MOUSE GAMETE INTERACTIONS DURING FERTILIZATION IN VITRO

Chlortetracycline as a Fluorescent Probe for the Mouse Sperm Acrosome Reaction

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ABSTRACT

We have developed an assay for detecting the acrosome reaction in mouse sperm using chlortetracycline (CTC) as a fluorescent probe. Sperm known to be intact with nonreacted acrosomes show CTC fluorescence in the presence of Ca^{2+} over the anterior portion of the sperm head on the plasma membrane covering the acrosome. Sperm which have undergone the acrosome reaction do not show fluorescence on the sperm head. Mouse sperm bind to zonae pellucidae of cumulus-free eggs in vitro in a Ca^{2+}-dependent reaction; these sperm are intact by the CTC assay. Intact sperm bind to mechanically isolated zonae under the same conditions: the egg is apparently unnecessary for this initial reaction. Sperm suspensions, in which >50% of the motile population had completed the acrosome reaction, were prepared by incubation in hyperosmolal medium followed by treatment with the divalent cation ionophore, A23187. Cumulus-free eggs challenged with such sperm suspensions preferentially bind intact sperm; acrosome-reacted sperm do not bind. We conclude that the plasma membrane of the mouse sperm is responsible for recognition of the egg's zona pellucida and that the obligatory sequence of reactions leading to fusion of mouse gametes is binding of the intact sperm to the zona pellucida, followed by the acrosome reaction at the zona surface, followed in turn by sperm penetration of the zona.

KEY WORDS   acrosome reaction · mammalian gamete recognition · zonae pellucidae · mouse sperm · chlortetracycline · fluorescent probe

Although many studies have been aimed at elucidating the various reactions which sperm and egg must undergo during fertilization in mammals, agreement on the precise sequence of these reactions has not yet been reached (11, 27). The preliminary reactions for sperm can be grouped into two consecutive processes. The first process, capacitation (1, 14), normally occurs during the sperm's residence in the female reproductive tract. It is still poorly understood, primarily due to the

lack of easily identifiable morphological correlates. The process is reversible (15) and is thought to be an obligatory prerequisite for the second reaction, the acrosome reaction, in which dramatic and predictable structural alterations occur (3, 8, 10). During the acrosome reaction, a portion of the plasma membrane which previously surrounded the acrosome, a cap-like organelle overlying the anterior section of the sperm head, is discarded. The inner acrosomal membrane is then exposed to the external environment and becomes the apical limiting membrane of the sperm head. It is well established that sperm which have penetrated the zona pellucida of the egg and lie within the perivitelline space have undergone an acrosome reaction (2, 11, 16, 30, 40). However, the physiological starting point for the acrosome reaction has not yet been identified. Does the reaction occur before or after the sperm binds to the zona? If it occurs before binding, then the reaction by which the sperm recognizes the zona pellucida and binds to it—a species-specific reaction—occurs via the sperm's inner acrosomal membrane. If it occurs after binding, then the recognition reaction is effected by the plasma membrane.

Our previous investigations of the binding of mouse epididymal sperm to the zonae pellucidae of mouse eggs in vitro demonstrated that the sperm's binding ability develops rapidly, requires extracellular Ca^{2+} and is reversed by EGTA (33). Ultrastructural studies indicated that only intact mouse sperm—those which had not undergone the acrosome reaction—were observed at the zona surface shortly after insemination, whereas limited numbers of fully acrosome-reacted sperm were bound to zonae recovered at much longer times after insemination (32). These observations led us to propose that a fertile mouse sperm binds to the zona in the intact configuration and, once bound, undergoes the acrosome reaction and penetrates the zona. In this scheme, it is the plasma membrane of the sperm which is responsible for specific recognition of the zona, not the inner acrosomal membrane. The scheme further predicts that mouse sperm, which have undergone the acrosome reaction and are limited anteriorly by the inner acrosomal membrane, are incapable of binding to zonae. This prediction can be tested by binding studies in which eggs are challenged with a suspension of motile mouse sperm containing a substantial fraction with reacted acrosomes. However, two experimental requirements must be met to obtain a definitive test. The first requirement is a

reliable assay for the acrosome reaction in a suspension of motile mouse sperm. These gametes have a particularly small acrosome, and the acrosome reaction is difficult to observe and quantitate by standard optical methods. The second requirement is a method to induce consistently the acrosome reaction in a substantial fraction of the sperm in suspension. In this paper, we describe the development of methods to fulfill these requirements and their use in evaluating our prediction concerning the sequence of sperm membrane reactions immediately before fertilization.

MATERIALS AND METHODS

Media

For handling mouse gametes, two types of media were used. The first, a complete culture medium (CM) which supports mouse fertilization in vitro (25), was a modified Krebs-Ringer bicarbonate medium containing sodium pyruvate (1 mM), sodium lactate (25 mM), glucose (5.56 mM), and bovine serum albumin (20 mg BSA/ml; Fraction V, Sigma Chemical Co.. St. Louis, Mo.) at pH 7.4. For some experiments, the NaCl content of CM was increased from 120 to 180 mM (HS-CM). The Ca^{2+} concentration for both CM and HS-CM was 1.7 mM. The other type of medium used was a simple buffer of Tris (20 mM) and NaCl (130 mM) at pH 7.4 (TN). TNC was a modified version of TN, which contained Ca^{2+} at a concentration of 1.7 mM; the NaCl concentration in TNC was decreased to 126 mM to maintain the same osmolality.

Guinea pig sperm were exposed to the minimal capacitating medium (MCM) of Barros (7), either in the presence of Ca^{2+} (MCM) or in its absence (CF-MCM). MCM is a completely defined medium containing NaCl (105 mM), NaHCO$_3$ (25 mM), sodium lactate (20 mM), sodium pyruvate (0.25 mM), and CaCl$_2$ (1.7 mM) at pH 7.4.

