation for 120 min, the motility of spermatozoa treated with 100 μmol DOG l⁻¹ or 100 μmol OAG l⁻¹ did not differ from DMSO controls. Incubation of wallaby spermatozoa in the presence of 100 μmol DiC₈ l⁻¹ led to significant loss of motility by 60 min (*P* < 0.01) and essentially all spermatozoa were immotile after 120 min (*P* < 0.01). DMSO-treated control wallaby spermatozoa

Fig. 4. Percentage acrosome loss (●) and percentage motility (▼) of spermatozoa from (a) common brushtail possums (*Trichosurus vulpecula*) and (b) tammar wallabies (*Macropus eugenii*) after incubation for 7, 14, 21 or 28 min with 100 μmol l⁻¹ of the diacylglycerol DiC₈ (1,2-dioctanoyl-*sn*-glycerol) plus 5 μmol calcium ionophore l⁻¹ (A23187). (○, ▽) are DMSO controls. Possum acrosome loss and sperm motility are significantly different from controls after 7 min (*P* < 0.001). Wallaby acrosome loss is significantly different from control after 7 min (*P* < 0.001). Wallaby sperm motility is significantly different from controls after 14 min (14 min *P* < 0.001; 21 min *P* < 0.05; 28 min *P* < 0.01).

showed little loss of motility after incubation for 120 min and the motility of spermatozoa treated with 100 μmol DOG l⁻¹ and 100 μmol OAG l⁻¹ did not differ from controls.

Ultrastructure of DiC₈-induced acrosomal loss

The ultrastructure of the sperm head and its overlying plasma membrane appeared normal in DMSO-treated control spermatozoa (Fig. 6a). The surface of the acrosome of spermatozoa incubated for 5 min in 100 μmol DiC₈ l⁻¹ was covered by many membrane vesicles (Fig. 6b). The morphology of the vesicles, and the more osmophilic character of the membrane component adjacent to the acrosome (Fig. 6b insert), suggested that they were a hybrid of plasma membrane and outer acrosomal membrane produced by multiple point fusions. Membrane vesiculation was limited to the acrosomal region; the plasma membrane over the remainder of the surface of most spermatozoa remained intact (Fig. 7). After 10 or 30 min incubation in 100 μmol DiC₈ l⁻¹, the acrosomal matrices were either highly vesiculated, or the acrosome contents and overlying plasma membrane were lost. The inner acrosomal membrane and the plasma membrane over the non-acrosomal surface remained intact in

Fig. 5. The percentage of spermatozoa of (a) common brushtail possums (*Trichosurus vulpecula*) and (b) tammar wallabies (*Macropus eugenii*) that were motile after incubation for 60 or 120 min with 100 $\mu\text{mol l}^{-1}$ of the diacylglycerols: (▼) DOG (1,2-dioleoyl-*sn*-glycerol); (■) OAG (1-oleoyl-2-acetyl-*sn*-glycerol) and (▲) DiC₈ (1,2-Dioctanoyl-*sn*-glycerol). (○) DMSO controls. Significantly different from controls: * $P < 0.05$, ** $P < 0.01$.

most spermatozoa that had lost the acrosome. Incubation with DiC₈ and 10 μmol calcium ionophore A23187 l^{-1} did not alter this pattern of ultrastructural change, but did result in more generalized cellular disruption than when spermatozoa were incubated with DiC₈ alone.

Discussion

The diacylglycerol DiC₈ (1,2-dioctanoyl-*sn*-glycerol) was found to induce acrosomal loss in 70% of wallaby spermatozoa and 40% of possum spermatozoa after incubation for 120 min. If 10 μmol calcium ionophore l^{-1} were present, the time required to reach these end points was reduced to 30 and 60 min, respectively. The diacylglycerol OAG (1-oleoyl-2-acetyl-*sn*-glycerol) produced some acrosomal loss when 10 μmol calcium ionophore l^{-1} was present, but not when ionophore was absent. The other diacylglycerol tested, DOG (1,2-dioleoyl-*sn*-glycerol), did not induce significant acrosomal loss in possum or wallaby spermatozoa, alone, or in combination with ionophore.

The finding that the three diacylglycerols tested in this study differed in their ability to induce acrosomal loss is consistent with previous studies. Diacylglycerol analogues differ in their ability to induce biological responses because derivatives with short, unsaturated fatty acid side chains compete for the binding sites of protein kinase C more effectively than those with satu-

Fig. 6. Electron micrographs of the anterior head of spermatozoa from tammar wallabies (*Macropus eugenii*) spermatozoa. pm: plasma membrane; oam: outer acrosomal membrane; iam: inner acrosomal membrane. (a) DMSO control incubated for 5 min. The intact plasma membrane is loosely applied to the outer acrosomal membrane on the smooth convex surface of the acrosome. (b) Spermatozoa incubated with 100 $\mu\text{mol l}^{-1}$ (1,2-dioctanoyl-*sn*-glycerol) for 5 min. The plasma membrane and outer acrosomal membranes have undergone multiple point fusions. There is also evidence of the early stages of vesiculation of the acrosomal matrix. (b Insert) Detail of plasma membrane outer acrosomal membrane point fusion. Note the difference in electron density of the two membranes. Scale bars: 0.5 μm (insert bar 0.07 μm).

rated fatty acid side chains (Ebeling *et al.*, 1985; Leach and Blumberg, 1989). However, the concentrations of DiC₈ required to induce the acrosome reaction in marsupial spermatozoa (50–100 $\mu\text{mol l}^{-1}$) were high compared with the range of concentrations used in other studies (0.1–100 $\mu\text{mol l}^{-1}$; Kramer *et al.*, 1987; Lee *et al.*, 1987; Burch, 1988; Roldan and Harrison, 1990b), suggesting a mode of action other than enzyme activation.

