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Calcium-dependent increase in adenosine 3′,5′-monophosphate and induction of the acrosome reaction in guinea pig spermatozoa

(capacitation/cyclic nucleotides/phosphodiesterase inhibitor/membranes)

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ABSTRACT **Experiments were designed to determine the interrelationship between cyclic AMP and Ca^{2+} during the processes of sperm capacitation and the acrosome reaction. In minimal culture media containing pyruvate and lactate as substrates, guinea pig spermatozoa required a minimum of 1.0–1.5 hr to capacitate in the presence of 1.7 mM Ca^{2+} and a minimum of 0.5–1.0 hr to capacitate in the absence of added Ca^{2+}. Sperm cyclic AMP concentrations were increased by as much as 30-fold within 0.5 min after addition of cells to various media containing Ca^{2+}, and the concentrations then remained increased for up to 4 hr. When the cells were added to several Ca^{2+}-deficient media, however, cyclic AMP concentrations increased only about 3-fold within 0.5 min and then returned to basal concentrations within 2 min. D-600, a calcium transport antagonist, completely blocked the Ca^{2+}-induced increase in sperm cyclic AMP concentrations. In contrast to capacitation, the acrosome reaction failed to occur in the absence of extracellular Ca^{2+}. After capacitation of spermatozoa in a Ca^{2+}-free medium, addition of Ca^{2+} caused an increase in sperm cyclic AMP concentrations within 1 min and a maximal number of spermatozoa showing an acrosome reaction within 10 min. The addition of 1-methyl-3-isobutylxanthine along with Ca^{2+} had a synergistic effect on the increase in cyclic AMP. Neither 1-methyl-3-isobutylxanthine nor 8-Br cyclic AMP induced an acrosome reaction in capacitated spermatozoa in the absence of Ca^{2+}, but both significantly decreased the time required for maximal expression of the acrosome reaction in the presence of Ca^{2+}. These results suggest that the sperm acrosome reaction is associated with both a primary transport of Ca^{2+} and a Ca^{2+}-dependent increase in sperm cyclic AMP concentrations. Because a cyclic AMP analogue did not induce an acrosome reaction in the absence of added Ca^{2+}, the increase in sperm cyclic AMP concentrations induced by Ca^{2+} probably reflects one of a number of Ca^{2+}-dependent events associated with the acrosome reaction.**

Spermatozoa present in freshly ejaculated semen of mammals lack the capacity to fertilize ova immediately (reviewed in refs. 1 and 2). Prior to acquisition of the ability to fertilize ova, spermatozoa undergo a series of changes termed "capacitation" (3, 4). The time of this capacitation process varies from species to species, but the end result is the same: the development of a sperm cell with the capacity to respond to stimuli with a physiological change called the "acrosome reaction" (5). The capacitation process does not appear to require external Ca^{2+} (6), although it may be influenced by the general composition of the sperm incubation medium (2, 7). The acrosome reaction, on the other hand, appears to be completely dependent on the presence of extracellular Ca^{2+} in both vertebrate (6, 8, 9) and invertebrate (10) spermatozoa. Based on various data (6, 11–13), it appears that altered Ca^{2+} permeability may represent one of the primary signals, or the only primary signal, for induction of the acrosome reaction in capacitated spermatozoa.

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Capacitation or the acrosome reaction or both also appear to be closely associated with an activation of sperm motility and metabolism in a number of different animals (6, 7, 14). Similar types of sperm motility and metabolic activations have been observed in response to added cyclic nucleotide phosphodiesterase inhibitors and cyclic AMP (15–18). These, as well as other observations, have led to speculation that cyclic nucleotides are involved in capacitation or the acrosome reaction or both (15, 19–21). In potential support of such a concept, Cornett and Meizel (21) have recently shown that catecholamines, agents that increase cyclic AMP concentrations in many tissues, have positive effects on the acrosome reaction rate in hamster spermatozoa. Garcia and Rogers (22), in contrast, have shown that dibutyryl cyclic AMP and various methylxanthines inhibit the acrosome reaction rate in guinea pig spermatozoa.

