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Detection of the Mouse Acrosome Reaction by Acid Phosphatase. Comparison With Chlortetracycline and Electron Microscopy

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ABSTRACT: The sperm acrosome is a uniquely regulated secretory vesicle containing several hydrolase enzymes, including acid phosphatase (AP). The exocytotic event that releases these enzymes, the acrosome reaction, is required for fertilization in mammals. Different methods have been described in the scientific literature for detection of the acrosome reaction: double and triple stains, fluorescent-lectin stains, monoclonal antibodies against acrosomal antigens (immunodetection techniques), Coomassie blue, differential interference contrast or phase contrast, flow cytometry, and chlortetracycline (CTC). In contrast, only 1 method to detect AP released by live and reacted sperm has been described in the literature thus far. In this work we compare 2 classical methods, CTC and transmission electron microscopy (TEM), with the assay of AP released from the acrosome. AP released during the acrosome reaction was measured in the culture medium. Enzyme remaining in nonreacted sperm cells was released by Triton X-100 treatment. This enzyme-based methodology shows an increase of AP in the culture media after the acrosome reaction and a corresponding decrease in the detergent-releasable enzyme. The AP assay thus permits the detection of the mouse acrosome reaction and compares well with the CTC and TEM methods. This method is performed on the whole sperm population and so avoids the observer error that is inherent in light microscopic methods.

Key words: Spermatozoa, acrosomal enzyme.

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The acrosome reaction (AR) is essential for sperm penetration of the zona pellucida (ZP). ZP penetration is a process that depends on enzymes contained in the acrosome. These enzymes are released during the AR by fusion of the acrosomal and plasma membranes. This event is triggered by the interaction of a ZP glycoprotein, ZP3, and a sperm receptor (Yanagimachi, 1994). One of the acrosomal enzymes liberated during the AR is acid phosphatase (AP; Gonzales et al, 1973). An assay for AP released during the AR could be an alternative marker for AR. This assay is performed with unfixed cells and could avoid some of the subjectivity that is intrinsic in determining the AR by light microscopy.

Several methods have been described for detection of the AR: double and triple stains (Talbot and Chacon, 1981), fluorescent-lectin stain (*Pisum sativum* agglutinin-fluorescein isothiocyanate (PSA-FITC; Morales and Cross, 1989), monoclonal antibodies against acrosomal antigens (immunodetection techniques) (Talbot and Chacon, 1982; Braun et al, 1991), Coomassie blue (Larson and Miller, 1999), differential interference contrast or phase contrast microscopy (Suarez et al, 1983), flow cytometry (Tao et al, 1993), and chlortetracycline (CTC; Ward and Storey, 1984). Most of the these methods are at the light microscopy level and involve visual counting by trained technicians. These methods are time-consuming, requiring evaluation of sperm heads one by one. Furthermore, the status of the AR in the sperm population is evaluated only by counting 100 sperm per condition. In most situations it would be useful to evaluate the entire sample at the same time. Finally, the most useful assay is one that can be performed on living cells (Wolf et al, 1985).

Indirect determination of AP lost from rat spermatozoa in order to assess the AR has been described in the scientific literature (Salzberger et al, 1992). In this report, AP was measured using the spectrophotometric techniques described in the diagnostics manual (procedure 104) provided by Sigma Chemical Company (St Louis, Mo).

In this work, enzyme levels were determined in 1) the culture medium, which represent AP lost from live and

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reacted sperm; and 2) AP remaining in the acrosome of nonreacted sperm. We used a fluorometric method previously described with high sensitivity to measure AP instead of the photometric method.

Our work was intended to develop the AP assay as a marker of the AR and to compare it with the classical CTC and ultrastructural (transmission electron microscopy; TEM) methods of detecting acrosomal AP.

Materials and Methods

Reagents

Reagents were from Sigma except for those used for electron microscopy, which were from Pelco (Redding, Calif).

