Effects of putative protease inhibitors on the acrosome reaction of sea urchin spermatozoa

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Summary. The acrosome reaction was induced by jelly coat factors, nigericin, or elevated pH. When spermatozoa were preincubated for 5 min in sea water maintained at pH 7.9 in the presence of 1 mm-phenylmethylsulphonyl-fluoride (PMSF), 1 mm-benzamidine, 0.1 mm-1-chloro-3-tosyl-amido-7-amino-2-heptanone (TLCK), 5 mm-diisopropylphosphofluoridate (DFP) or 5 mm-DFP that was previously hydrolysed, only DFP or its hydrolysis product(s) prevented formation of the acrosomal filament induced by jelly coat factors. When incubation with inhibitors was extended to 2 h only TLCK and its hydrolysis products inhibited the jelly-induced acrosome reaction. Only DFP significantly inhibited the acrosome reaction induced by elevated pH (9.0). Nigericin induced acrosome reactions in the presence of DFP or TLCK. These findings do not support the concept of an active role for acrosin in development of an acrosome reaction.

Introduction

The acrosome reaction of sea urchin spermatozoa is characterized by a sequence of events that include fusion of the acrosomal membrane with the plasma membrane, exocytosis of the acrosomal vacuole and polymerization of G-actin to form the acrosomal filament (Dan, 1967; Summers, Hylander, Colwin & Colwin, 1975). In many invertebrate spermatozoa, the membrane events are known to occur concomitantly with actin polymerization, and the acrosome reaction can be assessed by the presence of an extended filament (Tilney, Hatano, Ishikawa & Moore, 1973). The acrosome reaction appears to result in the exposure or release of various proteins, including a phospholipase (Conway & Metz, 1976), a protein responsible for sperm–egg adhesion, “bindin” (Vacquier & Moy, 1977) a protease similar to acrosin (Levine & Walsh, 1979) and an arylsulphatase (Hoshi & Moriya, 1980). A trypsin-like protease has been found ultrastructurally to be associated with the sea urchin sperm acrosomal vacuole (Green & Summers, 1980). In recent years, interest has focussed not only on the question of which acrosomal proteins may be intimately involved with the fertilization process, but also on which proteins may function in the induction of the acrosome reaction itself.

A factor associated with eggs (jelly coat) normally causes induction of the acrosome reaction in sea urchin spermatozoa (Popa, 1927; Dan, 1952, 1956). A protease similar to or identical with acrosin is released in response to these jelly coat factors and, based on protease inhibitor studies, Levine & Walsh (1979) have suggested that activation of this protease is required for successful completion of an acrosome reaction. Acrosin may be involved in the acrosome

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reaction in hamster spermatozoa (Meizel & Lui, 1976; Lui & Meizel, 1979), but is not required in the guinea-pig sperm acrosome reaction (Green, 1978; Vitug, Perreault & Rogers, 1979). Protease activation may therefore be associated with the successful completion of an acrosome reaction in general, or such activation may be required in spermatozoa from only some animals. For this reason, we have re-examined the effects of protease inhibitors on the sea urchin sperm acrosome reaction.

Materials and Methods

Sea urchins (Strongylocentrotus purpuratus) were purchased from Pacific Biomarine, Venice, California. The sea urchins were treated with an intracoelomic injection of approximately 1 ml 0-5 M-KCl and the gametes were then collected as described previously (Garbers, Sadduth & Hardman, 1975). All of the following operations were at 4°C. The spermatozoa were washed once by low-speed centrifugation (400 g; 5 min) to remove pigment and debris, followed by a higher-speed centrifugation (1000 g; 15 min) to sediment the spermatozoa. The spermatozoa were resuspended in sea water to concentrations ranging from 80–130 mg (wet wt) cells/ml (1 g wet packed sea urchin spermatozoa represents 0-5–1-0 × 10^11 cells and contains 100–125 mg protein: Gray, Drummond, Link, Hardman & Sutherland, 1976). Eggs were washed once by centrifugation (400 g; 5 min). A crude mixture of jelly coat and egg factors was obtained after sea urchin eggs were allowed to stand in sea water (20% suspensions, v/v) at pH 5-0 for 20 min, followed by centrifugation of the eggs (2000 g, 20 min). The pH of the resultant supernatant fluid was adjusted to 8-0, and the fucose content of the solution was then determined colorimetrically using the cysteine–sulphuric acid method of Dishe, Shettles & Osnos (1949).

