Phospholipase and lysophospholipase activities of goat spermatozoa in transit from the caput to the cauda epididymidis

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Summary. Phospholipase and lysophospholipase activities were assayed in goat epididymal spermatozoa. Lysophospholipase was 10 times more active than phospholipase, and both enzymes decreased in activity substantially in the transit of spermatozoa from the caput to the cauda epididymidis. A comparative study revealed that phosphatidyl-ethanolamine, -choline and -inositol and phosphatidic acid were hydrolysed by goat sperm phospholipase. Hydrolysis of phosphatidylethanolamine/lysophosphatidylcholine revealed the end products to be glycerophosphoethanolamine/choline but neither diglycerides nor lysophosphatidylethanolamine/lysophosphatidylcholine were detected.

Introduction

Glycolysable substrates are not available to mammalian spermatozoa in the epididymis. Therefore, for 'ripening or maturation' in the epididymis, spermatozoa must derive energy by the oxidation of other substrates, such as phospholipids which decrease markedly during the transit of spermatozoa from the caput to cauda region (Mann & Lutwak-Mann, 1981). There are, however, no studies of the phospholipases that release fatty acids from the phospholipids in the epididymis, although Scott & Dawson (1968) have described the hydrolysis of ethanolamine phosphoglycerides and phosphatidylinositol by ejaculated spermatozoa of rams and bulls. In this study, we have examined the phospholipase and lysophospholipase activities of spermatozoa in different regions of the goat epididymis.

Materials and Methods

Chemicals. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, glycerophospho-ethanolamine/-choline, snake venom (Crotalus atrox), PPO, POPOP and methyl esters of fatty acids were obtained from Sigma Chemicals Co., St Louis, MO, U.S.A. Lysophosphatidylcholine and lysophosphatidylethanolamine were purchased from Patel Chest Institute, New Delhi, India. Sodium [1-14C]acetate was obtained from Bhabha Atomic Research Centre, Bombay, India. Naphthalene (scintillation grade) was purchased from Koch-Light, Haverhill, Suffolk, U.K. All other reagents were of analytical grade.

Collection and washing of spermatozoa. Goat testes, purchased from the local slaughterhouse, were processed within 3–4 h of slaughter. The epididymides were separated from the testes and spermatozoa from the proximal caput and distal cauda regions were collected by the method of Henle & Zittle (1942). The spermatozoa were suspended in Krebs–Ringer bicarbonate buffer, pH 7.4 (Dawson, Elliott, Elliott & Jones, 1969) and centrifuged at 600 g for 10 min. The pellet was
washed twice more and finally suspended in the same buffer at a concentration of 1–2 × 10⁸ cells/ml. Sperm counts were made with a haemocytometer.

**Preparation of labelled substrates.** Labelled phospholipids were prepared by incorporation of sodium [1-¹⁴C]acetate into germinating soya bean seeds according to the procedure of Hoelzl & Wagner (1966). The extraction, purification and separation of individual phospholipids was carried out by methods described in detail by Jain & Anand (1975, 1976a). The purity of individual labelled phospholipid was tested by (i) co-chromatography with authentic sample and (ii) by using unlabelled phospholipid as a carrier for the labelled phospholipid. Separation on silica gel thin-layer plates by the two-dimensional technique of Rouser, Fleischer & Yamamoto (1970) gave single spots. The purity was confirmed by hydrolysing the labelled phospholipids with snake venom (Long & Penny, 1956) which gave only the corresponding lysophospholipid.

**Formation and analysis of methyl esters.** The methyl esters were prepared by BF₃-methanol reagent as described by Jain & Anand (1976b). These were run on a dual-column gas chromatograph (No. 304, Pye Unicam) equipped with flame ionization detector. The 1 m × 4 mm glass column was packed with 20% DEGS on diatomite C (100–120 mesh) and was kept isothermally at 198°C. The fatty acid methyl esters were eluted with nitrogen at a flow rate of 30 ml/min. Peaks were identified by comparing the retention time of the component fatty acids with those of known standards. Peak area was measured by triangulation. The fatty acids have been expressed in short-hand nomenclature, i.e. number of carbon atoms: number of double bonds.