In this study, the time requirement for capacitation is defined as the required duration of incubation of guinea pig spermatozoa before an acrosome reaction can be observed in response to either the continuous presence of Ca^{2+} or the addition of Ca^{2+}. Thus, capacitation as defined here represents a series of biochemical events that occur during an *in vitro* incubation and that result in the spermatozoa acquiring the ability to undergo an acrosome reaction.

MATERIALS AND METHODS

All glassware and instruments were treated with 1% detergent solution overnight, boiled for 10 min, and then thoroughly rinsed with tap water followed by deionized water before sterilization either by dry heat or autoclaving. Sterile, plugged, plastic pipets (Falcon Plastics, no. 7520) were used in preparing media.

Media. The media were always prepared from freshly distilled deionized water (resistance, ≈8 MΩ). The basic culture medium used was a minimal culture medium containing 102 mM NaCl, 1.7 mM $CaCl_2$, and 25.1 mM $NaHCO_3$. This basic medium was altered to contain sodium pyruvate (0.25 mM) and sodium lactate (20 mM) (PL-medium) (7). In one experiment, the medium of Biggers *et al.* as modified by Rogers and Yanagimachi (7) was utilized. When the minimal culture medium was modified, the osmolality was kept at approximately 306 mosmol/kg by adjusting the NaCl content. All of the media used contained, in addition, penicillin (100 units/ml) and streptomycin sulfate (50 μg/ml). Media were sterilized by passage through a Nalgene filter unit (0.45 μm in pore size) and gassed for 15 min with 5% CO_2/95% O_2 as described by Hoppe and Pitts (23). The media were stored at 4°C and used in experiments within 1 week after preparation; they were regassed just prior to use to adjust the pH to 7.6.

Chemicals. The chemicals used were obtained from the following sources: all common chemicals were from Mal-

Abbreviation: PL-medium, minimal culture medium with pyruvate and lactate as substrates.

linckrodt or Sigma; bovine serum albumin (fraction V from bovine plasma) from Armour Pharmaceutical Co. (Reheis Division, Phoenix, AZ); theophylline from Merck & Co.; magnesium chloride from Fisher; D-600 from Knoll A-G; and 1-methyl-3-isobutylxanthine from J. N. Wells (Department of Pharmacology, Vanderbilt University).

Sperm Preparation and Incubation Procedure. Adult male guinea pigs (Hartley strain) were anesthetized with ether and killed by cervical dislocation. A testis was removed, the distal portion of one cauda epididymis attached to the vas deferens was obtained, and the spermatozoa were isolated by flushing the lumen with approximately 3–4 ml of 0.9% NaCl. The spermatozoa were washed once by centrifugation (400 × *g*, 15 min) and resuspended in 0.9% NaCl. Aliquots (0.1 ml) of the sperm suspension were added to 0.9 ml of test medium in sterile polypropylene tubes with caps (Falcon Plastics, no. 2063). The final sperm concentration in each medium was 0.5×10^7 spermatozoa per ml. The tubes were placed horizontally on a shaker (Eberbach, Michigan), rotating at 60 strokes per min, in an incubator at 37°C with air as the atmosphere. After the addition of the spermatozoa, the pH of the media stabilized at approximately 8.3 within 15 min.

Determination of Sperm Motility and Acrosome Reaction. At various times during an incubation, small aliquots of the sperm suspension were transferred to a ceramic ring slide (Clay Adams) and mounted under a coverslip. Then at least 100 spermatozoa were examined to estimate the percentage of motile cells and the percentage of motile cells without a visible acrosomal cap by phase-contrast microscopy at ×160.

Cyclic Nucleotide Determination. The incubations were initiated by the addition of spermatozoa to the assay tubes and were stopped by the addition of 1 ml of 0.5 M perchloric acid. The acidified cell suspensions were then frozen and thawed five times before purification of the cyclic nucleotides on Dowex-50 columns (0.7 × 25.0 cm), as described by Schultz *et al.* (24). Parallel samples containing tracer amounts of cyclic [^{3}H]AMP or cyclic [^{3}H]GMP were also purified for estimation of sperm cyclic nucleotide recoveries (70–85%). The column fractions containing cyclic AMP or cyclic GMP were pooled, freeze-dried, and then dissolved in 1 ml of deionized water. The concentration of the cyclic nucleotides was determined by the radioimmunoassay method of Steiner *et al.* (25) as modified by Harper and Brooker (26).