Media for both mouse and guinea pig gametes were sterilized by Millipore filtration (0.2-μm filter).

Gametes

Detailed descriptions of the handling of mouse gametes were reported previously (33). A brief summary is given here. Unfertilized tubal mouse eggs were incubated for 10 min in CM containing 0.1% hyaluronidase (Type I, Sigma) to disperse the cumulus. Any remaining cumulus cells were removed mechanically. Completely denuded eggs were washed twice and stored in CM under sterile silicone oil (Dow Corning Corp., Midland, Miss.) at 37°C until egg collection was completed. When required, zonae pellucidae were removed manually by forcing completely denuded eggs through narrow-bore micropipettes; these isolated zonae were washed five times and similarly maintained in CM. Insemination of mouse eggs in vitro was accomplished according to the method of Wolf and Inoue (39), using a final inseminating sperm concentration of 1-3 × 10^5 cells/ml. Mouse sperm suspensions were prepared by mincing the excised caudae epididymides of a mature Swiss mouse in 0.4 ml TN. Particulate tissue was removed after allowing 10-15 min for sperm dispersion, and aliquots of the epididymal suspension were diluted 1:5 in the indicated medium (see text and figure legends).

Excised caudae epididymides of a mature guinea pig were held under silicone oil with a hemostat, which placed the epidid-

ymal contents under pressure. The engorged portions of the epididymis were pricked with a needle in several places, allowing extrusion of a nearly pure sperm suspension. Guinea pig sperm were diluted to a concentration of 10^8 cells/ml in either CF-MCM or MCM.

In experiments designed to examine sperm bound to the zona surface, mouse eggs were recovered 15–30 min after insemination. A one-step density gradient centrifugation technique (33) was used to remove sperm not specifically bound to zonae. The technique was modified from the original description by the omission of glutaraldehyde from the lower layer of the gradient, since the objective here was the examination of living specimens. (We have shown that eggs with sperm bound to the zona recovered in this way become fertilized [33].) This technique could not be employed for the recovery of isolated zonae with bound sperm due to their very low density. Instead, the isolated zonae were washed gently with a wide-bore micropipette to remove unbound sperm.

Fluorescence Methods

A chlortetracycline(CTC)-HCl stock solution of 500 μM was prepared daily in TN; the solution was maintained in the dark at 4°C and pH 7.4–7.5. Under these conditions, the fluorescence emission of CTC remained constant for several hours. Gametes were recovered at the times indicated (see text and figure legends), as described above. All solutions and materials in contact with the gametes were warmed to and maintained at 37°C. The gametes were introduced into a 20-μl drop of CM in the center of a prewarmed glass microscope slide with minimal increase in the drop's volume. Cysteine, at a final concentration of 2 mM, was added to the slide in a minimal volume. The CTC stock solution was diluted in TN and immediately applied to the gametes in a small volume to achieve a final concentration of 10–20 μM. The slide was covered with an inverted plastic petri dish and the gametes incubated with the probe at 37°C for 10 min. After incubation, a glass coverslip, to which a paraffin-wax: vaseline (1:19) mixture had been applied at the corners, was placed over the drop containing the gametes. In preparations containing intact eggs, the intense fluorescence of the egg cytoplasm due to CTC obscured the fluorescence of the sperm. To visualize the sperm, pressure was applied to the top of the coverslip, thereby rupturing the zonae at several sites, allowing the exit of the intensely fluorescent egg cytoplasm. This procedure did not appear to affect the sperm adversely, since most remained motile throughout slide preparation and examination. It also permitted visualization of sperm both at the zona surface and within the perivitelline space.

After preparation, the slide was examined immediately with an Aus Jena "Fluoval" microscope (Optical Apparatus Co., Ardmore, Penn.), equipped with phase contrast and epifluorescence optics. The excitation unit consisted of a Microscope Spectrum Analyzer (MSA) (Farrand Optical Co., Valhalla, N. Y.) fitted with 150 W Hg and Xe arc lamps. CTC fluorescence was excited at a wavelength of 390 nm, and the emission (λ_{max} at 520 nm) was observed in the epifluorescent mode through either a G247 or G249 guard filter (Aus Jena). For routine observation, the monochromator of the MSA unit was set at 390 nm with a 10-nm half band width. Both the Xe and Hg arcs were used initially. At this setting, a substantial portion of the high intensity Hg emission line at 405 nm is utilized, and the Hg arc proved superior to the Xe arc for this work. To obtain the increased intensity of excitation needed for photographic recording, the MSA monochromator was set in the full lamp transmit-

tance mode, and the light from the Hg arc was passed through a B22G (Aus Jena) filter with maximum transmittance at 410- and 90-nm half band width in tandem with a G241 (Aus Jena) filter with 380-nm cutoff. This filter combination allowed full utilization of the Hg emission at 405 nm. CTC fluorescence was photographed with a Nikon Microflex (M-35FA) system using either Kodak Tri-X or Ilford HP-5 film.

When the intensity and pattern of CTC fluorescence were compared to those of tetracycline, conditions for the use of this latter antibiotic were made identical to those for CTC, with the exception that the concentration of tetracycline used ranged from 10 to 170 μM.

Chemicals

CTC, tetracycline, EGTA, and cysteine were purchased from Sigma Chemical Co., A23187, a generous gift from Dr. Robert Hamill (Eli Lilly Co., Indianapolis, Ind.), was solubilized in dimethyl formamide (DMF) and used at a final concentration of 3 μM. The total DMF concentration in any preparation did not exceed 0.1%.

