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Enhanced Binding of Zona Pellucida Proteins to the Acrosomal Region of Intact Boar Spermatozoa in Response to Fertilizing Conditions: A Flow Cytometric Study[1]

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ABSTRACT

In this investigation we sought to determine whether sperm capacitation in vitro is accompanied by changes in the functional presence of zona binding sites on the plasma membrane of boar spermatozoa. During sperm incubation at 39°C in various modifications of a Tyrode's-based in vitro fertilization medium, the zona binding ability of individual spermatozoa was assessed with fluorescein-conjugated solubilized zona pellucida proteins, using a flow cytometer. Propidium iodide was routinely included to allow simultaneous assessment of membrane integrity; rhodamine-conjugated peanut agglutinin was used to assess acrosomal status. During incubation in the fertilization medium, a subpopulation of live acrosome-intact spermatozoa developed enhanced binding of the fluorescein-conjugated solubilized zona proteins. Microscopy revealed that the increase in cytometrically detected zona binding was paralleled by an increase in the area on the sperm head to which zona proteins bound, from the apical region to the whole of the acrosomal region. The changes were accelerated by phosphodiesterase inhibitors, were attenuated by omission of bicarbonate, and were completely inhibited by addition of EGTA. In the fertilization medium, numbers of sperm showing enhanced zona binding maximized after 60–90 min. This time course is somewhat similar to that reported by others for development of egg-penetrating ability in vitro. We suggest that the observed changes in zona binding ability bring about optimal sperm-egg attachment; they may also relate to induction of the acrosome reaction by zona pellucida components. In consequence, the zona binding changes may be an important part of the process by which the sperm acquires fertilizing ability as a result of capacitation.

INTRODUCTION

Before a spermatozoon can fuse with an oocyte, it needs to bind to and penetrate the zona pellucida, the extracellular matrix that surrounds the oocyte. To be able to penetrate the zona pellucida, the sperm have to undergo the acrosome reaction, an exocytotic membrane fusion event. There is considerable evidence that the fertilizing sperm undergoes the acrosome reaction in consequence of binding to components of the zona pellucida via specific receptors on the plasma membrane surface [1, 2]. Thus, correct interaction with the zona pellucida is crucial to the fertilization process.

Although spermatozoa fresh from the male reproductive tract can bind to the zona pellucida [3, 4], they do not have the immediate ability to undergo a zona-induced acrosome reaction [5] or to penetrate the zona pellucida [6]. These latter abilities develop in mammalian spermatozoa as a result of a priming process known as capacitation [2, 7]. Capacitation takes place in vivo in the female reproductive tract, but it can also be induced in vitro as a precondition for successful in vitro fertilization (IVF). Many cellular and biochemical changes that occur in sperm samples during such IVF incubations have been described [8]. However, relating a given change to the acquisition of fertilizing ability has proved difficult. The lengthy period needed for maximal fertilizing ability to develop within sperm samples subjected to in vitro "capacitating" treatments implies that the capacitation process may comprise a sequence of events. Moreover, sperm populations display heterogeneity, in that individual cells respond to capacitating conditions at widely different rates. It is not possible to detect the fertilizing ability of individual spermatozoa and therefore to match the changes that can be observed in individual cells to the acquisition of fertilizing ability. Thus, identification and ordering of the events within the capacitation sequence is rendered very problematical.

An alternative approach has been to use flow cytometry to examine the changes in individual cells within the sperm population that are brought about specifically by components or conditions essential to the acquisition of fertilizing ability. Among these, bicarbonate appears to play a primary role, having been shown to be needed for the acquisition of fertilizing ability in vitro ([9] and references therein), for the acquisition of the ability to undergo the acrosome reaction in response to zona pellucida components [1, 10–12], and for the expression of hyperactivated motility [13] (another parameter of capacitation, thought to be needed for zona penetration [14]). Moreover, bicarbonate has been shown to induce some specific changes in the sperm plasma membrane, with respect both to lipid architecture [15, 16] and to the reorganization of surface components [17]. The involvement of calcium in capacitation has also been demonstrated [18], but the details of its role have not yet been ascertained.

Despite the pivotal importance of binding of zona components for sperm-egg attachment and induction of the acrosome reaction, little is known about the effect of fertilizing conditions on the functional presence of zona receptors at the sperm plasma membrane surface. In this study, we have used flow cytometry to investigate the ability of boar sperm to bind fluorescein-conjugated solubilized zona pellucida proteins during the course of incubation under IVF conditions. In particular, we have noted the influence of the key components of IVF media, bicarbonate, and calcium.

MATERIALS AND METHODS

Chemicals

Unless stated otherwise, reagents (of analytical grade if available) were purchased from Sigma Chemical Co.

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TABLE 1. Composition of the variants of the Tyrode's-based medium used for sperm incubation.

Component	Variant					
	TB*	TBC	TB-Ca	TB+E	T	TC
15 mM HCO_3^-	+	+	+	+	−	−
4.5 mM Ca^{2+}	+	+	−	−	+	+
2 mM EGTA	−	−	−	+	−	−
2 mM caffeine	−	+	−	−	−	+

* Standard Tyrode's medium (TB) contained 15 mM $NaHCO_3$, 4.5 mM $CaCl_2$, 96 mM NaCl, 3.1 mM KCl, 0.3 mM KH_2PO_4, 0.4 mM $MgSO_4$, 5 mM glucose, 21.7 mM sodium lactate, 1 mM sodium pyruvate, 20 mM Hepes (adjusted to pH 7.6 at 25°C with NaOH, to obtain a pH of 7.4 at 39°C), 5 mg/ml BSA (Sigma A4378), 100 μg/ml kanamycin, 20 μg/ml phenol red, and 2.5 μg/ml propidium iodide (PI); all variants were prepared by mixing relevant amounts of stock solutions.

(Poole, Dorset, UK) or BDH (Merck Ltd., Poole, Dorset, UK). Percoll was obtained from Pharmacia Biotech Ltd. (St. Albans, Herts, UK). Tetramethylrhodamine isothiocyanate-labeled peanut agglutinin (TRITC-PNA) was derived from Vector Laboratories Ltd. (Peterborough, UK).

Media and Incubation Conditions

Variants of the modified Tyrode's medium used by Parrish et al. [19] were used for incubation of spermatozoa. The compositions of the variants are given in Table 1. Osmolality was maintained at 300 mOsmol/kg in all by adjusting the NaCl concentration. Variants containing $NaHCO_3^-$ (i.e., TB, TBC, TB-Ca, TB+E) were maintained in equilibrium with 5% CO_2 in air during incubations, whence all media had a nominal pH of 7.4 at 39°C (in practice, pH 7.3–7.4: this was achieved by adjusting EGTA and caffeine stocks to pH 7.4 with NaOH prior to inclusion and by maintaining all variants containing HCO_3^- in equilibrium with 5% CO_2; see [9]).

