Effects of Protease Inhibitors and Substrates on Motility of Mammalian Spermatozoa

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Abstract. A series of protease inhibitors were tested on the motility of human, rat, bull, and rabbit demembranated reactivated spermatozoa. Some inhibitors, including aprotinin, boc-gln-leu-lys-H, and D-phe-pro-arg-H, could inhibit motility as well as prevent initiation of motility. In general, with the exception of aprotinin, protease inhibitors were more potent in preventing the initiation of movement than in blocking motility of demembranated spermatozoa. Protease substrates could also block sperm motility. Of the substrates tested only those with arg or lys ester bonds were active. The inhibition of motility by protease substrates was reversible, as once spermatozoa hydrolyzed the added exogenous protease substrates, motility reappeared. The importance of ester bond in the inhibitory action of protease substrates was confirmed by experiments that showed the lack of effect of pre-hydrolyzed protease substrates.

The results suggest that a serine protease with lys and arg ester bond specificity is involved in the control of sperm motility. The fact that protease substrates also block motility of intact spermatozoa further emphasizes the physiological relevance of this new regulatory system.

PROTEASES fulfill two main functions: protein degradation as in protein turnover and protein limited degradation as in the activation or modulation of protein action. The importance of this second function is becoming more and more recognized. Several peptide hormones exist in a precursor form, and the action of specific proteases, often localized in the same storage organelles, yields active hormones (6). Proteases have also been shown to be involved in receptor-mediated events such as in the modulation of the β-adrenergic-receptor adenylate cyclase system (18) and in insulin action (1).

Acrosin, the most extensively studied protease associated with spermatozoa, is a trypsin-like protease involved in fertilization (21). Acrosin has been reported both in the acrosomal sac and on the inner acrosomal membrane where it exists in the form of proacrosin (14, 21). A different trypsin-like protease has also been isolated from bull spermatozoa. This enzyme can activate adenylate cyclase from rat brain (10) and human platelets (11). Kallikreins have been reported to affect sperm motility by generating kinins, which presumably act on motility (17). However, no protease has been directly implicated in sperm motility.

Recently, we have reported that aprotinin, a protease inhibitor, blocked the motility of demembranated reactivated mammalian spermatozoa (2, 3) without significantly affecting the force-generating dynein ATPase (8). In the present study, we investigated in more detail the possible involvement of a protease in sperm motility. We now report that a series of protease inhibitors as well as a series of protease substrates with lys or arg ester bonds inhibit the motility of both demembranated reactivated and intact spermatozoa. These results suggest that a serine protease modulates sperm motility.

Materials and Methods

Materials

The protease inhibitors soybean trypsin inhibitor (STI),[1] lima bean trypsin inhibitor (LBTI), leupeptin (*N*-acetyl-leu-leu-arginal), antipapain ([(*s*)-1-carboxy-2-phenylethyl]-carbamoyl-arg-val-arginal), *N*-α-*p*-tosyl-lysine chloromethyl ketone (TLCK), *l*-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), diisopropyl fluorophosphate (DFP), benzamidin; the protease substrates benzoyl-phe-val-arg-*p*-nitroanilide, *N*-α-benzoyl-arg ethyl ester, (BAEE), succinyl-phe-*p*-nitroanilide, *N*-benzoyl-tyr-*p*-nitroanilide; and Ficoll, Triton X-100, trypsin (2× crystallized), cAMP, bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). 2-[*N*-hydroxycarboxamido]-4-methylpentanoyl-arg-gly amide) (Zincov inhibitor), D-phe-pro-arg chloromethyl ketone (PPACK), *N*-α-benzyloxycarbonyl-lys thiobenzyl ester (BLT), and benzoyl-DL-arg-*p*-nitroanilide (BAPNA), were from Calbiochem-Behring Corp. (La Jolla, CA). Tosyl-gly-pro-arg-*p*-nitroanilide acetate (Chromozym TH), carbobenzoxy-val-gly-arg-*p*-nitroanilide acetate (Chromozym TRY), *S*-benzyl-cys-*p*-nitroanilide, vanadium-free ATP (disodium salt), phenylmethylsulfonyl fluoride, and aprotinin were obtained from Boehringer-Mannheim Diagnostics, Inc. (Montreal). Boc-gln-leu-lysinal and D-phe-pro-arginal were given generously by Dr. Gabor B. Makara, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary. Percoll was