RESULTS

Fluorescence Assay for Mouse
Sperm Acrosome Reaction

The antibiotic CTC is a fluorescent chelator of divalent cations, which markedly increases its fluorescence emission upon binding a divalent cation in a nonpolar environment (12, 13). It binds at low concentrations to mouse sperm and, in the presence of Ca^{2+}, emits fluorescence of variable intensity depending on its location. Fig. 1 shows the pattern of fluorescence displayed by sperm when CTC was applied to gametes recovered 15 min after insemination. Paired phase contrast and epifluorescent micrographs show that the mouse sperm bound at the zona surface (Fig. 1 a) exhibited a compartmentalized pattern of CTC fluorescence (Fig. 1 b). This distribution was observed consistently at this brief interval after insemination when sperm are known to be completely intact (32). Intense fluorescence was emitted from two areas, the midpiece and the area on the sperm head corresponding to the position of the acrosome, whereas the principal piece of the tail and the area corresponding to the post-acrosomal segment displayed very dim fluorescence. The intense CTC fluorescence emitted by the underlying egg cytoplasm was eliminated by rupturing the zona at several sites (Fig. 1 a and b, arrows) and extruding the cytoplasm to create a darkened background for viewing the sperm.

The characteristic CTC fluorescence pattern shown by intact sperm enabled us to answer the important ancillary question: does the egg partic-

ipate in the binding of intact mouse sperm to the zona pellucida? The answer is that the egg is unnecessary, in contrast to findings obtained with hamster gametes (22, 24). Sperm bound to isolated zonae (Fig. 1c) showed the same fluorescence pattern on the anterior portion of the head (Fig. 1d) as sperm bound to zonae of intact eggs (Fig. 1b). The pattern was easier to see since there was no background fluorescence from the egg. The typical bright fluorescence of the midpiece can be seen at the sperm's neck in this micrograph (Fig. 1d), but the motion of the flagellum, which is uneffected by CTC, obscured photographic recording of fluorescence distally. The Ca^{2+} requirement for sperm binding to isolated zonae (Fig. 2; Table I) is the same as those for binding to zonae of intact eggs. The use of the fluorescent probe in binding studies with isolated zonae defines this reaction in mouse gamete interaction as one which occurs between the surface of the intact membrane of the sperm head and the surface of the zona pellucida, with Ca^{2+} as the only exogenous factor required.

Previous ultrastructural studies of several mammalian species (2, 11, 16, 30, 40), including the mouse (42), have demonstrated that sperm which have entered the perivitelline space have undergone an acrosome reaction. In contrast, only intact mouse sperm which have not undergone the acrosome reaction were observed bound to zonae shortly after insemination (32). The pattern of CTC fluorescence of sperm located within the perivitelline space was therefore examined for comparison with that observed in sperm bound to the zona surface (Fig. 1b and d). For these experiments, mouse eggs were inseminated in the presence of the divalent cation ionophore, A23187 (3 μM), a treatment which permits as many as 50 sperm to penetrate the zona and enter the perivitelline space.[1] Under the usual conditions for fertilization in vitro, few extra sperm enter the perivitelline space; the use of A23187 greatly increases the probability of finding perivitelline sperm for examination. The mechanism of the ionophore effect is currently unknown, but its presence does not appear to alter markedly the kinetics of zona penetration by the sperm[1] or the gamete's ultrastructure (32). Gametes were recovered 2 h after insemination and thoroughly washed to remove

[1] Dr. Don P. Wolf, University of Pennsylvania School of Medicine, personal communication.

any sperm bound to the zona. Exposure to CTC produced a distribution of fluorescence notably different from that seen in sperm bound to zonae (Fig. 1): the bright fluorescence formerly localized in the anterior portion of the sperm head was no longer visible (Fig. 3b). The midpiece retained its intense fluorescence, thereby serving as a convenient internal control. Since the background fluorescence of the egg cytoplasm was unavoidable in this situation, the zona was again ruptured to permit the escape of the egg cytoplasm. The inset in Fig. 3a demonstrates that this sperm was located well within the perivitelline space: the sperm's tail is being carried through the tear in the zona by the outward flow of the egg cytoplasm.

CTC can therefore be used as a fluorescent probe for the acrosome reaction in motile mouse sperm. Intact sperm show bright fluorescence on the anterior portion of the sperm head corresponding to the position of the acrosome. Acrosome-reacted sperm do not show this fluorescence; the sperm head remains dark. The difference is sufficiently marked that the assay is reliable and readily quantitated with regard to percent sperm which have undergone the acrosome reaction.

Experiments were undertaken to extend this assay to guinea pig sperm, which have prominent acrosomes visible by phase contrast optics. The acrosome reaction in these sperm is readily induced in vitro (9, 36, 37). After exposure to CTC, the fluorescence pattern observed in guinea pig sperm was analogous to that in the mouse, with the difference that ~50% of the acrosome-reacted sperm appeared to acquire bright fluorescence in the post-acrosomal region. In all samples examined, however, the inner acrosomal membrane of the reacted sperm was nonfluorescent.

The occurrence of the acrosome reaction in suspended mouse sperm can be monitored by use of CTC in the absence of eggs. If mouse sperm were incubated for increasing lengths of time in either CM or TNC and then exposed to CTC, a predictable sequence of fluorescence patterns, reflecting the successive stages of the acrosome reaction, was observed. The sperm seen in Fig. 4a and b was recovered after a 10-min incubation and is completely intact, as indicated by the bright and uniform fluorescence limited to the acrosomal area of the sperm head. The first alteration in this CTC fluorescence pattern was the superimposition of discrete, intensely fluorescent spots on the already fluorescent acrosomal area (Fig. 4c and d). These spots appeared to coalesce gradually into larger

units with the simultaneous loss of the bright acrosomal background fluorescence (Fig. 4e and f). In the terminal phase of the reaction (Fig. 4g–j), all of the CTC fluorescence was lost from the sperm head.

