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Enzyme activity in anuran spermatozoa upon induction of the acrosome reaction

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Summary

At the time of sperm–egg fusion in *Discoglossus pictus*, a large amount of electron-dense material of an unknown nature is liberated from the sperm. In the present work we studied this material in *D. pictus* sperm, using an assay utilising strips of autoradiographic film as a gelatin substrate for proteolytic enzymes. Upon treatment with A23187, *D. pictus* sperm produced a large halo on the gelatin substrate, indicating the presence of enzymes released by the sperm at the time of the acrosome reaction. In contrast, *Xenopus laevis* sperm did not produce halos upon treatment with A23187. The use of protease inhibitors such as TLCK, leupeptin, chymostatin, SBTI and EACA strongly suggests that the *D. pictus* whole acrosome contains trypsin and chymotrypsin activity while an SBTI-sensitive activity is absent in a small portion of the acrosome, possibly the anteriormost region. Furthermore, the material released at the acrosome reaction also contains an EACA-inhibited activity, indicating the presence of plasminogen activator. We conclude that *D. pictus* sperm release proteolytic enzyme(s) that may act at the egg surface at the time of gamete fusion.

Keywords: Anurans, Fertilisation, Protease inhibitors, Proteolytic enzymes, Sperm acrosomes

Introduction

Several experimental approaches have shown that amphibian sperm contains enzymes with proteolytic activity (Raissman & Barbieri, 1969; Elinson, 1974; Raissman & Cabada, 1977; Iwao *et al.*, 1994; Mizoke *et al.*, 1999), similar to mammalian sperm (for a review see Barros *et al.*, 1996). The vitelline envelope (VE) lysin of the toad *Bufo bufo japonicus* is a trypsin-like protease (Yoshizaki & Katagiri, 1982; Yamasaki *et al.*, 1988). In this toad, as well as in *Bufo arenarum*, the VE lysin is able to digest the VE of uterine eggs and appear to be implicated in fertilisation, according to inhibition assays utilising protease inhibitors (Cabada *et al.*, 1978; Takamune *et al.*, 1986). Sperm extracts of the urodele *Cynops pyrrhogaster* and the anuran *Xenopus* have a similar proteolytic activity (Iwao *et al.*, 1994, 1995; Mizote *et al.*, 1999). *Cynops* sperm enzyme was putrified as a 100 and a 65 kDa protein with tryptic as well as chymotryptic properties and the ability to activate both *Cynops* and *Xenopus* eggs. Also *Bufo arenarum* sperm lysin is able to activate the corresponding egg (Cabada *et al.*, 1989). Therefore sperm-released enzymes appear to have a role both in the digestion of egg investments and in the process of sperm–egg fusion. However, in particular in the case of *Xenopus*, it is obscure how sperm interact with the VE and the egg plasma membrane, and sperm extracts are unable to activate either heterologous or homologous eggs (Mizote *et al.*, 1999). In this species, a receptor-ligand system is thought to be active at the time of fusion between egg and sperm, involving disintegrin at the sperm plasma membrane and integrins at the oolemma (Iawao & Fujimura, 1996; Shilling *et al.*, 1997, 1998), as for mammals (Blobel *et al.*, 1992; Almeida *et al.*, 1995; Chen & Sampson, 1999).

Sperm enzyme activity is thought to reside in the acrosome, both because of indirect evidence (for

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Cynops: Iwao *et al.*, 1994; for *Rana*: Penn & Gledhill, 1972) and because in other sperm, such as mammalian sperm, a tryptic protease, acrosin, is associated with the acrosome matrix (Hardy *et al.*, 1991).

In *Discoglossus pictus* fertilisation, ultrastructural observations indicated that a large amount of electron-dense material is liberated from sperm in the process of penetrating into the egg (Campanella *et al.*, 1997). It was hypothesised that enzymes released from the sperm acrosome are able to digest the egg glycocalyx and to uncover the sperm receptor in the egg plasma membrane (Maturi *et al.*, 1998). However, nothing is known about the enzymic nature of the material released from sperm as a consequence of the acrosome reaction (AR) in this species except for an early report that suggests the presence of an enzyme 'similar to pancreas enzymes' in the acrosome (Parat, 1933), and indirect evidence (Campanella *et al.*, 1997).