[1] *Abbreviations used in this paper:* BAPNA, benzoyl-DL-arg-*p*-nitroanilide; BLT, *N*-α-benzyloxycarbonyl-lys thiobenzyl ester; Chromozym TH, tosyl-gly-pro-arg-*p*-nitroanilide acetate; DFP, diisopropyl fluorophosphate; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; HBS, Hepes balanced saline, LBTI, lima bean trypsin inhibitor; STI, soybean trypsin inhibitor; TLCK, *N*-α-*p*-tosyl-lysine chloromethyl ketone; TPCK, *l*-1-tosylamide-2-phenylethyl chloromethyl ketone.

from Pharmacia Fine Chemicals (Dorval, Canada). Other chemicals used were at least of reagent grade. Protease inhibitors or substrates were solubilized in water or dimethyl sulfoxide (DMSO) or in a 1:1 mixture of DMSO and reactivation medium (described below). DMSO concentrations never exceeded 1% in the final assay conditions. At this concentration DMSO has no effect on sperm motility.

Reactivation of Demembranated Spermatozoa

Collection and preparation of ejaculated rabbit and of bull cauda epididymal spermatozoa was done as previously described (3, 4). Rat spermatozoa were obtained from cauda epididymides by mincing tissues in Gey's + BSA medium (2.5 ml/cauda). Sperm suspensions were filtered through cheesecloth to remove epididymal fragments and centrifuged at 200 *g* for 5 min at room temperature. The supernatant was discarded, the very soft pellet was resuspended in demembranation medium (0.1% Triton X-100, 200 mM sucrose, 25 mM potassium glutamate, 1 mM dithiothreitol [DTT] and 35 mM Tris-HCl, pH 8; 2.5 ml/original cauda) (2, 15), and immediately overlaid on 3.5% Ficoll in reactivation medium (4 ml/original cauda) devoid of Triton X-100. After a 5-min centrifugation at 300 *g* the soft pellet of spermatozoa was adjusted to the desired concentration with reactivation medium devoid of Triton X-100. Assays were run in the same medium.

Human semen collected by masturbation was obtained from volunteers. Only samples with more than 50% of progressive motility were processed for the various studies. The semen was allowed to liquify and was filtered through cheesecloth to remove any residual coagulum. Percoll density gradients were prepared as described by Lessley and Garner (12) with the following modifications: (*a*) volumes used were smaller, 0.5 ml of 95% Percoll buffered with Hepes balanced saline (HBS) + BSA (10 mM Hepes, 130 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 14 mM fructose, 1 mg/ml BSA adjusted to pH 8.0) was placed into a 15-ml conical sterile tube and overlayed with an 8-ml continuous linear gradient; (*b*) the gradient ranged from 15 to 65% Percoll buffered with HBS + BSA, since it permitted better recoveries than the 15–75% previously used. Filtered semen samples were overlaid on the Percoll gradients and centrifuged at room temperature for 45 min at 1,300 *g*. Spermatozoa were manually recovered from the 65–95% Percoll interface to the bottom of the tube.

Rabbit, bull, and human spermatozoa were demembranated and reactivated in the medium described above. Movement was reinitiated by the addition of 0.5 mM Mg·ATP (final concentration, made of 0.5 mM ATP and 0.5 mM $MgSO_4$). cAMP (50 μM, final concentration) was added to the demembranation medium when bull spermatozoa were used since it increased the beat frequency and the percentage of motile reactivated spermatozoa from the bull cauda epididymis (5, 13). cAMP was not used with spermatozoa from other species since it did not significantly improve the reactivation (5; unpublished results).

Substances to be tested were added either to already reactivated spermatozoa (Mg·ATP present in the medium before the addition of the test substance) or to spermatozoa in demembranation medium before the Mg·ATP-induced initiation of movement. Sperm motility was evaluated with an inverted microscope for the proportion of motile spermatozoa and the duration of reactivation (from the time Mg·ATP was added to the time at which 99% of spermatozoa stopped moving).

