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Cannabinoids Inhibit Fertilization in Sea Urchins by Reducing the Fertilizing Capacity of Sperm

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SCHUEL, H., M. C. CHANG, D. BERKERY, R. SCHUEL, A. M. ZIMMERMAN AND S. ZIMMERMAN. *Cannabinoids inhibit fertilization in sea urchins by reducing the fertilizing capacity of sperm.* PHARMACOL BIOCHEM BEHAV **40**(3) 609–615, 1991.—Delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN) inhibit fertilization in the sea urchin *Strongylocentrotus purpuratus* by reducing the fertilizing capacity of the sperm. Sperm fertility depends upon their motility, and their capacity to undergo the acrosome reaction upon encountering a specific ligand derived from the egg's jelly coat. The acrosome reaction involves exocytosis of the acrosomal granule at the apex of the sperm head and elongation of the acrosomal filament. This process exposes the sperm membrane that will attach to and fuse with the egg. Pretreatment of sperm with THC prevents the triggering of the acrosome reaction by solubilized egg jelly in a dose and time dependent manner. Motility of THC-treated sperm is not reduced compared to control sperm in sea water or vehicle dissolved in sea water. The adverse effects of THC on the acrosome reaction and sperm-fertilizing capacity are reversible. Studies with ionophores suggest that THC blocks the acrosome reaction by affecting event(s) in the stimulation-secretion coupling mechanism in the sperm preceding the opening of ion channels. Ultrastructural studies show that THC, CBD and CBN block the membrane fusion reaction between the sperm's plasma membrane and the acrosomal membrane that normally is elicited in response to stimulation by egg jelly to initiate the acrosome reaction. However, lipid deposits are found in the subacrosomal and centriolar fossae of cannabinoid treated sperm. The nuclear envelope is fragmented in close proximity to the lipid deposits within the subacrosomal fossa. These morphological observations suggest that cannabinoids may activate phospholipase(s) within the sperm. Biochemical studies show that THC activates phospholipase A_2 activity in sperm homogenates. Our studies show that cannabinoids reduce the fertilizing capacity of sea urchin sperm by preventing the induction of the acrosome reaction by egg jelly. The localized fragmentation of cellular membranes and the formation of lipid deposits within the sperm may be due to activation of phospholipase A_2 by cannabinoids. Metabolites derived from this membrane perturbation may inhibit triggering of the acrosome reaction by egg jelly and thereby inhibit fertilization.

Cannabinoids Delta-9-tetrahydrocannabinol Cannabidiol Cannabinol Sea urchin Sperm
Acrosome reaction Egg jelly coat Fertilization Marihuana Phospholipase A_2 Ionophores

IN mammalian systems, cannabinoids affect reproductive functions in both males and females. The effects include reduced secretion of pituitary gonadotropic hormones (2), suppression of ovulation in monkeys and rats (18,29), suppression of testosterone secretion in mice (12), reduction of sperm count and concentration of sperm in man (21), an increase in the incidence of abnormal sperm heads in mice (45), and a reduction in pregnancies carried to term (1). In studies on human populations of marihuana users or experiments on laboratory mammals, it is not possible to discriminate between reduced fertility resulting from a direct effect on fertilization and indirect effects on other reproductive functions or behaviors.

We are using sea urchin gametes as an in vitro model system to study the direct effects of cannabinoids on fertilization (7–8, 35, 37). Fertilization and embryonic development in sea urchins normally takes place externally in sea water. Large quantities of gametes can be readily collected from adult animals. The eggs undergo synchronous fertilization and development following insemination in vitro. These features make it possible to directly treat eggs and sperm in vitro with a drug to assess its effects on fertilization under carefully controlled laboratory conditions.