Discoglossus pictus sperm are 2.33 mm long, the head itself being about 900 μm long. Its anteriormost portion is constituted by the apical rod, a short specialisation of the acrosome, and posteriorly covered by the acrosome cap. The acrosome contains at least two ultrastructurally distinguishable constituents. The nucleus is located behind the apical rod; the acrosome cap covers it throughout its surface except its posterior edge. The acrosome cap is very thin at the sperm anteriormost region (0.02 μm) covering a 0.2 μm thick nucleus, while posteriorly, where the nucleus is about 1 μm thick, it doubles its size (Campanella *et al.*, 1979). Sperm are associated in register, entwined in bundles of 20 or more. When in contact with the egg jelly, sperm are freed from the bundles and enter the jelly coat, where the AR is triggered (Campanella & Gabbiana, 1979; Campanella *et al.*, 1997).

In this work we studied the material released upon induction of the acrosome reaction in *D. pictus* sperm, using an assay first developed in *Rana pipiens* sperm (Penn & Gledhill, 1972) whose acrosomal granule is quite inconspicuous (Poirer & Spink, 1971; Campanella, unpublished data). The assay is sensitive to small amounts of enzymes released by single sperm (Penn & Gledhill, 1972). Furthermore it allows the examination of a direct relationship between release of acrosome content and digestion of a substrate (see also Iwao *et al.*, 1994). The assay was also applied to *X. laevis* sperm.

Materials and methods

Adult *D. pictus* males were injected into the dorsal lymphatic sac with 200–250 IU of Profasi HP (Serono, Italy) diluted in amphibian Ringer's solution 100% (111 mM NaCl, 1.3 mM $CaCl_2$, 2 mM KCl, 0.8 mM Mg_2SO_4, in 25 mM HEPES-NaOH pH 7.4). Sperm bundles were obtained by squeezing the animals 24–48 h after hormone injection. Adult *X. laevis* males (Rettili, Varese, Italy) were decapitated under anaesthesia. Their testes were rinsed in 100% De Boer's solution (110 mM NaCl, 1.3 mM $CaCl_2$, 1.3 mM KCl, 5.3 mM tris-HCl pH 7.4) and minced in small pieces in 10% De Boer's solution to obtain motile sperm. Strips of autoradiographic film were used as gelatin substrate for proteolytic enzymes, according to Penn & Gledhill (1972). Briefly, the experimental procedure involved a 10 min exposure of Kodak A-25 autoradiographic films to light, followed by a 4 min treatment with Kodak D-19 developer and a 2 min fixation in sodium hyposulphite (1.98 M) and sodium bisulphite (1.64 M). Small strips of exposed film were then spread, emulsion side up, onto microscope slides.

For *D. pictus*, as it was previously shown that, in diluted saline release of sperm from their bundle is activated, drops of semen were added to 10% Ringer (for more details see Campanella *et al.*, 1997). Sperm suspensions were gently brushed onto the strips and then 50 μM calcium ionophore A23187 added (final concentration 25 μM) to trigger the AR (Gualtieri & Andreuccetti, 1996). The whole slide was covered with a coverslip, kept in a moisture chamber at 37 °C and viewed at regular intervals in a compound microscope.

Enzyme inhibitors were mixed with sperm suspension together with A23187 to obtain the following concentrations: 1 mM *N*-tosyl-lys-chloromethyl-ketone hydrochloride (TLCK; inhibitor of serine proteases), 1 mM leupeptin (inhibitor of trypsin-like and cystine proteases), 1 mM chymostatin (inhibitor of chymotrypsin-like serine proteases), 1 mg/ml soybean trypsin inhibitor (SBTI; inhibitor of trypsin, chymotrypsin, kallikrein and plasmin), 1 mM ε-amino-*n*-caproic acid (EACA; inhibitor of carboxypeptidase B and of the activator of plasminogen) and 1 mM E64 (inhibitor of cysteine proteases) (Sigma Biochemical, St Louis, MO). Table 1 summarises the enzyme inhibitors utilized and their inhibitory activities.

