Developmental Biology **237,** 222–231 (2001)
doi:10.1006/dbio.2001.0353, available online at http://www.idealibrary.com on IDEAL®

metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

pH Indicator and Its Relation to Sperm Capacitation

Tomoko Nakanishi,* Masahito Ikawa,* Shuichi Yamada,† Kiyotaka Toshimori,‡ and Masaru Okabe*,[1]

**Genome Information Research Center, Osaka University, Yamadaoka 3-1, Suita, Osaka, 565-0871, Japan; †Program for Promotion of Basic Research Activities for Innovative Biosciences; and ‡Department of Anatomy and Reproductive Cell Biology, Miyazaki Medical College, Miyazaki, 889-1692, Japan*

We previously targeted EGFP (a mutant of green fluorescent protein) to the lumen of the mouse sperm acrosome and reported the time course of EGFP release during the acrosome reaction. In the study reported here, we estimated the pH within the mouse sperm acrosome utilizing the pH-dependent nature of EGFP fluorescence. The average intra-acrosomal pH was estimated to be 5.3 ± 0.1 immediately after sperm preparation, gradually increasing to 6.2 ± 0.3 during 120 min of incubation in TYH media suitable for capacitation. Spontaneous acrosome reactions were noted to increase concomitantly with acrosomal alkalinization during incubation. We also demonstrated that acrosomal antigens detected by monoclonal antibodies MN7 and MC41 did not dissolve following the acrosome reaction in pH 5.3 media, but dissolved at pH 6.2. These data suggest that acrosomal alkalinization during incubation conducive for sperm capacitation may function to alter acrosomal contents and prepare them for release during the acrosome reaction. © 2001 Academic Press

Key Words: **mouse; sperm; capacitation; intra-acrosomal pH; acrosome reaction; acrosomal antigen; GFP.**

INTRODUCTION

The pH conditions within various cellular compartments are regulated to provide the optimal milieu for many cellular processes. In secretory pathways, posttranslational processing of secretory proteins and the cleavage of prohormones to produce active agents take place in a pH-dependent manner (Schmidt and Moore, 1995); in mitochondria, the pH gradient across the inner membrane drives ATP synthesis. Also, in sperm, the fertilizing ability seems to be regulated by adjusting cellular pH. For example, the time-dependent acquisition of fertilizing ability in the female reproductive tract or specific media is known as "capacitation" and is reported to correlate with the alkalinization of intracellular pH by introducing HCO_3^- into the sperm cytoplasm (Zeng *et al.,* 1996). In accordance with this notion, the increase in intracellular pH has been reported to cause a stimulation of soluble adenylyl cyclase and protein tyrosine phosphorylation, promoting sperm capacitation (Chen *et al.,* 2000; Visconti *et al.,* 1999). However, the regulation of sperm function by pH changes may not be limited in the cytosol. Sperm possess a structure called acrosome, a membrane-limited vesicle situated over the anterior of the sperm head. The acrosome is of particular importance because it contains hydrolytic enzymes that are considered to be involved in fertilization (Yanagimachi, 1994). In order to keep these enzymes stable, the intra-acrosomal pH is reported to be maintained at acidic levels when sperm reside in the male reproductive tract (Huang *et al.,* 1985; Meizel and Deamer, 1978). Following ejaculation into and capacitation within the female reproductive tract, the intra-acrosomal pH rises, leading to the activation of acrosomal enzymes. However, the precise change of intra-acrosomal pH in relation to capacitation and/or the acrosome reaction has remained unclear.

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has become a useful tool in molecular and cell biology. "Enhanced" GFP (EGFP), a mutant version of GFP, displays pH-dependent absorbance and fluorescence emission with apparent pKa values of 5.8 (Haupts *et al.,* 1998). This makes EGFP suitable as a noninvasive pH indicator for studies of pH regulation in intracellular compartments that cannot be labeled with conventional pH indicators (Han *et al.,* 1999; Llopis *et al.,* 1998).

[1] To whom correspondence should be addressed. Fax: (+81)-6-6879-8376. E-mail: okabe@gen-info.osaka-u.ac.jp.

0012-1606/01 $35.00
Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

We have reported the production of transgenic mouse lines in which EGFP was targeted to the interior of the sperm acrosome using the proacrosin signal peptide and N-terminal peptide and, using these "green sperm," the time required for EGFP to disperse during acrosome reaction was recorded (Nakanishi *et al.,* 1999). In the present paper, we have measured the intra-acrosomal pH of "green sperm" by utilizing the pH-dependent nature of EGFP fluorescence. Since the loading step of pH probes can be eliminated, this approach has a distinct advantage over methods requiring synthetic indicators (Meizel and Deamer, 1978). Furthermore, an immediate disappearance of the EGFP after the acrosome reaction made it possible to observe a relationship between the intra-acrosomal pH and the acrosome reaction in a noninvasive and real-time manner. To our knowledge, this is the first paper reporting an intra-acrosomal pH using transgenically expressed EGFP as an intrinsic pH indicator.

MATERIALS AND METHODS

Animals Used

The transgenic mouse line was produced by injecting a mixture of two kinds of transgenes into fertilized eggs obtained from B6C3F1 females mated with B6C3F1 males (SLC, Japan). The first construct used the mouse proacrosin promoter to direct the cell- and stage-specific expression of a transgene encoding the mouse proacrosin signal peptide with acrosin N-terminal peptide fused to the amino terminus of EGFP (*acr-EGFP*) (Nakanishi *et al.,* 1999). The other transgene was expressed by using a chicken β-actin promoter with the cytomegalovirus enhancer and encoded EGFP alone (*act-EGFP*) (Okabe *et al.,* 1997). A transgenic mouse line expressing green fluorescence both throughout the body from transgene *act-EGFP* and in the sperm acrosome from transgene *acr-EGFP* was established (B6C3F1 TgN[act/acr3-EGFP]17Osb). The transgenic mice were bred with B6C3F1 and the offspring were used for these experiments. Transgenes were inherited coordinately and stably by later generations. We used this coinjection system so that we could easily distinguish the transgene-inherited mice from wild-type animals by checking for body fluorescence derived from the *act-EGFP* transgene. Existence of Acr-EGFP inside sperm acrosome had no effect on the sperm-fertilizing ability (Nakanishi *et al.,* 1999).

Spectroscopic Studies of EGFP Fluorescence

To obtain the EGFP, muscle from *act-EGFP* transgenic mice (Okabe *et al.,* 1997) was homogenized in 0.9% saline (10% w/w) by using a Polytron PT1200 homogenizer (Kinematica, Switzerland). After the homogenate was centrifuged at 13,000*g* for 10 min, the supernatants were recovered and acetone was added to make 50% (v/v). The precipitate was removed by centrifugation. The supernatants containing EGFP were brought to 75% saturation by further addition of acetone and the precipitate obtained following centrifugation was dialyzed against 0.9% NaCl for fluorometry. In the same manner, Acr-EGFP was prepared from testes and epididymides of *acr-EGFP* transgenic mice (Nakanishi *et al.,* 1999). A spectrofluorometer FT-777 (Jasco, Japan) was used for fluorescence measurements of the EGFPs. The filters used for excitation were 488 ± 10 nm and 510 ± 10 nm for emission. Both EGFP and Acr-EGFP were diluted in calibration solutions containing 127–129 mM NaCl and 5 mM KCl, buffered with 20 mM citrate (pH 4.0, 4.5, 5.0, 5.5, and 6.0) or phosphate (pH 6.0, 6.5, 7.0, 7.5, and 8.0). The NaCl concentration was altered to adjust osmolarity between the citrate and phosphate buffers.