It was not possible to predict in advance the actual number of mouse sperm in a given suspension that would display this sequence of fluorescence changes. The variability appeared to reflect the individuality of sperm suspensions prepared from different males. This held true to a limited extent for the time course over which these changes occurred, although some generalizations about this parameter can be made. Fluorescence patterns similar to the sperm in Fig. 4b comprised the overwhelming majority of sperm observed during the first hour in suspension. During the next 2 h, variable numbers of sperm exhibited fluorescence patterns similar to those seen in Fig. 4d and f. Fluorescence patterns corresponding to fully reacted sperm (Fig. 4h and j) were rarely seen before 3 h of incubation.

The question of a Ca^{2+} requirement for the mouse acrosome reaction could not be answered with this assay. Exposure of sperm to CTC in the absence of added Ca^{2+} resulted in considerably reduced fluorescence emission (Fig. 4k and l). In this situation, the midpiece displayed weak fluorescence, while the sperm head was virtually invisible. The lack of fluorescence did not change as a function of length of incubation; mouse sperm exposed to CTC at different times in the absence of added Ca^{2+} exhibited an extremely faint fluorescent image. Addition of EGTA changes the fluorescence pattern observed with CTC plus Ca^{2+} to that observed with CTC in the absence of Ca^{2+}. The same dull image was obtained after incubation of sperm in TN plus 2 mM Mg^{2+}. Although
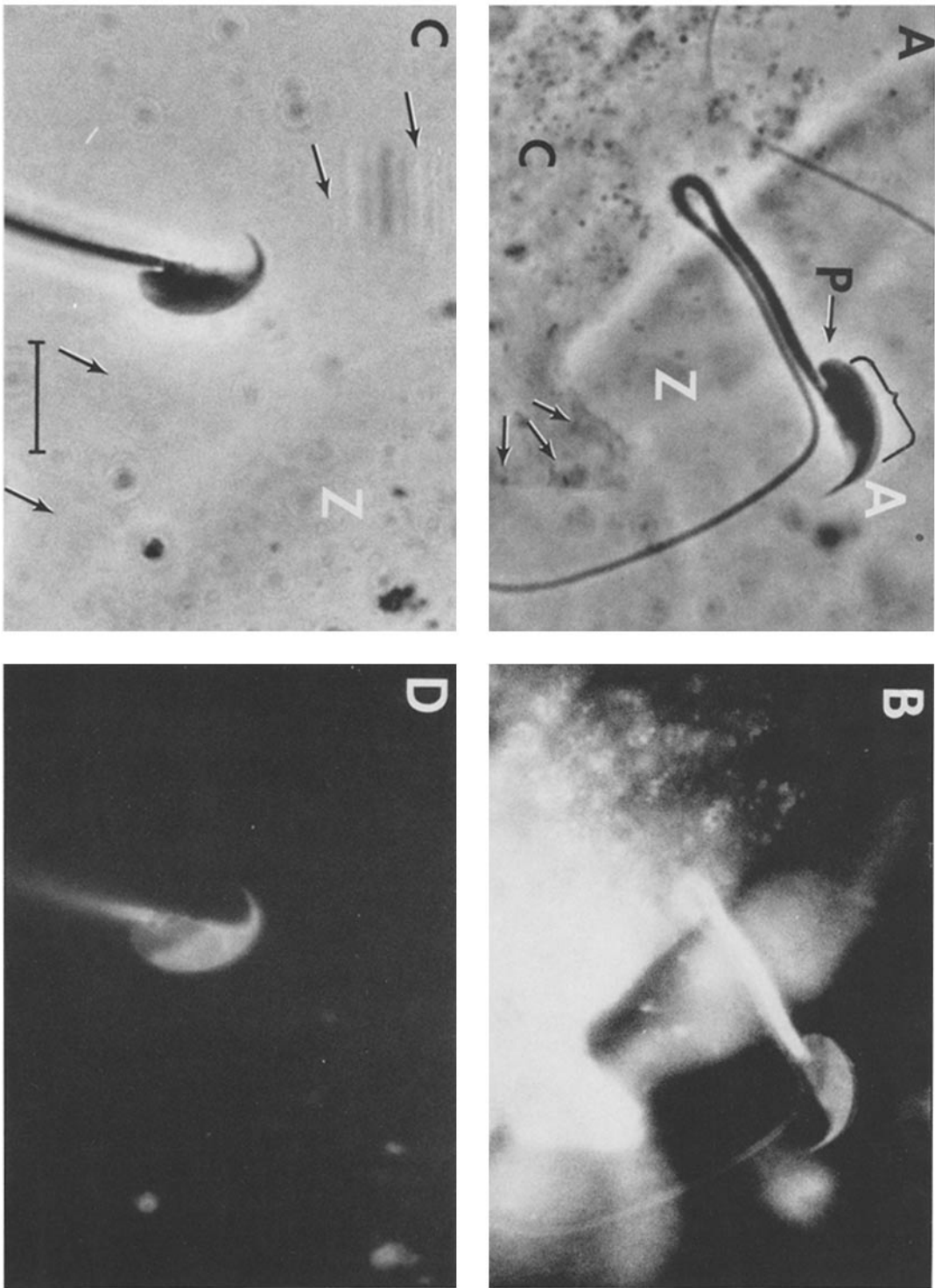
CTC will chelate Mg^{2+} as readily as Ca^{2+} and emit an intense fluorescent signal in a nonpolar environment (13), no perceptible fluorescence was displayed by mouse sperm in the presence of this divalent cation after exposure to CTC.

Several years ago, tetracycline was proposed as a fluorescent probe for the occurrence of capacitation in rabbit sperm (18, 19). The use of this antibiotic for that purpose was later questioned (38) on the basis that some of the observed tetracycline fluorescence changes did not correspond to the changes predicted to occur within the appropriate interval at known sites of capacitation. The question of whether tetracycline and CTC give similar results in the assay described here was therefore examined. Mouse sperm were exposed to tetracycline under conditions identical to those used for CTC. No fluorescent image resulted after the exposure of mouse sperm to 10 μM tetracycline, the concentration of CTC routinely used. Application of tetracycline at 170 μM provided only a faint fluorescent image of the midpiece alone; the sperm head generally did not exhibit any fluorescence with this compound. An occasional sperm displayed dull, uniform fluorescence over the entire sperm head; however, no regional fluorescence patterns were detected with tetracycline. Finally, these patterns did not change as a function of the length of sperm incubation. Tetracycline does not give the same results as CTC and is unsuitable as a fluorescent probe for the acrosome reaction.