Beltsville Thawing Solution (BTS) was used both as extender for fresh boar semen and as incubation medium in some experiments. BTS was made up according to Johnson et al. [20], with kanamycin (50 μg/ml) used as antibiotic instead of penicillin and streptomycin.

All media were passed through a 0.22-μm filter in order to reduce "noise" in the flow cytometric analyses.

Unless otherwise stated, incubations were carried out at a temperature that was measured as 38.5°C by conventional laboratory thermometers. However, to give the temperature as 38.5°C would imply a level of accuracy that could not be justified, therefore throughout this paper we have referred to the incubation temperature as 39°C.

Collection and Washing of Spermatozoa

Sperm-rich fractions of 25 ejaculates were collected from 4 Large White boars (proven breeders, 2–4 yr of age, 200–275 kg BW) belonging to the Babraham Institute's colony; collections were made from each boar once a week. After cooling slowly to room temperature (20–22°C), the semen was filtered through gauze to remove gel particles and diluted to a final concentration of 1.5×10^8 cells/ml in BTS. The diluted semen was stored at room temperature for up to 48 h.

Thirty minutes before start of the experiments, aliquots of 4.5×10^8 spermatozoa (3 ml of diluted semen) were layered over 4 ml 35% iso-osmotic Percoll-saline on 2 ml 70% Percoll-saline, and centrifuged for 5 min at 200 × *g* followed by 15 min at 900 × *g* [21]; the saline basal medium for the Percoll consisted of 137 mM NaCl, 2.5 mM KCl, 10 mM glucose, and 20 mM Hepes adjusted to pH 7.5 with NaOH. After centrifugation, the supernatant layers were aspirated to leave 0.5 ml "pellet plus supernatant," in which the loosely packed spermatozoa were readily resuspended.

Preparation of Fluorescein-Conjugated Solubilized Zona Pellucida Proteins

Zonae pellucidae were prepared from porcine ovaries obtained from a slaughterhouse. The ovaries were stored frozen until required and then thawed in an isolation buffer (2 mM EDTA, 10 mM Na_2HPO_4, 130 mM NaCl, 11 mM sodium citrate, 0.2% w:v polyvinyl alcohol [Sigma P-8136], pH 7.0). Oocytes were isolated from the ovaries, according to Dunbar et al. [22], on ice. Isolated oocytes were layered on top of 30 ml of 15% Percoll in isolation buffer and centrifuged for 45 min at 30 000 × *g* (8°C). A band of oocytes (visible at a height of two-thirds of the gradient column) was then aspirated. The oocytes were homogenized using a Potter-Elvehjem homogenizer. The zonae were collected on a 45-μm stainless steel screen, rinsed with the isolation buffer, and centrifuged twice for 10 min at 800 × *g*. The pure zonae were solubilized in 50 mM $NH_4HCO_3^-$ (pH 7.2, adjusted with acetic acid) by heating at 70°C for 30 min. The solubilized preparation was lyophilized and stored dry (with desiccant) at −20°C.

The purity of this porcine zona preparation was assessed using two-dimensional gel electrophoresis according to Topper et al. [23]. Material was solubilized in electrophoresis sample buffer under reducing conditions and subjected to isoelectric focusing in 8 M urea for the first-dimensional separation, and to SDS-PAGE in 7.5% poly-acrylamide gel for the second-dimensional separation. Proteins were visualized by the use of a silver-staining method.

The ability of the prepared zona proteins to inhibit binding of spermatozoa to the zona pellucida was investigated in a competition assay. Washed spermatozoa (2×10^6 cells/ml) were incubated in standard Tyrode's medium (TB, Table 1) at 39°C in plastic test tubes. After 2 h, 45-μl aliquants of the cell suspension were gently mixed with 5 μl of the solubilized zona pellucida proteins in the same medium at a final concentration of 100 μg/ml (or 0 μg/ml, as control), and incubation was continued in 50-μl droplets under paraffin oil. After 10 min, a volume of 50 μl of the same Tyrode's medium containing 10 oocytes (isolated according to Dunbar et al. [22]) was added to each sperm droplet, and incubation was continued for 60 min. The oocytes were washed to remove loosely bound spermatozoa by pipetting 5 times with a glass pipette and transferring them into fresh droplets of medium; the internal diameter of the pipette was twice the diameter of the oocytes. Finally, the oocytes were transferred to droplets of medium without BSA but containing 2.5% glutaraldehyde and fixed for 15 min. After treatment with 0.05% Nonidet P-40 and 10 μM propidium iodide (PI), the numbers of spermatozoa bound to the zonae were counted using a fluorescence microscope. Two separate experiments were carried out, using semen from 2 different boars, within each of which sperm-egg interactions at each level of zona protein were tested in duplicate.

Conjugation of zona proteins with fluorescein isothiocyanate (FITC) was performed by incubation of the proteins with FITC for 4 h at 20–22°C in a 100 mM borate buffer (pH 8.5; 1 mg/ml protein and 0.2 mg/ml FITC). Ex-

cess FITC was removed using a Centricon-30 centrifugal concentrator (Amicon Inc., Lexington, MA). The concentrated protein fraction (of approximately 400 μl) was then diluted fourfold with Hepes-buffered saline (150 mM NaCl, 20 mM Hepes; pH 7.4) and resubjected to centrifugal concentration. This washing procedure was repeated five times. The labeled protein preparation and the filtrates were stored at −20°C. The filtrate collected during the fifth washing step was used as a control for interference of "free" FITC during flow cytometry. The protein concentration of the obtained FITC-labeled solubilized zona protein (FITC-sZP) preparation was estimated in an assay using bicinchoninic acid [24].

Assessment of Binding of Zona Proteins

From the washed sperm preparations, aliquants of 15 μl that contained approximately 6×10^6 spermatozoa were transferred to 100 × 16-mm screw-capped conical-based plastic tubes (144AS: Bibby Sterilin Ltd., Stone, Staffs, UK), containing 1.985 ml of chosen prewarmed medium; the bicarbonate-containing media were in equilibrium with 5% CO_2. The suspensions were then mixed gently and put in a water bath to incubate at 39°C. After 5, 30, 45, 60, 90, 120, and 180 min of incubation, subsamples of 147 μl were taken and mixed with 3 μl of a 100 μg/ml FITC-sZP stock solution (in bicarbonate-free Tyrode's medium: T, Table 1); the final concentration of FITC-sZP was 2 μg/ml. Four minutes later, the spermatozoa were subjected to two-color flow-cytometric analysis to measure fluorescence of cell-associated FITC-sZP and PI simultaneously. Each time a tube of bicarbonate-containing medium was uncapped (for sperm addition or removal), the overlying (premoistened) 5% CO_2 gas phase was briefly replenished before recapping and continued incubation [21].

Pilot experiments showed that maximal binding of FITC-sZP was achieved in less than 4 min. Removal of residual free FITC-sZP was unnecessary because the flow cytometer only detects particle-associated fluorescence.