Incubation Media

TYH medium (Toyoda *et al.,* 1971) contained NaCl (119 mM), KCl (4.8 mM), $CaCl_2$ (1.7 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.0 mM), $NaHCO_3$ (25 mM), glucose (5.6 mM), sodium pyruvate (0.5 mM), and 4 mg/ml BSA. In experiments illustrated in Fig. 4, the $NaHCO_3$ concentration was adjusted to 11, 25, and 60 mM and, concomitantly, the pH of the medium was changed to pH 7.0, 7.5, and 8.0, respectively. Ca^{2+}-depleted TYH medium was prepared by removing $CaCl_2$ from TYH medium. In order to introduce Ca^{2+} into the medium, 170 mM $CaCl_2$ was added at a final concentration of 1.7 mM. MES-buffered TYH medium was prepared from TYH medium by adding 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES). The pH of the MES-buffered TYH medium was adjusted to different values (pH 5.3, 6.2, and 7.0) by using HCl and the osmolarity was adjusted to 295 mOsm, altering the NaCl concentration.

Sperm Incubation

Caudae epididymides were excised from *acr-EGFP* mice, minced, and submerged in 1 ml of medium (Figs. 2, 3, and 6 experiments: TYH medium; Fig. 4 experiment: 11, 25, and 60 mM $NaHCO_3$ TYH medium; Fig. 5 experiment: Ca^{2+}-depleted TYH medium; Fig. 7 experiment: MES-buffered TYH medium). Sperm were allowed to disperse for 10 min, after which 0.6 ml of the supernatant was incubated under mineral oil (Sigma Chemical Co., St. Louis, MO). Incubation was performed at 37°C under 5% CO_2 in air. *N,N′*-dicyclohexylcarbodiimide (DCCD; Kanto Chemical Co. Inc., Japan) and 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA; Sigma Chemical Co.) were dissolved in ethanol and DMSO, respectively, as 10,000-fold concentrated stock solutions and were diluted by TYH medium to make 10× stock solution immediately before use. 4,4′-diisothiocyanato-stilbene-2,2′-dislufonic acid (DIDS; Sigma Chemical Co.) was prepared daily in TYH medium as a 10× stock solution. These chemicals were added to sperm suspension immediately after preparation in TYH medium and their effects were analyzed periodically by flow cytometry.

Flow Cytometry

A FACScan flow cytometer (Becton Dickinson Co. LTD.) was used to investigate sperm fluorescence. EGFP, R-phycoerithrin (PE), and propidium iodide (PI) fluorescence were detected by using a 530/30 nm band-path filter, 585/42 nm band-path filter, and 650 nm long-path filter, respectively; data were collected for 10,000 events. To measure the sperm viability in the experiments depicted in Figs. 2–5, propidium iodide was added to a final concentration of 100 ng/ml.

Measurement of Intra-Acrosomal pH

Sperm suspensions prepared in TYH medium were subjected to intra-acrosomal pH analyses immediately after preparation or after a 120-min incubation. Initially, 20 μl of a sperm suspension was

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

added to 300 μl of calibration solution (described in the spectroscopy section) to make a final sperm concentration of approximately 1.0×10^4/ml and the resulting suspension was subjected to flow cytometry analysis. After the measurement of initial fluorescence, nigericin (K^+.H^+ ionophore: Sigma Chemical Co.), dissolved at 10 mM in ethanol, was added to the sperm suspension at a final concentration of 6.9 μM to equilibrate the intra-acrosomal pH to the extracellular pH. The ionophore caused the shifting of sperm fluorescence, bringing it to a standstill in less than 2 min at pH 6.0–8.0 and in 5 min at pH 4.0–5.5.

Immunocytochemical Analysis

Sperm (approximately 1.0×10^5/ml) incubated for 120 min in 0.6 ml of TYH medium or MES-buffered TYH medium (pH 5.3, 6.2, and 7.0) were induced to undergo the acrosome reaction by adding A23187 (Ca^{2+} ionophore; Sigma Chemical Co.) at a final concentration of 10 μM and incubating further. An aliquot (100 μl) was transferred into a 1.5-ml microcentrifuge tube at 3, 15, and 60 min after the incubation, and then centrifuged at 5,000 rpm for 5 min. The sperm pellets were washed in PBS once, treated for 10 min with 4% paraformaldehyde (Nacalai Tesque, Inc., Japan), followed by washing with PBS. For indirect immunofluorescent staining, the primary antibodies (diluted in PBS containing 5% newborn calf serum) were added to sperm and incubated for 3 h. Monoclonal antibodies against intra-acrosomal mouse 90-kDa (MN7) and 200-kDa proteins (MC41) were prepared as described previously (Tanii *et al.,* 1992, 1994). After washing with PBS, the sperm were treated with R-phycoerithrin (PE)-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) for 1 h. The sperm were washed once with PBS before subjecting them to flow cytometry analysis. In order to identify the total amount of the antigens inside acrosome (see Fig. 6B), sperm were first fixed with PLP solution for 20 min at 4°C. The PLP solution was prepared just before use by mixing 21.4 mg sodium periodate (10 mM), 7.5 ml of 0.1 M lysine/50 mM phosphate buffer (pH 7.5), and 2.5 ml of 4% paraformaldehyde. The sperm were then suspended in PBS containing 0.4 mg/ml saponin, 10% goat serum, and 5% newborn calf serum for 1 h to permeabilize their plasma membranes. Subsequently, sperm were stained as described above.

Statistical Analyses

Statistical analyses were performed by using a Student's *t* test using InStat (GraphPad Software Inc.). Values represented in the text indicate means ± SD.

RESULTS

Measurement of Intra-acrosomal pH

The pH-dependent fluorescence of EGFP and Acr-EGFP (EGFP fused with proacrosin signal peptide and acrosin N-terminal penta-peptide) was examined *in vitro.* The EGFP and Acr-EGFP were purified from muscle of *act-EGFP* transgenic mice and from male germ line cells of *acr-EGFP* transgenic mice, respectively, and were suspended in solutions of various pH values to measure the fluorescence intensity. In both EGFPs, the fluorescence followed a sigmoidal curve with increasing pH of the solutions (Fig. 1).