Conditions for the Occurrence of the Acrosome Reaction in Mouse Sperm In Vitro

Since unpredictable and relatively small num-

FIGURE 1 Paired phase contrast and epifluorescent micrographs of sperm bound to zonae and exposed to CTC. (A and B) Mouse sperm bound at the surface of the zona pellucida. The gametes were recovered 15 min after insemination by the stop-fix technique in the absence of glutaraldehyde and exposed to 10 μM CTC. The intense fluorescence of the egg cytoplasm often prohibited epifluorescent visualization of the sperm head. Creation of a small rupture (arrows) in the zona by the application of pressure to the top of the coverslip allowed the cytoplasm to escape, and provided a dark background for improved visualization. Note the bright, uniform fluorescence on the sperm head localized to the area occupied by the acrosome, in contrast to the dimly fluorescent adjacent area of the sperm head corresponding to the post-acrosomal segment. (C and D) Mouse sperm bound at the surface of a manually isolated zona pellucida. Sperm and isolated zonae were recovered 15 min after sperm addition, washed with a wide-bore micropipette and exposed to 10 μM CTC. The edge of the zona, which is difficult to resolve visually, is indicated by the arrows. The pattern of CTC fluorescence is identical to that seen in sperm bound to intact zonae recovered at this same interval after sperm addition and is readily visualized here due to the lack of background fluorescence contributed by the egg cytoplasm. Z, zona pellucida; C, egg cytoplasm; A, acrosomal region of the sperm head; P, post-acrosomal region of the sperm head. Bar, 10 μm.
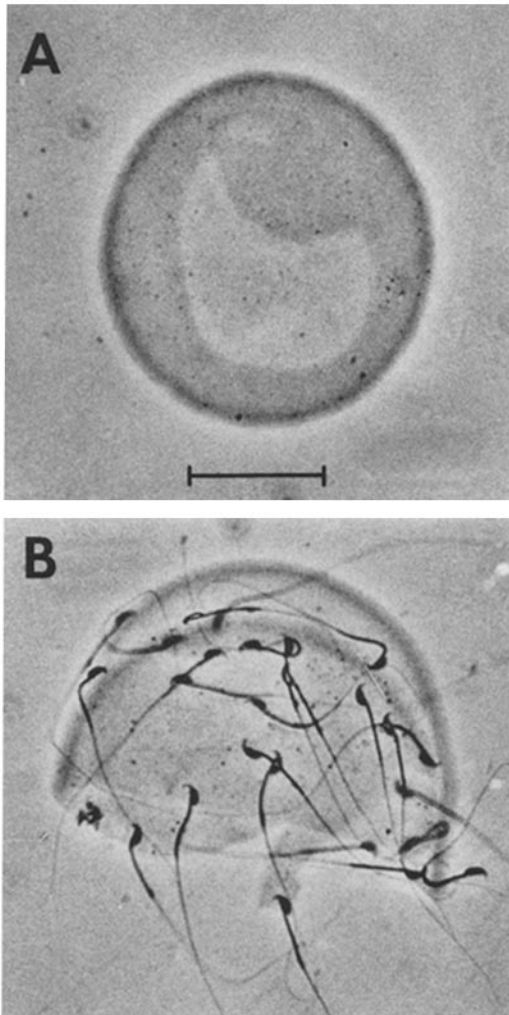
a sperm suspension results in the occurrence of the acrosome reaction in high numbers (21, 36, 37). The ionophore alone was not effective in the induction of the acrosome reaction in large numbers of mouse sperm incubated in either CM or TNC. Use of an incubation medium with increased salt concentration in combination with A23187, however, was consistently effective. Sperm were incubated in CM in which the NaCl concentration had been raised from 120 to 180 mM (HS-CM) for a minimum of 60 min, followed by the addition of A23187 (3 μM). Exposure of these sperm to CTC after a further incubation interval of 30 min consistently resulted in the occurrence of the acrosome reaction in at least 50% of the motile sperm population, as assayed with CTC. In some of the sperm suspensions examined, as many as 80% of the motile population had undergone the reaction. Neither HS-CM nor A23187 alone were capable of consistently producing this proportion of reacted mouse sperm.

Will Acrosome-Reacted Mouse Sperm Bind to the Zona Pellucida?

The development of the CTC fluorescence probe assay for the acrosome reaction and of the method for induction of this reaction in suspension with high salt medium plus A23187 permitted us to test the prediction that reacted mouse sperm will not bind to the zona of the egg. The rationale underlying the test was the following: If the particular membrane surface over much of the sperm head is irrelevant for recognition of and binding to the zona surface, then the proportions of intact and acrosome-reacted sperm bound to the zona should reflect the proportions of each in the population. Alternatively, if a specific membrane surface is required for this process, then those sperm

FIGURE 2 (A and B) Phase contrast micrographs of manually isolated zonae pellucidae incubated with mouse sperm in the absence (A) or presence (B) of 1.7 mM Ca^{2+}. Zonae were washed thoroughly in CM after isolation to eliminate contamination by the eggs. After incubation with sperm for 15 min, zonae were recovered, washed gently with a wide-bore micropipette, and examined for sperm binding. Sperm were preincubated for 60 min in (A) TN or (B) TNC. × 600.

