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Acrosin Accelerates the Dispersal of Sperm Acrosomal Proteins during Acrosome Reaction*

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Using homologous recombination, we have previously produced male mice carrying a disruptive mutation ($Acr^{-/-}$) in the acrosin gene. Although $Acr^{-/-}$ mouse sperm lacking the acrosin protease activity still penetrated the zona pellucida and fertilized the egg, the mutant sperm exhibited a delay in penetration of the zona pellucida solely at the early stages after insemination. To further elucidate the role of acrosin in fertilization, we have examined the involvement of acrosin in the acrosome reaction of sperm using the $Acr^{-/-}$ mutant mice. When the ability of sperm to adhere (attach) and bind to the zona pellucida of cumulus-free eggs was assessed *in vitro*, no significant difference was observed among $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ mouse sperm. Immunocytochemical analysis demonstrated that the release of several acrosomal proteins from the acrosome of $Acr^{-/-}$ mouse sperm was significantly delayed during the calcium ionophore- and solubilized zona pellucida-induced acrosome reaction, despite normal membrane vesiculation. These data indicate that the delayed sperm penetration of the zona pellucida in the $Acr^{-/-}$ mouse results from the altered rate of protein dispersal from the acrosome and provide the first evidence that the major role of acrosin is to accelerate the dispersal of acrosomal components during acrosome reaction.

The acrosome reaction of sperm, a fusion (vesiculation) event between the overlying plasma and outer acrosomal membranes, occurs following the binding of sperm to the zona pellucida (ZP),[1] an extracellular glycoprotein matrix surrounding the egg. This exocytotic reaction is required for fertilization, because only acrosome-reacted sperm are capable of penetrating ZP and of fusing with the egg plasma membrane (for review see Ref. 1). The acrosomal components, including hydrolytic enzymes, are released by the acrosome reaction and then interact initially with ZP to facilitate the sperm penetration of the glycoprotein matrix.

Acrosin, an endoprotease with a trypsin-like substrate specificity, is localized in the acrosomal matrix as an enzymatically inactive zymogen, proacrosin, that is then converted into the active form as a consequence of the acrosome reaction (2–4). The physiological role of acrosin in fertilization has long been believed to be the limited proteolysis of the ZP, thus enabling the sperm to penetrate the ZP. Using homologous recombination, we have successfully produced male mice carrying a disruptive mutation in the acrosin gene (*Acr*) and found that the mouse sperm lacking the acrosin protease activity ($Acr^{-/-}$) still penetrate ZP and normally fertilize the egg (5). These data provide evidence that acrosin is not essential for sperm penetration of the ZP. However, as compared with $Acr^{+/+}$ and $Acr^{+/-}$ mice, $Acr^{-/-}$ mouse sperm showed a delay in sperm penetration of the ZP solely at the early stages after insemination (5). A recent report using separate lines of $Acr^{-/-}$ mice (6) has confirmed that sperm lacking acrosin exhibit the delayed fertilization. Thus, these results imply that acrosin plays an important role prior to the sperm penetration of ZP, possibly at the time of the acrosome reaction, although the participation of acrosin in the ZP hydrolysis cannot be ruled out completely.

To elucidate the role of acrosin in fertilization, we have examined the involvement of acrosin in the acrosome reaction of sperm, using $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ male mice. Immunocytochemical analysis indicated that the release of several acrosomal proteins from the acrosome of $Acr^{-/-}$ mouse sperm during the acrosome reaction was significantly delayed. Thus, acrosin likely accelerates the dispersal of proteins from the sperm acrosome.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibodies against a mouse sperm protein (termed mAb OBF13) and an intra-acrosomal 155-kDa mouse protein (mAb MC101) were prepared as described previously (7, 8). Anti-mouse sp56 monoclonal antibody 7C5 (mAb sp56) was originally purchased from QED Biologicals (La Jolla, CA) by Dr. George L. Gerton and was kindly provided by him. Rabbit anti-recombinant mouse PH2O antiserum was a gift of Dr. Paul Primakoff. *p*-Aminobenzamidine and calcium ionophore A23187 were purchased from Sigma and Dojindo Laboratories (Kumamoto, Japan), respectively. $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ male mice were obtained by mating between $Acr^{+/-}$ males and females, as described previously (5).