To investigate the effect of temperature, similar incubations were carried out at 20°C instead of 39°C.

Assessment of the Acrosomal Status

Washed spermatozoa were incubated at 39°C in standard Tyrode's medium (TB) for 3 h. At set time points, subsamples of 147 μl were taken, mixed with 3 μl of TRITC-PNA (150 μg/ml) and, after 4 min, analyzed by flow cytometry. In these experiments, PI (whose fluorescence emission spectrum is similar to that of TRITC) was replaced with Hoechst 33258 (final concentration 2.5 μg/ml) to assess membrane integrity. Pilot studies confirmed that binding of TRITC-PNA was established within 1 min [17] and that the brief exposure to the lectin caused no detectable cell death (c.f. [17]).

PNA binds to sperm whose acrosomal components are exposed but does not bind to the plasma membrane of intact sperm [17, 25]; thus live cells with an intact acrosomal cap will be PNA-negative, whereas live cells undergoing an acrosome reaction will be PNA-positive. Such cells can be distinguished from acrosome-damaged cells because the latter will also stain with vital stains, such as Hoechst. In some experiments, FITC-sZP binding and TRITC-PNA binding were assessed together. TRITC-PNA was added 1.5 min before addition of FITC-sZP. Incubation was then continued for 2.5 min, after which the cells were subjected to three-color flow cytometry. The resultant binding of the probes used in combination was identical to the binding of the probes when used separately.

Parallel to the flow cytometric analysis of the sperm, the acrosomal morphology of TRITC-PNA-stained preparations was evaluated by fluorescence and phase contrast microscopy of the wet mounts. The spermatozoa were immobilized by addition of 0.8 mM formaldehyde [26], and acrosomes were assessed according to the criteria of Pursel et al. [27].

Flow Cytometric Analysis

Two-color flow cytometry was performed using a Facscan flow cytometer (Becton Dickinson, San José, CA), which collected fluorescence data in logarithmic mode, and forward and side light-scatter data in linear mode from 10 000 events per sample, at a rate of 500–1000 events per sec. Fluorescence of FITC was detected using the FL1 530/30-nm band-pass filter, and fluorescence of PI was detected using the FL3 650-nm long-pass filter. Data were analyzed using Facscan software. Through inspection of light-scatter data (indicative of size and cellular nature), small (non-sperm) events and large events (due mostly to sperm aggregates) were excluded from the analysis by gating. The data were then displayed as dot-plots of relative FITC and PI fluorescence, on which computerized quadrants were set up to partition the data into subpopulations from which the relative proportions of the sperm categories were calculated (see Fig. 2). The overall cell subpopulation patterns were very consistent in all experiments; however, there were slight day-to-day variations in channel response that stemmed from a combination of instrument performance and basal staining within individual sperm samples. Thus the channel limits for the quadrants were set daily (i.e., for a given day's experimental replicate), after visual inspection of the particular data set. Some dead cells were always detectable in any sperm sample (with relative PI fluorescence at least 10-fold greater than that of live cells); thus differentiation between the live and dead populations was always clear cut (in Fig. 2, quadrants I and II versus III and IV, respectively), and the dividing boundary was always set midway between the two groups. The dividing boundary between low and high FITC-sZP binding was always set just beyond the upper limit of the major live population seen after 5-min incubation (see division between quadrant I versus II in Fig. 2a).

Three-color flow cytometry was performed on a Facstar-Plus flow cytometer (Becton Dickinson). The fluorescent signals of Hoechst 33258 (excited at 361 nm with a UV laser and detected using a 390-nm long-pass filter), FITC-sZP (excited at 488 nm with an argon laser and detected using a 530/30-nm band-pass filter), and TRITC-PNA (excited with the argon laser and detected using a 580-nm long-pass filter) were collected from 10 000 events, in combination with forward light-scattering signals. Data were analyzed by procedures similar to those described above, using Lysis-II software (Becton Dickinson).

Statistical Analysis

Data were analyzed using a generalized linear mixed model [28]. Effects were introduced on the probit scale. Fixed effects were main effects for boars, time, media, and interaction between time and media. Random effects with corresponding components of variance were ejaculates within boars, tubes within ejaculates, and interaction between boars and media and between media and time. The random effects in the model account for correlation be-

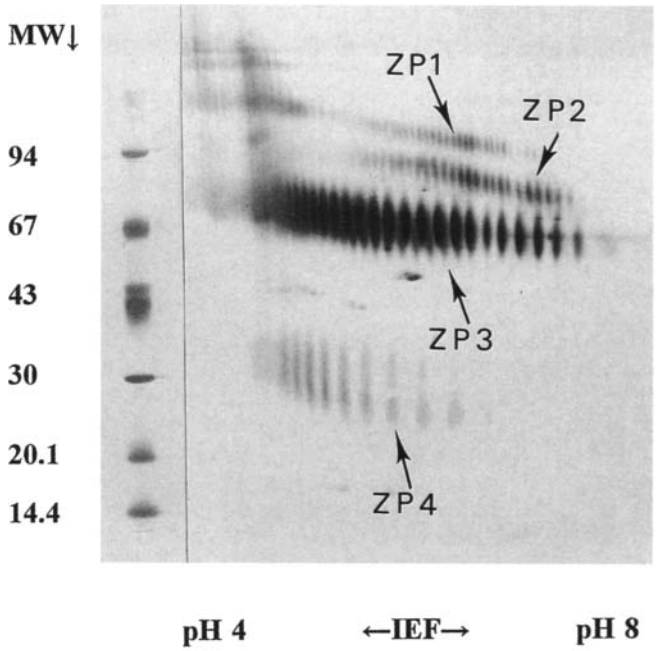

FIG. 1. Two-dimensional gel electrophoresis of solubilized porcine zona proteins. The protein preparation was isolated from porcine zonae pellucidae, as described in *Materials and Methods*, and then solubilized in electrophoresis sample buffer under reducing conditions. Isoelectric focusing in 8 M urea was used for the first-dimensional separation and SDS-PAGE in 7.5% polyacrylamide gel for the second-dimensional separation. Proteins were visualized by the use of a silver-staining method. For details of the method, see Topper et al. [23]. ZP1, ZP2, ZP3, and ZP4 indicate the major glycoprotein derivatives of the porcine zona pellucida [31]. Molecular weight markers ($\times 10^{-3}$) appear at left.

tween measurements on, for instance, the same ejaculate, or the same tube within an ejaculate. Boar main effects were entered as fixed effects and not as random effects because of the small number of boars in the experiment.

On the residual level, the variance (Var) of a fraction y (= percentage/100) was assumed to be a multiple of $\mu(1-\mu)$, where μ is the probability (conditional upon the random effects) for the expression of a given sperm property (e.g., a cell to be alive). Thus $\mathrm{Var}(y) = \phi\,\mu(1-\mu)$. The unknown dispersion parameter ϕ was estimated from the data and represented the combined effects due to sampling, counting, and other residual sources of variation. Overall significance tests were based on the Wald test [29]. Calculations were performed in the statistical programming language Genstat 5 [30].