bers of mouse sperm appeared to undergo the acrosome reaction when incubated in either CM or TNC alone, as assayed by the distribution of CTC fluorescence, a method was required for the consistent induction of the acrosome reaction in a large proportion of suspended sperm for use in zona binding determinations. In other mammalian systems, the addition of the ionophore A23187 to

TABLE I

Mouse Sperm Binding to Isolate Zonae Pellucidae

Medium*	Mean number of sperm/zona‡
CM	8.0 ± 0.70 (62)
TNC	9.5 ± 0.76 (66)
TN	0.07 ± 0.03 (75)

Data compiled from three replicate experiments.
* The same medium was used for both sperm preincubation (60 min) as well as for sperm and zonae incubation.
‡ Binding was assayed 15 min after sperm addition. Values are given ±SEM. Figures in parentheses give the actual number of zonae counted.
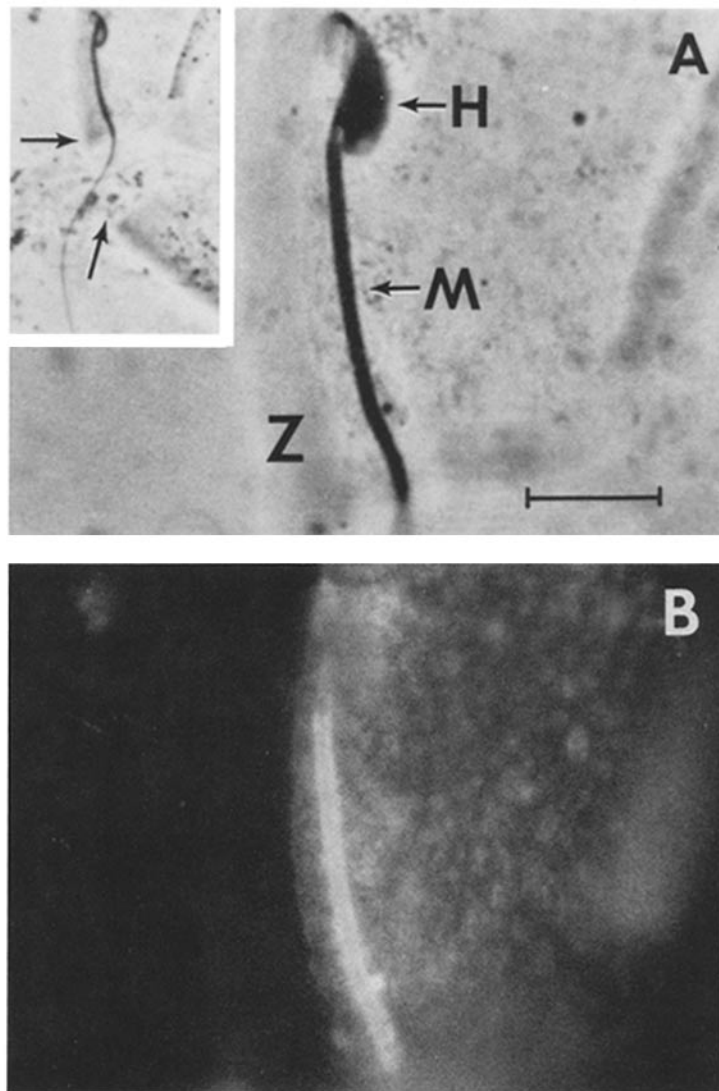
FIGURE 3 (*A* and *B*) Paired phase contrast and epifluorescent micrographs of a mouse sperm located within the perivitelline space. Gametes were inseminated in the presence of 3 μM A23187 and recovered 2 h after insemination. Exposure to 20 μM CTC resulted in emission of intense fluorescence from the sperm midpiece, while the entire sperm head is only barely visible with epifluorescence. The inset shows more clearly the position of the sperm within the perivitelline space; arrows point to the rupture in the zona, created to eliminate the background CTC fluorescence of the egg cytoplasm. Z, zona pellucida; M, sperm midpiece; H, sperm head. Bar, 10 μm.

having that membrane surface will be selectively bound, independent of their proportion in the population. Evidence that the latter case holds true for hamster gametes was obtained using scanning electron microscopy (22), although in those experiments the challenging sperm population consisted almost solely of acrosome-intact sperm.