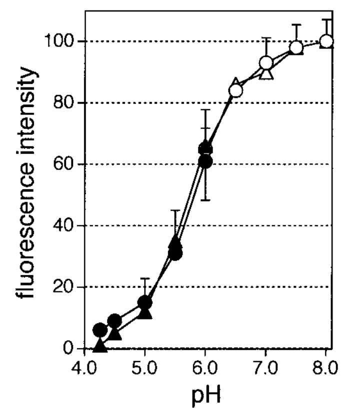

FIG. 1. The pH-dependent fluorescence of partially purified EGFP *in vitro* (▲, citrate buffer; △, phosphate buffer) and Acr-EGFP (●, citrate buffer; ○, phosphate buffer). The signal for each protein at pH 8.0 was normalized to 100. The data indicate mean ± SD of three independent experiments.

The intra-acrosomal pH of sperm was measured utilizing "green sperm" from *acr-EGFP* transgenic mice (Nakanishi *et al.,* 1999), which possessed Acr-EGFP inside acrosome as a soluble protein. Since the fluorescence intensity of Acr-EGFP changed corresponding to the environmental pH *in vitro,* acrosomal fluorescence was expected to increase when intra-acrosomal pH was raised and to decrease when it was lowered. Nigericin, which has been reported to equilibrate the intracellular pH with the extracellular pH as an H^+ ionophore (Beaumelle *et al.,* 1992; Kaneko *et al.,* 1991), was added to "green sperm" incubated in media of various pHs. The fluorescence intensity of live GFP-positive (acrosome-intact) sperm remained unaffected by suspending sperm in various extracellular pHs. The fluorescence intensities, however, were changed by the addition of nigericin, reflecting the extracellular pHs. The most significant decrease was observed at pH 4.5 and a little decrease at pH 5.0. On the contrary, the fluorescence increased at pH 5.5. This indicated that intra-acrosomal pH of freshly prepared sperm was between 5.0 and 5.5 (Fig. 2A). The alterations of fluorescence by nigericin were plotted in Fig. 2B. From this calibration curve, the intra-acrosomal pH was estimated at pH 5.3 ± 0.1 for freshly prepared sperm. The GFP-negative population represented live and acrosome-reacted sperm, which appeared when sperm were incubated in medium suitable for *in vitro* fertilization as shown in Fig. 2C. This population was increased by the addition of nigericin, especially at pH 4.5. The reason for this increase was unknown, but this was not observed at extracellular pHs close to the experimentally determined intra-acrosomal pH, indicating the phenomenon might not affect the measurement of intra-acrosomal pH.

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

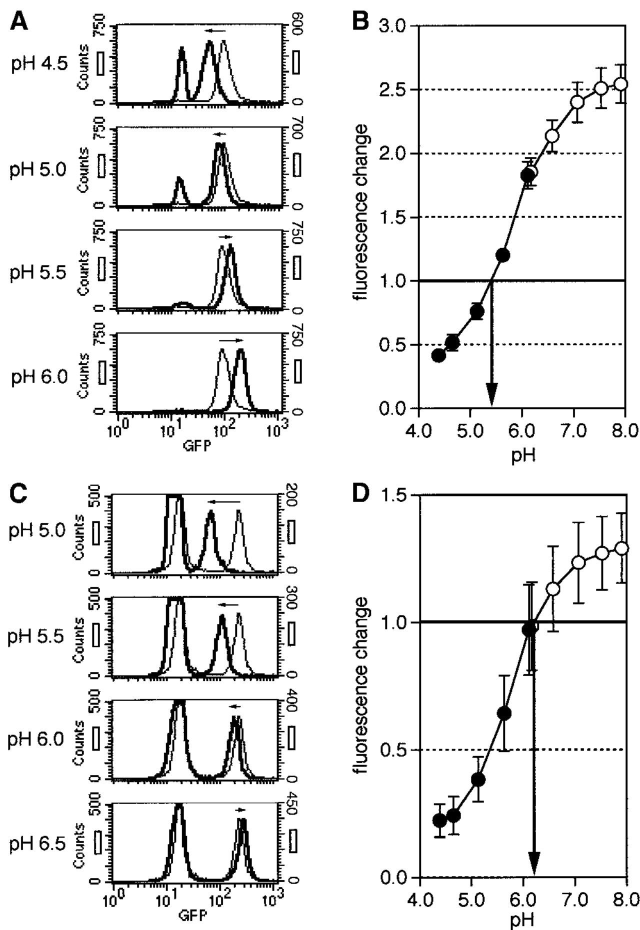

FIG. 2. Measurements of intra-acrosomal pH using "green sperm." (A) Freshly prepared "green sperm" were transferred to solutions of various pH values, nigericin was added, and the sperm were analyzed by flow cytometry. The fluorescence intensity of GFP-positive (acrosome-intact) population was changed by the addition of nigericin (shaded, before; line, after). Arrows indicate the amount and direction of the changes observed in each pH conditions. (B) The fluorescence intensities of the acrosome-intact population before and after nigericin addition (shown in A) were measured at each pH value and the ratios were plotted against the extracellular pH in which the experiments were performed. The intra-acrosomal pH of freshly prepared sperm was indicated to be pH 5.3 ± 0.1 as determined by the point where sperm fluorescence was not changed by the addition of nigericin (arrow). The data indicate mean ± SD of three independent experiments. ●, citrate buffer; ○, phosphate buffer. (C) A subpopulation of "green sperm," preincubated in TYH medium for 120 min, lost Acr-EGFP from acrosome due to spontaneous acrosome reactions (Nakanishi *et al.,* 1999) and appeared as GFP-negative peaks. The changes in fluorescence intensities of GFP-positive (acrosome-intact) populations by the addition of nigericin were analyzed as in (A) (shaded and line area represent before and after the addition, respectively) and indicated by arrows. (D) The fluorescence intensities of the acrosome-intact population before and after the nigericin addition (shown in C) were measured and the ratios were plotted as in (B). The intra-acrosomal pH of the incubated sperm estimated from the calibration curve (pH 6.2 ± 0.3) was indicated by an arrow. The data indicate mean ± SD of three independent experiments. ●, citrate buffer; ○, phosphate buffer. PI was added to the sperm suspensions and the sperm negative for PI fluorescence were analyzed throughout the experiments. Thus, degenerated sperm that had lost their membrane integrity (Garner *et al.,* 1994) were eliminated from the analyses.

Change and Regulation of Intra-Acrosomal pH during Incubation

When the same kind of experiment was performed on the sperm incubated for 120 min in TYH medium, the fluorescence of acrosome-intact sperm decreased even at pH 6.0 by the addition of nigericin and increased at pH 6.5 (Fig. 2C). This indicated that a significant increase in intra-acrosomal pH had taken place during the incubation. As shown in Fig. 2D, the average intra-acrosomal pH of incubated sperm was estimated to have risen to as high as 6.2 ± 0.3.