Fertilization is a process that begins with interaction of sperm with the egg's external coats and concludes with fusion of the male and female pronuclei within the fertilized egg (33,42). The mature sea urchin egg is surrounded by a jelly coat (comparable to the matrix of the cumulus oophorus in mammalian eggs). The vitelline envelope (comparable to the zona pellucida in mammals) is attached to the outer surface of the egg's plasma membrane, and contains species specific sperm receptors. Sea urchin sperm begin to swim actively upon spawning into sea water. Sea urchin sperm do not require capacitation as do mammalian sperm before fertilization can take place. Gamete interaction begins when sperm encounter components of the jelly coat which triggers the acrosome reaction. Acrosome-reacted sperm combine with receptors on the egg surface to activate the egg within 1–10 seconds after insemination in sea urchins (34). The activated sea urchin egg responds by elevating a fertilization envelope within a minute after insemination (33,42). The process of fertilization

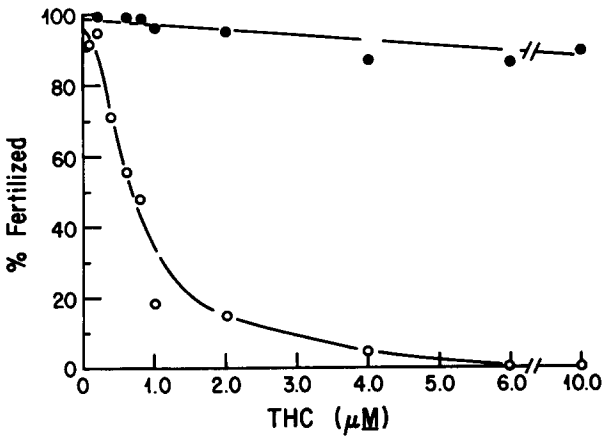

FIG. 1. Effect of pretreatment with THC (0.1–10 μM) for 5 min on fertility of *S. purpuratus* gametes. Egg pretreatment (●). Sperm pretreatment (○). N = 6 experiments. (37)

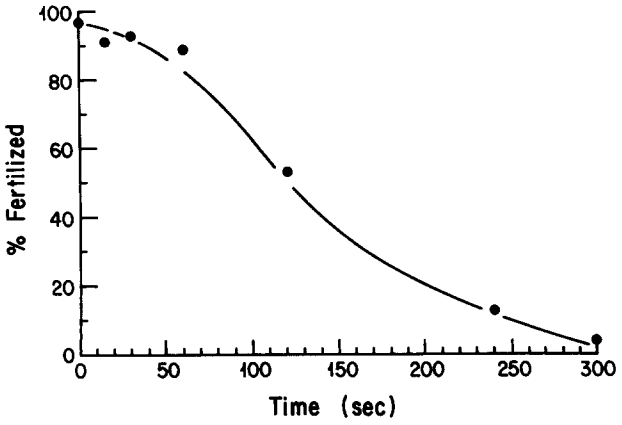

FIG. 2. Effect of duration of pretreatment with 10 μM THC on fertilizing capacity of sperm. A minimum sperm density was pretreated for 0–300 s in 5.0 ml of 10 μM THC prior to addition of untreated unfertilized eggs to the cultures. Zero time: eggs and sperm added simultaneously to 5.0 ml of 10 μM THC. N = 3 experiments. (37)

is completed in sea urchins with the fusion of the male and female pronuclei at 10–15 minutes after insemination.