Measurement of Sperm Binding to ZP—Fresh cauda epididymal sperm from male mice (2–3 months old) were dispersed in a 0.2-ml drop of modified Krebs-Ringer bicarbonate solution (TYH medium) containing glucose, sodium pyruvate, bovine albumin, and antibiotics (9) at 37 °C under 5% CO_2 in air until the medium became turbid (usually 15 min). The sperm were then capacitated by incubation at 37 °C under 5% CO_2 in air for 90 min. Female B6C3F1 mice (2–3 months old, Japan SLC, Inc., Shizuoka, Japan) were superovulated following intraperitoneal injections of pregnant mare serum gonadotropin and human chorionic gonadotropin at a 48-h interval. Eggs associated with cumulus

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[1] The abbreviations used are: ZP, zona pellucida; ABC, avidin-biotin peroxidase complex; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

cells were recovered approximately 15 h after human chorionic gonadotropin injection and placed under warm mineral oil in a plastic Petri dish containing TYH medium (0.2 ml), treated at room temperature for 5 min with hyaluronidase (Sigma Type I-S, 150 units/ml) to remove the cumulus cells, and washed with TYH medium. The eight cumulus-free eggs and two 2-cell embryos in TYH medium (5 μl) were incubated with 5 μl of the capacitated sperm suspension (2,000 sperm) at 37 °C for 30 min, transferred to a 100-μl drop of fresh TYH medium, and washed by pipetting (10–15 times). After fixation with 4% paraformaldehyde, the number of sperm bound to the egg ZP was counted using a Leica DMIRBE phytomicroscope. The 2-cell embryos were used as an internal negative control for nonspecific binding (10), and the number of the bound sperm per 2-cell embryo was less than 4 under the above conditions.

Calcium Ionophore-induced Acrosome Reaction—Capacitated cauda epididymal sperm (4×10^6 sperm/ml) in 0.2 ml of TYH medium were induced to undergo acrosome reaction by addition of calcium ionophore A23187 at a final concentration of 5 μg/ml followed by incubation at 37 °C under 5% CO_2 in air. An aliquot (50 μl each) was taken 3, 15, or 30 min after the incubation, transferred into a 1.5-ml microcentrifuge tube, and then centrifuged at 3,000 rpm for 10 min. The sperm pellets were resuspended in 50 μl of phosphate-buffered saline (PBS).

ZP-induced Acrosome Reaction—Solubilized ZP was prepared from the superovulated eggs of 2-month-old female ICR mice by the already described method (10) with a minor modification. Briefly, the cumulus-free, ZP-intact eggs (400–500) were incubated for 20 min in a bovine serum albumin-free acidified TYH medium (pH 2.2) at a concentration of 10 eggs/μl. After centrifugation at 13,000 rpm for 10 min, the supernatant containing the solubilized ZP (20 μl each) was neutralized with 16 μl of 62.5 mM Hepes/TYH medium (pH 7.7) containing 8 mg/ml bovine serum albumin to give a final pH of approximately 7.4. To the 36-μl solution, 4 μl of capacitated sperm suspension (1.5×10^7 sperm/ml) were added, and the mixture was then incubated for 60 min at 37 °C under 5% CO_2 in air.

Immunocytochemical Analysis of Sperm—Sperm suspensions were placed onto glass slides that had been coated with VECTABOND (Vector laboratories, Burlingame, CA), treated with PBS containing 4% paraformaldehyde or with PBS alone on ice for 30 min, and washed three times with PBS. For indirect immunofluorescent staining, the slides were incubated with primary antibodies diluted in PBS containing 5% fetal bovine serum overnight, washed with PBS, and treated with fluorescein isothiocyanate-conjugated goat anti-mouse IgA + IgG + IgM (Cappel, Durham, NC) for 4 h. After washing with PBS, the slides were observed using a Leitz DMRXE fluoromicroscope.