We then examined what kind of pumps were involved in the regulation of intra-acrosomal pH during incubation. A vacuolar-type H^+ ATPase (V-ATPase) located on the acrosomal membrane has been suggested to maintain the acidic condition of the acrosome (Kawa *et al.,* 2000; Working and Meizel, 1981). When "green sperm" were incubated with the H^+ ATPase inhibitor, DCCD, the intra-acrosomal pH of acrosome-intact sperm and the number of acrosome-reacted sperm reached a higher level than that found for control sperm (Fig. 3A). We next examined the involvement of the Na^+/H^+ exchanger (NHE) in controlling intra-acrosomal pH. The exchanger was known to be involved in the alkalinization of the cytoplasm of sperm (Garcia and Meizel, 1999). When the NHE inhibitor, EIPA, was added, a quick increase in the intra-acrosomal pH was observed, reaching a plateau (6.7 ± 0.1) by as early as 60 min of incubation. In the presence of EIPA, more sperm underwent spontaneous acrosome reactions compared to sperm without EIPA (Fig. 3B). These results suggested that, in addition to V-ATPase, NHE acted in the maintenance of the acidic intra-acrosomal pH. On the other hand, when DIDS, an inhibitor of the Cl^-/HCO_3^- exchanger (a member of the anion exchanger gene family) was added to a sperm suspension, alkalinization of the acrosome was completely blocked. Thus, the Cl^-/HCO_3^- exchanger expressed in sperm (Parkkila *et al.,* 1993) was demonstrated to function as a potential contributor to alkalinize the acrosome during incubation, as reported for the cytoplasm (Zeng *et al.,* 1996). DIDS also promoted the acrosome reaction (Fig. 3C).

Intra-Acrosomal pH and the Acrosome Reaction

Since the increase in intra-acrosomal pH was inhibited by the addition of the Cl^-/HCO_3^- exchanger inhibitor DIDS, we presumed the flux of HCO_3^- into the acrosome caused the pH elevation. When sperm were incubated in TYH medium containing 60 mM HCO_3^-, the overall increase in

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

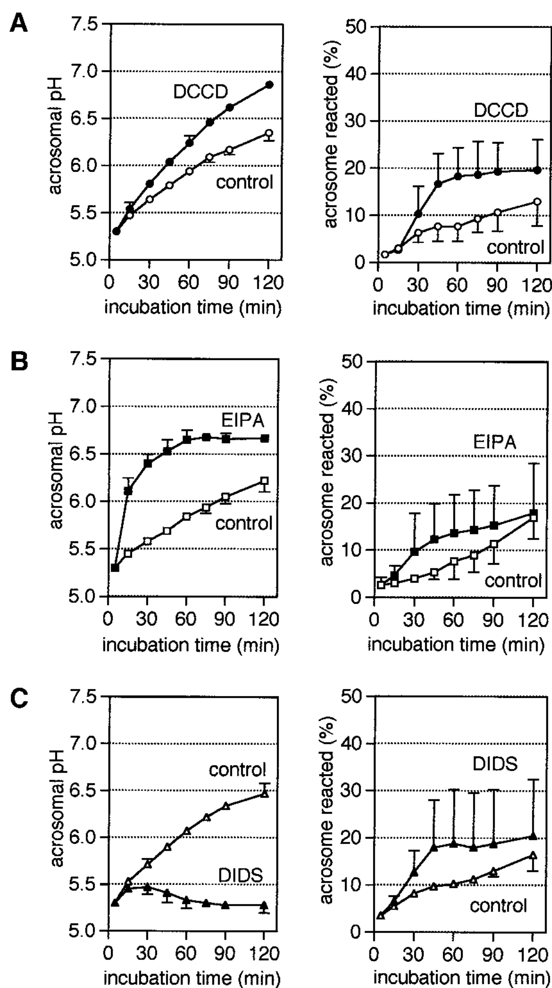

FIG. 3. Effects of DCCD (A), EIPA (B), and DIDS (C) on intra-acrosomal pH values and acrosome reactions. "Green sperm" were incubated in TYH medium for 120 min with (●, ■, ▲) or without (○, □, △) 20 μM DCCD, 1 μM EIPA, and 500 μM DIDS, respectively. A portion of the sperm suspension with or without each reagent was taken out periodically and was analyzed by flow cytometry. The change in intra-acrosomal pH values is shown in the left column. (Sperm fluorescence at 0 min and after nigericin addition at pH 7.5 were applied to pH 5.3 and pH 7.5, respectively. The rest of the pH values were estimated from the Acr-EGFP calibration curve shown in Fig. 1). The acrosome-reacted sperm populations measured in the same samples are shown in the right column. Each point represents the mean ± SD of three independent experiments.

intra-acrosomal pH (6.3 ± 0.2) after 120 min of incubation was not so different from that of sperm incubated in standard TYH medium (25 mM of HCO_3^-) (6.2 ± 0.3) (Fig. 4A). The increase in pH was, however, quicker than in standard TYH medium and the pH values reached pH 5.9 during the first 30 min of incubation. Concomitantly, the number of acrosome-reacted sperm (defined by the loss of fluorescence from the acrosome; Nakanishi *et al.,* 1999) increased rapidly (Fig. 4B). On the other hand, when sperm were incubated in TYH medium containing low HCO_3^- (11 mM), the intra-acrosomal pH after the incubation was the lowest (pH 6.0 ± 0.2) among the three conditions (Fig. 4A). Moreover, at a concentration of 11 mM HCO_3^-, the number of acrosome-reacted sperm was the lowest (13 ± 2%) and the timing of the appearance of such sperm was the slowest among all the conditions tested (Fig. 4B).

To examine the relationship of the intra-acrosomal pH and the acrosome reaction, we incubated sperm in Ca^{2+}-depleted TYH medium. In this condition, cytoplasmic Ca^{2+} was expected to become low because steady-state Ca^{2+} influx from outside the cell was blocked. As a consequence, sperm failed to undergo the acrosome reaction (Fig. 5A) as various papers indicated (Yanagimachi, 1994). Since cytoplasmic Ca^{2+} has been recognized as one of the major second messengers in regulation of NHE (Wakabayashi *et al.,* 1995) and NHE was shown to be involved in the acidification of acrosome (Fig. 3B), the depletion of Ca^{2+} caused a quick increase of intra-acrosomal pH and the resulting pH values were higher than those of sperm incubated in TYH medium (Fig. 5B).

