Volume 326, number 1,2,3, 149–152 FEBS 12677 July 1993

Progesterone action on the human sperm surface is potentiated by an egg-associated acrosin activator

C. Mendoza[a], J. Moos[b] and J. Tesarik[c,d]

[a]*Department of Biochemistry and Molecular Biology, University of Granada Faculty of Sciences, Campus Universitario Fuentenueva, 18071 Granada, Spain,* [b]*Institute of Molecular Genetics, Videnska 1078, 14220 Prague, Czech Republic,* [c]*American Hospital of Paris, 63 Boulevard Victor Hugo, 92202 Neuilly sur Seine, France* and [d]*INSERM, Unité 355, 32 rue des Carnets, 92140 Clamart, France*

Received 17 May 1993

The progesterone-induced Ca^{2+} influx and acrosomal exocytosis in human sperm are recently described examples of non-genomic steroid action on the cell surface. These progesterone effects are known to be inhibited by synthetic protease inhibitors. In this study we tested a hypothesis that a physiological activator of the sperm protease acrosin modulates the sperm response to progesterone. It was found that the activator augments the amplitude of the progesterone-induced Ca^{2+} transient and accelerates the progesterone-induced acrosomal exocytosis. These observations suggest a physiological significance of the modulation of nongenomic steroid effects by protease regulators.

Progesterone receptor; Plasma membrane; Nongenomic steroid effect; Calcium influx; Acrosin; Acrosome reaction

1. INTRODUCTION

The rapid Ca^{2+} influx and acrosome reaction (AR) occurring in human spermatozoa in response to progesterone [1,2] are increasingly used as a model for the study of nongenomic steroid effects on the cell. Studies using cell-impermeant progesterone conjugates [3–5] have shown that progesterone acts on the sperm plasma membrane, presumably on an atypical steroid receptor or a Ca^{2+} channel. The implication of a plasma membrane receptor in the progesterone action in human spermatozoa has been supported by recent observations that a typical sperm response can be induced by antibody-mediated crosslinking of progesterone-binding sites on the sperm surface [6] and that aggregation of the binding sites occurs rapidly after ligand binding [7]. However, virtually nothing is known about the mechanism by which the ligand-bound receptor operates the entry of Ca^{2+} into the sperm cell, a prerequisite for the membrane fusion events leading to the acrosomal exocytosis.

One of the interesting aspects of the progesterone-induced AR is the inhibition of this event by protease inhibitors [8]. It has been shown that protease inhibitors do not affect either progesterone binding to spermatozoa or the ensuing receptor aggregation [7]. Protease action may thus be involved in a process coupling the crosslinking of the ligand-bound receptor to the opening of a Ca^{2+} channel or to the activation of another mechanism of Ca^{2+} transport across the plasma membrane. The protease involved may be acrosin (EC 3.4.21.10) which becomes partly exposed, in its proenzyme form, on the sperm plasma membrane before the beginning of membrane fusion in the human sperm acrosome reaction; this externalized proacrosin is then preferentially converted into active forms of the enzyme [9]. It is to be reminded in this context that both progesterone [10] and a high-M_r proteoglycan acting as a potent proacrosin activator [11,12] are present in high concentrations in the periovulatory human cumulus oophorus, the outer part of the egg coat which the spermatozoon must traverse shortly before fertilization, at the time when the physiological AR is triggered.

In this study, we used a previously characterized [13] preparation of the proacrosin activator (PA) from the human cumulus oophorus and examined its effects on progesterone binding to the sperm surface and on the progesterone-induced Ca^{2+} influx and AR.

Correspondence address: J. Tesarik, INSERM, Unité 355, 32 Rue des Carnets, 92140 Clamart, France. Fax: (33) (1) 4632 4890.