The inhibitory concentrations were calibrated by exposing film strips to increasing concentrations of commercial enzymes (i.e. trypsin, Sigma) and by adding to the enzyme the corresponding inhibitors (i.e. TLCK or SBTI). Neither the Ringer nor calcium ionophore produce alteration of the gelatin substrate of the Kodak autoradiographic film.

Results

To test the effect of the acrosome reaction on the gelatin-coated strips, *D. pictus* sperm were exposed to 25 μm calcium ionophore A23187. Upon incubation for 10 min at 37 °C, a large halo was observed on the strip where sperm were located (Fig. 1*a*), indicating diges-

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Table 1 The inhibitors used and their inhibitory action

Inhibitor	Inhibitory action
TLCK	Irreversible inhibitor of serine-trypsin proteases. Also inhibits bromelain, endoproteinase Arg-C, endoproteinase Lys-C, papain, plasmin, thrombin and trypsin
Leupeptin	Reversible inhibitor of serine-trypsin proteases. Inhibits some cystein-like proteases including endoproteinase Lys-C, kallikrein, papain, thrombin and cathepsin B
SBTI	Reversible inhibitor of serine-proteases. Also inhibits trypsin and factor X
Chymostatin	Reversible inhibitor of chymotrypsin-like serine proteases including α-, β-, γ-, δ-chymotrypsin
E64	Irreversible inhibitor of cystein-proteases
ε-amino-*n*-caproic acid (EACA)	Inhibitor of carboxypeptidase B. Inhibitor of the activator of plasminogen

tion and degradation of the gelatin substrate. By utilising a small aliquot of spermatozoa, we observed that the extension of the halo produced on the gelatin strip is not as long as the sperm (about 200 μm in contrast to the 2.33 mm of the whole sperm length), indicating that the release of the enzyme occurs in a discrete region of sperm, such as the region where the acrosome is more conspicuous (Fig. 2*b*). For untreated sperm, similarly incubated on strips, halos were absent (Fig. 1*b*). On the contrary, *X. laevis* sperm, whether treated with A23187 or not, did not undergo the formation of a halo, even after several hours of incubation.

In experiments utilising enzyme inhibitors, incubation of sperm with TLCK or leupeptin on the gelatin-coated strips resulted in major inhibition of halo formation after AR induction (Fig. 2*c*, *d*; and compare with Fig. 2*b*). In contrast, incubation with SBTI caused partial inhibition of halo formation, as several circular spots of gelatin digestion were observed next to the reacted sperm (Fig. 2*f*). These data suggest that a partial inhibition of sperm AR enzymes occurs with SBTI and that a discrete segment of sperm is insensitive to this inhibitor.

While the thiol-protease inhibitor E64 is not able to inhibit sperm enzyme action on gelatin (Fig. 2*h*), chymostatin has a good inhibitory activity (Fig. 2*e*). Similarly, by using EACA, an inhibitor of plasminogen activator (Huarte *et al.*, 1993), the gelatin was affected by the sperm-released enzymes except in tiny regions visible within or around spermatozoa (Fig. 2*g*).