Unless otherwise stated, numerical data are expressed as means ± SD.

RESULTS

Characteristics of the Solubilized Zona Protein Preparation

The electrophoretic pattern of the solubilized porcine zona protein preparation is shown in Figure 1. It resembled that reported by Koyama et al. [31]: under reducing conditions, 4 zona protein families (ZP1, ZP2, ZP3, ZP4) were resolved, and only very low levels of other (contaminating?) proteins could be detected.

Incubation of spermatozoa with the solubilized zona preparation led to a significant reduction in the numbers of sperm binding to the zona pellucida, from an average of 266 ± 84 to 52 ± 28 spermatozoa per zona (4 ejaculates tested, using 20 oocytes for each sperm sample; $p < 0.05$). The effect indicated that the preparation used for the study was indeed able to interact with the sperm receptors responsible for sperm-zona attachment.

Binding Capacity of Spermatozoa for FITC-sZP during Incubation in Tyrode's Medium

At the start of incubation at 39°C, all membrane-intact (i.e., live) sperm showed approximately the same level of FITC-sZP fluorescence (Fig. 2a,, in which the live cells are designated by quadrants I and II). However, in the standard Tyrode's medium TB (i.e., in the presence of 15 mM HCO_3^- and 4.5 mM Ca^{2+}), a subpopulation of the live sper-

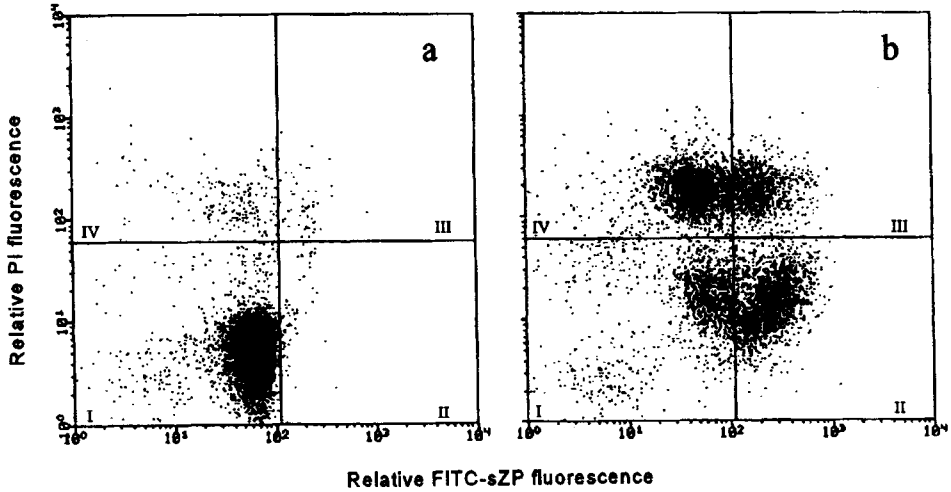

FIG. 2. Flow cytometric dot plots of sperm populations labeled with FITC-sZP. Washed spermatozoa were incubated in standard Tyrode's medium (TB, Table 1) at 39°C; at the stated times, samples of the suspension were mixed briefly with FITC-sZP and analyzed by use of two-color flow cytometry (PI was included in the incubation medium as a viability stain). Fluorescent signals of cell-bound FITC-sZP and PI were collected in a logarithmic mode. After collection of data, signals from debris and sperm aggregates were excluded from further analysis, after which computerized quadrants were set according to the dot-plot patterns of relative FITC-sZP and PI fluorescence signals. For details of all procedures, see *Materials and Methods*. **a**) After 5-min incubation; **b**) after 180-min incubation. Cells within quadrants I and II are live (PI-negative); cells within quadrants III and IV are dead (PI-positive); cells within quadrant I are those defined as low-FITC-sZP-binding live; cells within quadrant II are those defined as high-FITC-sZP-binding live.

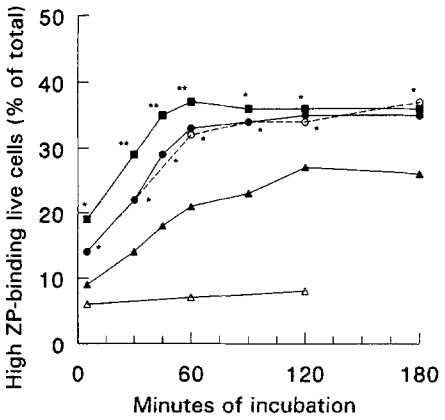

FIG. 3. Effect of bicarbonate, calcium, and caffeine on the development of a subpopulation of intact boar spermatozoa with altered zona binding abilities. Washed spermatozoa were incubated at 39°C in various modifications of a Tyrode's medium (see Table 1): solid circles, TB (4.5 mM Ca^{2+} and 15 mM HCO_3^- present); open circles, TB-Ca (15 mM HCO_3^- present but no supplementary Ca^{2+}); squares, TBC (4.5 mM Ca^{2+}, 15 mM HCO_3^- and 2 mM caffeine present); solid triangles, T (4.5 mM Ca^{2+} present but no bicarbonate); open triangles, TB+E (15 mM HCO_3^- and 2 mM EGTA present). PI was included in all media as a viability stain. At intervals, subsamples were mixed briefly with FITC-sZP and subjected directly to two-color flow cytometry. After signals from debris and sperm aggregates were excluded, the percentage of the total population that constituted the live cells with enhanced cell-bound FITC-sZP fluorescence was calculated for each sample (i.e., those cells falling within quadrant II in Fig. 2). The mean results of 25 ejaculates from 4 boars are presented (except for t = 45 and t = 180 min, which are mean values from subsets of 14 and 8 ejaculates, respectively, from the same 4 boars). $*p < 0.05$; $**p < 0.01$.

matozoa developed that showed clearly increased FITC-sZP binding ability (Fig. 2b, quadrant II). This subpopulation increased rapidly over the first 60 min and thereafter leveled off (Fig. 3); after a 120-min incubation in TB, the size of the high FITC-sZP-binding live population, expressed as a proportion of the total sperm population, was 35 ± 9%, averaged over all ejaculates (n = 25).

Incubation in TB also caused sperm death (compare Fig. 2, a and b, quadrants III and IV), an effect of fertilization-supporting media reported in previous studies [17, 21] and considered to be an inevitable eventual outcome of capacitation [7]. (It should be noted that cell death was considerably lower in media T and TC (Fig. 4); T and TC do not support fertilization [9]). The proportion of dead cells in the sperm samples increased particularly rapidly during the early stages but continued to rise throughout the 3-h period (Fig. 4A). Although some of the dead cells showed clearly enhanced zona binding (see upper right quadrant in Fig. 2b), such cells were not considered to be of physiological importance, for, being dead, they would clearly not be able to fertilize. Our further studies therefore focused essentially only on changed zona binding ability within the live sperm population.