Collection of Gametes and Capacitation

Mouse sperm was obtained by cutting the isolated cauda epididymides of mature Balb-c outbred mice under HM, which is a modified Krebs-Ringer bicarbonate medium (HM-Hepes buffered). HM was prepared by thawing 5 mL of 3× HM containing 25 mM Hepes, 109 mM NaCl, 4.77 mM KCl, 1.19 mM $MgSO_4 \cdot 7H_2O$, 1 mg/mL glucose, 3.7 μL Na-lactate (60% syrup), 1.19 mM KH_2PO_4, and adding 1.65 mg sodium pyruvate, 1.5 mL of 17 mM $CaCl_2$ and 8.5 mL of double distilled water. Finally, the pH was adjusted to 7.3 with 10 M NaOH (Visconti et al, 1995). The pieces of epididymis were removed after 10 minutes and the sperm were washed. To evaluate how many washes were needed to rid the sperm of epididymal AP, the samples were washed 3 times with HM by centrifugation at 120 × *g* for 10 minutes, and the supernatants were assayed for AP. The results indicated that 2 washes were sufficient (data not shown). To capacitate (cap) mouse sperm, the sperm pellet was resuspended, adjusted to 1×10^6 sperm/mL, and incubated in a modified Krebs-Ringer bicarbonate medium (HMB) for 1 hour at 37°C and 5% CO_2. HMB was made from 15 mL of HM media plus 31.5 mg $NaHCO_3$ and 3 mg/mL bovine serum albumin (BSA, Fraction V; Sigma, pH 7.3; Visconti et al, 1995). Some pellets were suspended in HM to perform experiments at zero time or under noncapacitated conditions (noncap).

Sperm motility was checked several times in each experiment in 40 μL of the sperm suspension viewed at 400× in a Bausch & Lomb light microscope to identify the number of living cells. Experiments in which the fraction of living cells was less than 70% were not considered.

Induction of the Acrosome Reaction

In different sets of experiments, the following reagents were added to the sperm suspension in the final 15 minutes of incubation: 1 μM (P1) or 10 μM (P10) progesterone or 10 μM calcium ionophore (A23187). Control groups were assayed without reagents (see below) or with the addition of the vehicle dimethyl sulfoxide (DMSO). The doses used in this study were selected from the literature, from preliminary experiments, or both (Roldan et al, 1994; Murase and Roldan, 1996).

Enzyme Activity Assay

The samples used for enzyme activity were processed as described above and came from the same set of experiments used for CTC. The suspensions of capacitated or noncapacitated sperm were centrifuged (200 × *g* for 10 minutes) and the supernatants were kept on ice (SN1). The sperm pellets were resuspended in HM with 1% Triton X-100, vortexed, and incubated for 15 minutes at 4°C. Finally, the samples were centrifuged at 9300 × *g* for 10 minutes and supernatants (SN2) and pellets were kept on ice. The activity of the enzymes was measured fluorometrically (Barret and Heath, 1977) using the corresponding 4-methylumbelliferyl as substrate at a concentration of 0.03 mg/mL in 0.05 M citrate buffer, pH 4.5. After incubation for 30 minutes at 37°C, the reaction was stopped by adding 1 mL of 0.4 M glycine buffer, pH 10.4. One unit of activity represents the amount of enzyme that catalyzes the release of 1 nmol of 4-methylumbelliferone/mL per hour (Belmonte et al, 1998). The culture medium, HM, was used as the blank to zero the spectrofluorometer. Medium plus 1% Triton X-100, ionophore, and HMB media were checked for any fluorescence signal, but no difference was found (data not shown).

Beta-glycerophosphate, a well-known inhibitor of AP (Barret and Heath, 1977), was included in control experiments, and no AP activity was measured at concentrations of 30 μM in either SN1 or SN2.

Proteases could potentially decrease the measured AP activity. Therefore, we incorporated another control to check for protease activity by adding various protease inhibitors to the medium. Control experiments were performed in the presence of 10 μM leupeptin and 10 μM aprotinin, neither of which modified the measured enzyme activity (data not shown).

Enzyme activity measured in the supernatants from wash steps showed that the second wash contained 24% and the third 22% of the activity found in the first supernatant. The difference between the second and third washes was not significant; thus, we limited ourselves to 2 washes in order to optimize the integrity of the sperm cells. Sperm samples from the wash steps were processed for TEM to check the integrity of the cells.