RESULTS

Sperm Cyclic AMP Concentrations During Capacitation and Acrosome Reaction. Spermatozoa were highly motile in the 0.9% NaCl and thus effects on cyclic AMP concentrations were not related to an initiation of sperm motility. When guinea pig spermatozoa were incubated in the PL-medium, a large proportion of the spermatozoa underwent an acrosome reaction after an incubation period of 1–3 hr (Fig. 1). At 3 hr, approximately one-half of the motile spermatozoa had generally shown an acrosome reaction. Basal concentrations of cyclic AMP in guinea pig spermatozoa (≈2 pmol/10^7 cells) were in close agreement with cyclic AMP concentrations found in other mammalian sperm cells (17, 18). The sperm cyclic AMP concentrations rapidly and markedly increased after transfer to the PL-medium (approximately 10-fold after 5 min) but then decreased with time; the concentration appeared to increase again, however, during the acrosome reaction (1.5–2 hr). The cyclic AMP concentrations remained increased approximately 3-fold during the remainder of the incubation period. The spermatozoa maintained good motility throughout the incubation period; this is represented as a percentage and does not reflect possible different patterns of motility during the acrosome reaction as described earlier by Yanagimachi and Usui (6). Cyclic GMP was not detected in the spermatozoa under these conditions (cyclic GMP in excess of 0.2 pmol/10^7 cells would have been detectable).

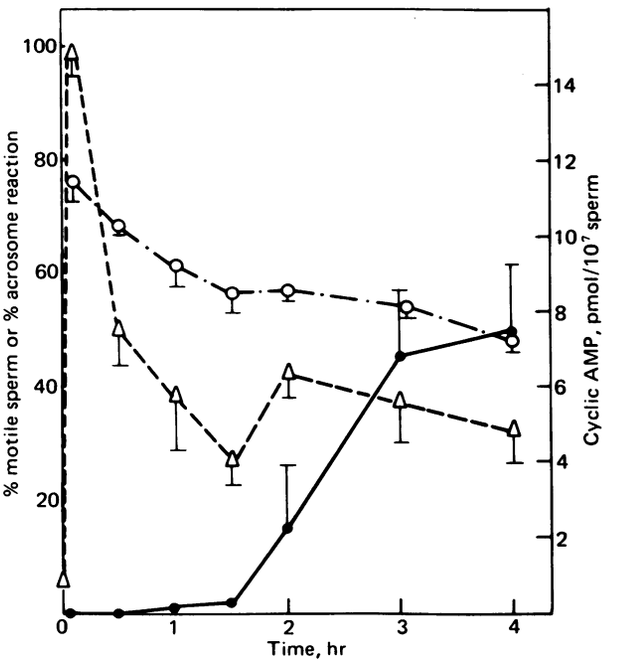

FIG. 1. The percentage motility (○), percentage acrosome reaction (●), and concentration of cyclic AMP (△) of guinea pig spermatozoa incubated in PL-medium. Values shown are the mean ± SEM (n = 5).

To determine if a relationship existed between the rapid increase in guinea pig sperm cyclic AMP concentrations and capacitation or the acrosome reaction, spermatozoa were incubated in a modified medium (7) in which the acrosome reaction is considerably delayed (not observed until about 8 hr). Cyclic AMP concentrations again increased approximately 10-fold after 5 min of incubation and then declined to basal concentrations after 1 hr and remained at basal or less than basal concentrations over the remaining incubation period of 10 hr (Fig. 2). The sperm motility declined markedly throughout the

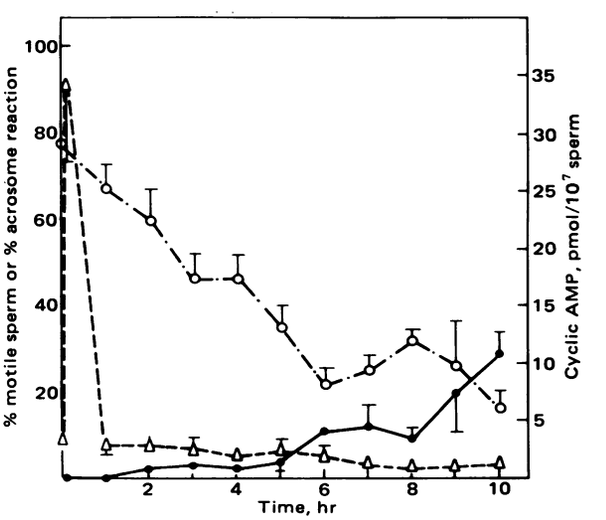

FIG. 2. Guinea pig sperm percentage motility (○), percentage acrosome reaction (●), and cyclic AMP concentration (△) during incubation in modified medium (7). Values shown are the mean ± SEM (n = 5).