Influences of Bicarbonate/CO_2, Phosphodiesterase Inhibitors, and Ca^{2+} on FITC-sZP Binding

When the same 25 ejaculates were incubated in bicarbonate-free Tyrode's medium (T, Table 1), the rate at which the subpopulation of intact cells with enhanced zona binding capacity was formed was decreased (Fig. 3; compare also Fig. 4C with Fig. 4A). Moreover, the final size of that subpopulation (after 120-min incubation) was significantly smaller than in the presence of bicarbonate: 27 ± 9% ver-

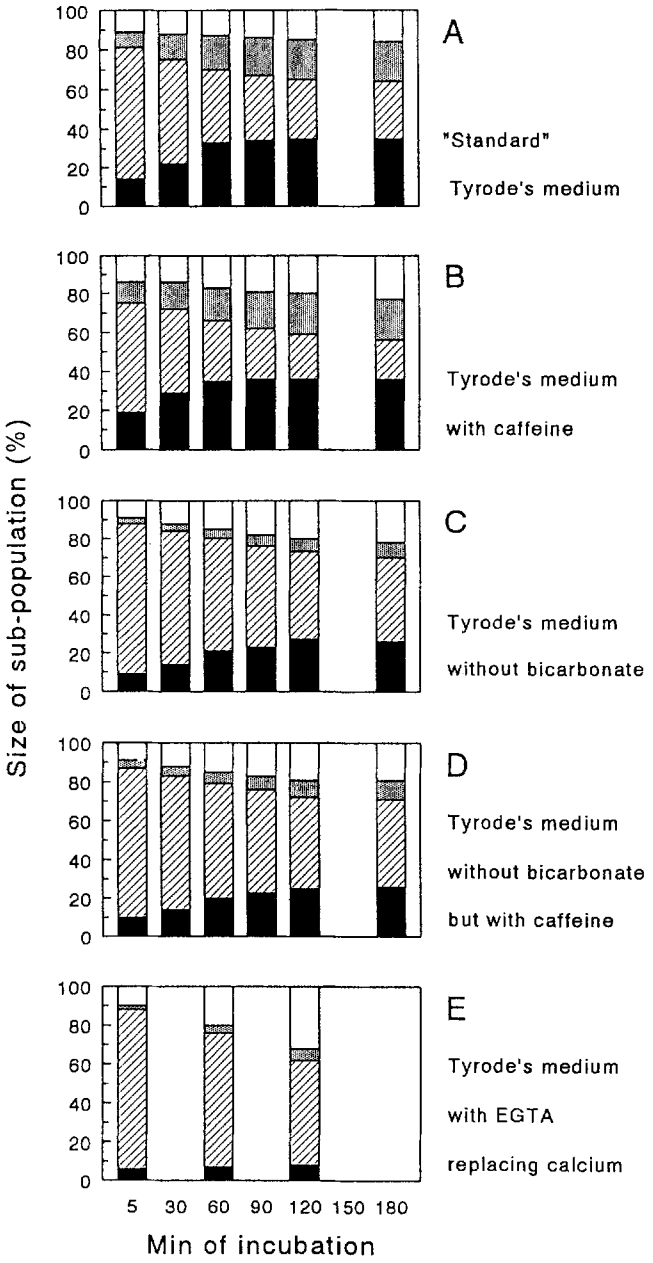

FIG. 4. Relative changes in sperm subpopulations during incubation in various modifications of Tyrode's medium. Washed spermatozoa were incubated at 39°C in variants of a modified Tyrode's medium (see Table 1): **A**) TB (4.5 mM Ca^{2+} and 15 mM HCO_3^- present); **B**) TBC (4.5 mM Ca^{2+}, 15 mM HCO_3^- and 2 mM caffeine present); **C**) T (bicarbonate-free but calcium-supplemented); **D**) TC (bicarbonate-free but caffeine- and calcium-supplemented); **E**) TB+E (Ca-deficient bicarbonate-containing medium supplemented with 2 mM EGTA). PI was included in all media as a viability stain. At intervals, subsamples were mixed briefly with FITC-sZP and subjected to two-color flow cytometry. The data were analyzed to yield the percentages of the total sperm population constituted by live cells with low cell-bound FITC-sZP fluorescence (cross-hatched bars), live cells with high FITC-sZP fluorescence (solid bars), dead cells with high FITC-sZP fluorescence (dotted bars), and dead cells with low FITC-sZP fluorescence (open bars). (These four categories were represented by quadrants I, II, III, and IV in Fig. 2; see *Materials and Methods* for analytical procedures.) The mean results of 25 ejaculates from 4 boars are presented (except for t = 180 min, which are mean values from a subset of 8 ejaculates from the same 4 boars).

sus 35 ± 9%; n = 24 (one missing value), $p \leq 0.05$. The effect of omitting bicarbonate was seen as even greater if considered in terms of the proportion of live cells developing the enhanced zona binding, since cell death was lower in the absence of bicarbonate. The differences between the media could not be ascribed to any alteration in external pH, as this was maintained at 7.4, regardless of the presence or absence of bicarbonate.

Inclusion of the phosphodiesterase inhibitor caffeine (medium TBC) led to an acceleration of the formation of the subpopulation of intact cells with enhanced zona binding capacity (Fig. 3; see also Fig. 4B). The size of this subpopulation was significantly higher at 30 min and 60 min than in the absence of caffeine (at 30 min, in relation to the total sperm population, 29 ± 5% versus 22 ± 6% respectively; n = 25 in both), although by 90 min, differences due to caffeine addition were no longer evident. In the absence of bicarbonate, however, caffeine (medium TC) had no significant effect on the development of enhanced sZP binding (compare Fig. 4, C and D). Inclusion of isobutylmethylxanthine (IBMX), another phosphodiesterase inhibitor, in the standard Tyrode's medium (tested on 3 ejaculates) had effects very similar to those of caffeine (data not shown).

Omission of Ca^{2+} from the Tyrode's medium (medium TB-Ca, resulting in a residual free Ca^{2+} concentration of 10–50 μM: [18, 32]) did not affect the development of the subpopulation of sperm with enhanced zona binding (Fig. 3): there was no discernable difference when compared to the medium TB containing added calcium (n = 25). However, if 2 mM EGTA was added to chelate residual Ca^{2+} (medium TB+E), the development of the subpopulation was significantly inhibited (Fig. 3; also Fig. 4E): the size of the subpopulation of intact cells with enhanced FITC-sZP binding, in a set of four ejaculates tested with and without EGTA, were, respectively, 8 ± 2% and 34 ± 8% after 120 min. In samples of sperm from the same four ejaculates to which 5 mM $CaCl_2$ was added after 30-min incubation in TB+E, the subpopulation of intact cells with enhanced FITC-sZP binding increased rapidly, reaching a size of 32 ± 9% by 120 min (data not shown). Notably, although the inclusion of EGTA in the Tyrode's medium prevented the development of zona binding ability, it did not reduce sperm death (compare Fig. 4, E with A).