Cannabinoids Inhibit Fertilization

We studied the effects of delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN) on fertilization in the sea urchin *Strongylocentrotus purpuratus* (37). The effects of THC on gamete fertility were assessed by pretreating eggs or sperm with THC (0.1–10 μM) for 5 min prior to insemination (Fig. 1). We used a minimal sperm density (0.6 to 1.8×10^6 cells/ml) that was just sufficient to fertilize 90–100% of the eggs in sea water. The elevation of the fertilization envelope normally is completed in fertilized eggs within 60 s after insemination (33). The percent of eggs fertilized was determined by scoring eggs with elevated fertilization envelopes in egg cultures fixed at 10 minutes postinsemination. A minimum of 100 eggs were counted at random from each culture. THC-pretreated eggs did not show a reduction in receptivity to untreated sperm. However, pretreatment of sperm with THC reduced their capacity to fertilize untreated eggs. The concentration of THC that reduced sperm fertility by 50% was 1.1 ± 1.1 μM. At 10 μM THC, sperm fertility was reduced by 99.8 ± 0.4%. In all of our studies, vehicle controls containing equivalent amounts of ethanol and propylene glycol dissolved in sea water did not significantly affect fertilization or sperm function.

The reduction in the fertilizing capacity of sperm as a result of duration of pretreatment with 10 μM THC was evaluated. Minimal sperm densities were pretreated with 10 μM THC for 15–300 s prior to the addition of untreated eggs to the cultures. The fertilizing capacity of the sperm was reduced with increasing exposure time to 10 μM THC (Fig. 2). The pretreatment time required to reduce sperm fertility by 50% was 129.3 ± 43.0 s.

We also found that CBD and CBN inhibit fertilization by reducing the fertilizing capacity of sperm. The adverse effect of THC on sperm fertilizing capacity was reversible. Sperm in THC swam as actively as controls incubated in sea water or sea water containing vehicle for several hours.

These observations show that THC, CBD and CBN directly affect the process of fertilization. Cannabinoids inhibit fertiliza-

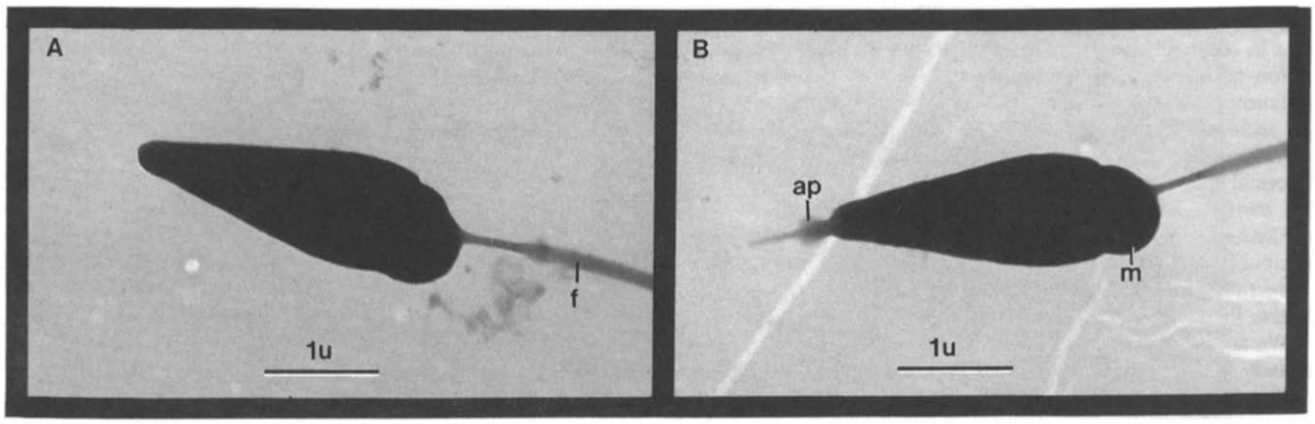

FIG. 3. Transmission electron micrographs of whole mount preparations of sperm on formvar coated grids. Acrosomal process (ap); flagellum (f); mitochondrion (m). (35) (A) Unreacted sperm fixed after incubation in sea water. (B) Acrosome reacted sperm fixed 3 min after exposure to solubilized egg jelly (1.2 μg fucose/ml final). The acrosomal process (ap) is present at the apex of the sperm head.