Abbreviations: AR, acrosome reaction; BSA-FITC, fluorescein isothiocyanate-labeled bovine serum albumin; $[Ca^{2+}]_i$, intracellular free Ca^{2+}; FACS, fluorescence-activated cell sorting; indo 1-AM, acetoxymethyl ester of indo 1; PA, proacrosin activator; progesterone-BSA-FITC, progesterone conjugated with BSA-FITC; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Materials*

Ionophore A23187, fluorescein isothiocyanate-labeled bovine serum albumin (BSA-FITC), progesterone, progesterone conjugated with BSA-FITC (progesterone-BSA-FITC), acetoxymethyl ester of indo 1 (indo 1-AM), 4′-acetamidophenyl 4-guanidinobenzoate, fluorescein isothiocyanate-labeled *Pisum sativum*, agglutinin, electrophoresis reagents, and M_r standards were purchased from Sigma (La

Verpillière, France). Proacrosin activator (PA), isolated from the human cumulus oophorus intercellular matrix [13], was a gift from Dr. J. Drahorad (Institute of Molecular Genetics, Prague, Czech Republic).

2.2. *Sperm source and preparation*

Semen samples were obtained by self-masturbation from 25 healthy volunteers, and spermatozoa were washed from seminal plasma and incubated for in vitro capacitation as described [14]. The same conditions were used for subsequent sperm incubation with different agents in individual experiments.

2.3. *Application of probes and stimuli*

The following probes and stimuli were used: ionophore A23187, progesterone, progesterone-BSA-FITC, indo 1-AM, and PA. Ionophore A23187, free and conjugated progesterone, and indo 1-AM were applied as described [6,15] including corresponding controls. PA was added to incubation media at a concentration of 1 mg/ml.

2.4. *Electrophoresis and gel enzymography*

To visualize individual components of the sperm proacrosin/acrosin system, SDS-PAGE of proteins extracted from sperm pellets and sperm-conditioned media was performed in polyacrylamide gels copolymerized with gelatine followed by renaturation of the separated proteins and visualization of bands of gelatinolysis in the gels [16].

2.5. *Fluorescence-activated cell sorting (FACS)*

The progesterone binding to the sperm suface was evaluated with the use of an Ortho Cytoron (Ortho Diagnostics, Tokyo, Japan) cell sorter after sperm incubation with progesterone-BSA-FITC [15]. The specifically labeled sperm subpopulation was identified using control incubations with BSA-FITC as described [7,15].

2.6. *Estimation of intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$*

Changes in $[Ca^{2+}]_i$ after addition of stimuli were evaluated by spectrofluorimetric analysis of suspensions of indo 1-loaded sperm cells using the previously described equipment and methodology [6].

2.7. *Evaluation of sperm movement*

The percentage of motile spermatozoa and kinetic parameters of sperm movement were determined in a fully automated, computer-controlled cell movement analyzer (Model IVOS; Hamilton-Thorn Research, Danvers, MA, USA) with the use of the previously described parameter setup and measurement conditions [14].

2.8. *Evaluation of the acrosome reaction and sperm cell viability*

The stimulus-induced acrosome reaction was evaluated using the fast AR measure index [17] after staining sperm acrosomes with fluorescein isothiocyanate-labeled *Pisum sativum* agglutinin using a method allowing a simultaneous evaluation of the acrosome reaction and sperm viability [18].

2.9. *Statistics*

Statistical analyses were performed with the use of StatView II (Abacus Concepts, Berkeley, CA, USA) statistical package. Maximal values of $[Ca^{2+}]_i$ after addition of different stimuli were compared by analysis of variance and Student's *t*-test. Percentual values (% progesterone-binding spermatozoa, % acrosome-reacted spermatozoa) were transformed by arc-sine before comparing by paired and unpaired *t*-tests.