By introducing Ca^{2+}, sperm previously incubated without Ca^{2+} were induced to acrosome react. When Ca^{2+} was added to sperm suspensions incubated for 30 min in the Ca^{2+}-depleted medium (estimated intra-acrosomal pH per sperm suspension: 5.55 to 5.74), an average of 20% of sperm responded to the Ca^{2+} addition by undergoing an acrosome reaction. When the incubation period was prolonged to 60

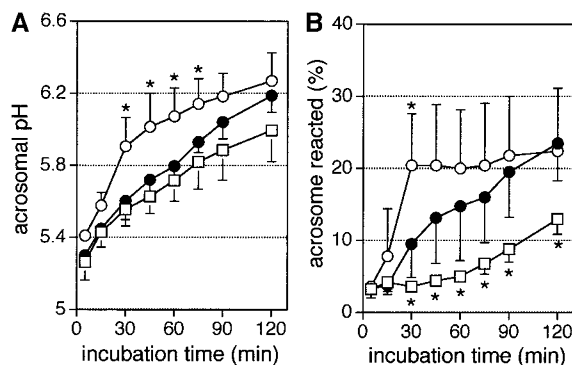

FIG. 4. Acrosomal status of "green sperm" incubated in TYH medium with various concentrations of HCO_3^- (□, 11 mM; ●, 25 mM; ○, 60 mM). (A) Alteration of intra-acrosomal pH was indicated. The values at 0 and 120 min were estimated by subjecting sperm to nigericin equilibration experiments as shown in Fig. 2. The other values from 15 to 90 min were calculated utilizing the Acr-EGFP calibration curve shown in Fig. 1. (B) The occurrence of spontaneous acrosome reactions was indicated. The results represent the mean ± SD of at least four independent experiments. An asterisk indicates a difference within an indicated time point in intra-acrosomal pH or the percentage of acrosome reactions from the sperm in 25 mM HCO_3^-, $P < 0.05$.

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

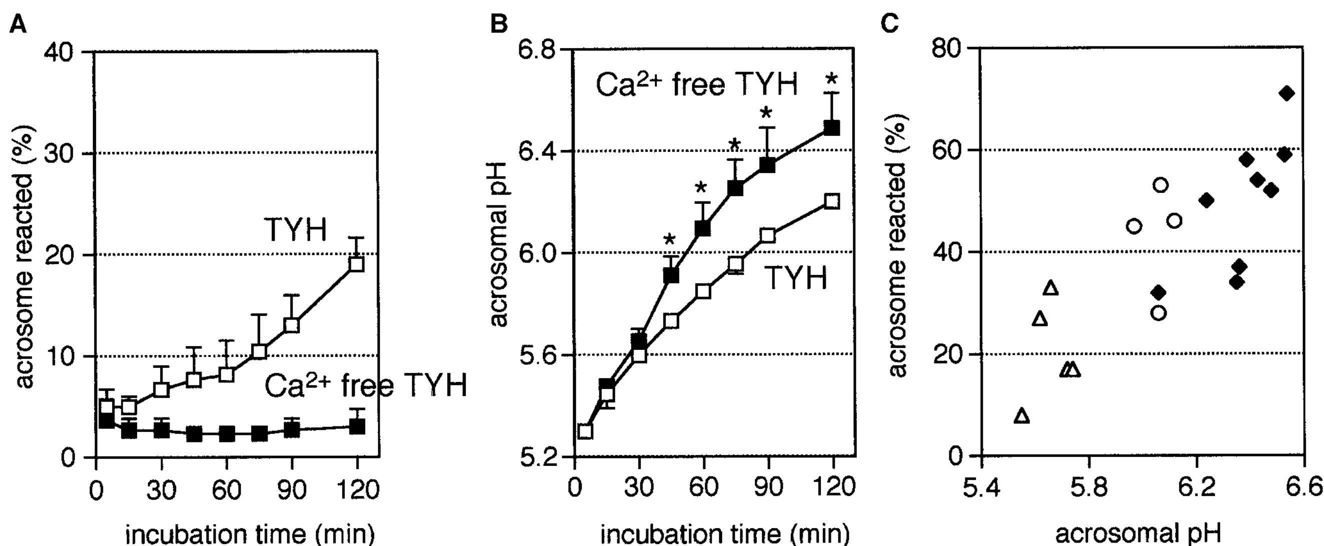

FIG. 5. Increase of intra-acrosomal pH during incubation in Ca^{2+}-depleted medium and acrosome reaction after Ca^{2+} addition. (A, B) Sperm incubated in TYH medium with (□) or without Ca^{2+} (■) were analyzed at the indicated time periods. The increase in acrosome-reacted sperm and the intra-acrosomal pH are shown in (A) and (B), respectively. (The pH values represent estimated values from calibration curve shown in Fig. 1.) The results represent the mean ± SD of three independent experiments. *, Significantly different from TYH medium, $P < 0.05$. (C) Acrosome reactions were induced by adding Ca^{2+} to sperm incubated for 30 (△), 60 (○), and 120 min (◆) in Ca^{2+}-depleted medium. The percentages of acrosome-reacted sperm after the addition of Ca^{2+} were plotted against the intra-acrosomal pH of sperm before Ca^{2+} addition in five, four, and nine independent experiments at 30, 60 and 120 min, respectively.

and 120 min, the pH values within the acrosomes increased and ranged from 5.97 to 6.12 and 6.06 to 6.54, respectively. The average percentages of sperm that had undergone acrosome reactions following the addition of Ca^{2+} were 43% and 50% for 60- and 120-min incubated sperm, respectively. The relationship between the estimated intra-acrosomal pH before Ca^{2+} addition and the subsequent percentage of sperm that had undergone spontaneous acrosome reactions following the addition of Ca^{2+} is shown in Fig. 5C. As the incubation periods were extended, the intra-acrosomal pH values increased and more sperm underwent acrosome reactions following the addition of calcium.

The Relationship of Intra-Acrosomal pH and the Status of Acrosomal Protein

To induce acrosome reactions in sperm that had been preincubated for 120 min in TYH medium (pH 7.5), the Ca^{2+} ionophore, A23187, was added. Two portions of the sperm suspension were separated from the suspension at 0, 3, 15, and 60 min after the ionophore addition and each portion was fixed, washed with PBS, and incubated with monoclonal antibodies (mAbs) against MN7 or MC41 (a 90-kDa protein localized to the entire mouse acrosomal matrix and a 200-kDa protein localized to the cortex region of the mouse anterior acrosomal matrix, respectively; Tanii *et al.,* 1992, 1994). The sperm were subjected to flow cytometric analysis to determine acrosomal integrity with Acr-EGFP and the amount of antigen with R-phycoerithrin (PE)-conjugated anti-mouse IgG (Fig. 6A). When the acrosome reaction was traced by the loss of Acr-EGFP, an average of 80% and 99% of the sperm had lost their Acr-EGFP within 3 min and 15 min after the ionophore addition, respectively. In accordance with our previous observation, when sperm were analyzed by flow cytometry at any time point, they were separated into two peaks: sperm with strong GFP signals (representing acrosome-intact GFP-positive sperm) or sperm with negligible GFP signals (representing acrosome-reacted, GFP-negative sperm) (GFP column in Fig. 6B). Few cells were detected in the range between these two extremes, probably because the Acr-EGFP was dispersed within 3 s of the onset of acrosomal exocytosis in individual sperm (Nakanishi *et al.,* 1999). On the other hand, when we examined the disappearance of MN7 and MC41, we found that their behavior was very different from Acr-EGFP (MN7 and MC41 columns in Fig. 6B). Considerable amounts of these antigens were shown to remain on acrosome-reacted sperm examined 3 min after the addition of ionophore. The amounts of antigens were, however, decreased gradually during the following 60 min of incubation after the ionophore addition.

