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BENZAMIDINE AS AN INHIBITOR OF PROACROSIN ACTIVATION IN BULL SPERM

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Summary

Epididymal and ejaculated sperm contain a zymogen form of acrosin (acrosomal proteinase, EC 3.4.21.10) which is converted to active enzyme prior to fertilization. Benzamidine at concentrations greater than 10 mM has been shown to inhibit the conversion of proacrosin to acrosin. Based on this inhibition, a procedure was developed for extracting and quantitating the proacrosin content of bull sperm. Sperm were isolated from semen and washed by centrifugation through 1.3 M sucrose and the outer acrosomal membrane removed by homogenization. When 25 mM benzamidine was added to the semen and wash solutions, 98% or more of the acrosin activity in the sperm homogenate was present as proacrosin. Proacrosin can be extracted from the sperm homogenate by dialysis at pH 3, which solubilized the proenzyme and removed benzamidine. Benzamidine has been useful in isolating proacrosin and provides a new method for studying the activation of proacrosin in intact sperm.

Neutralization of sperm extracts, after removal of benzamidine, resulted in rapid activation of proacrosin with a pH optimum of 8.5, and activation was complete within 15 min over a pH range of 7.0 to 9.5. Rapid activation also occurred during the washing of sperm in the absence of benzamidine, and this activation correlated with a swelling of the acrosomal membrane. This rapid activation appears to result from a small amount of acrosin activity consistently present in the sperm extract. These results indicate an autocatalytic conversion of proacrosin to acrosin and suggest that disruption of the acrosomal membrane may trigger this activation.

Introduction

The acrosome of mammalian sperm contains a trypsin-like proteinase, acrosin (acrosomal proteinase, EC 3.4.21.10), which is thought to aid in the pene-

tration of the ovum by sperm at the time of fertilization [1]. Because of the importance of this enzyme in fertilization, it has been the subject of extensive research which has been recently reviewed by McRorie and Williams [1] and Zaneveld et al. [2]. Recently, Meizel and Huang-Yang [3] have partially purified a zymogen form of acrosin from rabbit testes which were homogenized in 0.25 N H_2SO_4. Subsequently, proacrosin has been demonstrated in acid extracts of epididymal rabbit sperm [4] and ejaculated boar sperm [5,6]. Partially purified preparations of proacrosin undergo conversion to active enzyme at natural to slightly basic pH. The conversion is stimulated by Ca^{2+} and inhibited by Zn^{2+} and appears to be autocatalytic [4]. Based on these results, Meizel and Mukerji [4] have suggested that activation of proacrosin is required prior to fertilization and may occur during capacitation in the female tract. It is therefore of interest to determine the properties of proacrosin and the mechanism of its activation both with purified zymogen and in intact sperm.

Seminal plasma contains protein inhibitors of acrosin which interfere with studies of both the zymogen and the active enzyme [1]. It is thus desirable to wash sperm prior to extraction of proacrosin or prior to the investigation of its activation in intact sperm. However, handling of sperm during washing has the potential of inducing zymogen activation. While proacrosin activation is partially prevented by immediately titrating the sperm sample to acidic pH (3—6), this procedure also solubilizes the proenzyme and destroys the integrity of the sperm acrosome. This report presents evidence that benzamidine, a competitive inhibitor of acrosin [7] and trypsin [8], inhibits proacrosin activation. A procedure is described for washing sperm and extracting proacrosin which allows estimation of the degree of enzyme activation.

Materials and Methods

Chemicals

All chemicals were reagent grade. Sucrose was purchased from Mallinckrodt; fructose, *N*-benzoyl arginine ethyl ester BzArgOEt and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanusulfonic acid (HEPES) were from Sigma Chemical Co.; and benzamidine was a product of Aldrich Chemical Co. The concentration of benzamidine solutions was determined spectrophotometrically using a molar extinction coefficient of 813 at 268 nm.