Chlortetracycline Method

The CTC method was performed following the protocol of Kholkute et al (1995). Briefly, the CTC solution was made by dissolving CTC-HCl at a concentration of 500 μM in a buffer containing 20 mM Tris HCl, 130 mM NaCl, and 5 mM cysteine, pH 7.8. Fresh CTC solution was made before each assay. Fifty μL of sperm suspension from each condition was mixed with 50 μL of CTC. After a few seconds, sperm were fixed by the addition of 8 μL of 12.5% glutaraldehyde in PBS (20 mM phosphate buffer, 150 mM NaCl pH 7.4). The sperm suspension was washed by centrifugation in PBS at 120 × *g* for 10 minutes. The pellet was then resuspended in 100 μL PBS, and 7 μL of this suspension was placed on a clear slide with a drop of 10 mg/mL n-propyl gallate and 50% glycerol (all in PBS). Spermatozoa were examined for CTC fluorescence at a magnification of 600× on a Nikon microscope (Optiphot-2; excitation at 400–440 nm, emission at 455 nm). A total of 100 spermatozoa were scored on each slide. Two CTC staining patterns were observed: 1) head fluorescence with a fluorescence-free band in the post-

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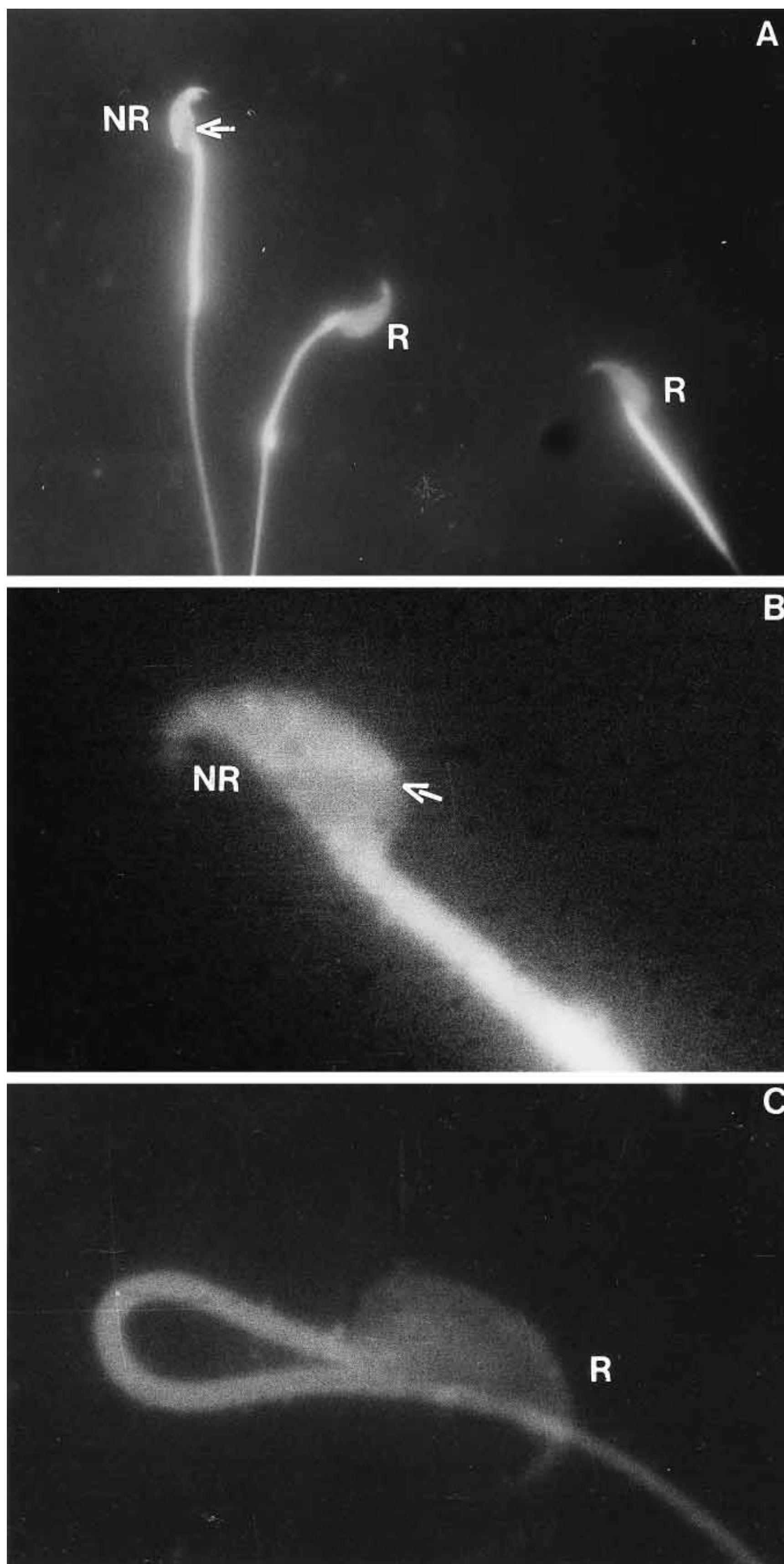