incubation period, from an initial value of 78% to 16% at 10 hr. Only 4–6% of the total spermatozoa present showed an acrosome reaction and thus it would be difficult to determine whether or not cyclic AMP was altered in the reactive cells at the time of the acrosome reaction. Nevertheless, it seems clear that the initial rapid increase in cyclic AMP is not associated with the length of time required for capacitation and that such an increase is not capable of causing an acrosome reaction in uncapacitated cells.

Sperm Cyclic AMP Concentrations During Short-Term Incubation. Because an increase in sperm cyclic AMP concentration was apparent within 5 min when the spermatozoa were added to either the PL-medium or the modified medium (7), spermatozoa were added to various modifications of the minimal culture medium in order to determine if a component of the medium was responsible for the initial rapid increase in the cyclic AMP concentration. When spermatozoa were added to the PL-medium, the sperm cyclic AMP concentrations increased approximately 30-fold after 0.5 min of incubation and then began to decline immediately (Fig. 3). When the substrates pyruvate and lactate were eliminated, the sperm cyclic AMP concentration also increased but not to the level observed in the presence of the substrates. In the absence of added Ca^{2+}, however, the increase in cyclic AMP concentration was markedly suppressed. When PL-medium containing 10 μM EDTA or 1 mM Mg^{2+} and no added Ca^{2+} was used, after 0.5 min of incubation, the cyclic AMP concentration increased 3-fold and then it declined rapidly to basal concentrations. Thus, the initial cyclic AMP increase appeared to be almost completely dependent on Ca^{2+}.

Because the guinea pig spermatozoa would capacitate normally in either the presence or absence of added Ca^{2+} (6), the Ca^{2+}-induced increase in cyclic AMP appeared to be unrelated to the capacitation process.

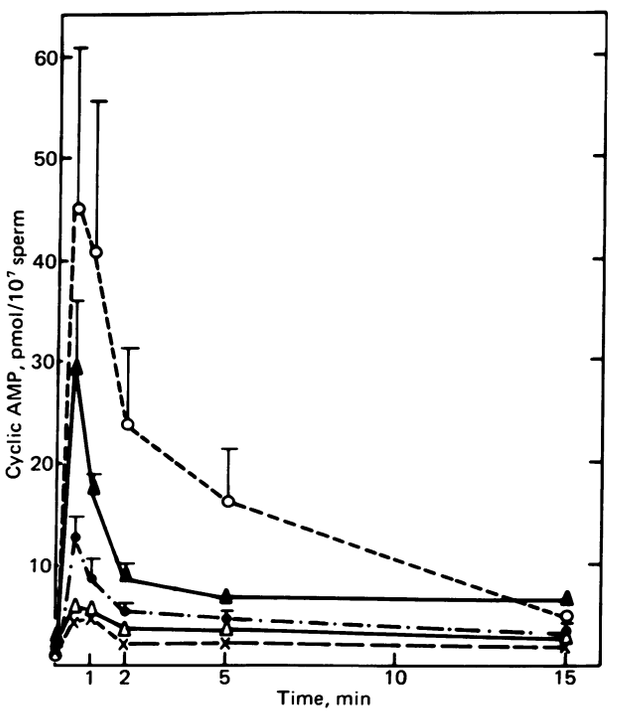

FIG. 3. Guinea pig sperm cyclic AMP concentrations during incubation in various modifications of minimal culture medium: ○, PL-medium; ▲, standard minimal culture medium; ●, Ca^{2+}-free PL-medium; △, Ca^{2+}-free Pl-medium containing 1 mM Mg^{2+}; X, Ca^{2+}-free PL-medium containing 10 μM EDTA. Values shown are the mean ± SEM (n = 4). When vertical bars are not shown, the SEM lies within the area occupied by the symbol.