Preparation of sperm

Semen was collected from five Holstein bulls using an artificial vagina. Sperm were washed by a modification [9] of the procedure of Garbers et al. [10]. 15 ml of semen were layered on 20 ml of 1.3 M sucrose/0.15 M NaCl and centrifuged for 30 min at full speed in an IEC model HNS clinical centrifuge at room temperature. The sperm pellets were resuspended in approximately 18 ml of 0.15 M NaCl/5 mM HEPES, pH 7.0, using a Pasteur pipette. A second washing step was carried out by layering the sperm suspensions of 20 ml 1.3 M sucrose/0.15 M NaCl and centrifuging for 20 min at 20 000 rev./min in a Beckman SW27 rotor at 0—4°C. The washed sperm were resuspended in 10 ml 0.15 M NaCl/mM HEPES, pH 7.0, and homogenized [9]. Sperm homogenates were either frozen or stored under refrigeration at 0—4°C.

Assay of acrosin and proacrosin

Acrosin was assayed spectrophotometrically at 37°C using BzArgOEt as substrate [11]. The standard reaction mixture contained 0.6 mM BzArgOEt, 0.1 M $CaCl_2$ and 40 mM HEPES, pH 7.5, in a final volume of 2.0 ml. The reaction was initiated by addition of enzyme and the increase in absorbance at 253 nm recorded using a Gilford recording spectrophotometer equipped with Beckman optics. A molar extinction coefficient of 1150 was used to convert the change in absorbance to nmoles BzArgOEt hydrolyzed. In most cases benzamidine was removed from sperm homogenates prior to assay by dialysis against 100 volumes of 1 mM HCl. Samples were dialyzed overnight at 4°C with one change of the dialyzate. Sperm were removed from the sample by centrifugation in a clinical centrifuge and the acid extract used for assay of acrosin or proacrosin activity.

Activation of proacrosin

Proacrosin present in sperm homogenates (bound to the sperm) was activated by dialysis against 100 volumes of 0.15 M NaCl/5 mM HEPES, pH 7.0. Samples were dialyzed overnight at 0—4°C with one change of dialyzate. The solubilized proacrosin present in acid extracts was activated by incubation of the extract with an equal volume of 0.1 M buffer at various specified pH valves. The buffers used for this experiment were, cacodylate (pH 6.0 and 6.5), HEPES (pH 7.0 to 8.5), and glycine (pH 9.0 to 10.0). The final pH was recorded, and the samples were incubated at room temperature. At 5 and 15 min, 0.01 ml aliquots were assayed for acrosin activity using the assay conditions outlined above.

Results

Washing bull sperm by centrifugation through 1.3 M sucrose removes the cytoplasmic droplets and seminal plasma and also loosens the plasma and outer acrosomal membranes [9]. Subsequent homogenization removes these membranes and exposes acrosomal enzymes, including acrosin. Sperm homogenates prepared by this procedure have a high acrosin activity which is largely bound to the sperm [9]. Since no significant increase in activity occurs during storage for several days at neutral pH and 0—4°C, acrosin present in these homogenates appears to be fully activated. A 50 to 100% increase in acrosin activity results from preincubation of the sperm homogenate at pH 3.0 prior to analysis at pH 7.5, suggesting the presence of residual seminal plasma acrosin inhibitors [12]. For this reason, the acrosin activity of sperm homogenates was normally determined after dialysis against 1 mM HCl.

The effectiveness of benzamidine as an inhibitor of bull sperm acrosin was determined for both the insoluble enzyme present in sperm homogenates and soluble enzyme prepared by dialysis at pH 3.0. Fig. 1 presents a double-reciprocal plot of data obtained using a sperm homogenate. Benzamidine is a competitive inhibitor of BzArgOEt hydrolysis with a K_i of approximately 0.025 mM. The K_m for BzArgOEt hydrolysis is approximately 0.1 mM. Soluble preparations of acrosin were also inhibited competitively by benzamidine and gave similar kinetic constants.