Immunoperoxidase staining was also carried out by the avidin-biotin peroxidase complex (ABC) method (11) using a Vectastain Elite ABC kit (Vector Laboratories). The sperm samples on slides with or without fixation with 4% paraformaldehyde were treated with 0.3% hydrogen peroxide in methanol for 30 min, washed with PBS containing 0.1% Tween 20, and blocked with 1.5% normal goat serum in PBS for 30 min and with an avidin/biotin blocking kit (Vector laboratories) for 30 min at room temperature. The slides were incubated in a primary antibody solution at room temperature for 3 h, washed three times with the above blocking solution, and then treated with biotin-conjugated goat anti-mouse IgG + IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) or anti-rabbit IgG (Vector laboratories) for 30 min followed by an ABC solution containing horseradish peroxidase-conjugated avidin (Vector laboratories) for 30 min. After washing with PBS, the sperm samples were stained using 3,3′-diaminobenzidine as a chromogen, mounted, and viewed under an Olympus BX50 microscope.

RESULTS

As described previously (5), a remarkable delay in the sperm penetration of ZP was observed in $Acr^{-/-}$ mouse on *in vitro* fertilization within 60 min after insemination. To examine whether the delay is caused by a reduced ability of $Acr^{-/-}$ mouse sperm for adhesion (attachment) or binding to ZP, the cumulus-free, ZP-intact eggs were incubated with capacitated sperm. No significant difference of the ability to adhere to ZP was apparently observed among $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ mouse sperm at 15 and 30 min after insemination. Moreover, the sperm binding to ZP at 30 min after the incubation were found to be 28.5 ± 9.7, 38.2 ± 9.2, and 26.7 ± 7.7 (means of sperm numbers/egg ± S.D.) in $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ mice, respectively. These data indicate that $Acr^{-/-}$ mouse sperm still possess a normal capacity for adhesion and binding to ZP and imply that the delay in the sperm penetration of ZP in $Acr^{-/-}$ mouse is implicated in a physiological event occurring after the sperm bind ZP.

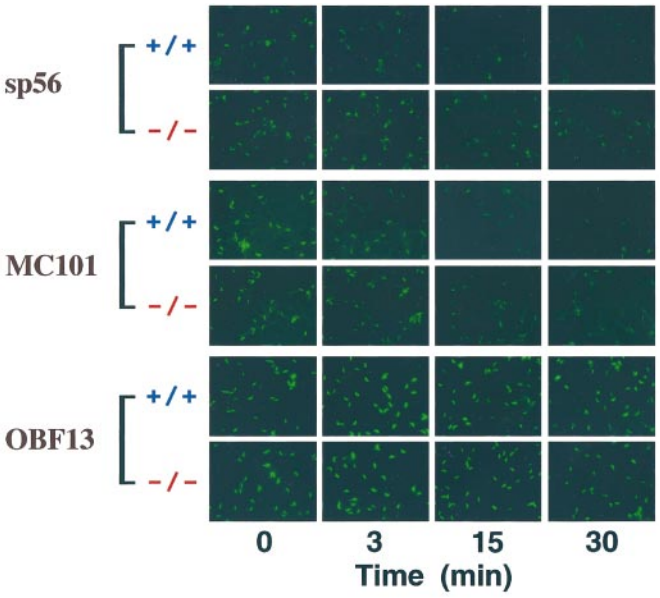

FIG. 1. **Indirect immunofluorescent staining of mouse sperm following calcium ionophore A23187 treatment.** Capacitated cauda epididymal sperm (4×10^6 sperm/ml) from $Acr^{+/+}$ (+/+) and $Acr^{-/-}$ (−/−) mice were incubated in 0.2 ml of TYH medium containing calcium ionophore A23187 (5 μg/ml) at 37 °C under 5% CO_2 in air. The sperm suspension (50 μl each) was taken at 3, 15, or 30 min after the incubation and incubated first with mAb sp56, mAb MC101, or mAb OBF13 on glass slides followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgA + IgG + IgM. Note that the immunofluorescent signals in mAb sp56 and mAb MC101 disappeared from the $Acr^{+/+}$ sperm acrosome as the incubation time elapsed, whereas the signals still remained in the noticeable numbers of the $Acr^{-/-}$ sperm acrosome even after the 30-min incubation. mAb OBF13 initially immunostained the acrosomal cap region, and the signals quickly spread over the sperm head in both $Acr^{+/+}$ and $Acr^{-/-}$ mouse sperm.