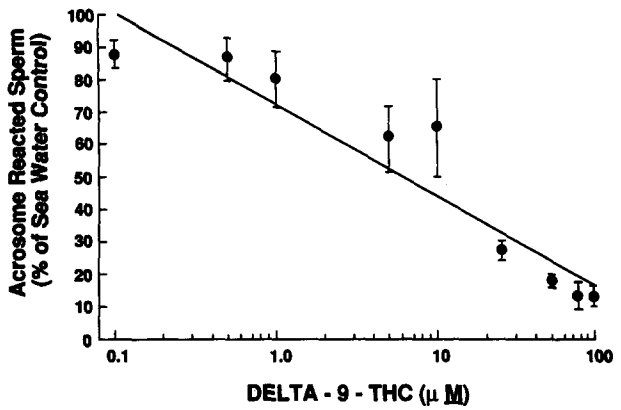

FIG. 4. Effect of pretreatment with 0.1 to 100 μM THC for 5 min on induction of the acrosome reaction by egg jelly. Sperm were fixed 3 min after stimulation with solubilized egg jelly (1.2 μg fucose/ml final). Sperm density: $2.7 \pm 0.1 \times 10^8$ cells/ml. Data points: mean values ± SEM. N = 9 experiments. Coefficient of regression: .89. (35)

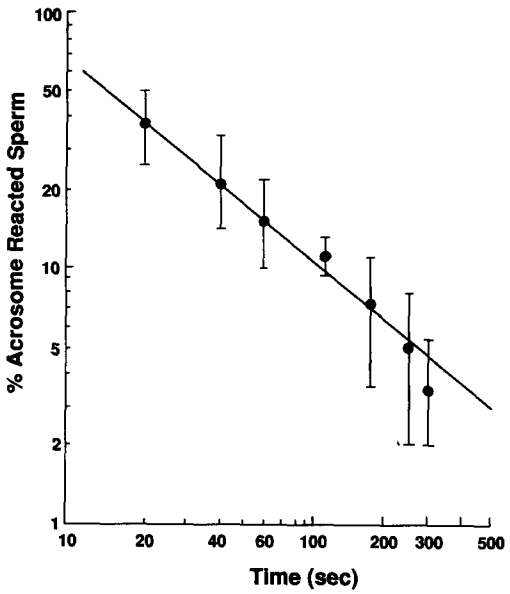

FIG. 5. Effect of duration of pretreatment with 100 μM THC on induction of the acrosome reaction by egg jelly. Data points: mean values ± SEM. N = 7 experiments. Coefficient of regression: .98. (35)

tion in the sea urchin by reducing the fertilizing capacity of the sperm. The receptivity of the eggs to sperm does not appear to be affected. Cannabinoids are thought to affect cell function by perturbing phospholipids in biological membranes (22,23) or by reacting with a specific cannabinoid receptor (15,26). Similar factors may account for the differences in the responses of the sea urchin sperm and egg to cannabinoids.

Cannabinoids Inhibit the Acrosome Reaction

Sperm fertility depends upon: 1) their motility; and 2) the acrosome reaction which enables the sperm to attach to, activate, and penetrate the egg. Fertilization in sea urchins normally takes place within seconds after insemination (33,34). Since sperm swim actively in THC for several hours, it is unlikely that impaired motility could account for their inability to fertilize eggs.

The acrosome reaction involves exocytosis of the acrosomal granule at the apex of the sperm head and formation of the acrosomal process by polymerization of G-actin in the subacrosomal fossa into F-actin. The acrosome reaction is essential for fertilization since it exposes the sperm membrane at the apex of the acrosomal process that will attach to and fuse with the egg (13,41). The acrosome reaction normally is triggered during gamete interaction at fertilization by a ligand (fucose-sulfate glycoconjugate) derived from the egg's jelly coat with a species specific receptor on the sperm surface (25,39).