**Assay of phospholipases and lysophospholipases.** The activities of A₁ and A₂ phospholipases (EC 3.1.1.32 and EC 3.1.1.4) and lysophospholipases (EC 3.1.1.5) were assayed using ¹⁴C-labelled phospholipids/lysophospholipids. Sonicated substrates (0.7 µmol containing 0.5–1 × 10⁵ c.p.m.) in 5 mM-Tris–HCl buffer, pH 8.6 were incubated with spermatozoa (1–2 × 10⁸) from the caput or cauda epididymis in presence of 5 mM-CaCl₂ at 37°C with occasional shaking. The reaction was terminated by adding 5 ml chloroform:methanol (2:1, v/v) and the lipids were extracted as described by Jain & Anand (1975, 1976a). Fatty acids released by the hydrolysis of phospholipids/lysophospholipids were separated on silica gel plates with hexane:diethyl ether:acetic acid (70:30:1, by vol.) as the developing solvent. Oleic acid served as the fatty acid carrier and the fatty acid spot was scraped off into the counting vial containing 10 ml of the scintillation mix (0.2 g POPOP, 4.0 g PPO, 60 g naphthalene, 20 ml ethylene glycol, 100 ml methanol, 880 ml dioxane). The samples were counted in a Packard liquid scintillation spectrometer (Bray, 1960).

**Results and Discussion**

The hydrolysis of [¹⁴C]phosphatidylethanolamine by goat spermatozoa removed from the caput and cauda regions was linear in relation to the time of incubation and to sperm concentration (Text-fig 1). The results for hydrolysis of phosphatidylethanolamine and lysophosphatidylethanolamine were similar (data not shown). Maximum hydrolysis was observed between neutral and alkaline pH (7–9). Calcium was not essential but it stimulated the hydrolysis of phosphatidylethanolamine and maximum activity was observed at 14 mM-calcium concentration. EDTA at 5 mM final concentration did not inhibit the phospholipase activity. A comparative study of phospholipase activity catalysing the hydrolysis of various phospholipid substrates is presented in Table 1. The enzyme activity decreased substantially as the spermatozoa travelled from the caput to the cauda region. The extent of decrease for the four phospholipids studied was 64% for phosphatidylethanolamine, 81.5% for phosphatidylcholine, 77% for phosphatidylcholinol and 79% for phosphatidic acid.

For the identification of hydrolysis products, phosphatidylethanolamine/-choline were hydrolysed in Tris–HCl buffer, pH 8.6 with caput spermatozoa as a source of phospholipases.
### Table 1. Hydrolysis of phospholipids/lysophospholipids by goat caput and cauda spermatozoa*

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Caput</th>
<th>Cauda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylethanolamine</td>
<td>504.39 ± 80.56</td>
<td>182.50 ± 46.75</td>
</tr>
<tr>
<td>Phosphatidylycholine</td>
<td>456.80 ± 57.97</td>
<td>84.22 ± 6.84</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>276.67 ± 56.23</td>
<td>62.55 ± 16.84</td>
</tr>
<tr>
<td>Phosphatic acid</td>
<td>263.08 ± 52.72</td>
<td>54.00 ± 10.45</td>
</tr>
<tr>
<td>Lysophosphatidylethanolamine</td>
<td>4853.00 ± 219.10</td>
<td>1584.00 ± 89.44</td>
</tr>
</tbody>
</table>

* The substrates were hydrolysed using goat caput and cauda sperm cells (1–2 × 10^8 in 2 ml assay mixture) at a substrate concentration of 0.7 µmol containing 0.5–1 × 10^5 c.p.m. The values are mean ± s.e.m. of 5 samples assayed in duplicate. Each sample was a pooled preparation of epididymal spermatozoa obtained from at least 5–6 goats.