acrosomal region, which is characteristic of capacitated and unreacted sperm (Figure 1A and B); and 2) either a weakly homogeneous fluorescence or absence of fluorescence that was characteristic of acrosome-reacted cells (Figure 1A and C).

Transmission Electron Microscopy

Aliquots of sperm obtained from each experiment were fixed in 2% glutaraldehyde in PBS for 2 hours at room temperature. The samples were washed twice in PBS by centrifugation-resuspension at 750 × *g* for 10 minutes. The sperm pellets were resuspended in 200 μL PBS and an equal volume of 2% OsO_4 overnight at 4°C. The cells were then centrifuged (750 × *g* for 10 minutes) and the pellets were dehydrated in graded ethanol-acetone. Finally, the pellets were embedded in Epon 812 (Pelco). Thin sections were obtained with an Ultracut R ultramicrotome (Leica, Austria), stained by routine uranyl-Pb techniques, and observed in an Elmiskop I (Siemens) transmission electron microscope.

Statistical Methods

All statistical analyses and graphics were performed with SigmaPlot software (version 1.02[a], Jandel Corporation, San Rafael, Calif) except the one-way analysis of variance (ANOVA) test. Differences were considered to be statistically significant when $P < .05$. The values reported in the text and figures are means ± SD (range). The comparison between methods was made by Student's *t*-test. In addition, data were also analyzed with one-way ANOVA with $P = .05$ for multiple comparison.

Results

Acid Phosphatase Activity

Initially, the total AP activity was assayed. Total activity was considered to be the sum of the action in the culture media from the suspension of capacitated or noncapacitated sperm (SN1) and the supernatant from sperm treated with Triton X-100 (SN2). The AP activity was measured at 15, 30, 60, and 90 minutes of incubation in the absence or presence of Triton X-100. The total AP activity was similar at those times under these conditions (data not shown). This finding implies that the enzyme was stable in our experiments. We also examined whether the AP increased with increasing numbers of sperm. The total activity increased linearly between 1×10^5 and 1×10^6 sperm/mL (Figure 2). The sperm samples obtained from the wash steps showed no disruption of the sperm cells under electron microscopy observations (data not shown). These observations indicated that the cells were intact at the beginning of the experiment and implied that the amount of enzyme was not lost in the washing steps.

AP Activity in Capacitated and Noncapacitated Sperm

The activity was measured in the supernatants (SN) pre- (SN1) and post-Triton X-100 (SN2), and in the final pellets of noncapacitated (noncap; Figure 3) and capacitated sperm (cap; Figure 4). SN1 corresponds to the enzyme released from the acrosome into the culture medium during the incubation period as a result of the AR. SN1 in cap sperm (37% ± 5) shows more AP activity compared with noncap sperm (24% ± 3%, $P = .05$; compare Figures 3 and 4). SN2 corresponds to the enzyme activity released by disruption of the plasma membrane with Triton X-100 and thus likely represents the fraction of nonreacted sperm. Note that the AP activity was higher in noncap sperm (SN2 = 74% ± 4%) compared with cap sperm (SN2 = 55% ± 2%, $P < .05$; Figures 3 and 4). No significant differences were observed between the pellets (noncap and cap, $P > .05$; Figures 3 and 4). The DMSO control did not show any difference compared with the control group (data not shown).

AP Activity in the Culture Medium of Sperm That Have Undergone the Acrosome Reaction

AP released from reacted sperm into the culture medium (SN1) at time zero was 24% (± 2%; Figure 5). This represents the enzyme released by spontaneous reaction and some remaining from the epididymal fluid. Under capacitating conditions (control) the activity was 41.3% (± 3.0%; Figure 5). Activity was further increased by adding A23187 (65.4% ± 7.0%) or progesterone (P1, 63.7% ± 6.0%; or P10, 70% ± 13.0%) to the sperm suspension (Figure 5). Significant difference were found between control and A23187 ($P < .05$), P1 ($P = .05$), and P10 ($P < .05$; Figure 5). No significant differences were found between A23187, P1, and P10. AP remaining in the nonreacted sperm released by Triton X-100 treatment (SN2) showed a decrease that was proportional to the increase in SN1 activity (data not shown).