Experiments were done at room temperature (20°C) unless indicated.

Assay Conditions for Intact Spermatozoa

The effects of various substances that affected reactivated spermatozoa were also studied on intact spermatozoa incubated in Gey's + BSA or HBS + BSA buffers. Percentage and duration of motility were criteria to evaluate the effects of these substances.

Results

Effects of Protease Inhibitors on the Reactivation of Mammalian Spermatozoa

In the first series of experiments, protease inhibitors were added to demembranated reactivated spermatozoa (Mg·ATP added before the addition of the inhibitor) from rabbits, rats, bulls, and humans (Table I). Of the three macromolecular compounds (*Group A*) tested, only aprotinin could inhibit sperm motility at concentrations ranging from 0.5 to 2.6 μM for the four species tested. The inhibitory effect of aprotinin was not always permanent. At concentrations of aprotinin slightly above (5–10%) that required to inhibit motility, the movement, after being arrested, usually reinitiated by itself after a delay of ~5 min for bull spermatozoa and of 1–2 h for rat spermatozoa. This motility lasted at least as long as in the

*Table I. Concentrations of Protease Inhibitors Needed to Block the Motility of Demembranated Reactivated Spermatozoa**

	Inhibitory concentrations (μM)			
Protease inhibitors	Rabbit	Rat	Bull	Human
Group A				
STI	≫50	≫50	≫50	≫50
LBTI		≫100	≫100	≫120
Aprotinin	0.5	1.3	0.9	2.6
Group B				
Leupeptin	≫200	≫1,300	≫1,200	≫1,200
Antipapain		≫1,000	≫1,200	≫1,200
Boc-gln-leu-lys-H		45	109	79
D-phe-pro-arg-H		310	215	610
Group C				
TPCK		≫1,300		
TLCK		≫2,250	2,000	2,250
PPACK‡		≫1,300		
Group D				
Zincov inhibitor§		≫1,300		
Benzamidin		≫28,000	≫20,600	≫10,000
DFP		≫90,000		

* Sperm were demembranated and reactivated with 0.5 mM Mg·ATP. The concentration of protease inhibitors needed to block completely and instantaneously sperm motility was then determined. Each value represents the mean of at least three different experiments. The symbol ≫ indicates that the concentration tested had no inhibitory action on sperm motility and that no inhibitory action could be expected near this level.

‡ D-phe-pro-arg chloromethyl ketone.

§ 2-(*N*-hydroxycarboxamido)-4-methylpentanoyl-arg-gly amide.

control demembranated reactivated sperm incubated without aprotinin, that is 15–20 min for bull spermatozoa and 3–4 h for rat spermatozoa. However, at higher concentrations, the inhibition was permanent within the time frame mentioned above.

Two of the four tripeptides with a terminal aldehyde (arg-H or lys-H) tested (*Group B*) inhibited the motility of reactivated spermatozoa (Table I). Depending on the species investigated, boc-gln-leu-lys-H was 2–7.7 times more potent than D-phe-pro-arg-H. TLCK, one of the three chloromethyl ketone tested (*Group C*), could also block the motility but at very high concentrations. Benzamidin and DFP (*Group D*), two potent protease inhibitors, had no effect on motility at concentrations as high as 28 and 90 mM, respectively, even after 10 min of contact with spermatozoa.

In the second series of experiments, protease inhibitors were added to demembranated spermatozoa before the addition of Mg·ATP to test the effect of these substances on the initiation of motility (Table II). Under these conditions spermatozoa were generally more sensitive to the effect of protease inhibitors. STI at 3.7 μM prevented the reactivation of human spermatozoa but had no effect on initiation of motility in the three other species investigated. Aprotinin, at concentrations very similar to those used to inhibit the motility of already reactivated spermatozoa (Table I), could also prevent the reactivation of spermatozoa in all four species (Table II). The inhibitory potency of tripeptides with terminal aldehyde was much higher when added to demembranated spermatozoa before the addition of Mg·ATP than after the initiation of motility. Leupeptin could prevent the motility of rabbit and human sperm, whereas boc-gln-leu-lys-H and D-phe-pro-arg-H were up to 10-fold more potent. TLCK was also more potent in preventing sperm reactivation than in blocking already reactivated sperm. These data suggested that a protease was possibly involved in sperm motility.