Induction of the acrosome reaction

All experiments were performed at 15–16°C in artificial sea water composed of 454 mM-NaCl, 9-7 mM-KCl, 24-9 mM-MgCl_2, 9-6 mM-CaCl_2, 27-1 mM-MgSO_4, 4-4 mM-NaHCO_3 and Tris base adjusted to pH 7-9 with HCl (normal sea water). The time, or order of addition of various protease inhibitors, is specified in the legend to Table 1. Normally, incubation mixtures contained 890–940 μl sea water, 10 μl inhibitor or control solvent (isopropanol or dimethylformamide) and 4–7 mg (wet wt) of spermatozoa.

Assessment of the acrosome reaction

After various periods of incubation, 1 ml 2% glutaraldehyde in normal sea water was added to the incubation mixtures and spermatozoa were examined by phase-contrast microscopy at ×1250 magnification (oil immersion). The spermatozoa were scored as reacted (fully extended filament) or unreacted (no visible filament), and 100 of each sample were assessed.

Chemicals

Phenylmethylsulphonylfluoride (PMSF), diisopropylphosphofluoridate (DFP), benzamidine HCl, 1-chloro-3-tosyl-amido-7-amino-2-heptanone (TLCK) and leupeptin were obtained from Sigma (St Louis, Missouri). Stock solutions of 0-5 M-DFP in isopropanol, 0-5 M-PMSF in dimethylformamide, 0-1 M-PMSF in isopropanol, 0-1 M-benzamidine, 0-01 M-TLCK or 2 mM-leupeptin in water were made and used within 15 min of preparation. p-Toluene-sulphonyl-L-arginine methyl ester HCl and trypsin were from Sigma.

Hydrolysis of inhibitors

TLCK (10 mM) was hydrolysed by treatment with sodium hydroxide (0-5 M) overnight. The pH of the hydrolytic products was then adjusted to 7-8. DFP (5 mM, final concentration) was
added to sea water (pH 7.9) or sodium hydroxide (0.5 M) in a capped test-tube and incubated at 23°C for 12 h or 2 days. The pH of the hydrolytic products was then adjusted to 7.8. The ability of TLCK, DFP or their hydrolysis products to inhibit trypsin was assayed using a procedure described by Walsh (1970). The effectiveness of hydrolysis was also monitored by high pressure liquid chromatography (Beckman, model 100) using reverse phase (ultrasphere ODS, 25 cm × 4.6 mm column) with 100 mM-potassium phosphate, pH 7.4 (aqueous), and acetonitrile (organic) solvents.

Results

Induction of the acrosome reaction in the presence of protease inhibitors

Using an experimental procedure similar to that of Levine & Walsh (1979), sea urchin spermatozoa were incubated for 5 min in sea water (20 mM-Tris, pH 7.9) containing inhibitors (Table 1). Jelly coat factors (or 500 mM-NaCl as a control) were then added and the suspensions were incubated for an additional 1 min. Under these conditions, only DFP or its hydrolysis products prevented the formation of the acrosomal filament; sperm motility was not inhibited. When sea water was buffered with 5 mM-Tris (the concentration of buffer used by Levine & Walsh (1979)), PMSF effectively blocked the acrosome reaction (Table 1). PMSF is rapidly converted to phenylmethylsulphonic acid and the formation of this acid causes a decrease in pH; this decrease (to values as low as 7.0) was evident in the presence of 5 mM-Tris. The acrosome

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Δ Acrosomal filaments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Jelly Factors</td>
</tr>
<tr>
<td>None</td>
<td>74 ± 6(4)</td>
</tr>
<tr>
<td>1% Isopropanol</td>
<td>72 ± 6(3)</td>
</tr>
<tr>
<td>1 mM-PMSF</td>
<td>63 ± 4(3)</td>
</tr>
<tr>
<td>1 mM-PMSF*</td>
<td>0(3)</td>
</tr>
<tr>
<td>5 mM-PMSF†</td>
<td>76(2)</td>
</tr>
<tr>
<td>1 mM-Benzamidine</td>
<td>60(2)</td>
</tr>
<tr>
<td>1 mM-Benzamidine†</td>
<td>77(2)</td>
</tr>
<tr>
<td>0.1 mM-TLCK</td>
<td>61(2)</td>
</tr>
<tr>
<td>1 mM-TLCK†</td>
<td>7(2)</td>
</tr>
<tr>
<td>0.33 mM-TLCK (hydrolysed)†</td>
<td>15(2)</td>
</tr>
<tr>
<td>5 mM-DFP</td>
<td>11 ± 4(4)</td>
</tr>
<tr>
<td>5 mM-DFP (hydrolysed)</td>
<td>12 ± 7(4)</td>
</tr>
<tr>
<td>0.2 mM-Leupeptin†</td>
<td>73(2)</td>
</tr>
</tbody>
</table>

Table 1. Effect of protease inhibitors on the acrosome reaction of sea urchin spermatozoa

Values are mean ± s.e.m. for the no. of determinations indicated in parentheses.
* 5 mM-Tris buffer in sea water was utilized.
† Stock sperm suspensions (99 µl or 90 µl) were preincubated for 2 h with 1 or 10 µl inhibitor solution at the listed concentrations at 4°C. Jelly factors or sea water were added to give a final volume of 1 ml and the spermatozoa were then fixed with glutaraldehyde after 1 min incubation at 15°C.