3. RESULTS

In a previous study [12], we described an increase in the acrosin enzymatic activity in the presence of PA. In this study, we extended these observations by examining the effect of PA on the gelatinolytic activity of individual compounds of the proacrosin/acrosin system. When the acrosin release and activation were stimulated by incubation of spermatozoa with ionophore A23187 followed by SDS-PAGE of proteins from sperm pellets (Fig. 1A, lane A) and sperm-conditioned media (Fig. 1A, lane B) in gelatine-copolymerized gels, gelatinolytic activity was only detected in the positions of 3 distinct proteins bands (51–55 kDa, 45–49 kDa, and 34–38 kDa). The apparent M_r of these proteins corresponded

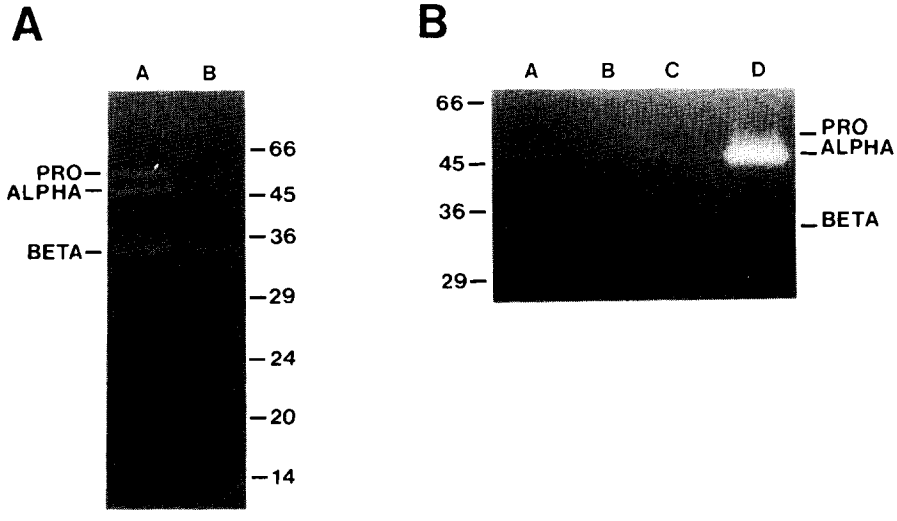

Fig. 1. Characterization of the effect of sperm incubation with PA on the molecular conversions in the proacrosin/acrosin system. Gel A shows the gelatinolytic activity of proteins from sperm pellet (lane A) and sperm-conditioned medium (lane B) after the treatment of spermatozoa with ionophore A23187. Gel B shows the same type of preparation for proteins from sperm pellet (lane A) and sperm-conditioned medium (lane B) after control incubation in capacitating medium without any addition, and for proteins from sperm-conditioned medium (lane C) and sperm pellet (lane D) after sperm incubation for 30 min with PA (1 mg/ml). Positions of the principal acrosin forms, proacrosin (PRO), α-acrosin (ALPHA), and β-acrosin (BETA), and of M_r standards are marked on opposite sides of both gels.

to the three main forms of the human proacrosin/acrosin system – proacrosin, α-acrosin, and β-acrosin [19,20]. In control-incubated spermatozoa, the gelatinolytic activity was present in traces in both sperm pellets and medium supernatants (Fig. 1B, lanes A and B). The inclusion of PA in the sperm incubation medium did not change the pattern of gelatinolysis in sperm-conditioned media (Fig. 1B, lane C), but it increased markedly and selectively the gelatinolytic activity in the position of α-acrosin detected in the sperm pellets (Fig. 1B, lane D). No gelatinolytic activity was detected in PA itself. The incubation with PA did not affect either sperm viability or parameters of sperm movement or the frequency of the acrosome reaction (data not shown).

The presence of PA in the sperm incubation medium did not influence either the size of the sperm subpopulation capable of binding progesterone-BSA-FITC to the cell surface or the intensity of the fluorescence signal emitted by the labelled cells (Fig. 2). The percentage of progesterone-binding cells was similar to that described previously [6,15], both in the presence (9.6 ± 1.0%; mean ± S.E.M., $n = 5$) and in the absence (9.4 ± 0.9%; mean ± S.E.M., $n = 5$) of PA. The positions of the peak and median fluorescence channels of the specifically labeled sperm subpopulation (Fig. 2) were not influenced by the presence of PA.