Preincubation of wallaby spermatozoa with suboptimal concentrations of DiC₈ (10 $\mu\text{mol l}^{-1}$ or 20 $\mu\text{mol l}^{-1}$) before addition of A23187 (10 $\mu\text{mol l}^{-1}$) led to little acrosomal loss ($10.5 \pm 3\%$; 60 min each treatment). This was not statistically different from spermatozoa incubated in 10 or 20 $\mu\text{mol l}^{-1}$ DiC₈ alone (data not shown). No significant acrosomal loss was detected when possum or wallaby spermatozoa were treated

Fig. 7. Electron micrographs of spermatozoa from tammar wallabies (*Macropus eugenii*) after incubation in $100 \mu\text{mol DiC}_8 \text{ l}^{-1}$ (1,2-dioctanoyl-*sn*-glycerol) for 5 min. The main figure is of sections of sperm heads and mid-pieces (scale bar: $1.0 \mu\text{m}$). The insert is a higher power detail of a region of the principal piece of a sperm tail from the same treatment group (scale bar: $0.25 \mu\text{m}$). A: acrosome; M: mitochondria of the mid-piece; arrowheads: plasma membrane. In the sperm heads the plasma membrane overlying the acrosome is undergoing vesiculation due to multiple point fusions with the underlying outer acrosomal membrane and there is some vesiculation of the acrosomal matrices. The non-acrosomal plasma membrane of the sperm heads and the plasma membrane of the mid-pieces and principal piece are intact without any evidence of vesiculation or fusion.

with $10 \mu\text{mol A23187 l}^{-1}$ alone for 1–4 h (data not shown). These findings agree with earlier work from this laboratory which found that acrosomal loss by possum and wallaby spermatozoa was not induced by A23187 (Mate and Rodger, 1991).

Sperm motility was rapidly compromised in the presence of calcium ionophore (5 or $10 \mu\text{mol l}^{-1}$) after incubation for 28 min, and it is unlikely that many spermatozoa were viable after incubation for 30 min. However, despite the deleterious effects of ionophore treatment, a significant number of wallaby spermatozoa had lost their acrosome (7 min; Fig. 4b) before a significant decline in motility was detected (14 min; Fig. 4b). The statistically significant acrosomal loss, found for possum and wallaby spermatozoa incubated with OAG and ionophore for 60 min, is likely to reflect major cellular disruption rather than a diacylglycerol/ionophore-induced acrosome reaction. Of the three diacylglycerols tested, only DiC_8 led to significant loss of motility when used without A23187. However, this occurred only after relatively long incubation periods (wallaby: 60 min; possum: 120 min; data not shown).

The ultrastructure of the acrosomal loss induced by DiC_8 , point fusions of the outer acrosomal membrane and overlying plasma membrane, was essentially identical to the physiological acrosome reaction described for a broad range of placental mammals (reviewed by Yanagimachi, 1988). The ultrastructure of the vesiculation observed in wallaby spermatozoa treated with DiC_8 suggested that, as in placental mammals, they were formed of hybrids of plasma membrane and outer acrosomal membrane. Onset of point fusion between the outer acrosomal membrane and its overlying plasma membrane was detected after incubation for 5 min with DiC_8 . Significant loss of motility was not detected until 30 min exposure to the diacylglycerol.

After 30 min incubation, most spermatozoa had lost all traces of the outer acrosomal membrane and acrosomal matrix, although the inner acrosomal membrane and adjacent nuclear envelope were intact (a detailed ultrastructural examination of the DiC_8 -induced acrosomal loss will be the subject of a subsequent paper). The non-acrosomal plasma membrane of spermatozoa undergoing acrosomal loss remained intact.

The manner of acrosomal loss induced by DiC_8 treatment was quite different from that seen when possum and wallaby spermatozoa were treated with the detergent Triton X-100 (Sistina et al., 1993a) or lysophosphatidylcholine (Sistina et al., 1993b). In these cases, the first structure disturbed was the plasma membrane and it was affected over the entire surface of the spermatozoa. Disruption of the acrosomal matrix and membranes followed, but there was no evidence of plasma membrane–outer acrosomal membrane fusion. These studies and our earlier work on marsupial spermatozoa have demonstrated that the acrosome and its membrane are unusually stable compared with the acrosomes of the spermatozoa of placental mammals (Mate and Rodger, 1991; Rodger et al., 1991). We have not observed outer acrosomal membrane instability/fusogenicity in any of our previous work.

The mechanism of acrosomal loss induced by DiC_8 in marsupial spermatozoa remains unclear. The effective concentrations of DiC_8 were much higher than those used in studies of the acrosome reaction in placental mammals and in other phosphoinositide pathway systems (e.g. Roldan and Harrison, 1990b). Yet the morphology of acrosomal loss induced mimicked that seen in the physiological acrosome reaction of placental mammals and not that seen when the marsupial acrosome is lost after disruptive treatments (Sistina et al., 1993a, b). A companion paper (Mate and Rodger, 1993) has examined the mechanism of the DiC_8 -induced acrosomal loss and concluded that it was most likely that the diacylglycerol acted as a direct membrane fusogen.

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