Effects of Protease Substrates on the Reactivation of Mammalian Spermatozoa

To investigate the type of protease that might be involved in sperm motility, various protease substrates were tested on the motility of rabbit, rat, bull, and human demembranated spermatozoa (Table III). Substrates with lysine ester bond (*A*, Table III) or arginine ester bond (*B–F*) could completely and instantaneously (within 5–10 s) inhibit motility. Other substrates (*G–I*) with phenylalanine, tyrosine, or cysteine ester bonds had no effect on motility. The concentrations of the various trypsin-like protease substrates (*A–F*) that were needed to inhibit motility were very similar for the four species investigated. However, there was an exception since the BLT concentration required to block human sperm motility (230 μM) was 10-fold that needed to inhibit the mobility of rabbit, rat, and bull spermatozoa (22–25 μM).

While the inhibitory concentration of the various substrates (chromogenic substrates with nitroanilide, substrates *B–E* of Table III) was being tested, it was noted that the medium yellowed with time and that this phenomenon was associated with the reappearance of sperm motility. The inhibitory effect of nonchromogenic substrates like BLT could also be reversed with time. The phenomenon of substrate hydrolysis and reappearance of motility was constantly observed with rat spermatozoa (length of reactivation is 3–4 h) but was less often noted with other species as their reactivation durations (between 10 and 20 min) were probably too short compared with the time required to hydrolyze protease substrates. The fact that motility was restored once the substrate was hydrolyzed by demembranated spermatozoa suggested the importance of

*Table II. Concentrations of Protease Inhibitors That Can Prevent the Initiation of Motility of Demembranated Spermatozoa**

Protease inhibitors	Inhibitory concentrations (μM)			
	Rabbit	Rat	Bull	Human
Group A				
STI	≫50	≫50	≫50	3.7
LBTI		≫65	≫65	≫65
Aprotinin	0.5	1.5	0.9	2.8
Group B				
Leupeptin	98	≫1,300	≫200	12
Antipapain		≫650	≫650	≫650
Boc-gln-leu-lys-H		5	25	8
D-phe-pro-arg-H		138	120	196
Group C				
TPCK		≫1,300		
TLCK		≫1,300	380	1,660
PPACK‡		≫1,300		
Group D				
Zincov inhibitor§		≫1,300		
Benzamidin		≫28,000	≫23,000	≫10,000
DFP		≫90,000		

* Protease inhibitors were added to the demembranation medium at the given concentrations, and after a 15-s contact with spermatozoa, 0.5 mM Mg·ATP was added. Values are mean of at least three different experiments. The symbol ≫ indicates that the concentration tested had no inhibitory action on sperm motility and that no inhibitory action could be expected near this level.

‡ D-phe-pro-arg chloromethyl ketone.

§ 2-(*N*-hydroxycarboxamido)-4-methylpentanoyl-arg-gly amide.

*Table III. Concentrations of Protease Substrates Needed to Block the Motility of Reactivated Spermatozoa**

	Protease substrates	Inhibitory concentrations (μM)			
		Rabbit	Rat	Bull	Human
A	*N*-α-benzoyl-carbonyl-lys-thiobenzyl ester (BLT)	22	25	23	228
B	Benzoyl-phe-val-arg-*p*-nitroanilide	73	58	69	107
C	Tosyl-gly-pro-arg-*p*-nitroanilide (Chromozym TH)	278	312	286	332
D	Carbobenzoxy-val-gly-arg-*p*-nitroanilide (Chromozym TRY)	278	335	278	400
E	*N*-benzoyl-DL-arg-*p*-nitroanilide (BAPNA)	377	472	422	520
F	*N*-α-benzoyl-arg-ethylester (BAEE)	7,300	9,000	9,300	7,800
G	Succinyl-phe-*p*-nitroanilide	≫2,600	≫2,600		
H	*N*-benzoyl-tyr-*p*-nitroanilide	≫1,720	≫1,700		
I	*S*-benzoyl-cys-*p*-nitroanilide	≫1,750	≫1,600		

* Spermatozoa were demembranated and reactivated with 0.5 mM Mg·ATP. The concentration of protease substrates needed to block completely and instantaneously the motility was then determined. Values are mean for at least three different experiments. The symbol ≫ indicates that the concentration tested had no inhibitory action on sperm motility and that no inhibitory action could be expected near this level.