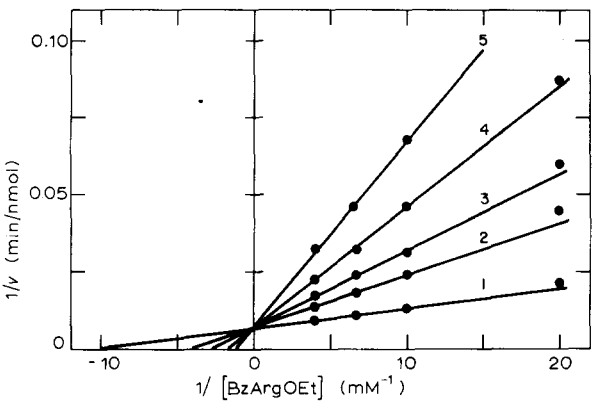

Fig. 1. Inhibition of bull sperm acrosin by benzamidine. Acrosin was assayed as described in Methods, using the indicated concentrations of BzArgOEt and benzamidine. Each point represents the average of duplicate determinations. The sperm homogenate had an acrosin activity of 1400 nmol/min/10^8 sperm under standard assay conditions.

The effect of benzamidine on activation of proacrosin was tested by adding varying concentrations of benzamidine to semen. The sperm were then washed by centrifugation through sucrose and homogenized, using solutions containing the same concentration of benzamidine, as described in Methods. Acrosin activity was measured in the acid extract obtained by dialysis at pH 3.0 and centrifugation to remove sperm. Benzamidine concentrations between 0.1 and 50 mM inhibited proacrosin activation during the preparation of sperm homogenates, while proacrosin was fully activated in the absence of benzamidine. At 2.5 mM benzamidine, less than 5% of the proacrosin was activated and at 25 and 50 mM benzamidine, less than 1% was activated. Below 2.5 mM a variable degree of activation was observed. Based on these results 25 mM benzamidine was selected for further studies.

The proacrosin content of sperm homogenates and pH 3.0 extracts was determined by measuring the increase in acrosin activity resulting from activation of the proenzyme. Sperm homogenates were activated by dialysis at pH 7.0, and acid extracts were activated by neutralization to pH 7—7.5. Table I presents typical results for a sperm homogenate prepared with 25 mM benzamidine. Before dialysis, acrosin activity could not be detected in the sperm homogenate, while after dialysis the rate is comparable to the control sample prepared without benzamidine. Acrosin activity was also measured in acid extracts of these samples. After dialysis at pH 3.0, the control sample had approximately twice the activity of the sperm homogenate, due to dissociation of acrosin inhibitor [12], and less than 5% of the activity is present in the sperm pellet after extraction and centrifugation. The low rate observed in the treated sample was less than 1% that of the control. Activation of this extract by neutralization yielded an activity similar to that in the sperm homogenates and control.

To determine which step or steps in the washing procedure are responsible for activation of proacrosin, 25 mM benzamidine was added at different stages in the procedure and the washing completed in the presence of 25 mM benzamidine. As seen in Table II, addition of benzamidine to semen or to the first

TABLE I

MEASUREMENT OF PROACROSIN IN HOMOGENATES OF BULL SPERM

The sperm homogenate was prepared as described in Methods using 25 mM benzamidine, and a control homogenate was prepared in the absence of benzamidine. Proacrosin in the sperm homogenate was directly activated by dialysis at pH 7.0. Alternatively, an extract was prepared by dialysis at pH 3.0, and the solubilized proacrosin activated by adjusting the pH to 7.2 and incubating at room temperature for 30 min. Acrosin activity is expressed as nmol/min/10^8 sperm.

Sample	Acrosin activity	
	Benzamidine treated	Control
Sperm homogenate	ND *	753
pH 7.0 dialysis	726	765
pH 3.0 supernate **	8.1	1407
pH 3.0 pellet **	ND *	52
pH 3.0 supernate incubated at pH 7.2	745	732

* Below the limit of detection.