Physiological studies. We studied the effects of cannabinoids on the triggering of the acrosome reaction by solubilized egg jelly (35). Sperm ($2.5–3.0 \times 10^8$ cells/ml) were pretreated with THC (0.1–100 μM) for 0–5 min before the addition of solubilized egg jelly. The sperm cultures were fixed 3 min later. The acrosome reaction was assayed by examining whole mount preparations of sperm in a transmission electron microscope (14). The incidence of acrosome reacted sperm was scored on the basis of the presence of the acrosomal process at the apex of the sperm (IC_{50}) head (Fig. 3). A minimum of 100 sperm cells was counted at random from each culture. Pretreatment of sperm with THC for 5 minutes produced a concentration dependent inhibition of the acrosome reaction (Fig. 4). The concentration of THC required to prevent the acrosome reaction in 50% of treated sperm (IC_{50}) compared to sea water controls was estimated to be 6.6 μM based on analysis of the data by curvilinear regression. The IC_{50} for blockade of the acrosome reaction by THC is within the same order of magnitude as the IC_{50} to reduce sperm fertilizing capacity. Pretreatment of sperm with 100 μM THC significantly reduced the egg jelly induced acrosome reaction by $88.9 \pm 2.3\%$ ($p<0.01$) compared to vehicle pretreated controls (N = 16 experiments). Under these conditions, vehicle did not significantly affect the capacity of the sperm to undergo the acrosome reaction upon subsequent stimulation by soluble egg jelly compared to sea water pretreated controls ($p<0.4$). The time dependence was evaluated by pretreating sperm with 100 μM THC for 0–5 min prior to addition of egg jelly to the cultures (Fig. 5). THC significantly inhibited the acrosome reaction by 33% ($p<0.02$) at zero time (simultaneous exposure of sperm to THC and egg jelly), and by 93% ($p<0.01$) after 5 min pretreatment (data not shown). The pretreatment time required for 50% inhibition of the acrosome reaction with 100 μM THC was estimated to be 20.8 s compared to sea water controls at zero time (Fig. 5). These results show that the onset of the blockade of the jelly-induced acrosome reaction by THC is very rapid and increases progressively with preincubation time. Sperm cultured in 100 μM THC for 8 minutes were observed to swim as actively as control sperm cultured in sea water or vehicle. CBD and CBN inhibited the acrosome reaction in a manner similar to THC (35).

We evaluated the reversibility of the adverse effects of THC on the acrosome reaction and sperm fertilizing capacity. When sperm were pretreated with 100 μM THC to suppress the triggering of the acrosome reaction by egg jelly in over 90% of treated sperm (see Fig. 4), there was a corresponding reduction in their capacity to fertilize eggs. The capacity of the sperm to undergo the acrosome reaction upon stimulation by egg jelly was restored to normal levels along with their capacity to fertilize eggs within 5–10 minutes after THC was removed (35).

Ionophores are known to bypass the receptor-ligand reaction with egg jelly to trigger the acrosome reaction in sperm by opening ion channels associated with the stimulation response coupling mechanism: calcium influx is promoted by A23187 and