Capacitated $Acr^{+/+}$ and $Acr^{-/-}$ mouse sperm were treated with calcium ionophore A23187, and the time course of protein release from the acrosome was monitored by indirect immunofluorescent staining using two monoclonal antibodies against mouse acrosomal proteins (mAb sp56 (12, 13) and mAb MC101 (8); Fig. 1). mAb OBF13 (7), which immunoreacts both with a protein located at the acrosome cap region of capacitated sperm and with the same protein redistributed over the entire sperm head after acrosome reaction (14), was also used as a control. The immunofluorescent staining patterns of mAb sp56 and mAb MC101 disappeared from the $Acr^{+/+}$ sperm acrosome as the incubation time elapsed, whereas the signals still remained in noticeable numbers of the acrosome of $Acr^{-/-}$ mouse sperm even at 30 min after addition of the ionophore. As expected, mAb OBF13 initially immunostained the acrosomal cap region, and the signals quickly spread over the sperm head. There was no difference of the immunostaining pattern in mAb OBF13 between $Acr^{+/+}$ and $Acr^{-/-}$ mouse sperm. Thus, these observations clearly demonstrate that the dispersal of the antigenic proteins recognized by mAb sp56 and mAb MC101 is delayed only in $Acr^{-/-}$ mouse sperm, despite apparently normal membrane vesiculation during the acrosome reaction.

To assess the acrosomal status more clearly, capacitated sperm with or without ionophore treatment were stained by the ABC method (11) using four different antibodies, including polyclonal anti-PH2O antibody (Fig. 2). As found in indirect immunostaining, some of the stained signals in $Acr^{+/+}$ and $Acr^{-/-}$ mouse sperm were lost from the acrosome by the iono-