Location of FITC-sZP Binding

Microscopical examination showed that the increase in FITC-sZP binding as detected by flow cytometry was accompanied by an extension of the area on the sperm head to which zona proteins were seen to bind: from the apical ridge to the entire acrosomal area (Fig. 5).

Relation between FITC-sZP Binding and Acrosomal Status

The increased binding of FITC-sZP, shown in the previous section to take place over the acrosomal region of the head, could have been the result of exposure of acrosomal material through the occurrence of spontaneous acrosome reactions. In particular, (pro)acrosin has been shown to bind zona proteins [33, 34]. Thus, in a set of four ejaculates, the acrosomal status was assessed by staining with TRITC-PNA after 3 h of incubation at 39°C in medium TB. It was found that, on average, only 9 ± 2% of the total sperm population showed TRITC-PNA binding, whereas after 20-min incubation at 39°C in the presence of 0.3 μM ionophore A23187 (to induce acrosome reactions in part of the sperm population), 39 ± 5% of sperm from the same four ejaculates showed PNA binding.

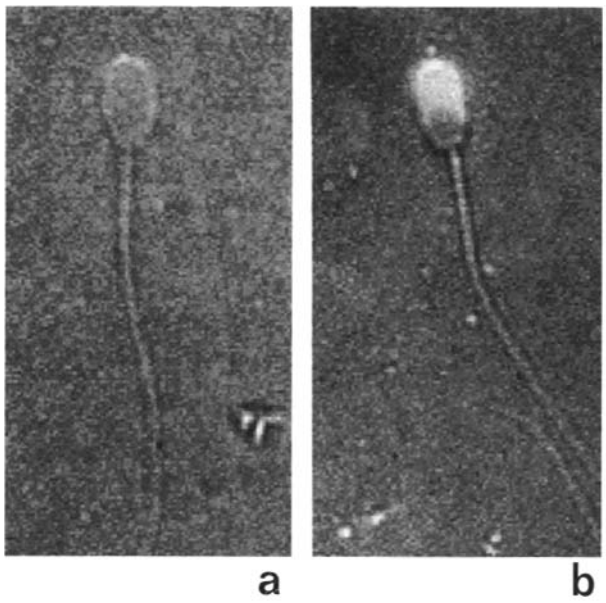

FIG. 5. Spermatozoa stained with FITC-sZP after different periods of incubation under IVF conditions. Washed spermatozoa were incubated at 39°C in medium TB (i.e., in the presence of 4.5 mM Ca^{2+} and 15 mM bicarbonate/CO_2). PI was included in the medium as a viability stain. After incubation, cells were mixed briefly with FITC-sZP and immobilized with a low concentration (0.8 mM) of formaldehyde [26]; samples were then observed on a 38°C warm-stage by fluorescence microscopy. Preliminary tests showed that formaldehyde did not interfere with FITC-sZP binding. **a**) PI-negative spermatozoon after 5 min of incubation and **b**) after 120 min of incubation.

If sperm samples were stained with both TRITC-PNA and FITC-sZP, 81% (75–86%) of the live subpopulation (Hoechst-negative) that showed enhanced sZP binding could be classified as unstained by PNA, whereas, in samples treated with ionophore to induce acrosome reactions, essentially none of the live high-sZP-binding population remained unstained (Fig. 6).

Microscopical investigations (fluorescence in combination with phase contrast) showed that PNA-negative cells were acrosome-intact (results not shown). Spermatozoa that bound PNA were at different stages of acrosomal reaction but still in the possession of a perceptible acrosomal cap or remnants thereof. Rarely, cells were seen that had lost their acrosomal cap completely, and these cells were dead.

All these results indicated that enhanced binding of FITC-sZP was not linked to spontaneous acrosome reactions.

Assessment of Acrosin Involvement in FITC-sZP Binding

To further eliminate the possibility that enhanced zona protein binding might be due to exposure of acrosin, sperm were incubated for 3 h at 39°C in medium TB and then treated with 5 μg/ml of a FITC-labeled rabbit anti-porcine acrosin antibody. This antibody, generously provided by Dr. Larry Johnson (U.S. Department of Agriculture, Beltsville, MD), reacts specifically with acrosin and proacrosin on Western blots of sperm extracts (unpublished results). In tests on 2 independent ejaculates, flow cytometric analysis of the antibody-treated sample showed that only an average of 6% of the total sperm population (9% of the live cells) showed binding of the antibody. However, after treatment of parallel samples with 1% Nonidet P-40 (to permeabilize