Spermatozoa (50 µl) were added to normal sea water (20 mM-Tris) containing inhibitor (or solvent as a control) and were preincubated for 5 min. Jelly factors (2–4 µg fucose/ml, final concentration) were added to give a final volume of 1 ml and the spermatozoa were then fixed with glutaraldehyde after 1 min or the pH of the sperm suspensions was determined after 1 min. Spermatozoa (50 µl) were added to the high pH sea water (20 mM-Tris, pH 9.0) containing an inhibitor (or solvent as a control) to give a final volume of 1 ml and were incubated for 5 min. Concentrations of inhibitor or isopropanol are those in the final incubation mixture (except for † treatments). The values are the mean ± s.e.m. The numbers in parentheses are the number of determinations.
reaction of sea urchin spermatozoa is highly pH-dependent (Text-fig. 1) and thus the previously reported effects of PMSF (Levine & Walsh, 1979) may have been due to its effects on pH. Assessment of dose–response curves indicated that DFP was 3–4-fold more effective as an inhibitor of the sperm acrosome reaction than was hydrolysed DFP (Text-fig. 1).

When spermatozoa were preincubated for 2 h with inhibitors only TLCK and its hydrolysis products prevented an acrosome reaction induced by jelly coat factors (Table 1).

Treatment of spermatozoa with PMSF, benzamidine, or TLCK in sea water containing 20 mM-Tris adjusted to pH 9.0 did not prevent formation of the acrosomal filament (Table 1). DFP, however, did cause a decrease in the percentage of reacted spermatozoa while hydrolysed DFP was completely ineffective.

Treatment of spermatozoa with DFP (5 mM) for 5 min or with TLCK (1 mM) for 2 h failed to prevent acrosome reactions induced by 60 µM-nigericin (not shown).

Discussion

It has been suggested that acrosin is required for formation of an acrosomal filament in sea urchin spermatozoa (Levine & Walsh, 1979). Of various inhibitors tested by Levine & Walsh (1979), PMSF and DFP effectively inhibited the jelly-induced acrosome reaction. In the studies reported here, we were unable to repeat the PMSF observation of Levine & Walsh (1979) unless the pH of the seawater was allowed to decrease in the presence of PMSF. Only low percentages of acrosome reactions, as judged by filament extensions, occur at pH values below 7.5 in S. purpuratus (this study) or other sea urchin species (Gregg & Metz, 1976; Collins & Epel, 1977). Therefore, PMSF does not appear to be an effective inhibitor of the acrosome reaction, even though previous work showed that it could inhibit acrosin of sea urchin spermatozoa (Levine, Walsh & Fodor, 1978). DFP and its hydrolysis products inhibited the jelly-induced acrosome reaction. DFP was more active, however, and the difference could represent specific acrosin inhibition by DFP. Nevertheless, DFP is highly reactive and is known to inhibit enzymes other than proteases (Jansen, Fellows Nutting, Jang & Balls, 1949). DFP or its hydrolysis products failed to inhibit filament extension induced by nigericin. Therefore, if DFP is believed to inhibit the jelly-induced acrosome reaction by virtue of a specific inhibition of acrosin, an explanation of the nigericin data is required. One of the strongest arguments would be that nigericin causes an acrosome reaction by a mechanism which is completely different from that of jelly. Although this is possible, nigericin is known to require extracellular Ca<sup>2+</sup> for its effects (Schackmann,
Eddy & Shapiro, 1978), to increase the uptake of 45Ca2+ (Schackmann et al., 1978), to elevate cyclic AMP concentrations (Garbers & Kopf, 1980) and to activate sperm heads so that they can subsequently activate eggs (Garbers, 1981); these same effects are observed with egg jelly (Garbers & Kopf, 1980).

The present results indicate the limitations of the methodology used to support the suggestion that activation of a protease is required for successful completion of an acrosome reaction. Whether or not activation of acrosin is an absolute requirement for the early events of the acrosome reaction in sea urchin spermatozoa, therefore, remains uncertain, although in our estimation it is at present difficult to cite any data that support such an hypothesis.

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References