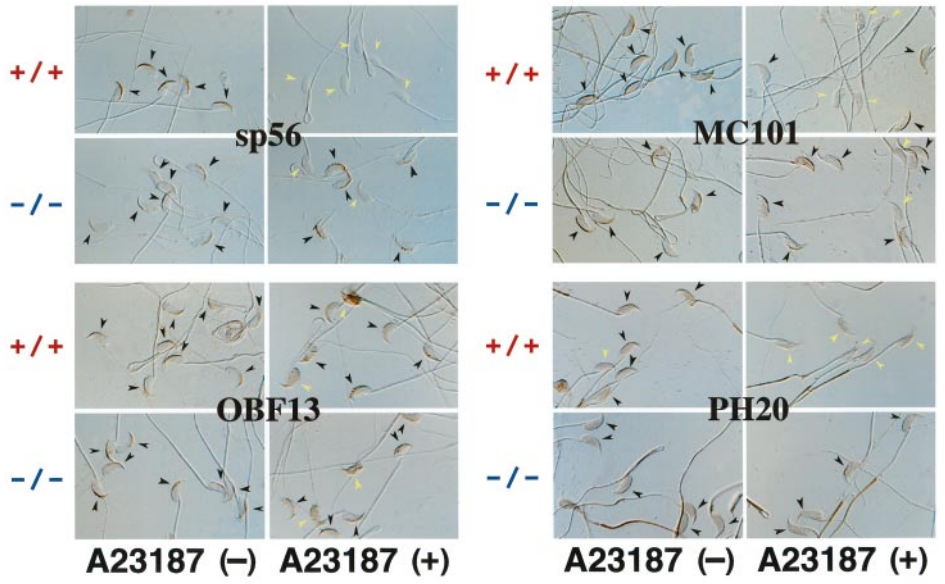

FIG. 2. **ABC immunostaining of mouse sperm following calcium ionophore A23187 treatment.** Capacitated cauda epididymal sperm from $Acr^{+/+}$ (+/+), $Acr^{+/-}$, and $Acr^{-/-}$ (−/−) mice were incubated at 37 °C in TYH medium containing calcium ionophore A23187 and sampled at time intervals as described in the legend to Fig. 1. The sperm were reacted with mAb sp56, mAb MC101, mAb OBF13, or anti-mouse PH2O antibody on glass slides and then with biotin-conjugated goat anti-mouse IgG + IgM or anti-rabbit IgG. After treatment with peroxidase-conjugated avidin, the sperm samples were stained using 3,3′-diaminobenzidine as a chromogen. The sperm incubated with the ionophore for 3 min (mAb MC101 and mAb OBF13) or for 30 min (mAb sp56 and anti-PH2O antibody) are shown (not shown for $Acr^{+/-}$ mouse sperm). The sperm are visually divided into two groups by the following criteria: (i) sperm that still contain the antigens recognized by the antibodies in the acrosome in spite of the fact that they are acrosome-intact, initiate the acrosome reaction, or have already acrosome-reacted (*black arrowheads*) and (ii) sperm that contain no signal of the antigens in the acrosome (the acrosome reaction has been already accomplished, and the antigens have been completely dispersed, *yellow arrowheads*).

phore treatment (data not shown for $Acr^{+/-}$ mouse sperm). The sperm were divided into two groups by the following criteria: (i) sperm that still contained the antigens recognized by the antibodies in the acrosome, in spite of the fact that they were acrosome-intact, and initiated the acrosome reaction or had already acrosome-reacted and (ii) sperm that contained no signal of the antigens in the acrosome. When anti-PH2O antibody was used, several regions of sperm were stained; the acrosome and sperm head were strongly and weakly stained, respectively. This result may be due to the fact that PH2O is present in the sperm as two forms: a soluble form locating in the acrosome, and a membrane-anchored form on the plasma and inner acrosomal membranes (15, 16). Although the sperm tail was also stained by anti-PH2O antibody, the signal may be nonspecific.

As shown in Fig. 3, the sperm acrosome immunostained by four antibodies following ionophore treatment was quantified according to the above criteria. The number of the acrosome immunostained rapidly decreased in $Acr^{+/+}$ and $Acr^{+/-}$ mouse sperm with the passage of time, and approximately 90% of the sperm did not show any signal in the acrosome after the 30-min ionophore treatment. However, the disappearance of the signals in $Acr^{-/-}$ sperm acrosome was obviously delayed when mAb sp56, mAb MC101, and anti-PH2O antibody were used. In the case of mAb OBF13, no significant difference in the pattern of the signal disappearance was found among $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ mouse sperm. These results confirm the delay of the dispersal of the acrosomal proteins in $Acr^{-/-}$ mouse sperm during the ionophore-induced acrosome reaction. The number of the acrosome stained by the four antibodies was estimated to be 79–86% of total $Acr^{+/+}$, $Acr^{+/-}$, or $Acr^{-/-}$ mouse sperm without the ionophore treatment. The reduced numbers may result from the possibility that 14–21% of the sperm have undergone the spontaneous acrosome reaction during capacitation. Moreover, almost 40% of $Acr^{+/+}$ and $Acr^{+/-}$ mouse sperm without the ionophore treatment exhibited no signal in

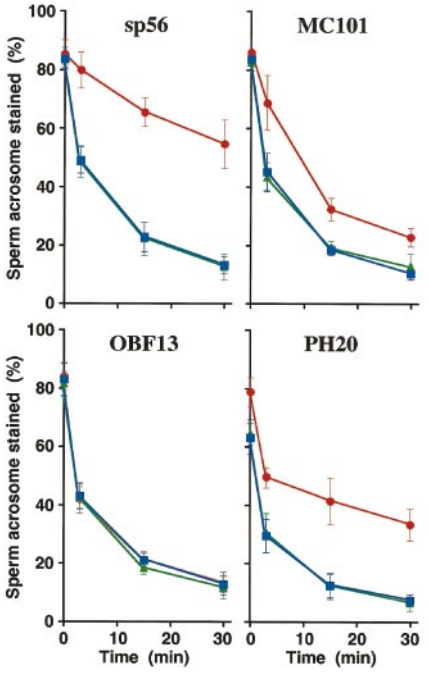

FIG. 3. **Time course of protein dispersal from sperm acrosome following treatment of calcium ionophore A23187.** Capacitated cauda epididymal sperm from $Acr^{+/+}$ (*blue square*), $Acr^{+/-}$ (*green triangle*), and $Acr^{-/-}$ (*red circle*) mice were treated with calcium ionophore A23187, sampled at time intervals, and immunostained by the ABC method (11) as described in Fig. 2. The 300 sperm were selected at random and divided into two groups by the criteria shown in Fig. 2. The number of sperm, which still contained the antigens recognized by the antibodies in the acrosome, in spite of the fact that they were acrosome-intact, initiated the acrosome reaction, or had already acrosome-reacted (*black arrowheads* in Fig. 2), was counted. Data are expressed as the means ± S.D., where $n \geq 3$.