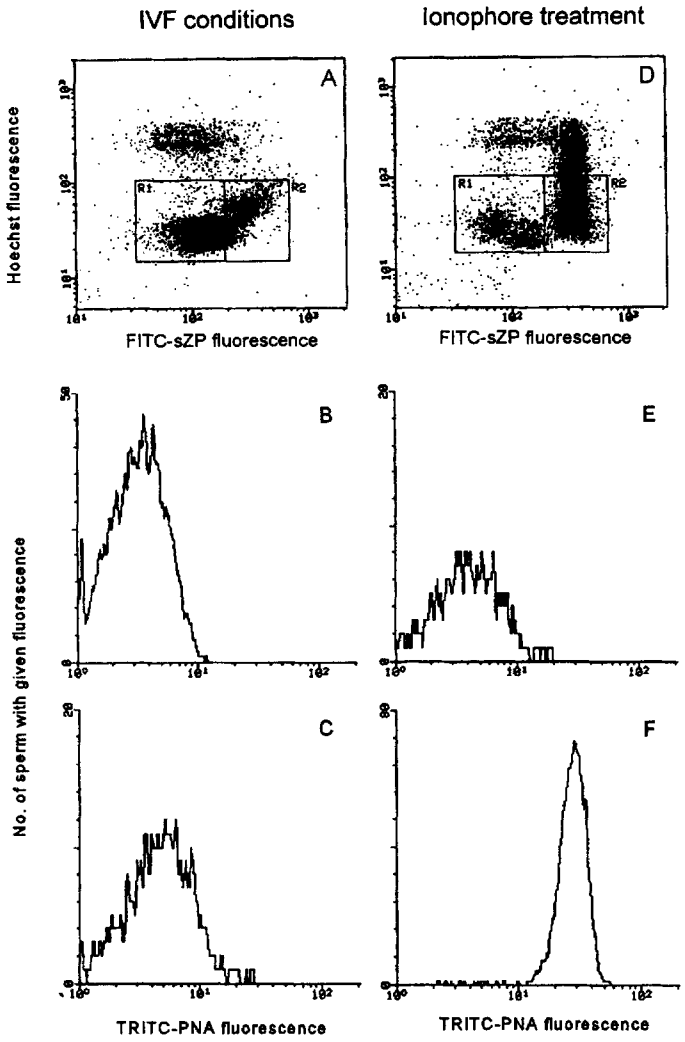

FIG. 6. Simultaneous assessment of zona binding capability and exposure of acrosomal contents after incubation in Tyrode's medium under IVF conditions: comparison with ionophore treatment. Washed spermatozoa were incubated at 39°C in Tyrode's medium TB (Table 1) for 3 h; Hoechst 33258 was included in the medium in place of PI to monitor cell viability. A sample of the suspension was mixed first with TRITC-PNA, shortly afterwards FITC-sZP was added, and then the sample was subjected to three-color flow cytometric analysis (see *Materials and Methods* for full experimental details). As a comparison, spermatozoa were incubated at 39°C for 20 min in medium TB in the presence of ionophore A23187 (0.3 μM final concentration), then stained as above with TRITC-PNA and FITC-sZP, and analyzed by flow cytometry. **A–C** relate to spermatozoa incubated in Tyrode's medium alone; **D–F** relate to spermatozoa incubated with ionophore. In **A** and **D**, fluorescence data relating FITC-sZP binding to Hoechst staining are presented; computerized windows were set (empirically) on the data in **A** to select separately live sperm displaying low sZP binding (R1) and live sperm showing high sZP binding (R2); **D** shows the sperm events selected by the same window settings. Histograms **B** and **E** display the TRITC-PNA binding levels in the R1 populations (live sperm showing low binding of FITC-sZP). **C** and **F** show TRITC-PNA binding levels in the R2 populations (live sperm showing high binding of FITC-sZP). Note that the sperm in **F** (high sZP-binding after ionophore treatment) show uniformly high PNA binding (interpreted as due to exposure of acrosomal contents after the induction of acrosome reactions). However, only low PNA binding is seen in the high-sZP-binding sperm population in **C** (incubated under IVF conditions).

acrosomes and expose acrosin), 79% of the spermatozoa were found to bind the FITC-labeled anti-acrosin. When incubated spermatozoa were treated with unlabeled anti-acrosin (50 μg/ml final concentration) for 10 min before staining with FITC-sZP, binding of the latter was not inhibited, and the two subpopulations of live sperm (i.e., low-sZP-binding and high-sZP-binding) were still detected.

Other Control Experiments

During incubation at 39°C in BTS, a storage extender for boar semen (containing bicarbonate, but also the calcium chelator EDTA), no subpopulation with enhanced FITC-sZP binding was formed (tested on two different ejaculates; data not shown). Binding of FITC-sZP was not itself blocked by components of BTS: when cells were incubated in medium TB for 2 h and a subpopulation with enhanced binding had developed, the capacity of the sperm in this subpopulation to bind FITC-sZP was not diminished after 10-fold dilution of the cell suspension in BTS.

Development of enhanced binding of FITC-sZP to spermatozoa did not occur at 20°C in any medium under any circumstance tested.

In order to check the specificity of FITC-sZP binding, subsamples of sperm suspensions were incubated with 75 μg/ml unlabeled sZP for 4 min, just before the addition of FITC-sZP. Binding of FITC-sZP to the spermatozoa was blocked with the unlabeled sZP (tested in a set of 2 ejaculates).

The fluorescent signal of the FITC-labeled probes was exclusively caused by the labeled probes themselves and not by "free" FITC. Spermatozoa did not show any binding of fluorescent material after they had been exposed to the filtrates that were collected after ultrafiltration of FITC-sZP (see *Materials and Methods*).

Control experiments were also performed in which, parallel to incubation with FITC-sZP, spermatozoa were incubated and analyzed with FITC-labeled BSA, FITC-labeled ovalbumin, or FITC-labeled transferrin (these proteins were labeled using the same procedure as for sZP). The intensity of cell-bound fluorescence after addition of the FITC-labeled "control" proteins to incubated spermatozoa (tested on a subset of three ejaculates) was at least 4.5 times lower than the FITC-sZP fluorescence. No subpopulation with enhanced binding of the control proteins was detected after incubation at 39°C.

DISCUSSION

In this paper we demonstrate that incubation of boar spermatozoa under IVF conditions results in a subpopulation of live cells that show increased capacity to bind FITC-sZP on the plasma membrane surface. Cellular fluorescence resulting from addition of FITC-sZP was not due to labeling by any contaminating free FITC; moreover, fluorescence increases were inhibited by preincubation of the sperm with unlabeled sZP. The binding of zona proteins by the sperm was specific: incubation of sperm with a number of FITC-labeled control proteins, i.e., BSA, ovalbumin, and transferrin, resulted in only negligible fluorescence of the sperm, and the binding capacity for these proteins did not increase during incubation of the sperm under IVF conditions. Furthermore, the amount of bound FITC-sZP on the sperm was not diminished by dilution of the labeled cells in medium without FITC-sZP.

It has been shown that the acrosomal proteinase acrosin binds zona proteins [33, 34], and thus the increase in FITC-sZP binding during incubation might have resulted from the occurrence of spontaneous acrosome reactions and the consequent exposure of acrosin at the sperm surface. However, the large majority of the sperm in the high-sZP-binding live subpopulation were acrosome-intact, acrosin could

not be detected on their surface, and binding of FITC-sZP was not inhibited by antibodies against acrosin. Thus increased sZP binding was not due to preoccurring acrosome reactions. Binding might have resulted from acrosome reactions induced by the addition of the FITC-sZP at the time of the analysis (since zona proteins can induce the acrosome reaction—see *Introduction*), but pilot experiments (results not shown) indicated that although the sZP preparation could induce acrosome reactions rapidly when reconstituted as a solid matrix, in soluble form it could induce acrosome reactions only at concentrations above 75 μg/ml during lengthy (1 h) incubations. The final concentration of FITC-sZP during our staining protocol was only 2 μg/ml, and the sperm were exposed to this for only 4 min before flow cytometric analysis.

Thus our flow cytometric approach, which uses minimally low concentrations of zona material, which obviates the need for pretreatment or washing of the cells (thereby minimizing cell damage), and which enables analysis of some 10 000 cells in less than 15 sec, would seem to provide a sensitive and quantitative evaluation of binding of zona pellucida components to the surface of intact spermatozoa. By including the viability probe PI in our media, we were able to distinguish between live and dead sperm in our suspensions, and thereby, crucially, to focus on the live cells; only the latter could be expected to participate in the fertilization process. Enhanced sperm death during incubation in bicarbonate-containing Tyrode's based media (i.e., under fertilizing conditions) has been reported previously [17, 21]. Indeed, it has been proposed that capacitation (necessarily induced by IVF conditions) is an ongoing destabilization process that inevitably and eventually leads to cell death [7]. At present, the most likely explanation for the two levels of zona binding that developed in the dead cell population (upper right quadrant in Fig. 2b) is that these represented terminally degenerated cells derived from each of the two live subpopulations.