Table IV. Effect of Hydrolysis on the Capacity of Serine Protease Substrates to Inhibit the Motility of Rat Reactivated Spermatozoa

Conditions	Motility rating*
Control	+++
Substrates at 2 IC_{100}‡	0
Substrates at 2 IC_{100} + trypsin $\xrightarrow{2\ min}$ + STI§	+++
Substrates at 2 IC_{100} + (STI + trypsin)‖	0
STI + trypsin¶	+++

* Motility was quantified visually on a 0 to ++++ scale: 0, no motility, +, <25% motility, ++, 25–50% motility, +++, 50 to 75% motility, ++++, >75% motility.

‡ Substrates were used at twice the concentration needed to inhibit motility (IC_{100}, concentration that will inhibit 100% of sperm) (Table III). Substrates tested were BLT, Chromozym TH, Chromozym TRY, benzoyl-phe-val-arg-*p*-nitroanilide, and BAPNA.

§ A trypsin concentration that can completely hydrolyze the various substrates within 2 min was selected. After hydrolysis, a threefold excess of STI was added to complex and inhibit trypsin molecules.

‖ STI and trypsin, at the maximal concentrations used above, were mixed before their addition to each substrate. Under these conditions, protease substrates were not hydrolyzed.

¶ STI and trypsin, at the maximal concentrations used above, were mixed before their addition to demembranated reactivated spermatozoa.

*Table V. Inhibitory Concentrations of Protease Substrates as a Function of the Mg·ATP Concentration Used for Reactivation of Spermatozoa**

Substrates	Mg·ATP	Inhibitory concentrations
	mM	*μM*
BLT	0.5	24
	2.5	30
	5.0	35
Chromozym TH	0.5	244
	2.0	476
	5.0	654
Benzoyl-phe-val-arg-*p*-nitroanilide	0.5	107
	2.5	123
	5.0	145

* Spermatozoa were demembranated and reactivated with Mg·ATP at the given concentrations, and the concentrations of protease substrates needed to completely block motility were determined.

an intact ester bond in the mode of action of these motility inhibitors. It also emphasized the fact that the motile apparatus (the axoneme) of spermatozoa had suffered no permanent damage by these substances.

The importance of ester bonds in the inhibitory action of serine protease substrates was further investigated by adding prehydrolyzed substrates to demembranated reactivated rat spermatozoa (Table IV). Substrates (Table III, *A–E*) at twice their inhibitory concentrations were hydrolyzed to completion by trypsin. A threefold excess of STI was then added to complex and inhibit trypsin molecules, and the whole mixture was added to reactivated sperm. The five substrates tested lost their inhibitory effects on motility after hydrolysis. Similar results were obtained with bull, rabbit, and human spermatozoa. Controls, (STI + trypsin), and (STI + trypsin) added to substrates showed that the complex STI–trypsin had no effect on reactivated sperm or on protease substrates. Therefore, the ester bond appears to be essential for the inhibitory action of protease substrates.

As in the case of aprotinin (3), the inhibitory effects of BLT, benzoyl-phe-val-arg-*p*-nitroanilide, and Chromozym TH, at the concentrations given in Table III, could be reversed and motility reinitiated by the immediate addition of high Mg·ATP concentrations (2.5–5 mM final concentrations). Conversely, when rat spermatozoa were reactivated with higher Mg·ATP concentrations, the concentrations of BLT and Chromozym TH needed to block motility increased (Table V). Similar results were obtained with bull and human spermatozoa. The influence of Mg·ATP, thus motility, was once more emphasized by results showing that the amount of protease substrates needed to prevent the reactivation was 2–3-fold lower than the amount needed to block the movement of already reactivated spermatozoa (data not shown).