** The supernate and pellet obtained by dialysis of the sperm homogenate followed by centrifugation in a clinical centrifuge.

sucrose solution effectively prevented activation of proacrosin. The most dramatic increase in acrosin activity was obtained when sperm were resuspended at ambient temperature after the first centrifugation, while the addition of benzamidine at later points in the wash procedure resulted in smaller increases in acrosin activity. When the entire wash procedure is performed at 0—4°C, activation was delayed until the final resuspension and homogenization steps were performed. Sperm at each stage of washing were viewed by phase contrast microscopy to detect changes in acrosomal morphology. After the first centrifugation through 1.3 M sucrose and resuspension in buffered saline, the acro-

TABLE II

ACTIVATION OF PROACROSIN DURING WASHING OF BULL SPERM

A pooled sample of bull semen was divided into six equal aliquots and the sperm washed and homogenized as described in Methods. Benzamidine was added to one aliquot of semen to a final concentration of 25 mM. For the remaining aliquots 25 mM benzamidine was introduced at successively later steps in the procedure. Once benzamidine was introduced all remaining steps were carried out in the presence of 25 mM benzamidine. The first wash step and resuspension were performed both at room temperature and at 0—4°C. Acrosin activity was measured after dialysis at pH 3.0 and centrifugation to remove sperm. Acrosin activity is expressed as nmoles/min/10^8 sperm. Percent zymogen was calculated by subtracting the amount of active enzyme in each sample from the amount of acrosin present in homogenates prepared in the absence of benzamidine.

1st addition of benzamidine	Ambient temperature (0—4°C)			
	Acrosin activity	% zymogen	Acrosin activity	% zymogen
Semen	25	97.5	7	99.3
Sucrose, 1st wash	20	98.1	16	98.4
Saline, 1st resuspension	141	86.2	24	97.6
Sucrose, 2nd wash	883	13.4	439	65.6
No benzamidine	1020	—	990	—

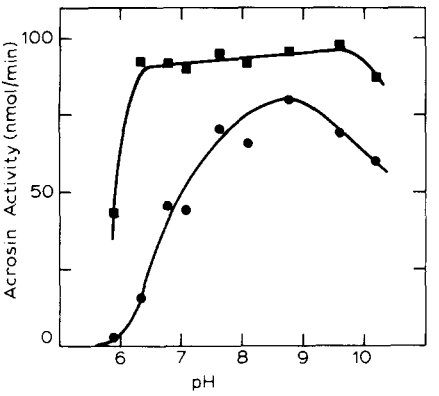

Fig. 2. pH dependence of proacrosin activation. A solubilized preparation of proacrosin was obtained by washing and homogenizing bull sperm in the presence of 25 mM benzamidine followed by dialysis at pH 3.0. Aliquots were mixed with an equal volume of 0.1 M buffer of varying pH and the acrosin activity measured in the standard assay after 5 (•) and 15 (▪) min incubation. The pH of each sample was measured after mixing.

somal membrane remained attached to most sperm but was visibly swollen. At this stage, the outer acrosomal membrane was removed from less than 5% of the sperm. After the second sucrose wash and homogenization, the acrosomal membrane is removed from greater than 95% of the sperm. The same results were obtained whether the first wash step was performed at room temperature or at 0—4°C.

The activation of proacrosin was further studied using a solubilized preparation of zymogen. A sperm homogenate, prepared with 25 mM benzamidine, was dialyzed at pH 3.0 to solubilize proacrosin and remove benzamidine. After removal of sperm by centrifugation, activation was carried out at various pH valves as described in Methods. Fig. 2 presents the results of this experiment. After 5 minutes of incubation acrosin activity was present in samples incubated at pH 6.5 to 10.5, with highest activity occurring at pH 8.5, and after 15 min incubation activation is complete at all but the extreme pH valves. The amount of activity, expressed on the basis of 10^8 sperm, was the same as found in a sample of sperm homogenate dialyzed at pH 7.0, indicating that essentially all of the proenzyme was solubilized by dialysis to pH 3.0.

Discussion

Stambaugh and Buckley [7] reported that benzamidine is a competitive inhibitor of acrosin isolated form rabbit sperm. The results presented here confirm this finding and demonstrate that benzamidine also inhibits the activation of proacrosin during washing and homogenization of bull sperm. Both soluble acrosin and acrosin bound to the sperm head are inhibited by benzamidine with equal efficiency, and the K_i of $2.5 \cdot 10^{-5}$ M found for benzamidine inhibition of bull sperm acrosin is in good agreement with that reported by Stambaugh and Buckley [7] using rabbit sperm. When sperm are isolated in the presence of benzamidine at concentrations greater than 10 mM, the activation of proacrosin is effectively inhibited, while some activation occurs at lower concen-

trations. A concentration of 25 mM benzamidine was selected for routine preparation of proacrosin extracts.