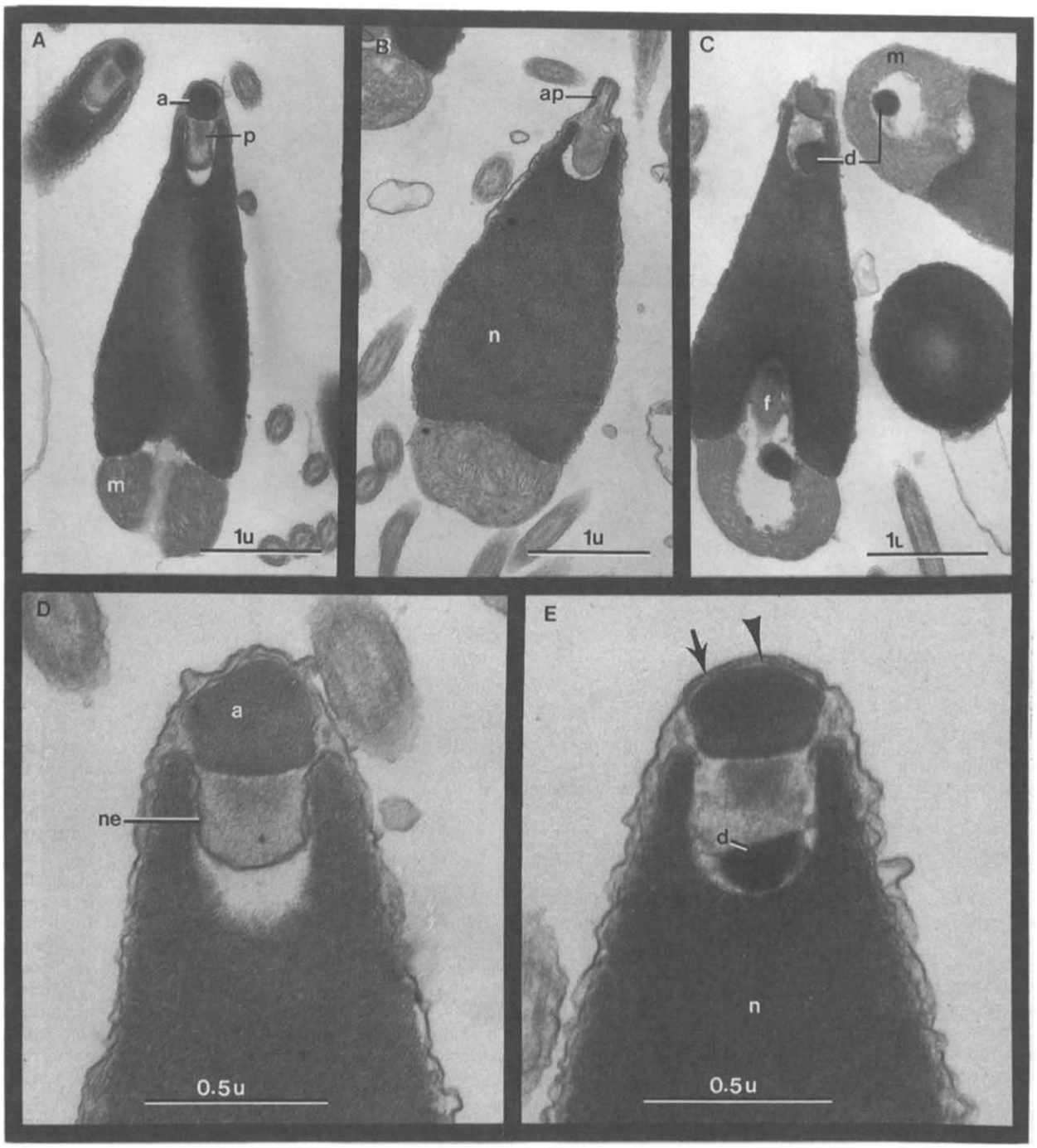

FIG. 6. Ultrastructure of sperm fixed after incubation in: vehicle dissolved in sea water (A and D); vehicle dissolved in sea water and stimulated with egg jelly (B); 100 μM THC and stimulated with egg jelly (C and E). Nucleus (n); Mitochondrion (m); Flagellum (f); Acrosome (a); Acrosomal process (ap); Profilactin (monomeric actin) in subacrosomal fossa (p); Nuclear envelope (ne); Electron dense deposit (d); Plasma membrane (arrow); Acrosomal membrane (arrow head) (7).

ionomycin (20,31); potassium efflux is promoted by nigericin (32); NH_4OH elevates the internal pH of the sperm (12). Monensin causes sodium influx but does not by itself induce the acrosome reaction (20). THC did not inhibit the acrosome reaction induced by 10 μM ionomycin and 50 μM nigericin, but partially inhibited (20–30%) the acrosome reaction induced by 50 μM A23187 and 2 mM NH_4OH. Addition of monensin (25 μM) to egg jelly or to A23187 did not overcome the THC inhibition. We conclude that THC affects event(s) in the stimulus-response coupling mechanism of the sperm that precede the opening of ion channels (35).