To investigate the relationship of pH and the nature of MN7 and MC41, sperm were preincubated for 120 min in MES-TYH media at three different pH values (pH 5.3, 6.2, and 7.0) and were then induced to undergo acrosome

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

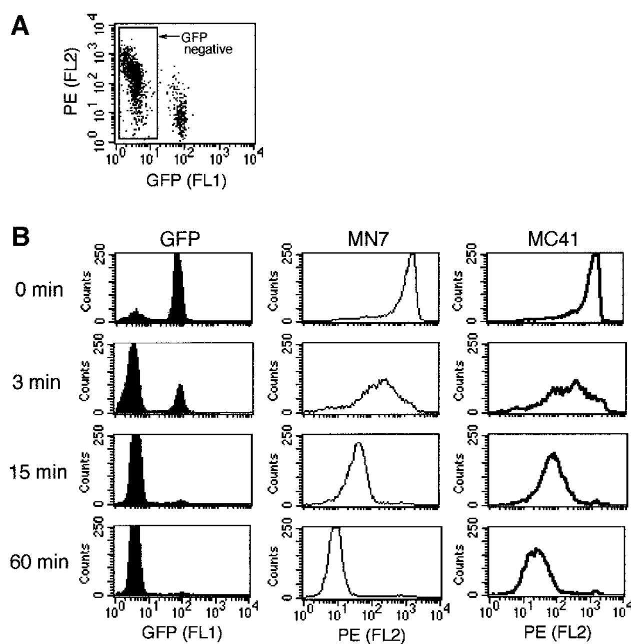

FIG. 6. Delayed loss in reactivity of mAb-MN7 and mAb-MC41 from acrosome-reacted sperm. "Green sperm" were preincubated in TYH medium (pH 7.5) for 120 min, and were induced to undergo acrosome reactions by adding Ca^{2+} ionophore A23187. Sperm were fixed with 4% paraformaldehyde and stained with mAb-MN7 or mAb-MC41 at the indicated time points. (A) A representative case of two-dimensional flow cytometric dot-plot pattern of "green sperm" stained with mAb-MN7 using PE-conjugated anti-mouse IgG. (B) Entire sperm population shown in (A) was analyzed in the GFP column. The sperm were separated into GFP-positive (acrosome-intact) and -negative (acrosome-reacted) populations. The reactivity of mAb-MN7 or mAb-MC41 with "acrosome-reacted" sperm populations (square area in A) was analyzed and indicated in the MN7 and MC41 columns. The GFP and MN7 columns represent a set of data using the same specimen. The data for MC41 column were obtained by using a different portion of the same sperm suspension used for MN7 measurement. The matching GFP column for MC41 was not shown because they were equal to the GFP column indicated. At the 0-min time point, the reactivities with antibodies were measured by using fresh sperm permeabilized with saponin (see Materials and Methods).

reactions using Ca^{2+} ionophore A23187. Sixty minutes after the addition, sperm were stained with mAb-MN7 or mAb-MC41; GFP-negative (acrosome-reacted) sperm were then analyzed as in the experiment shown in Fig. 6. When sperm were preincubated in pH 5.3 for 60 min, both antigens did not disappear from acrosome-reacted sperm after the ionophore addition. On the other hand, at pH 7.0, a significant amount of the antigen reactivity was lost during the 60 min of incubation and only 9 ± 4% and 15 ± 6% for MN7 and MC41, respectively, were detected on acrosome-reacted sperm in comparison to the amount found on sperm at pH 5.3. The antigens also disappeared from sperm at pH 6.2 (27 ± 6% and 20 ± 2% for MN7 and MC41, respectively, remained on sperm) (Fig. 7).

DISCUSSION

EGFP As an Intra-Acrosomal pH Indicator

In the present paper, we have utilized "green sperm" for the measurement of intra-acrosomal pH. Since the mature sperm were translationally inactive, the amount of Acr-EGFP inside the acrosome would not change during sperm incubation. Moreover, when H^+ ionophore nigericin was used to equilibrate the intra-acrosomal pH with media ranging from pH 4.0 to 8.0, the fluorescence intensity of Acr-EGFP that accumulated in condensed conditions inside the acrosome followed a sigmoidal curve with increasing extracellular pH (data not shown) as diluted EGFPs *in vitro* (Fig. 1). These data indicated that the increase in fluorescence of Acr-EGFP within the acrosome could be attributed to an increase in intra-acrosomal pH.

Previously, the intra-acrosomal pH of hamster sperm had been estimated by methods based on monoamine distribution between membrane-enclosed volumes. These experiments suggested that the sperm acrosomes maintained pH gradients in the range of pH 3.21–3.46 and pH 4.7–5.1, using

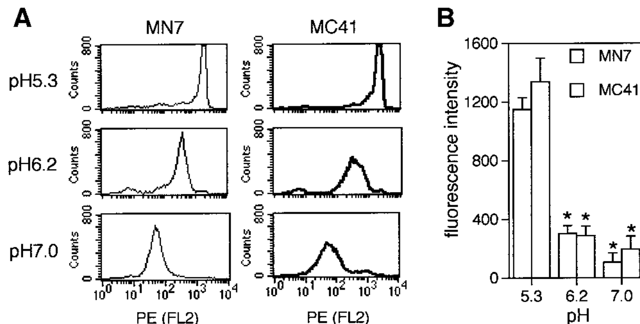

FIG. 7. Effect of pH on the disappearance of MN7 and MC41 antigens from sperm acrosomes. (A) "Green sperm" incubated for 120 min in MES-buffered TYH medium (pH 5.3, 6.2, and 7.0) were induced to undergo acrosome reactions by the addition of Ca^{2+} ionophore A23187. Sixty minutes after the addition, the reactivity of mAb-MN7 or mAb-MC41 with acrosome-reacted sperm (GFP-negative population) was analyzed by a flow cytometry using a different portion of the same sperm suspensions. The data indicate a typical pattern of the histograms. (In a separate experiment, intra-acrosomal pHs before Ca^{2+} ionophore addition were estimated at 4.2, 5.8, and 6.1, respectively, utilizing a method described in Fig. 3.) (B) The average fluorescence intensities indicating the amount of antigen on acrosome-reacted sperm 60 min after the ionophore addition were calculated and illustrated as mean ± SD of three independent experiments. Acrosome-intact sperm were eliminated from counting by gating out the GFP-positive sperm. *, $P < 0.001$ when comparing the values from pH 6.2 and pH 7.0 with the value from pH 5.3.

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

9-aminoacridine and ^{14}C-methylamine, respectively (Meizel and Deamer, 1978). The variations may be derived from the fact that the pH was estimated by calculations based on two assumptions: (1) the dye was localized exclusively in the acrosome, and (2) the estimated acrosomal volume was accurate.

In the present method, transgenic technology was used to exclusively localize EGFP inside acrosome as an intrinsic pH probe (Nakanishi *et al.,* 1999) and the determination of acrosomal volume was not necessary for the measurement of intra-acrosomal pH. Moreover, by combining PI staining with "green sperm," the intra-acrosomal pH measured in the present experiment solely reflected the change occurred in "live" and "acrosome-intact" sperm. From these considerations, we conclude that a more accurate estimation of the pH within the acrosomes of freshly prepared sperm was achieved with our method.