Discussion

Our data indicate that substances released by *D. pictus* sperm upon the induction of the AR are enzymes, as they are able to digest a gelatin matrix and enzyme inhibitors impair this ability. Given that TLCK, leupeptin, SBTI and chymostatin are capable of inhibiting halo formation of the gelatin matrix, it can be concluded that the enzymes have tryptic and chymotryptic activity. This is in agreement with data on *Bufu bufo japonicus* (Yamasaki *et al.*, 1988), *Bufo arenarum* (Raissman & Cabada, 1977), *Cynops* and *Xenopus* (Mizote *et al.*, 1999) showing that a serine protease with tryptic activity is present in sperm extracts. Moreover, the chymotryptic activity we found in *D. pictus* sperm can be compared with a similar activity found in *Cynops* sperm (Mizote *et al.*, 1999). *D. pictus* shares several properties with urodele species (Campanella *et al.*, 1991). Although the experimental approach we used allowed the observation of sperm at a low resolution, the morphology of the halo developed by sperm and the location along the sperm strongly suggest that the enzymes are located in the acrosome cap. By comparing the halos produced upon exposure to SBTI with the complete inhibitory activity obtained with the other inhibitors, it can be further hypothesised that the whole acrosome contains trypsin and chymotrypsin activity while the SBTI-sensitive activity is absent in a small portion of the acrosome. The apical rod at the tip of the acrosome is a good candidate for such SBTI-resistant release. This is in agreement with the complex morphology of the acrosome cap, which has two types of granular content, and of the apical rod, which has a fine granular content and several sheets of membranes (Campanella & Gabbiani, 1979). Furthermore, our experimental approach allows us to conclude that *D. pictus* sperm contain EACA-sensitive enzymes, such as the plasminogen inhibitor. Interestingly urokinase can be involved in sperm–egg interaction in a co-operative action with integrin (Huarte *et al.*, 1993; Chapman, 1977) and integrins are thought to be the sperm receptors in vertebrate eggs (Almeida *et al.*, 1995; Shilling *et al.*, 1997, 1998).

The complexity of the enzyme content of *D. pictus* spermatozoa deserves more study. However, the present data suggest that the enzymes packed in the acrosome may have functions directly linked to specific substrates that the sperm interact with during egg investment penetration and egg membrane interaction (see also Hardy *et al.*, 1991). Ultrastructural evidence found that component B, the inner acrosome matrix component, is released before compound A, in close proximity to the egg surface (Campanella *et al.*, 1997). Our results support the hypothesis that the material released by *D. pictus* sperm at the time of fusion with the egg (Campanella *et al.*, 1997) is constituted by