Response of Spermatozoa to the Addition of Ca^{2+}. The number of motile spermatozoa and the incidence of the acrosome reaction was determined before and 10 min after the addition of Ca^{2+} (final concentration 5 mM) to guinea pig spermatozoa preincubated for different periods of time in Ca^{2+}-free PL-medium containing 1 mM Mg^{2+}. The addition of magnesium to the Ca^{2+}-free medium was necessary to maintain optimal sperm motility. There was no difference in the percentage of motile spermatozoa for the different treatments (Fig. 4A), although the addition of calcium to the medium after a preincubation of 0.5 hr did appear to enhance the

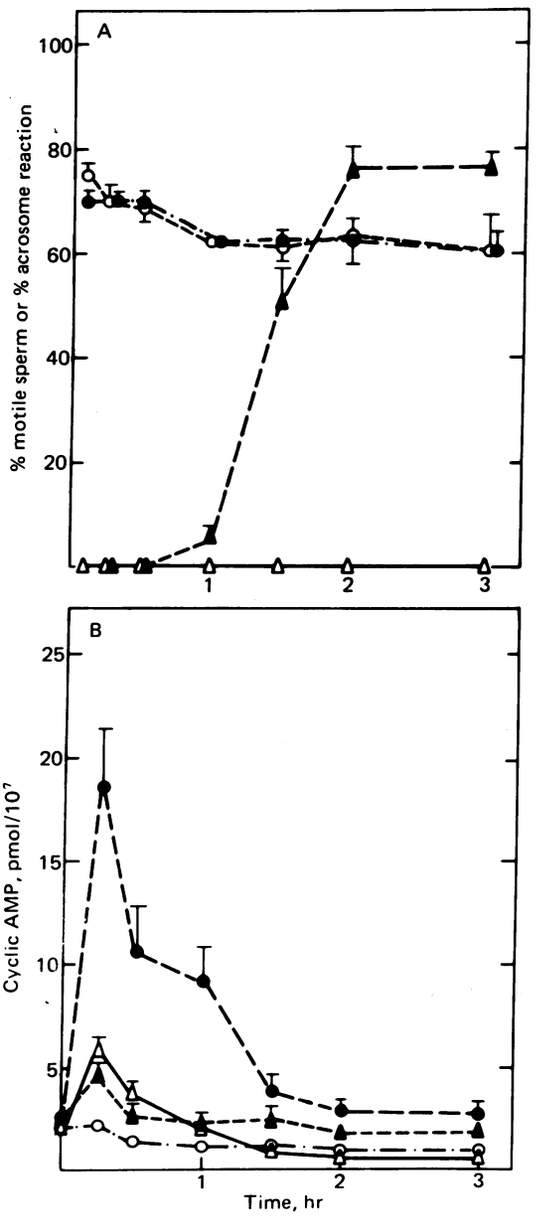

FIG. 4. Spermatozoa were preincubated for various times in PL-medium containing 1 mM Mg^{2+} and no added Ca^{2+}. Data are shown as mean ± SEM. (A) Percentage of motile spermatozoa before (○) and 10 min after (●) the addition of Ca^{2+} to a final concentration of 5 mM (50 μl of 100 mM $CaCl_2$) (n = 4) and percentage of motile spermatozoa without acrosomal caps before (△) and 10 min after (▲) the addition of Ca^{2+} (n = 4). (B) Sperm cyclic AMP concentrations 1 min after the addition of various agents. All substances were added in 50-μl aliquots from a stock solution to give the final concentration indicated: ○, water (50 μl, control) (n = 8); △, 5 mM Ca^{2+} (n = 5); ▲, 0.25 mM 1-methyl-3-isobutylxanthine (n = 4); ●, 5 mM Ca^{2+} plus 0.25 mM methylisobutylxanthine (n = 4).