Intra-Acrosomal pH and Acrosome Reaction

In the female reproductive tract, mammalian sperm undergo a unique maturational step known as capacitation, a prerequisite for fertilization. During capacitation, various biochemical and biophysical changes are reported to occur in sperm such as an efflux of cholesterol from sperm membrane and a change in mobilization of ions across the membrane. Recently, it was reported that a HCO_3^- influx into the cytoplasm causes an increase in cytosolic pH (Zeng *et al.,* 1996), and results in the stimulation of the cAMP/PK-A pathway and protein tyrosine phosphorylation (Chen *et al.,* 2000; Visconti *et al.,* 1999). However, one of the disadvantages in these experiments was that researchers could not distinguish whether the changes derived from the acrosome-reacted, or the acrosome-intact population. It would be possible to solve this problem if the "green sperm" would be used in the future experiments.

In the present investigation, the alteration of the intra-acrosomal pH during incubation was observed only in "live" and "acrosome-intact" sperm and its relationship to acrosome reaction was investigated. However, we measured the intra-acrosomal pH and not the pH of the cytoplasm where membrane fusion between plasma and outer acrosomal membranes takes place during acrosome reaction. Therefore, the alkalinization inside the acrosome may not directly affect the membrane fusion events involved in the acrosome reaction. As a matter of fact, when we used DIDS to maintain a low intra-acrosomal pH, spontaneous acrosome reaction was enhanced. However, without the inhibitor, the intra-acrosomal pH increased gradually during incubation of sperm in conditions suitable for capacitation and, concomitantly, spontaneous acrosome react increased (Fig. 4). Moreover, the higher the intra-acrosomal pH of sperm incubated in Ca^{2+}-depleted medium, the more sperm were induced to acrosome react by introduction of Ca^{2+} (Fig. 5). In conclusion, these results demonstrate that, as one of the important steps in sperm capacitation, the intra-acrosomal pH could be an indicator of the readiness of individual sperm to undergo the acrosome reaction.

Intra-Acrosomal pH and Protein Excretion

During the fertilization process, it is assumed that sperm utilize their enzymes and surface proteins to ascend the female reproductive tract (Cho *et al.,* 1998), to pass through the vestments surrounding the eggs (Yanagimachi, 1994), and to fuse with the plasma membranes of the eggs (Cho *et al.,* 1998). Since these steps are separated in terms of time, it must be necessary for different components of sperm to be expressed on their surface in proper amounts at the proper time. In fact, it is reported that proteins such as dipeptidyl peptidase, autoantigen 1, and soluble hyualuronidase are swiftly released from acrosome following acrosome reaction while proacrosin is released gradually from acrosome (DiCarlantonio and Talbot, 1988; Hardy *et al.,* 1991). One possible mechanism causing sequential release could be a distinctive association of acrosomal proteins to the segmented acrosomal matrix (NagDas *et al.,* 1996a,b), while another possibility is proteolysis of the acrosomal matrix, thereby facilitating the release of acrosomal contents (Westbrook-Case *et al.,* 1994; Yamagata *et al.,* 1998). In neurons and endocrine cells, the acidic environment inside secretory vesicles maintains neuropeptides and peptide hormones in a concentrated form (Yoo, 1996), which is optimal for storage. However, physiological alkalinization inside secretory vesicles takes place before exocytosis and alters the physical state of intra-vesicular contents in preparation for release (Han *et al.,* 1999). In guinea pig sperm, it has been reported that a morphologically intact but membrane-free acrosomal matrix remained morphologically stable at pH 5.2; however, it dissolved rapidly at pH 7.0. This dispersion of acrosomal matrix was prevented by trypsin inhibitors (Huang *et al.,* 1985). Recent reports have stressed the importance of intermediate states during the acrosome reaction, speculating that the sequential dissolution of the acrosomal matrix (AM50 and AM67) plays a key role in the events of sperm-zona interactions, enabling sperm to release from their initial point of attachment and then move through the zona pellucida without losing contact with the eggs' extracellular matrix (Kim *et al.,* 2001a,b). We found that the delayed secretion (or modification) from the acrosome was not limited to certain antigens such as AM50 and AM67, but is also applicable to MN7 and MC41 (Fig. 6).

Along these lines, the intra-acrosomal pH was related to the disappearance of acrosomal components recognized by mAb-MN7 and mAb-MC41 (Tanii *et al.,* 1992, 1994). As shown in Fig. 7, it was possible to induce acrosome reaction at pH 5.3 (intra-acrosomal pH of freshly prepared sperm) as monitored by the release of Acr-EGFP. However, the antigens were not released from the acrosome using this condition. On the other hand, the antigens disappeared from the acrosome when the acrosome reaction was induced at pH 7.5 after incubating the sperm in TYH medium for 120

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

min. However, it took 60 min for the antigens to disappear completely at pH 7.5 (Fig. 6). Combining these findings, it was assumed that if the intra-acrosomal pH did not increase during 120 min of incubation, the rate of antigen disappearance must have been delayed considerably. Our findings suggest that the increase in intra-acrosomal pH could be one of the necessary steps for sperm capacitation, resulting in the alteration of the acrosomal contents before acrosome reaction.

The "green sperm" with EGFP in their acrosomes could be utilized in innumerable experiments requiring monitoring of the acrosomal status noninvasively. The mice also provide clues towards solving the mechanism of fertilization by breeding with many types of mutant mouse lines defective in fertilization.

ACKNOWLEDGMENTS

We thank Dr. George L. Gerton for critically reading the manuscript. All experiments were performed with the consent of the Animal Care and Use Committee of Osaka University. This work was supported by grants to M.I. (11780533) and M.O. (11234203) from the Ministry of Education, Science, Sports and Culture and a grant to M.O. (H10-genome-012) from the Ministry of Health and Welfare.

REFERENCES

Beaumelle, B., Bensammar, L., and Bienvenue, A. (1992). Selective translocation of the A chain of diphtheria toxin across the membrane of purified endosomes. *J. Biol. Chem.* **267,** 11525–11531.

Chen, Y., Cann, M. J., Litvin, T. N., Iourgenko, V., Sinclair, M. L., Levin, L. R., and Buck, J. (2000). Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor [see comments]. *Science* **289,** 625–628.

Cho, C., Bunch, D. O., Faure, J. E., Goulding, E. H., Eddy, E. M., Primakoff, P., and Myles, D. G. (1998). Fertilization defects in sperm from mice lacking fertilin beta. *Science* **281,** 1857–1859.

DiCarlantonio, G., and Talbot, P. (1988). Evidence for sequential deployment of secretory enzymes during the normal acrosome reaction of guinea pig sperm *in vitro*. *Gamete Res.* **21,** 425–438.