CTC Method in Sperm Spontaneously Reacted or Induced to React

The AR in noncap sperm cells (0 time) was 25.6% (± 1.0%), but in cap sperm (control) the AR increased to 33.3% (± 2.0%; Figure 6). When A23187 or P10 were added to the sperm suspension the AR reached 66.5% (±

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Figure 1. Mouse spermatozoa incubated in HMB media for 60 min and stained by the CTC method. The spermatozoa show a bright fluorescent zone over the acrosome region that contrasts clearly with the postacrosomal region (arrow) in capacitated, nonreacted sperm (NR) **(A–B)**. A conspicuous equatorial line can be seen at higher magnification (arrow; **B**). Capacitated, reacted mouse sperm (R) display a homogeneous fluorescence over the sperm head. Note that no difference in fluorescence can be seen between the acrosome and postacrosome regions (**A–C**). Magnification: 400× **(A)**, 600× **(B)** and **(C)**.

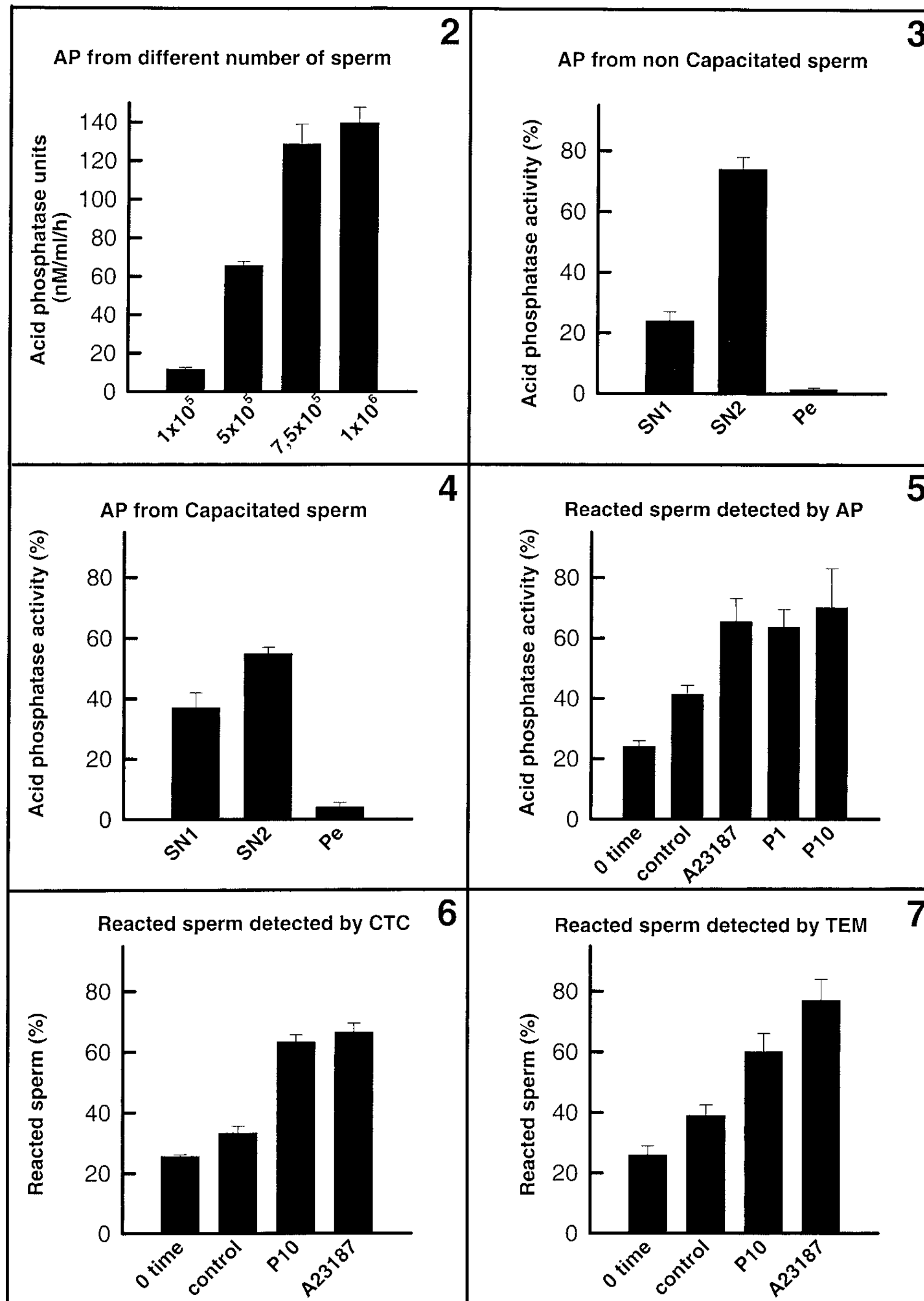

Figure 2. Increasing the number of capacitated mouse sperm (1×10^5 to 1×10^6 sperm/mL) increased AP activity linearly from 15 to 140 nM/mL per hour. The bars represent the total AP activity of the sample (sum of SN1 and SN2). Similar results were obtained with noncapacitated sperm (data not shown). Each bar represents the mean ± SD of at least 6 experiments. Figure 3. AP activity in the media from 1×10^6 noncapacitated spermatozoa/mL was 3 times greater in the Triton X-100 extract of the sperm cells (SN2) compared with the activity in SN1. The remaining pellet (Pe) had low activity. SN1, SN2, and Pe AP activities are expressed as a percentage of the total activity. Each bar represents the mean ± SD of at least 6 experiments. Figure 4. AP activity from 1×10^6 capacitated spermatozoa/mL was only 18% greater after Triton X-100 treatment of the sperm cell (SN2) compared with SN1. The remaining pellet also had a low activity (Pe). SN1, SN2, and Pe AP activities are expressed as a percentage of the total activity. Each bar represents the mean ± SD of at least 6 experiments. Figure 5. Reacted sperm detected by AP in 1×10^6 sperm/mL. AP activity (SN1) originating from capacitated sperm was increased by the addition of ionophore (A23187, 10 μM) or progesterone (P1 = 1 μM; P10 = 10 μM) compared with time zero and control samples. Control corresponds to capacitated sperm SN1 without additions and 0 time corresponds to noncapacitated sperm SN1 also without any treatment. Each bar represents the mean ± SD of at least 6 experiments. Figure 6. Reacted sperm detected by CTC. Acrosome reaction was also increased by the addition of P10 and A23187. Each bar represents the mean ± SD of at least 3 experiments. A total of 100 spermatozoa were scored on each treatment. Figure 7. Reacted sperm detected by TEM. Acrosome reaction was also increased by the addition of P10 and A23187. Each bar represents the mean ± SD of at least 3 experiments. A total of 100 spermatozoa were scored on each treatment.

3.0%) or 63.3% (± 2.0%), respectively (Figure 6). Progesterone was used at 10 μM in the AP assay because this concentration produced a large difference in AP compared with the SN1 control.

Transmission Electron Microscopy of Sperm Spontaneously Reacted or Induced to React

Most of the sperm heads in capacitated sperm were surrounded by the plasma membrane and intact acrosome, but in others, some degree of AR was observed. The number of reacted sperm was as follows: in noncap (0 time), 26% (± 3.0%); in cap (control), 39.0% (± 3.5%); in A23187, 77% (± 7%); and in P10, 60% (± 6%; Figure 7). The AR varied from complete vesiculization and a total disappearance of acrosomal content to merely a swelling of the acrosomal content (Figure 8). We considered to be truly reacted only those sperm that presented a complete loss of acrosomal content and a well-determined equatorial segment (Figure 8).

Statistical Study

One-way ANOVA revealed insignificant differences between the 3 methods (comparisons were made with the percentage of the AR sperm detected by AP, CTC, and TEM in noncap, cap, P, and A23187).

Discussion

The AR is a phenomenon that has been studied by several laboratories with different objectives: to analyze the signal transduction pathway that triggers this reaction, to consider the role of this event in the fertilization process, and to characterize the molecules involved in membrane fusion. In all these cases, however, the precise and rapid evaluation of true AR is crucial.