Effects of Protease Substrates on the Motility of Intact Spermatozoa

The effects of serine protease substrates were also tested on the motility of intact spermatozoa (Table VI). A 2-min period of contact was conceded before observation to allow these substances to permeate the sperm membrane. With the exception of rabbit sperm, BLT could block the motility of intact spermatozoa at a concentration similar to that required to inhibit motility in demembranated sperm models. Other serine protease substrates were also effective in interrupting intact sperm motility though at a concentration 2–6-fold

*Table VI. Concentrations of Protease Substrates Needed to Block the Motility of Intact Spermatozoa**

Protease substrates	Inhibitory concentrations (μM)			
	Rabbit	Rat	Bull	Human
N-α-benzoyl-carbonyl-lys-thiobenzyl ester (BLT)	1,370	31	31	487
Tosyl-gly-pro-arg-*p*-nitroanilide (Chromozym TH)	1,980	1,670	1,670	566
Carbobenzoxy-gly-pro-arg-*p*-nitroanilide (Chromozym TRY)	1,650	1,670	≫3,100	740
N-benzoyl-DL-arg-*p*-nitroanilide (BAPNA)	1,760			734
N-α-benzoyl-L-arg-ethylester	≫17,600		≫16,700	≫2,840

* Protease substrates were added to motile intact sperm suspended in HBS + BSA or Gey's + BSA medium and the minimal amount needed to block motility within 2 min was determined. ≫, the concentration tested had no inhibitory action on sperm motility, and no inhibitory action could be expected near this level.

higher than that needed to stop motility of demembranated reactivated spermatozoa (Table III). However, similar to demembranated models, prior hydrolysis of protease substrates resulted in the loss of capacity to inhibit intact sperm motility (data not shown).

Discussion

In this paper, we have shown that a series of protease inhibitors and protease substrates can affect the motility of spermatozoa. These results raise the possibility that a protease may be involved in the regulation of sperm motility.

With the exception of aprotinin, protease inhibitors were more effective in preventing initiation of motility than in blocking the motility of reactivated spermatozoa. For instance, boc-gln-leu-lys-H was 4–10-fold more active in preventing than in stopping motility. Another inhibitor, leupeptin, had no effect on motile spermatozoa but was highly potent in preventing the initiation of sperm reactivation. These data may suggest either that ATP and protease inhibitors interact with a common receptor molecule or that the binding and hydrolysis of ATP affect the affinity of the protease inhibitors for its receptor. We have reported a similar phenomenon with a factor from the seminal plasma (4). This factor turned out to be a dynein ATPase inhibitor (4). However, aprotinin, the most potent inhibitor of motility at 40–80-fold the concentration needed to block motility, has only marginal effect (25% decrease) on dynein ATPase (8).

Even though either lys or arg residues were constantly present in the structure of protease inhibitors that could block motility at significantly low concentrations, others that possessed the same amino acids were inactive. Aprotinin, the most potent inhibitor, has one lys and two arg at its active site. Two of the small peptides with terminal arginal or lysinal residues blocked motility, whereas antipapain with a terminal arginal and others such as D-phe-leu-lys-H, boc-D-phe-leu-lys-H, and boc-D-phe-phe-lys-H (data not shown) had no inhibitory effect at concentrations up to 1.3 mM.

DFP, a known irreversible serine protease inhibitor, had no effect on the motility of demembranated spermatozoa. This inhibitor normally acts by forming a covalent bond with the active site of protease (16). Thus, the lack of effect observed could be due to the time required for this chemical reaction. The shortening of the reactivation time observed with rat spermatozoa incubated in the presence of DFP from 2–3 h to 15 min is consistent with this hypothesis.

In addition to protease inhibitors, serine protease substrates with arg or lys residues could also block sperm motility (Table III), thus suggesting that a sperm-associated protease preferred the exogenously added substrates to its endogenous substrates. This concept was supported by results which showed that once the exogenously added substrates were hydrolyzed by the demembranated sperm preparation, motility reinitiated and that trypsin-hydrolyzed substrates had no effect on motility (Table IV). The reversibility of the motility blockade observed in the above experiments further indicated that no permanent damage was done to the sperm motility apparatus when protease substrates were added. By contrast to protease substrates with basic amino acid ester bonds, substrates with tyr, phe, and cys ester bonds had no effect on motility at concentrations up to 2 mM (Table III). These results emphasized the specificity of the sperm-associated protease.