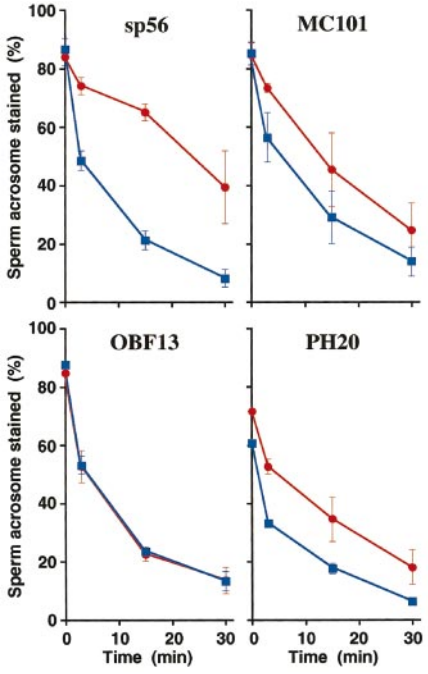

FIG. 4. **Time course of protein dispersal from sperm acrosome following treatment of calcium ionophore A23187 in the presence of *p*-aminobenzamidine.** Capacitated cauda epididymal sperm from $Acr^{+/+}$ mice were treated with calcium ionophore A23187 in the presence (*red circle*) or the absence (*blue square*) of 1 mM *p*-aminobenzamidine, sampled at time intervals, and immunostained by the ABC method (11). The number of sperm, which still contained the antigens recognized by the antibodies in the acrosome in spite of the fact that they were acrosome-intact, initiated the acrosome reaction, or had already acrosome-reacted (*black arrowheads* in Fig. 2), was counted as described in the legend to Fig. 3. Data are expressed as the means ± S.D., where $n \geq 3$.

the acrosome stained by anti-PH2O antibody. The reason for the unusual observation is not clear at the present time.

p-Aminobenzamidine, a competitive inhibitor for trypsin and acrosin, has been reported to inhibit not the acrosome reaction itself, but the dispersal of the acrosomal matrix and sperm penetration of ZP (17–20). When the release of proteins, which were recognized by four antibodies, from the acrosome of $Acr^{+/+}$ mouse sperm treated with ionophore was examined in the presence of 1 mM *p*-aminobenzamidine, the patterns of the signal disappearance were similar to those in $Acr^{-/-}$ mouse sperm in the absence of the acrosin inhibitor (Figs. 3 and 4). These data verify the delayed release of several proteins from the $Acr^{-/-}$ sperm acrosome during ionophore-induced acrosome reaction (Fig. 3).

Because calcium ionophore A23187 is not a physiological inducer of acrosome reaction, we examined protein release from sperm acrosome following solubilized ZP treatment (Table I). Approximately 19–25% of $Acr^{+/+}$ mouse sperm lost the signals stained by mAb sp56, mAb MC101, and mAb OBF13 from the acrosome at 60 min after addition of the solubilized ZP. However, the number of the unstained sperm was still 7–8% in $Acr^{-/-}$ mouse when mAb sp56 and mAb MC101 were used. Thus, the $Acr^{-/-}$ mouse sperm possess the reduced ability for the protein dispersal from the acrosome during the ZP-induced acrosome reaction.