Taken together, these findings show that cannabinoids reduce the fertilizing capacity of sperm by blocking the triggering of the acrosome reaction by egg jelly.

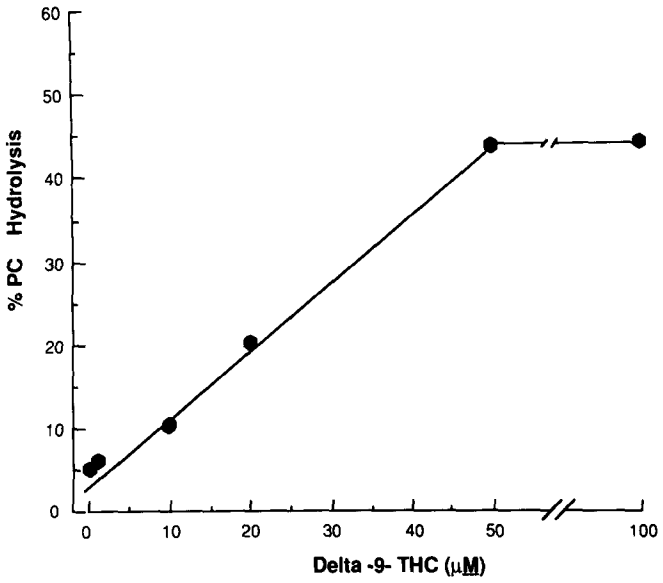

FIG. 7. Activation of phospholipase A_2 activity in sperm homogenate by THC (1–100 μM). Sperm were sonicated in 0.3 M sucrose containing 10 mM HEPES at pH 7.4. Enzyme assay contained 1-stearoyl-2-[1-^{14}C] arachidonyl phosphatidylcholine as substrate, 125 mM Tris buffer at pH 8.0, and sperm homogenate (100 μg protein). Free radioactive arachidonic acid assayed after 30 min incubation at 17°C. N = 4 experiments. (8)

Ultrastructural studies We conducted ultrastructural studies to gain further insight into the mechanism of action of cannabinoids on the acrosome reaction (7). Sperm were pretreated with 100 μM THC for 5 min, stimulated with egg jelly, and fixed 3 min later (Fig. 6). This concentration of THC was used initially because it blocked the acrosome reaction in over 90% of treated sperm (see Fig. 4). In control sperm that were not stimulated with egg jelly, the plasma membrane, acrosomal membrane, and the nuclear envelope are intact (Fig. 6A). The acrosome reaction (exocytosis of the acrosomal granule and elongation of the acrosomal process at the apex of the sperm head) has been completed in control sperm following stimulation with egg jelly (Fig. 6B). In THC-pretreated sperm that are stimulated by egg jelly, the acrosomal granule is intact and there is no sign of elongation of the acrosomal process (Fig. 6C). However, electron dense deposits are present within the subacrosomal and centriolar fossae of the sperm. The acrosomal membrane and the plasma membrane in THC-treated sperm are morphologically intact (Fig. 6E) and similar to those seen in unstimulated control sperm (Fig. 6D). The electron dense deposit in THC-treated sperm is adjacent to the fragmented nuclear envelope within the subacrosomal fossa (Fig. 6E). The electron dense deposits are lipid because they lack a limiting membrane, are extractable with chloroform:methanol (2:1) which is known to remove lipids from cells (19), and stain positively for lipid with thymol and farnesol (44). CBD and CBN produced similar morphological effects on sperm (7).