The washing and homogenization procedure used here was selected because it not only separates sperm from other semen components, but also loosens and removes the outer acrosomal membrane [9]. This has the advantage of exposing acrosomal enzymes making their extraction easier and allowing for their direct measurement. The proacrosin and acrosin present in sperm homogenates can then be solubilized and quantitated by dialysis at pH 3.0. The use of benzamidine in combination with this procedure has aided in the isolation and purification of proacrosin and has proved to be a valuable method for studying proacrosin activation in intact sperm. This procedure has been used to quantitate the proacrosin content of sperm from several mammalian species (Polakoski, K.L. and Zahler, W.L., in preparation).

Meizel and Mukerji [4] studied the activation of partially purified proacrosin from rabbit sperm and concluded that activation occurs by an autocatalytic mechanism. A similar conclusion was reached by Polakoski and Zaneveld [6] in studies of a homogeneous preparation of boar preacrosin. Our results provide further support for this mechanism. The pH dependence for activation of bull sperm proacrosin is similar to the pH dependence of acrosin, and benzamidine, a competitive inhibitor of acrosin activity, also inhibits proacrosin activation. In addition, a small but detectable amount of acrosin activity is consistently present in sperm extracts prepared using benzamidine. This activity may result from a small amount of acrosin normally present in ejaculated bull sperm or may be intrinsic to proacrosin. In either case, it is likely that this activity is responsible for the rapid activation of proacrosin after neutralization of sperm extracts and during washing in the absence of benzamidine.

The results of Meizel and co-workers [3,4] and of Polakoski and Zaneveld [6] indicate that activation of proacrosin occurs after ejaculation, and Meizel and Mukerji [4] have suggested that activation may be part of capacitation. Our results show that proacrosin is rapidly activated during washing of sperm, and that this activation correlates with a swelling of the outer acrosomal membrane. These results further support the possibility that the small amount of acrosin activity present in sperm promotes acrosin activation and suggest that activation is triggered by changes in the permeability or integrity of the acrosomal membrane. It is therefore possible that proacrosin activation takes place during the acrosome reaction rather than during capacitation.

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References

1 McRorie, R.A. and Williams, W.L. (1974) Annu. Rev. Biochem. 43, 777—803
2 Zaneveld, L.J.F., Polakoski, K.L. and Schumacher, G.F.B. (1975) in Cold Spring Harbor Conferences on Cell Proliferation (Reich, E., Rifkin, D.B. and Shaw, E., eds.), Vol. 2, pp. 683—706, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
3 Meizel, S. and Huang-Yang, Y.H.J. (1973) Biochem. Biophys. Res. Commun. 53, 1145—1150
4 Meizel, S. and Mukerji, S.K. (1975) Biol. Reprod. 13, 83—93
5 Polakoski, K.L. (1974) Fed. Proc. 33, 1308 (abstr.)
6 Polakoski, K.L. and Zaneveld (1976) in Methods in Enzymology (Lorenz, L., ed.) Vol. 45, Academic Press, New York, in the press
7 Stambaugh, R. and Buckley, J. (1972) Biochim. Biophys. Acta 284, 473—477
8 Mares-Guia, M. and Shaw, E. (1965) J. Biol. Chem. 240, 1579—1585
9 Zahler, W.L. and Doak, G.A. (1975) Biochim. Biophys. Acta 406, 479—488
10 Garbers, D.L., Wakabayashi, T. and Reed, P.W. (1970) Biol. Reprod. 3, 327—337
11 Schwert, G.W. and Takenaka, Y. (1955) Biochim. Biophys. Acta 16, 570—575
12 Polakoski, K.L., Zaneveld, L.J.D. and Williams, W.L. (1971) Biochem. Biophys. Res. Commun. 45, 381—386