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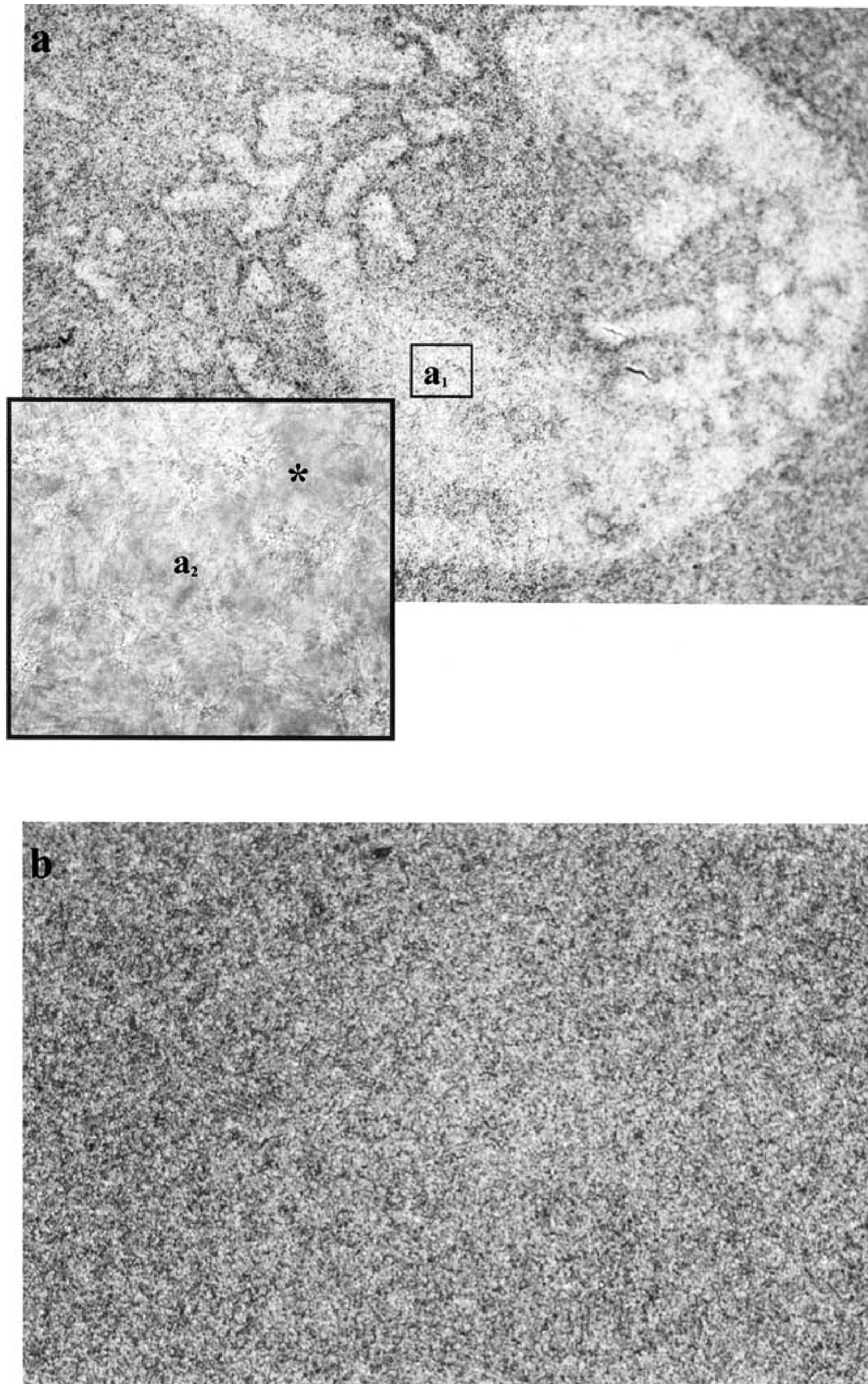

Figure 1 (*a*) Effect of material released from A23187-treated-sperm on gelatin-coated strips. A large area where gelatin was digested is seen, corresponding to the sperm. ×60. The inset a_2 is a magnification of the detail a_1, showing sperm (asterisk) in the region where gelatin was digested. ×160. (*b*) Control strip treated with Ringer-diluted sperm ×220.