We believe that the increase in sZP binding ability that takes place within the live spermatozoa is physiologically significant. We envisage that such increases in zona binding ability relate to optimal attachment of the sperm to the zona pellucida, thus improving chances for fertilization. The increased zona binding ability may also reflect sensitization of the sperm to induction of the acrosome reaction by zona components. Although noncapacitated spermatozoa can bind to the zona pellucida [3, 4], incubation under IVF conditions has been shown to develop or increase the ability of sperm to undergo the zona-induced acrosome reaction [1, 10–12] and to fertilize in vitro [9]. The development of these latter abilities seems to depend on the same presence of Ca^{2+} (c.f. [18]) and bicarbonate that we found in the present study to be prerequisites for the increase of the capacity to bind zona proteins. Moreover, the time needed for the increase of the capacity to bind zona proteins is of the same order as the time needed for acquisition of the ability to penetrate the zona pellucida during pig IVF (e.g., [6]). Thus, the observed increase in the binding of zona proteins could well reflect changes that are necessary for effective sperm-zona interaction. It is noteworthy that the conditions optimal for pig IVF (i.e, incubation in the caffeine-supplemented bicarbonate-containing Tyrode's medium TBC; see [9]) were also those that led to the most rapid development of enhanced zona binding ability as found in this study.

The nature of the sperm surface receptors that mediate the binding of sZP is unknown. Töpfer-Petersen and Calvete [35] have proposed that the class of small proteins known as "spermadhesins" may play an important role in zona binding of boar sperm; these proteins, which interact strongly with zona components, are produced by the male reproductive tract and bind to the sperm head surface, in particular to the acrosomal region, at ejaculation. However, it has been found that most of the spermadhesin activity dissociates from the sperm during capacitation [36], a characteristic that does not accord with the zona binding changes we have observed. On the other hand, the individual members of the spermadhesin family are not released uniformly, and it may be that those with greatest affinity for the zona may be retained or even unmasked. Several other putative zona receptors have been described in different sperm species [35, 37]. The boar sperm membrane protein AP_z identified by Peterson and Hunt [38] is apparently distributed over the whole sperm surface [39], and its involvement in the changes we have described thus seems doubtful. Hardy and Garbers [40] have isolated a boar sperm membrane protein that confers specific binding ability with respect to the pig zona pellucida; this protein is expressed primarily in haploid spermatids and contains a region that may be involved in oligomerization, as well as mucin-binding regions [41], but its distribution on the sperm cell has not yet been defined.

The mature spermatozoon is not considered to have any significant capacity for de novo expression of internally localized membrane components. Thus the increased capacity for binding sZP and the extension of the surface area over which binding occurred would seem to be due to exposure, activation, or increased affinity of already existing structures in the sperm membrane. These alterations could result respectively from unmasking, from oligomerization, or from changes of the physical microenvironment during dynamic reorganization of the plasma membrane architecture. Ashworth et al. [17] have described alterations in specific lectin-binding sites on boar sperm that develop during incubation under conditions of incubation essentially similar to those used in the present study; the lectin-binding changes seemed to be related to loss of seminal plasma-derived coating proteins and to concomitant unmasking of underlying components. However, IVF conditions also induce dynamic reorganization of the plasma membrane lipid bilayer [16], and changes in the lipid architecture could affect the ability of membrane components to migrate to new locations within the bilayer, with resultant altered binding characteristics. Gadella and colleagues [42] have demonstrated a migration of glycolipids within the boar sperm plasma membrane, from the apical ridge towards the equatorial subdomain, which parallels closely our own observations of the extension of the surface on which sZP binding occurred. The glycolipid migration was also dependent on Ca^{2+} and took place during incubation under IVF conditions with a time course similar to the sZP binding change we have described.

The mechanism of bicarbonate's action in increasing zona binding capability remains to be elucidated. The relatively slow time course of the binding change suggests that it results from a multistep process. Given the accelerating effects of caffeine or IBMX in the presence of bicarbonate, it could be that bicarbonate acts through direct stimulation of adenylyl cyclase ([43]; see also [7]), but how the putative rise in cAMP might bring about the bicarbonate-dependent changes in membrane lipid organization [15, 16] or dissociation of surface components [17] is at present quite unknown. Similarly, the site of action of Ca^{2+} is un-

certain. Sperm that had been incubated for 2 h in the standard Tyrode's medium were able to bind FITC-sZP binding even after dilution in BTS (which contains EDTA); thus FITC-sZP binding itself is not dependent on calcium. Calcium's role must therefore be in the development of the binding ability. Since this development appears to parallel changes in the sperm's surface coating, calcium may well act externally. However, development of the binding sites could also involve an intracellular process dependent on calcium that is brought into play after a membrane reorganization that enhances a calcium influx. EGTA would prohibit such an influx, or could even lead to a decrease in cytoplasmatic calcium levels [44].

Our flow cytometry analysis of the changes in sZP binding ability of individual cells within the live sperm population has revealed considerable heterogeneity between cells in their speed of response to IVF conditions. One subgroup of sperm underwent the changes relatively rapidly, within the first 60 min of incubation, whereas the rest of the population underwent the changes at disparate and much slower rates (or not at all, within the time-frame of our experiments). At all time points, two live subpopulations could be distinguished clearly; thus the change in zona binding ability within any given individual sperm could be deduced to be rapid. The heterogeneity in rate of various sperm responses to IVF conditions and the either/or characteristic of this response has been noted in previous studies (see [7]), as has been the increase in sperm death brought about by IVF conditions [7, 21]. While zona binding ability is not directly associated with membrane instability (compare effects of EGTA on these respective characteristics), the parallel enhancing effects of bicarbonate on both suggest that they may depend on a common core mechanism. It has been proposed that capacitation is necessarily a destabilizing process, and that the capacitated state represents a "window" degree of instability [7]. Although there was no evidence to suggest that increased zona binding in itself resulted in enhanced instability, the concomitance of the zona binding changes and of cell death in response to IVF conditions was in accord with the general concept of capacitation as a destabilizing process.

As well as noting the heterogeneity of response within a sperm population, we also noted considerable variation between ejaculates. A particular point of interest is that sperm in the ejaculates described above, taken from boars in the Babraham Institute colony, showed attenuated but perceptible increases in sZP binding during incubation in the absence of bicarbonate. However, in later experiments on ejaculates from boars at a Dutch AI station, the percentage of live sperm with increased zona binding capacity barely rose at all during incubation in the absence of bicarbonate, remaining on average below 10% (46 ejaculates of 18 boars, manuscript in preparation). Given the biphasic nature of the overall response, the question arises as to whether the cells that undergo rapid changes represent the competent population or whether a slow population response is more desirable (better survival in vivo?). Regardless of the answer, such variations in development of what we consider to be a physiologically relevant characteristic suggest that acquisition of zona binding ability under IVF conditions might be a useful parameter for assessing the fertilizing ability of boar semen samples.

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