The localized fragmentation of the nuclear envelope and the lipid deposits in the subacrosomal and centriolar fossae were seen in sperm after treatment with 5–100 μM THC for 1–10 minutes. The presence of lipid deposits in sperm treated with 5 μM THC for 5 minutes and with 100 μM THC for 1 minute is consistent with conditions required to inhibit the egg jelly-induced acrosome reaction in 50% of treated sperm. The lipid deposits disappeared from THC-treated sperm after the drug was removed by washing under experimental conditions where the sperm regained their capacity to undergo the acrosome reaction and to fertilize eggs.

These morphological studies suggest that cannabinoids may inhibit the acrosome reaction via a mechanism involving the activation of phospholipase(s) within the sperm.

Biochemical studies. Sea urchin sperm are known to contain phospholipases A, C, and D (11, 16, 17, 28, 40). Work on mammalian somatic cells indicates that cannabinoids may exert their biological effects by activating phospholipase A_2 to liberate free arachidonic acid from membrane phospholipids (4, 5, 9, 43). In collaboration with Dr. S. G. Laychock, we have obtained evidence that addition of THC to homogenates of *S. purpuratus* sperm activates phospholipase A_2 activity (8). Phosphatidylcholine with ^{14}C-arachidonic acid in the second acyl position was the substrate in these enzymatic studies. Phospholipase A_2 activity was activated by THC in a concentration dependent manner (Fig. 7). The role of calcium in supporting phospholipase A_2 activity was studied using the specific calcium chelator ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid (EGTA). The THC-stimulated phospholipase A_2 activity in the sperm homogenate was completely inhibited by EGTA, which shows that it is a calcium-dependent enzyme. These findings, taken together with the morphological observations described above, suggest that the adverse effects of THC on sperm fertilizing capacity may be promoted via the activation of phospholipase A_2 within the sperm.

Conclusion

Our studies show that cannabinoids (THC, CBD, and CBN) can directly affect the process of fertilization in sea urchins. The biological effects of THC on sea urchin sperm are not directly related to the psychoactive properties of these substances, since CBD and CBN, which are not psychoactive (27), affect sperm function in a manner similar to THC. Cannabinoids inhibit fertilization by reducing the fertilizing capacity of the sperm. Cannabinoids reduce the fertilizing capacity of sperm by inhibiting the acrosome reaction that normally is stimulated by a specific ligand in the jelly coat of the egg. Studies with ionophores suggest that THC blocks the acrosome reaction by affecting event(s) in the stimulation-secretion coupling mechanism of the sperm preceding the opening of ion channels. Ultrastructural studies of cannabinoid-treated sperm show that inhibition of the acrosome reaction is associated with the localized fragmentation of the nuclear envelope and the formation of lipid deposits within the subacrosomal and centriolar fossae of the sperm. These morphological observations suggest that cannabinoids may cause the activation of phospholipase(s) within the sperm. Biochemical studies on sperm homogenates show that THC can activate phospholipase A_2 activity.

Free arachidonic acid that is liberated from membrane phospholipids by the action of phospholipase A_2 can be oxidized within cells to produce extremely potent bioregulatory products such as prostaglandins and leukotrienes (24,30). This process is known as the arachidonic acid cascade. Cannabinoids activate the arachidonic acid cascade in other cell systems, and this may be a common cellular mechanism for the diverse biological effects of cannabinoids (3–6). We propose that activation of the arachidonic acid cascade may be responsible for the blockade of the acrosome reaction and the reduction of the fertilizing capacity of sea urchin sperm caused by cannabinoids. Our proposal is

consistent with previous studies implicating arachidonic acid derived metabolites in modulating gamete interactions during fertilization (33, 36, 38). Bioregulatory metabolites derived from the actions of phospholipases C and D in the sperm also may be involved. Elucidation of the molecular mechanisms responsible for these phenomena in sea urchin sperm may provide the basis for understanding how cannabinoids affect other cellular systems.

ACKNOWLEDGEMENT

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