The addition of PA to suspensions of living indo-loaded spermatozoa did not produce perceptible changes in $[Ca^{2+}]_i$ (Fig. 3A). However, the addition of PA together with progesterone caused a significant amplification of the progesterone-induced Ca^{2+} influx as compared with progesterone alone (Fig. 3B and C). The peak $[Ca^{2+}]_i$ elevation above the baseline value after the addition of progesterone combined with PA and of progesterone alone was 420 ± 20 nM and 120 ± 10 nM, respectively (both are mean ± S.E.M., $n = 5$; $P < 0.01$).

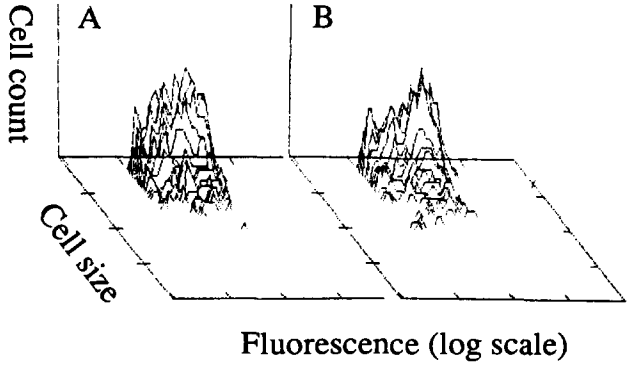

Fig. 2. FACS analysis of the effects of PA on progesterone-BSA-FITC binding to living spermatozoa. (A) Capacitated spermatozoa were incubated for 30 min with progesterone-BSA-FITC (20 μg conjugate/ml, corresponding to 3 μM progesterone) in the presence of PA (1 mg/ml). (B) Capacitated spermatozoa were incubated with progesterone-BSA-FITC as in A for the omission of PA. Only the specifically labeled sperm subpopulation is shown.

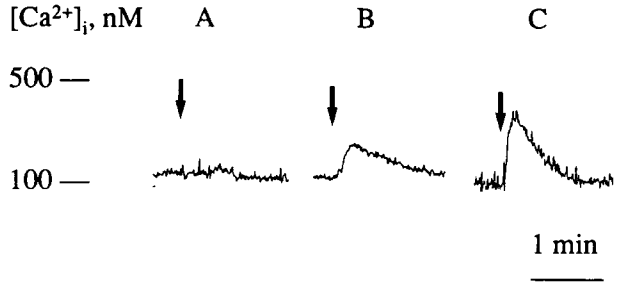

Fig. 3. Spectrofluorimetric analysis of the effects of PA on $[Ca^{2+}]_i$ in living sperm cells in the absence and in the presence of progesterone. Sperm cells loaded with indo 1-AM were stimulated with 1 mg/ml PA alone (A), with 3 μM progesterone alone (B), or with a combination of both (C) in the course of the measurement of fluorescence emission. The excitation and emission wavelengths were 331 nm and 410 nm, respectively. The time of stimulus addition is marked with an arrow. $[Ca^{2+}]_i$ was estimated after correction for cell autofluorescence as described [6].

The comparison of the fast AR measure index (indicator of a recently occurred AR) determined at different times after the addition of progesterone combined with PA with that after the addition of progesterone alone (Fig. 4) showed a significant acceleration of the exocytotic response to progesterone in the presence of PA. However, the percentage of recently reacted spermatozoa converged towards the same plateau both in the presence and in the absence of PA (Fig. 4).

4. DISCUSSION

Previous findings [7,8] have documented an inhibitory effect of protease inhibitors on the events induced by the action of progesterone on the sperm surface. This study demonstrates a positive, enhancing effect of a physiological activator of a sperm protease upon the sperm response to the hormone. Since the pioneering work reported from the laboratory of S. Meizel [21,22], acrosin has been suspected to act early in the induction of the acrosomal exocytosis, possibly before the beginning of membrane fusion. However, it was only recently that the demonstration of immunoreactive acrosin on the plasma membrane of still acrosome-intact spermatozoa and of the activation of this externalized acrosin by human follicular fluid [9] showed a possible mode of acrosin implication. The acrosin-activating component of the follicular fluid [11] is probably identical with PA from the cumulus oophorus [12]; the latter has been partly characterized as a high-M_r proteoglycan [13].