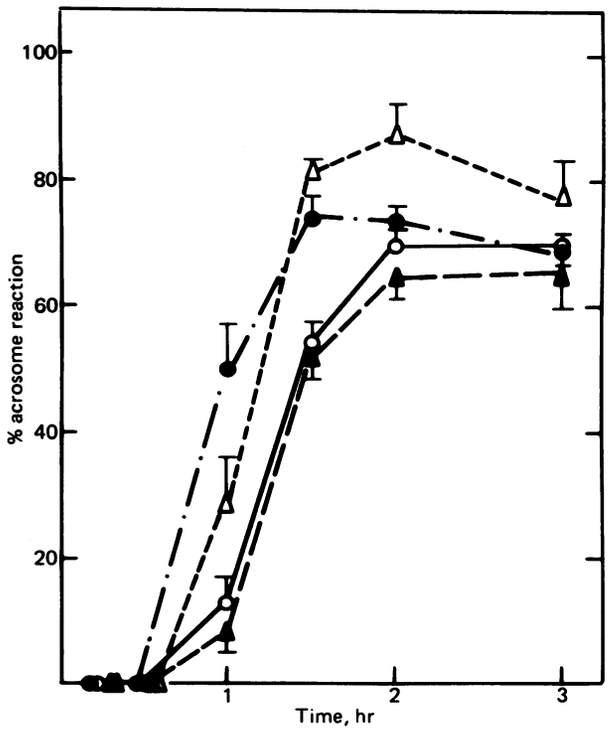

FIG. 5. Effect of various treatments on guinea pig sperm acrosome reaction. Spermatozoa were preincubated with the various agents from time zero, and Ca^{2+} (final concentration, 5 mM) was added at the times indicated and the percentage of motile spermatozoa without acrosomal caps was assessed 10 min later. The medium used in all treatments was Ca^{2+}-free PL-medium with 1 mM Mg^{2+}. The sperm motility was not different for any of the treatments. Symbols: ○, control medium (n = 15); ●, 5 mM 8-Br cyclic AMP (n = 4); △, 1 mM 1-methyl-3-isobutylxanthine (n = 4); and ▲, 5 mM 8-Br AMP (n = 3). Data are shown as the mean ± SEM.

vibration frequency of the sperm tail. When the spermatozoa were incubated in Ca^{2+}-free PL-medium containing 1 mM Mg^{2+}, no acrosome reaction was observed during the 3-hr incubation period. However, when Ca^{2+} was added to the medium after a preincubation period of >0.5 hr, the acrosome reaction occurred within 10 min. At 2 hr, approximately 75% of the motile spermatozoa showed an acrosome reaction within 10 min after the addition of calcium. Preincubation of spermatozoa in the Ca^{2+}-free medium also shifted the time–response curve for maximal expression of the acrosome reaction to approximately 1 hr earlier than the time of its maximal expression in spermatozoa incubated in the medium containing Ca^{2+} (Fig. 1).

The sperm cyclic AMP concentrations before and after the addition of Ca^{2+} (5 mM) to spermatozoa were also determined. After a small initial increase in cyclic AMP concentration upon dilution into the Ca^{2+}-free medium, the sperm cyclic AMP concentration remained at basal levels (Fig. 4*B*). The addition of Ca^{2+} (5 mM) to the sperm suspension resulted in an increase in cyclic AMP concentrations within 1 min. The simultaneous addition of 1-methyl-3-isobutylxanthine (0.25 mM), a cyclic nucleotide phosphodiesterase inhibitor, and Ca^{2+} to the sperm suspension resulted in synergistic effects on cyclic AMP concentration. It appears, therefore, that the effect of Ca^{2+} on cyclic AMP metabolism may be on the rate of formation of cyclic AMP (i.e., adenylate cyclase).

Effects of D-600. The Ca^{2+}-transport antagonist D-600 was added in the presence of Ca^{2+} to determine whether or not it could block the Ca^{2+}-induced increase in sperm cyclic AMP concentration. Experiments were designed as described in Fig. 4*B* except that D-600 (100 μM) was added 15 sec prior to the addition of Ca^{2+}. Because the apparent Ca^{2+} effect on cyclic AMP was maximal at the early incubation times, all D-600 and Ca^{2+} additions were at a fixed time point of 5 min. Although D-600 did not appear to affect sperm motility, it completely blocked the Ca^{2+}-induced increase in sperm cyclic AMP concentrations (data not shown).