Eggs were inseminated with sperm from one of three possible preincubation regimes: incubation for 90 min in TNC or CM, or in HS-CM for 60 min plus 3 μM A23187 for 30 min to induce the acrosome reactions. Eggs were recovered by the stop-fix technique in the absence of glutaraldehyde, at 30 min post-insemination, and exposed to
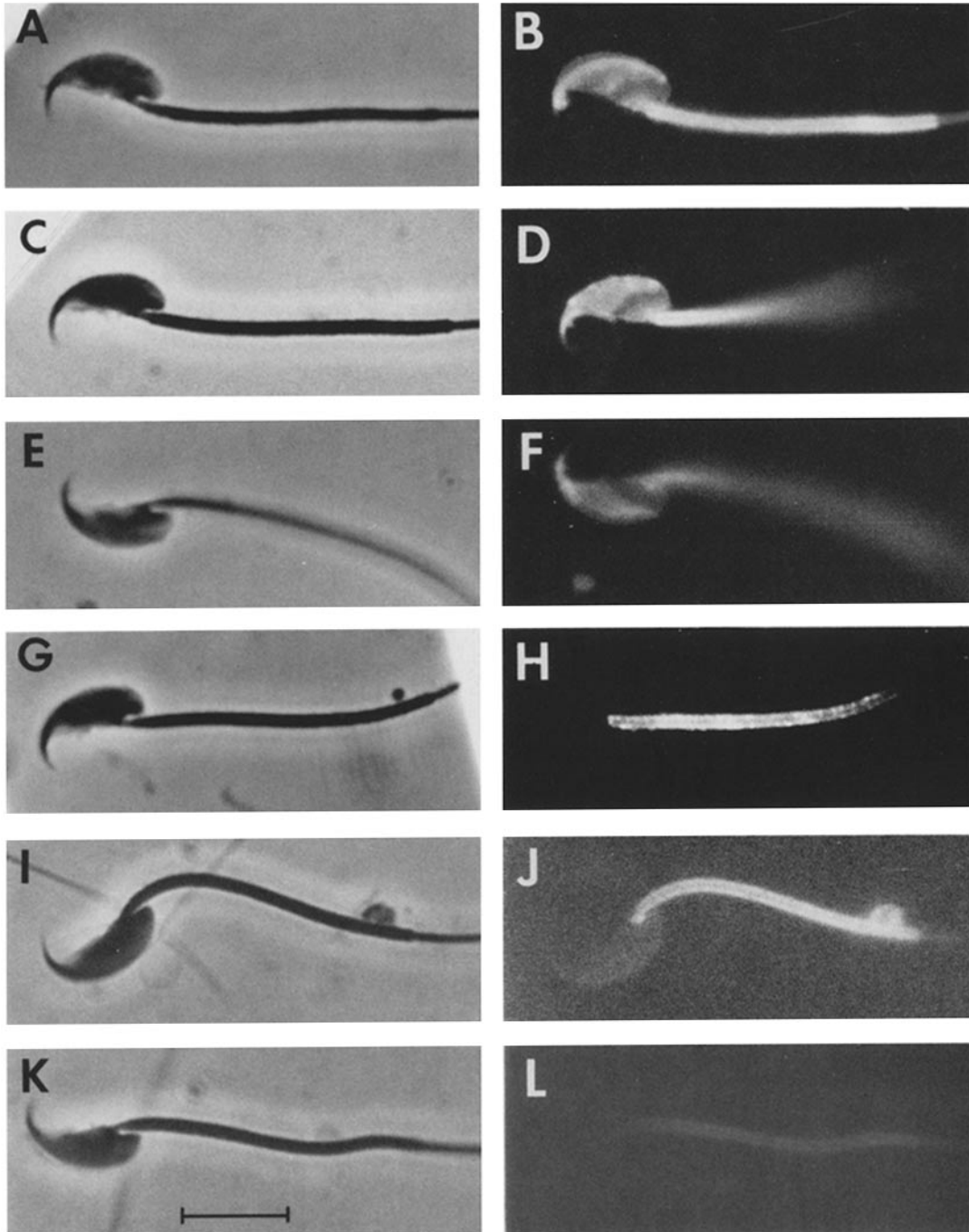
FIGURE 4   Paired phase contrast and epifluorescent micrographs of motile mouse sperm in suspension exposed to CTC. Panels *A* through *J* show the successive stages of the acrosome reaction from the fully intact to the fully reacted state. Throughout the reaction, the CTC fluorescence of the midpiece remains quite bright. (*A* and *B*) Completely intact sperm displaying the CTC fluorescence distribution characteristic for that state. Note the bright and uniform fluorescence emitted from the acrosomal area of the sperm head, in contrast to the poorly fluorescent post-acrosomal segment. (*C* and *D*) An early stage of the

TABLE II

Status of the Acrosome of Mouse Sperm Bound to Zonae

Insemination medium	Sperm preincubation*	CTC fluorescence category‡			
		1	2	3	4
		%	%	%	%
A. CM	HS-CM/A23187	93	6	1	0
B. CM	CM	91	7	2	1
C. TNC	TNC	92	6	1	1

Eggs recovered by the stop-fix technique in the absence of glutaraldehyde at 50 min postinsemination and exposed to 10 μM CTC. The values represent the percentage of sperm in the CTC fluorescence category, as defined below, for the stated combination of insemination medium and sperm preincubation conditions in the experimental sets A, B, and C. The total number of gametes examined in each experimental set were as follows: A, 305 sperm, 81 eggs; B, 281 sperm, 76 eggs; C, 227 sperm, 68 eggs.

* Sperm were preincubated for 90 min in (A) HS-CM for 60 min, plus 3 μm A23187; (B) CM; or (C) TNC.

‡ CTC fluorescence category: (1) Completely intact sperm, with bright uniform fluorescence over the acrosomal area abruptly adjacent to very dim fluorescence over the post-acrosomal segment (see Fig. 4b). (2) Sperm in the initial phase of the acrosome reaction, with intensely fluorescent spots of fluorescence superimposed upon the already fluorescent acrosomal area, giving rise to a mottled appearance of the acrosome (see Fig. 4d). (3) Sperm in a very late stage of the acrosome reaction—with few bright spots of fluorescence lying on a weakly fluorescent acrosomal area (see Fig. 4f). (4) Completely reacted sperm, with very weakly fluorescent or nonfluorescent sperm head (see Fig. 4h and j).

10 μM CTC. Epifluorescent examination of the sperm bound to these eggs provided the results shown in Table II. Over 90% of bound sperm were intact, as assayed by the CTC fluorescence distri-

bution. Preferential binding of intact sperm occurred even when the eggs were inseminated with sperm in which >50% of the population were known to have undergone the acrosome reaction. The test of our prediction is positive: mouse sperm with intact membranes bind to zonae pellucidae, but acrosome-reacted sperm do not.