Within the four species tested, the concentrations of protease substrates needed to block sperm motility were similar. However, there was an exception to this phenomenon. BLT, the most potent substrate, inhibited motility of rabbit, rat, and bull spermatozoa at concentrations ranging between 22 and 25 μM, whereas a concentration of 230 μM was required to stop human spermatozoa. This peculiarity of human spermatozoa was first thought to be caused by the presence of Percoll (colloidal silica particles coated with polyvinylpyrolidone) present in the medium used to isolate spermatozoa. This possibility was rejected since human spermatozoa, simply washed in HBS + BSA buffer and then reactivated, had their motility blocked by 210 μM BLT. It was neither due to the presence of extra spermatozoal proteases, as human spermatozoa washed through Percoll gradients are essentially devoid of other cellular elements and debris so frequently found in human semen.

As in the case of aprotinin (3), the concentrations of protease substrates needed to block the motility of demembranated spermatozoa varied according to the concentration of Mg·ATP used for reactivation (Table V). In addition, once motility was initiated by Mg·ATP (0.5 mM) and then blocked by protease substrates (Table III), motility could be reinitiated by the rapid addition of more Mg·ATP (2.5–5 mM). Furthermore, spermatozoa appear to be more sensitive to the effects of protease substrates in the absence of Mg·ATP; the concentration of substrates needed to prevent reactivation was two- to threefold lower than that needed to block the motility of already reactivated spermatozoa (data not shown). The possibility that protease substrates would act as dynein ATPase inhibitors was rejected since BLT did not inhibit bull dynein ATPase, even at a concentration of 480 μM (unpublished results). Nevertheless the results may suggest that a sperm-specific protease, Mg·ATP, and dynein ATPase have some interactions.

When reactivated, rat spermatozoa were inhibited by 1.5

μM aprotinin, and motility usually reinitiated after 1–1.5 h, lasting for 2–2.5 h. This observation raised the possibility that aprotinin would block motility acting as a substrate for the motility-related protease. It is known that the lys^{15}-ala^{16} bond present at the active site of aprotinin is subject to hydrolysis by catalytic amounts of proteases and that this hydrolyzed bond is also subject to thermodynamically controlled resynthesis (20). If a trypsin-like protease, such as starfish trypsin 1, very rapidly and with high specificity, hydrolyzes the lys^{15}-arg^{16} bond and, at the same time, dissociates from aprotinin quickly, resynthesis is prevented and hydrolyzed aprotinin is found in the medium (7). It is possible that the protease involved in sperm motility has these properties and uses to some extent aprotinin as a substrate. We have observed, with reactivated human and rabbit spermatozoa, that the inhibition caused by aprotinin could be reversed by further addition of DTT (concentration in the reactivation medium increased to 2.5 mM) (unpublished results). Furthermore, when aprotinin was incubated with 2.5 mM DTT for 1 h at room temperature, the amount of aprotinin needed to block motility of human reactivated spermatozoa doubled. DTT alone did not affect the reactivation. These data would also support the hypothesis that aprotinin is substrate for the motility-related protease since DTT can reduce disulfide bonds and since once the cys^{14}-cys^{38} disulfide bond of aprotinin is reduced, the rate of hydrolysis of the lys^{15}-ala^{16} bond is facilitated (9, 19).

Results with demembranated reactivated sperm would suggest that permeable substrates like BLT or Chromozym TH block motility of intact spermatozoa via a mechanism compatible with their mode of action of reactivated sperm, that is by inhibiting a sperm-associated specific serine protease.

In conclusion, the inhibitory effects of protease inhibitors and substrates on sperm motility strongly suggest that a serine protease with lys and arg ester bond specificity is involved in the control of sperm motility. This may imply that spermatozoa must have a second enzyme to repair the ester bond hydrolyzed by the protease. Alternatively, the same protein structure could carry both enzymatic functions, hydrolyzing and repairing the same bond. The localization of these two enzymatic activities, as well as the nature of the endogenous substrates involved, remain to be established. The fact that protease substrates also block motility of intact spermatozoa from four mammalian species further emphasizes the physiological relevance of this new regulatory system.

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