The acrosin activation cascade involves sequential transformation of proacrosin into α-acrosin and β-acrosin followed by further cleavage of β-acrosin into smaller, mostly enzymatically inactive fragments [16,19,20,23]. In this study, we show that PA acts selectively on the first step of this cascade reaction. This step

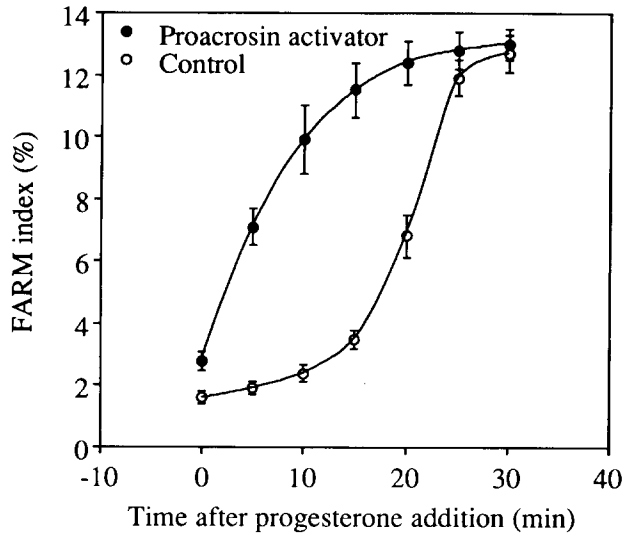

Fig. 4. Cytochemical analysis of the effect of PA on the progesterone-induced AR. Capacitated spermatozoa were mixed with 3 μM progesterone at time 0 in the presence or absence of PA. At the times indicated, samples of sperm suspensions were smeared on microscope slides, permeabilized with methanol, and stained with fluorescein isothiocyanate-labeled *Pisum sativum* agglutinin. The fast AR measure (FARM) index was calculated as described [17]. At least 200 cells were evaluated in each preparation. Data are mean ± S.E.M. of 5 replicates. The differences between both groups are significant ($P < 0.01$) between the 5-min and 20-min time points.

is known to imply an intrazymogen, proteolysis-independent mechanism of proenzyme conversion [24] and to be selectively inhibited by a sperm protein that does not inhibit the acrosin proteolytic activity [25]. It may be pertinent to note in this context that, unlike acrosin activation in a cell-free system, α-acrosin is remarkably stable when associated with living spermatozoa and may thus be the main form of acrosin implied in fertilization [26].

The observed amplification of the progesterone-induced Ca^{2+} influx and the acceleration of the progesterone-induced AR by PA, in the absence of a direct effect of PA on $[Ca^{2+}]_i$, can be best explained by the action of PA on the externalized acrosin on the sperm plasma membrane even though the possibility of an acrosin-independent action of PA cannot be definitevely excluded. The present data have shown that the PA action does not involve an increase in the number of hormone-responsive cells. Since the ligand-induced progesterone receptor aggregation in human spermatozoa is not sensitive to protease inhibitors [7], the PA-activated acrosin may influence an event downstream of the receptor crosslinking. This action is not necessarily direct; in fact, acrosin might activate another membrane enzyme which, in turn, would sensitize a Ca^{2+} channel or another Ca^{2+} transporting mechanism to the action of progesterone. Phospholipase A_2 [27] and ornithine decarboxylase [28] are some possible candidates for such a mediator.

Even though the exact mechanism of the amplification of the progesterone effect on human sperm by PA is not known, the present data have provided a further insight into the recently recognized phenomenon of the steroid action on the sperm surface. The physiological nature of this amplification is supported by the presence of both progesterone and PA in the egg coat at the time of fertilization. The possibility of protease involvement in the mechanism of steroid action is to be revisited in other cell types in which steroids act on the cell surface.

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