Different techniques had been developed in previous studies to evaluate the acrosomal status. Most of these procedures involve counting acrosome-reacted profiles sperm by sperm, which entails a substantial subjective component that leads to variability. In addition, it is supposed that the 100 sperm counted are representative of the entire sample. Some of these techniques are sensitive to variations in stain intensity, which results in additional variability in the discrimination of reacted and nonreacted sperm. Such errors could be avoided by using a more objective technique applied to the entire sperm sample. Furthermore, the time consumed by the methodology is also a consideration. Another possible objection to these techniques is that some of them use fixed cells (Wolf et al, 1985). These ideas prompted us to evaluate whether the AP technique would avoid some of the negative aspects pointed out above, and if it is a useful means for measuring mouse sperm AR.

A previous report indicated that AP released from the sperm acrosome can be used to assess the AR in rat spermatozoa (Salzberger et al, 1992). It has also been reported that AP activity was localized by cytochemical detection at the ultrastructural level, between the outer acrosomal membrane and the inner surface of the periacrosomal plasmalemma in mouse spermatozoa (Poirier, 1975).

The AP assay represents a simple procedure that has the advantage of measuring the entire sample. AP activity was measured simultaneously in 1) the culture media, which is representative of AP released by the AR (SN1); and 2) the media containing AP that was liberated by detergent (SN2). This process offers the possibility of comparing both pre-reaction and post-reaction AP activity. For example, SN2 (post-Triton X-100) was higher than SN1 in noncapacitated sperm. But in capacitated sperm the SN1 increased while the SN2 decreased proportionately, due to spontaneous AR. It is also notable that AP released from reacted sperm into the culture medium (SN1) at time zero was lower than that of the capacitated condition (control), indicating that the released activity increased during the capacitation period. Furthermore, adding ionophore or progesterone to the sperm suspension substantially increased the activity of released AP. All these increments are related to the occurrence of spontaneous or induced AR.

In previous papers the AR was demonstrated by CTC or PSA-FITC, each of which detected chemical groups on the surface of sperm (Khön et al, 1997). But the test described here was designed to use AP release as a direct measure of the AR. It is interesting to remark that the ultrastructure of the sperm is well preserved and that the reacted sperm show a well-defined equatorial segment. In addition, it should be noted that we also found a good correlation between the 3 methods used in this work. Some small differences between the AR score in the 3 methods could be due to the sensitivity of the methods themselves.

Another source of AP was the epididymal fluid (Nikkanen and Vanha-Perttula, 1976). AP activity decreased in the wash media and was very low in the final wash. This activity might remain on the sperm surface and thus would give rise to the basal activity in the AP assays (noncap).

In conclusion, this procedure is an alternative method to evaluate true AR in whole sperm population. It is also an objective technique that avoids observer error. In addition, it is quick to perform compared with optical methods.