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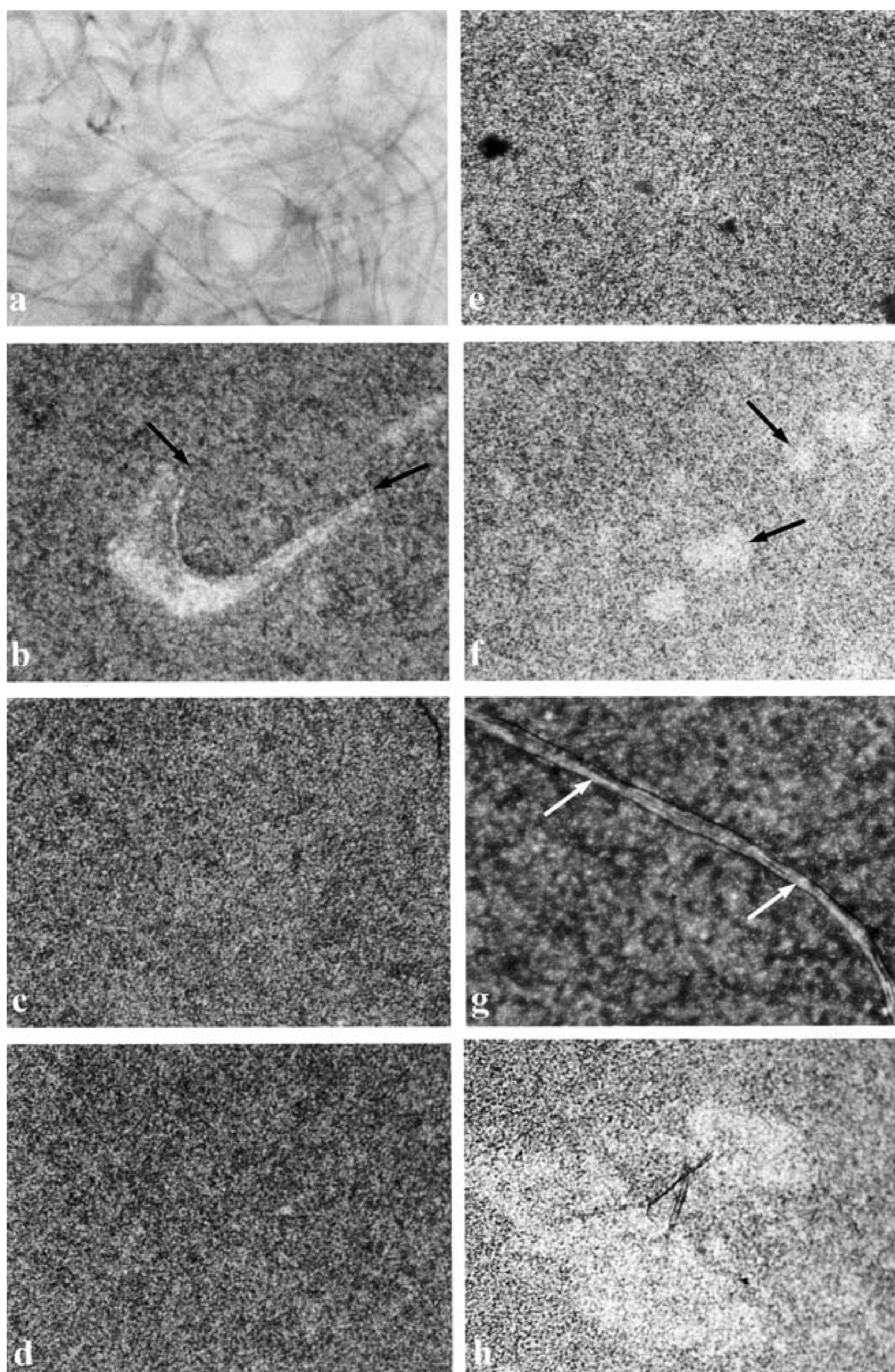

Figure 2 A23187-treated sperm on the gelatin-coated strips. (*a*) As seen in Nomarski DIC optics. ×450. (*b*) As seen with normal optics. A halo is seen along part of the sperm length (arrows). ×220. (*c*) Sperm exposed to TLCK: no halo formation occurred, as in (*d*) where sperm were incubated with leupeptin, and (*e*) where the inhibitory action of chymostatin is seen. ×220. (*f*) SBTI-exposed sperm. The inhibitory action is exerted on sperm but there are small regions where halos are present (arrows). ×220. (*g*) EACA-exposed sperm. Inhibition occurred except for tiny spots of gelatin digestion along sperm bundles (small arrows). ×850. (*h*) Sperm exposed to E64: no inhibition occurred and large halos of digested gelatin are seen on the strip. ×220.

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trypsin and chymotrypsin-like enzyme(s) with further urokinase activity, that have a role in membrane interaction.

In contrast, *Xenopus* sperm do not release chemicals able to digest gelatin upon treatment with calcium ionophore that is able to provoke the AR in general and in amphibians in particular (Gualtieri & Andreuccetti, 1996; Martinez & Cabada, 1996). This finding may indicate, however, that sperm are not responsive to A23187, or that the assay we used is not sensitive enough to the relatively small amounts of proteolytic enzymes found in *Xenopus* sperm extracts (Mizoke *et al.*, 1999). Alternatively, it may show that the enzymes are not located in the acrosome cap of this species.

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