Garcia, M. A., and Meizel, S. (1999). Regulation of intracellular pH in capacitated human spermatozoa by a Na^+/H^+ exchanger. *Mol. Reprod. Dev.* **52,** 189–195.

Garner, D. L., Johnson, L. A., Yue, S. T., Roth, B. L., and Haugland, R. P. (1994). Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. *J. Androl.* **15,** 620–629.

Han, W., Li, D., Stout, A. K., Takimoto, K., and Levitan, E. S. (1999). Ca^{2+}-induced deprotonation of peptide hormones inside secretory vesicles in preparation for release. *J. Neurosci.* **19,** 900–905.

Hardy, D. M., Oda, M. N., Friend, D. S., and Huang, T. T., Jr. (1991). A mechanism for differential release of acrosomal enzymes during the acrosome reaction. *Biochem. J.* **275,** 759–766.

Haupts, U., Maiti, S., Schwille, P., and Webb, W. W. (1998). Dynamics of fluorescence fluctuations in green fluorescent protein observed by fluorescence correlation spectroscopy. *Proc. Natl. Acad. Sci. USA* **95,** 13573–13578.

Huang, T. T., Jr., Hardy, D., Yanagimachi, H., Teuscher, C., Tung, K., Wild, G., and Yanagimachi, R. (1985). pH and protease control of acrosomal content stasis and release during the guinea pig sperm acrosome reaction. *Biol. Reprod.* **32,** 451–462.

Kaneko, K., Guth, P. H., and Kaunitz, J. D. (1991). *In vivo* measurement of rat gastric surface cell intracellular pH. *Am. J. Physiol.* **261,** G548–G552.

Kawa, G., Yamamoto, A., Yoshimori, T., Muguruma, K., Matsuda, T., and Moriyama, Y. (2000). Immunohistochemical localization of V-ATPases in rat spermatids [In Process Citation]. *Int. J. Androl.* **23,** 278–283.

Kim, K. S., Cha, M. C., and Gerton, G. L. (2001a). Mouse sperm protein sp56 is a component of the acrosomal matrix. *Biol. Reprod.* **64,** 36–43.

Kim, K. S., Foster, J. A., and Gerton, G. L. (2001b). Differential release of guinea pig sperm acrosomal components during exocytosis. *Biol. Reprod.* **64,** 148–156.

Llopis, J., McCaffery, J. M., Miyawaki, A., Farguhar, M. G., and Tsien, R. Y. (1998). Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc. Natl. Acad. Sci. USA* **95,** 6803–6808.

Meizel, S., and Deamer, D. W. (1978). The pH of the hamster sperm acrosome. *J. Histochem. Cytochem.* **26,** 98–105.

NagDas, S. K., Winfrey, V. P., and Olson, G. E. (1996a). Identification of hydrolase binding activities of the acrosomal matrix of hamster spermatozoa. *Biol. Reprod.* **55,** 1405–1414.

NagDas, S. K., Winfrey, V. P., and Olson, G. E. (1996b). Proacrosin-acrosomal matrix binding interactions in ejaculated bovine spermatozoa. *Biol. Reprod.* **54,** 111–121.

Nakanishi, T., Ikawa, M., Yamada, S., Parvinen, M., Baba, T., Nishimune, Y., and Okabe, M. (1999). Real-time observation of acrosomal dispersal from mouse sperm using GFP as a marker protein. *FEBS Lett.* **449,** 277–283.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. (1997). "Green mice" as a source of ubiquitous green cells. *FEBS Lett.* **407,** 313–319.

Parkkila, S., Rajaniemi, H., and Kellokumpu, S. (1993). Polarized expression of a band 3-related protein in mammalian sperm cells. *Biol. Reprod.* **49,** 326–331.

Schmidt, W. K., and Moore, H. P. (1995). Ionic milieu controls the compartment-specific activation of pro-opiomelanocortin processing in AtT-20 cells. *Mol. Biol. Cell* **6,** 1271–1285.

Tanii, I., Araki, S., and Toshimori, K. (1994). Intra-acrosomal organization of a 90-kilodalton antigen during spermiogenesis in the rat. *Cell Tissue Res.* **277,** 61–67.

Tanii, I., Toshimori, K., Araki, S., and Oura, C. (1992). Appearance of an intra-acrosomal antigen during the terminal step of spermiogenesis in the rat. *Cell Tissue Res.* **267,** 203–208.

Toyoda, Y., Yokoyama, M., and Hoshi, T. (1971). Studies on the fertilization of mouse eggs *in vitro*. I. *In vitro* fertilization of eggs by fresh epididymal sperm. *Jpn. J. Anim. Reprod.* **16,** 147–151.

Visconti, P. E., Stewart-Savage, J., Blasco, A., Battaglia, L., Miranda, P., Kopf, G. S., and Tezon, J. G. (1999). Roles of bicarbonate, cAMP, and protein tyrosine phosphorylation on capacitation and the spontaneous acrosome reaction of hamster sperm. *Biol. Reprod.* **61,** 76–84.

Wakabayashi, S., Ikeda, T., Noel, J., Schmitt, B., Orlowski, J., Pouyssegur, J., and Shigekawa, M. (1995). Cytoplasmic domain of the ubiquitous Na^+/H^+ exchanger NHE1 can confer Ca^{2+} responsiveness to the apical isoform NHE3. *J. Biol. Chem.* **270,** 26460–26465.

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.

Westbrook-Case, V. A., Winfrey, V. P., and Olson, G. E. (1994). A domain-specific 50-kilodalton structural protein of the acrosomal matrix is processed and released during the acrosome reaction in the guinea pig. *Biol. Reprod.* **51,** 1–13.

Working, P. K., and Meizel, S. (1981). Evidence that an ATPase functions in the maintenance of the acidic pH of the hamster sperm acrosome. *J. Biol. Chem.* **256,** 4708–4711.

Yamagata, K., Murayama, K., Okabe, M., Toshimori, K., Nakanishi, T., Kashiwabara, S., and Baba, T. (1998). Acrosin accelerates the dispersal of sperm acrosomal proteins during acrosome reaction. *J. Biol. Chem.* **273,** 10470–10474.

Yanagimachi, R. (1994). Mammalian fertilization. *In* "The Physiology of Reproduction, Second Edition" (E. Knobil and J. D. Neill, Eds.), pp. 189–317. Raven, New York.

Yoo, S. H. (1996). pH- and $Ca^{(2+)}$-dependent aggregation property of secretory vesicle matrix proteins and the potential role of chromogranins A and B in secretory vesicle biogenesis. *J. Biol. Chem.* **271,** 1558–1565.

Zeng, Y., Oberdorf, J. A., and Florman, H. M. (1996). pH regulation in mouse sperm: identification of $Na^{(+)}$-, $Cl^{(-)}$-, and $HCO3^{(-)}$-dependent and arylaminobenzoate-dependent regulatory mechanisms and characterization of their roles in sperm capacitation. *Dev. Biol.* **173,** 510–520.

Received for publication January 25, 2001
Revised May 8, 2001
Accepted June 6, 2001
Published online July 30, 2001

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.