DISCUSSION

The utility of CTC as a fluorescent probe for assaying the acrosome reaction results from the localized fluorescence of the probe bound to the intact mouse sperm head in the presence of Ca^{2+}. The fluorescence disappears when the sperm undergoes the acrosome reaction. The intense fluorescence signal of CTC is elicited upon chelation of a divalent cation, such as Ca^{2+} or Mg^{2+}, in a nonpolar environment (12, 13): The probe actually reports the location of divalent cations in membranes. In mouse sperm, the physiological divalent ion is Ca^{2+}. Fluorescence in the absence of added divalent cation was very weak (Fig. 4k and l); either endogenous Ca^{+2} or trace levels of Ca^{2+} in TN may be responsible for the dim fluorescence emitted from the sperm's midpiece. The addition of Mg^{2+} to sperm in TN did not increase the CTC fluorescence over that seen in TN, while EGTA in amounts sufficient to chelate the Ca^{2+} in TNC entirely prevented the CTC fluorescence. Any proposal for the mechanism of fluorescence loss must account for the observation that intense fluorescence is emitted only when the $CTC-Ca^{2+}$ complex is membrane-bound (12). Loss of CTC fluorescence could result from the release of membrane-bound Ca^{2+} from the sperm head, due to loss of specific Ca^{2+} binding groups on the membrane. An alternative proposal, that better takes into consideration the regionalization of the CTC fluorescence, is that the $CTC-Ca^{2+}$ complex binds

acrosome reaction. Intensely bright spots of fluorescence appear superimposed on the already fluorescent acrosomal area. The post-acrosomal segment remains dimly fluorescent. (*E* and *F*) An intermediate stage of the acrosome reaction. The intense spots of CTC fluorescence appear to coalesce into larger spots while the bright background fluorescence of the acrosome gradually disappears. As before, the faint post-acrosomal segment fluorescence remains unaltered. (*G-J*) Completely acrosome reacted mouse sperm. The CTC fluorescence is entirely gone from the sperm head. In some specimens (*H*), even the outline of the sperm head is invisible with epifluorescence, while in other reacted sperm (*J*), a very faint uniform fluorescence permits the sperm head to be discerned. (*K-L*) Mouse sperm exposed to CTC in the absence of Ca^{2+}. Very weak fluorescence is emitted from the midpiece alone. Sperm incubated in (*A* and *B*) CM; (*C* and *D*) TNC; (*E* and *F*) TNC; (*G* and *H*) TNC; (*I* and *J*) CM; and (*K* and *L*) TN. Final CTC concentration: (*A-J*) 10 μM; (*K* and *L*) 20 μM. Bar, 10 μm.

specifically to the plasma membrane overlying the acrosomal, but not post-acrosomal, region. Given the close apposition of the plasma and outer acrosomal membranes, the CTC-Ca^{2+} complex may also be bound to the latter (31). Loss of fluorescence would then result quite simply from the loss of these membranes which occurs as the main feature of the acrosome reaction. Such a restricted localization of the chelate-cation complex is feasible due to the highly compartmentalized organization of spermatozoa (20).

A similar suggestion regarding the localization of Ca^{2+} in the sperm head has been made by Singh et al. (34) to account for the consistent disappearance during the acrosome reaction of a portion of ^{45}Ca^{2+} associated with bovine sperm. Additional support for this localization of the CTC-Ca^{2+} complex overlying the acrosome is found in micrographs of reacting guinea pig sperm exposed to pyroantimonate (20). This agent also chelates cations, but then forms an insoluble precipitate rather than a fluorescent compound. In reacting sperm, all of the cation-pyroantimonate precipitate is located within membranous vesicles surrounding the sperm head. Although this reagent does not have a strict cation specificity, the experiments nonetheless demonstrate the absence of cation binding sites on the newly exposed inner acrosomal membrane.

Use of CTC as a fluorescent probe also showed a striking localization of the Ca^{2+}-CTC complex in the midpiece region of mouse and guinea pig sperm. This observation agrees closely with the Ca^{2+} distribution in bull sperm exposed to 0.17 mM Ca^{2+}, as determined by Babcock et al. (6), using electron microanalysis. They found that the midpiece was 3.5-8 times increased over background, while the content of the other regions was near background. The question of whether the Ca^{2+} was actually located in the mitochondria under these conditions was answered in the affirmative, based on both electron microanalysis (6) and previous biochemical studies (4, 5). Yet the sperm plasma membrane in rabbit (35) and human (29) sperm seems quite impermeable to Ca^{2+}. Further investigation is required to clarify this problem; CTC should prove to be a useful fluorescent probe in this investigation.

The effectiveness of the HS-CM medium in the induction of the acrosome reaction in mouse sperm in vitro was not altogether unexpected, since media of high ionic strength (28) have been utilized previously to enhance mouse sperm membrane

destablization. Nor was the need for A23187 surprising, since this ionophore has been employed for the induction of the acrosome reaction in other mammalian systems (22, 36, 37), and the dependency of the acrosome reaction upon extracellular Ca^{2+} in both invertebrate (see reference 17) and mammalian (37, 41) sperm has been well established. However, it was not anticipated that sequential exposure of mouse sperm to these conditions would be required, although a fairly similar system has very recently been reported by Lui and Meizel (26) for the synchronous induction of the acrosome reaction in hamster sperm. If influx extracellular Ca^{2+} is the event common to all sperm for the initation of the acrosome reaction, then it is not unreasonable to suspect that this same goal may be attained by slightly different routes demanding very particular experimental conditions for gametes from different species.

The selective binding of intact sperm to zonae, particularly when the eggs were confronted with a large population of motile, acrosome-reacted sperm, allowed the identification of the plasma membrane as the sperm surface responsible for recognition of and binding to the zona pellucida. This finding has several interesting implications. It defines the obligatory sequence for the membrane reactions of fertile mouse sperm immediately before sperm-egg fusion: binding of the sperm to the zona surface is followed by the acrosome reaction, and then zona penetration. The acrosome reaction, for the successful spermatozoon, occurs at the zone surface. The occurrence of the reaction at this location neither implies nor negates the activity of a specific zonal or vitelline initiating factor. The interaction at the zona could be restricted to the binding reaction. The acrosome reaction would then be viewed as the culmination of endogenous processes initiated before binding to the zona. The zona pellucida is the site of a major barrier to interspecific fertilization (23, 40), suggesting the existence of a specific receptor for the zona in sperm. We have demonstrated here that this putative receptor resides in the mouse sperm plasma membrane.

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