TABLE I
Dispersal of acrosomal proteins from acrosome following treatment of sperm with solubilized zona pellucida

Male mouse	Sperm acrosome unstained by monoclonal antibody[a]		
	mAb sp56	mAb MC101	mAb OBF13
	%		
Wild type ($Acr^{+/+}$)	20.7 ± 6.2	25.0 ± 7.0	18.7 ± 3.3
Mutant ($Acr^{-/-}$)	8.7 ± 2.9	7.3 ± 0.5	28.7 ± 10.7

[a] ZP from superovulated eggs was solubilized in 20 μl of a bovine serum albumin-free acidified TYH medium (pH 2.2) and neutralized with 16 μl of 62.5 mM Hepes/TYH medium (pH 7.7) containing 8 mg/ml bovine serum albumin. To the solubilized ZP solution, 4 μl of capacitated sperm suspension (1.5×10^7 sperm/ml) were added. The mixture was incubated for 60 min at 37 °C under 5% CO_2 in air and then subjected to immunocytochemical analysis according to the ABC method (11) as described the legend to Fig. 2. A solution containing the acidified TYH medium and Hepes/TYH medium without ZP (36 μl) was used in all experiments as a control to measure the protein dispersal from the sperm acrosome due to spontaneous acrosome reaction. Data are calculated by subtracting the values in the control experiments and expressed as the means ± S.D. of three individual experiments.

DISCUSSION

This paper demonstrates that the dispersal of acrosomal proteins from the acrosome is delayed in $Acr^{-/-}$ mouse sperm during either calcium ionophore-induced or solubilized ZP-induced acrosome reaction (Fig. 3 and Table I). These data are consistent with the fact that only $Acr^{-/-}$ mouse sperm show a delayed sperm penetration of ZP at the early stages of *in vitro* fertilization (5). Therefore, it may be concluded that acrosin plays a major role in acceleration of the dispersal of acrosomal proteins *in vivo* and that the decreased ability of $Acr^{-/-}$ mouse sperm to disperse acrosomal proteins is responsible for the delayed sperm penetration of ZP. However, the patterns of the signal disappearance from the acrosome in $Acr^{-/-}$ mouse sperm were distinguished from one another by the antibody used (Fig. 3). In particular, approximately 50% of $Acr^{-/-}$ mouse sperm still had the mAb sp56-positive signal in the acrosome even at 30 min after the ionophore treatment. This differential dispersal of the acrosomal proteins from the acrosome may be correlated with the localization and associated state of each of the proteins within the acrosomal matrix, as reported previously (13, 17, 21, 22), if the protein release from the acrosome during the ionophore-induced acrosome reaction totally reflects the *in vivo* phenomenon.

In guinea pig, the sperm acrosome can be ultrastructurally separated into three domains (M1, M2, and M3) by electron density (23). Proacrosin is preferentially localized in the most electron-dense domains (M2 and M3) presumably in a state firmly associated with other matrix proteins (17, 21, 22, 24, 25), whereas some acrosomal proteins, including soluble PH2O and autoantigen 1, have been reported to associate weakly with the matrix (22, 26–28). Thus, acrosin, which is converted from proacrosin by autoactivation during the acrosome reaction, probably prefers to hydrolyze a core protein(s) of a stable acrosomal matrix, such as AM50 (25, 29) and AM67 (13) in guinea pig sperm, so that the acrosomal proteins, including the partially digested core proteins, would be readily released. Moreover, it is still reasonable to consider the possibility that acrosin possesses a selectivity for each of the acrosomal proteins in the matrix dispersal. If so, we can speculate that acrosin may act to destroy the physiological function of other acrosomal proteins and/or to give a function to latent forms of the proteins by proteolysis as well as to hydrolyze the core proteins forming the acrosomal matrix during acrosome reaction.

Our data provide the first evidence that the role of acrosin is to accelerate the dispersal of acrosomal components during acrosome reaction. However, we should not consider that the protein dispersal is modulated solely by acrosin, because the acrosomal proteins are released with a time delay, and their dispersal is not completely blocked in the absence of acrosin (Fig. 3 and Table I) or in the presence of *p*-aminobenzamidine (Fig. 4). Moreover, we have found that $Acr^{-/-}$ mouse sperm are incapable of fertilizing the egg in the presence of *p*-aminoben-

zamidine.[2] Therefore, a protease(s) sensitive to the inhibitor must be present in the sperm to enable it to penetrate the ZP. This protease may compensate for the insufficient function(s) of the $Acr^{-/-}$ mouse sperm due to the lack of acrosin. Thus, to elucidate the sperm function in fertilization, the identification and characterization of the novel sperm protease(s) remains to be accomplished.

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