Effects of 8-Br Cyclic AMP and 1-Methyl-3-isobutylxanthine on the Acrosome Reaction. Guinea pig spermatozoa were preincubated in Ca^{2+}-free PL-medium containing 1 mM Mg^{2+} and, in addition, 5 mM 8-Br cyclic AMP or 5 mM 8-Br AMP or 1 mM 1-methyl-3-isobutylxanthine. None of the added agents was capable of causing the induction of an acrosome reaction in the absence of added Ca^{2+}. However, in response to the addition of Ca^{2+}, 8-Br cyclic AMP shifted the time of maximal expression of the acrosome reaction to approximately 30 min earlier than the control; 8-Br AMP had no effect (Fig. 5). The phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine also shifted the time of maximal response by about 30 min. The percentage of motile spermatozoa was not altered by any of the treatments. When 8-Br cyclic AMP or 1-methyl-3-isobutylxanthine was added at the same time as Ca^{2+} (1 hr) instead of at the start of the incubation, both agents continued to increase the incidence of the acrosome reaction as compared to the control. These data, although positive, were considerably more variable than when the agents were incubated with the spermatozoa for longer periods of time; this may be due to permeability problems.

DISCUSSION

These experiments estimate cyclic AMP concentrations in spermatozoa during capacitation and the acrosome reaction. The media used in these experiments were previously shown to be capable of supporting a physiological acrosome reaction of guinea pig spermatozoa as established by fertilization of oocytes (7) and ultrastructural morphology (6, 27). With the minimal culture medium used in the experiments reported here, sperm motility was maintained at levels of 50% or higher during 3-hr incubations in either the presence or the absence of Ca^{2+}. Maintenance of a constant high motility was necessary because the experiments were designed to correlate a physiological change with a biochemical feature (cyclic AMP concentration). The increase in the sperm cyclic AMP concentration measured in these experiments also did not appear to be associated with the initiation of sperm motility or with an increase in sperm motility, although the pattern of motility may have changed. The spermatozoa were highly motile after collection from the lumen of the epididymis and maintained good motility in 0.9% NaCl. These results are in contrast to the situation with hamster spermatozoa with which added Ca^{2+} has been shown to increase epididymal sperm cyclic AMP concentrations and to initiate sperm motility (28). With respect to sperm capacitation and the acrosome reaction, the primary and secondary biochemical events are not well understood (2, 7, 14, 21, 27). It seems clear from a number of studies, however, that Ca^{2+} is required in the extracellular medium in order for an acrosome reaction to occur (6, 11–13); Ca^{2+} does not appear to be required for capacitation (6).

From the data presented in this manuscript, it can be suggested that one biochemical effect of Ca^{2+} on spermatozoa is the induction of increased cyclic AMP concentrations. Ca^{2+} addition induces an increase in sperm cyclic AMP concentrations and D-600 blocks these Ca^{2+}-induced increases. The biochemical mechanism of the Ca^{2+}-induced increase of sperm cyclic AMP is not yet known. It could be due to activation of adenylate cyclase or to inhibition of cyclic nucleotide phos-

phodiesterase, but the latter seems less likely because the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine potentiates the calcium effect. Because a calcium-dependent regulator protein (calmodulin) has been shown to activate adenylate cyclase of brain (29) and to be present in spermatozoa (30, 31), possibly in the acrosomal area (32), the activation of an acrosomal membrane adenylate cyclase (33) by a Ca^{2+}–calmodulin complex seems possible. The results also suggest that more than one Ca^{2+}-dependent process is responsible for induction of the guinea pig sperm acrosome reaction. An example of another Ca^{2+}-dependent event, the conversion of the zymogen precursor proacrosin to the active protease acrosin, has been suggested as necessary for the dispersal of the acrosomal contents during the sperm acrosome reaction (34, 35).

It appears that a cyclic AMP increase by itself will not induce an acrosome reaction. This is supported by the observation that, despite a rapid and marked increase in cyclic AMP concentrations within 0.5 min in a number of different incubation media, the acrosome reaction failed to occur unless the spermatozoa were capacitated. Furthermore, when 8-Br cyclic AMP was added to capacitated spermatozoa in the absence of added Ca^{2+}, it did not cause an acrosome reaction. Because added 8-Br cyclic AMP and 1-methyl-3-isobutylxanthine did cause a change in the time of maximal expression of the acrosome reaction, the alteration of sperm cyclic AMP concentrations may represent a required, although secondary component, of the acrosome reaction.

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