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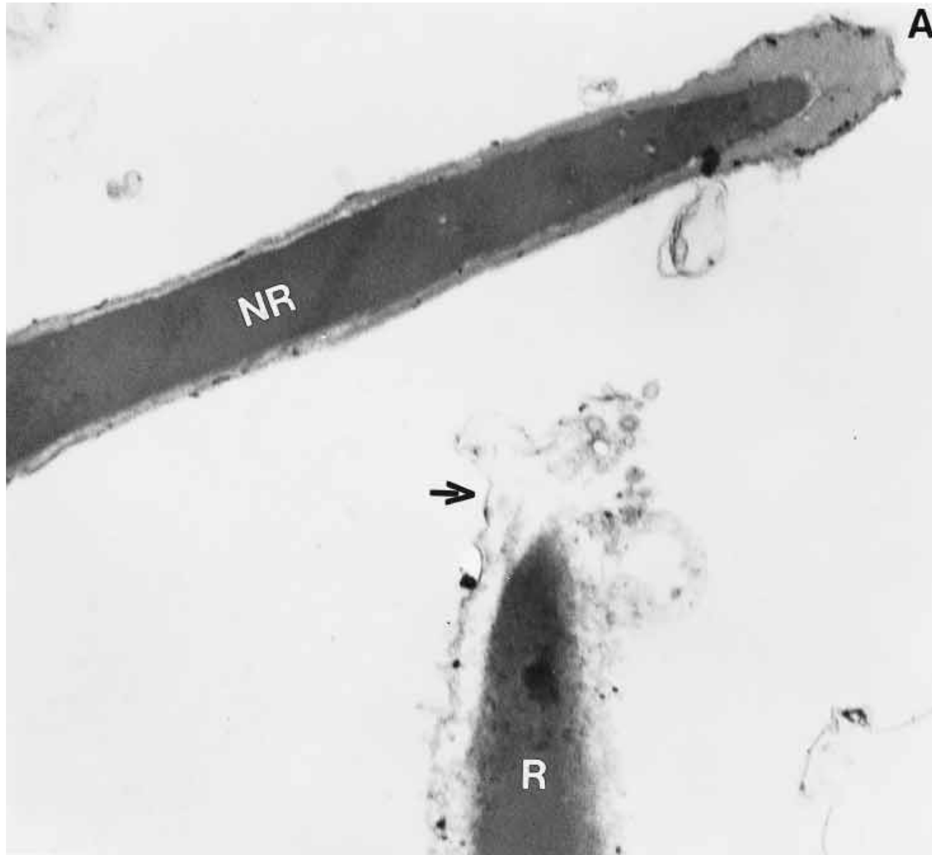
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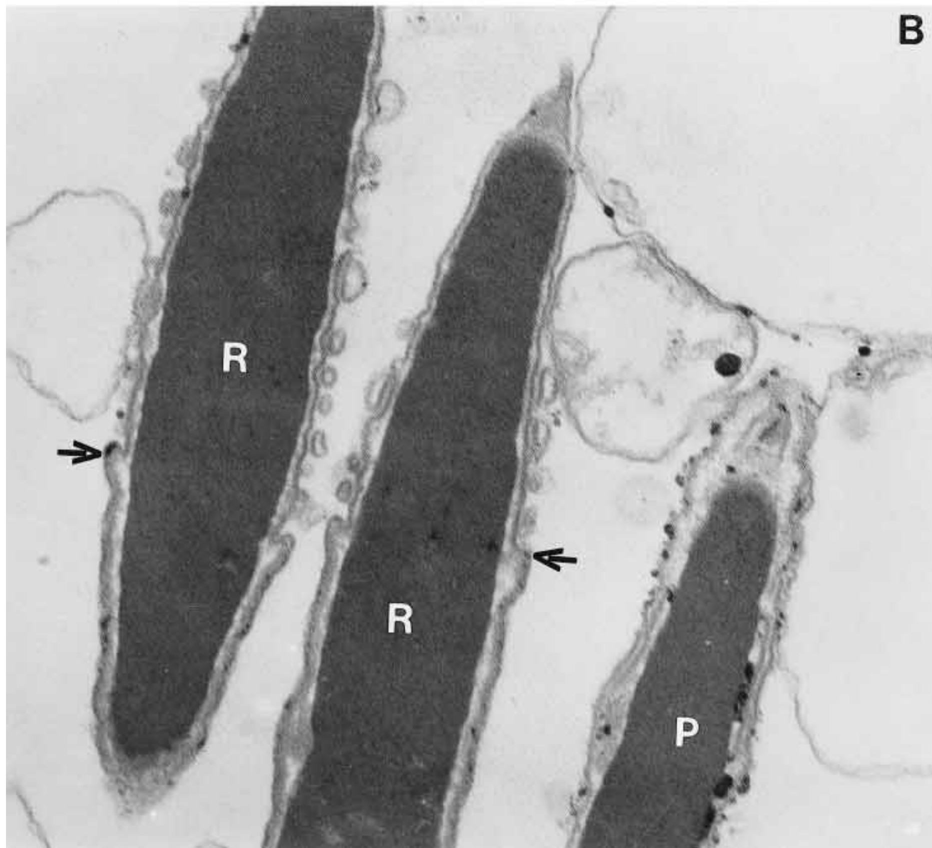
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and Irene Britten for her reading. This paper is dedicated to the memory of O. Arango, who died 12 September 1998.

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Figure 8. Electron micrograph of sperm incubated in HMB. A nonreacted sperm (NR) maintains the acrosome content and its membranes **(A)**. Note the membrane vesicles and electron lucent acrosome content present at the anterior zone of the head indicating a reacting spermatozoa (arrow; **A**). Full-reacted sperm (R) show vesicles and a clearly established equatorial segment (arrows; **B**) and another partially reacted (P; **B**). NR and P sperm were scored as nonreacted but R sperm were scored as reacted sperm. Magnification: 6000× **(A)**, 8500× **(B)**.