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**Text-fig. 1.** Hydrolysis of phosphatidylethanolamine by phospholipases from the spermatozoa of goats in relation to the time of incubation (a) and enzyme concentration (b). Standard assay conditions were used for the hydrolysis.

Phosphatidylethanolamine/-choline and their hydrolysis products were extracted with n-hexane and the remaining water layer was taken to dryness under vacuum and finally dissolved in a small volume of distilled water. The hydrolysis products arising as a result of phospholipase action on phosphatidylethanolamine or phosphatidylycholine in the hexane and water layers were identified as follows. The hexane layer was separated on silica gel thin-layer plates using two solvent systems. When the plates were developed with hexane:diethyl ether:acetic acid (70:30:1, by vol.), no spot corresponding to authentic diglycerides was observed and when the developing solvent was chloroform:methanol:25% NH₄OH (65:25:5, by vol.) (Rouser *et al*., 1970), lysophosphatidylethanolamine/-choline and their hydrolysis products were identified.
ethanolamine/-choline could not be detected. The water layer was resolved by paper chromatography using phenol saturated with water as the solvent, a single spot containing phosphorus was detected which gave a positive reaction with ninhydrin/Dragendorff’s reagent depending on the phosphatidylethanolamine/-choline used initially for hydrolysis. It was identified as glycerophosphoethanolamine/glycerophosphocholine by co-chromatography with authentic samples. These results suggest that (i) goat epididymal spermatozoa contain phospholipase A₁ and phospholipase A₂ or (ii) one of the two phospholipases plus lysophospholipase. Lysophospholipase was assayed using [14C]lysophosphatidylcholine as the substrate and was found to be 10 times more active than the phospholipase activity. This enzyme activity also decreased during the transit of goat spermatozoa from the caput to the cauda epididymidis (Table 1).

Our next concern was to decide about the positional specificity of goat sperm phospholipases and use was made of sodium deoxycholate which inhibits, in many cases, lysophospholipases (Magee, Gallai-Hatchard, Sanders & Thompson, 1962). The effect of increasing concentrations of sodium deoxycholate presented in Text-fig. 2 revealed that, in addition to lysophospholipase, phospholipase activity of goat spermatozoa is also inhibited. At the concentration of 5 mM-sodium deoxycholate chosen, lysophospholipase was inhibited 95% and phospholipase 77% for the hydrolysis of phosphatidylcholine by caput spermatozoa. Simultaneously, phosphatidylcholine was hydrolysed by snake venom phospholipase, the specificity of which is well established and liberates the unsaturated fatty acids esterified at the Sn-2 position (Hanahan, Brockerhoff & Barron, 1960). Analysis of hydrolysis products in two cases gave a lysophosphatidylcholine spot with snake venom enzyme but no such spot was observed with goat sperm enzyme. Variation in the conditions by using different concentrations of sodium deoxycholate, shorter incubation times of 1 or 2 min and hydrolysis of phosphatidylcholine at two pH values (7 and 9) did not help to detect the lysophosphatidylcholine spot. The results of analysis of methyl esters of fatty acids released by goat sperm phospholipases (Table 2) were also inconclusive to decide whether phospholipase A₁ or phospholipase A₂ or both accompany lysophospholipase in goat spermatozoa. Ferber, Munder, Fischer & Gerisch (1970) have reported that sodium deoxycholate is not always effective in inhibiting the lysophospholipase and this is presumably what has happened with goat epididymal spermatozoa. Other methods therefore need to be used to determine the positional specificity of the phospholipases which accompany lysophospholipase, and we are now attempting to fractionate the phospholipase activity of goat epididymal spermatozoa.

Text-fig. 2. The effect of sodium deoxycholate on the activities of phospholipases and lysophospholipase of goat caput spermatozoa. The activities were determined in standard assay conditions.
Table 2. Composition of fatty acids released from phosphatidylcholine by goat caput spermatozoa and snake venom*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Phosphatidylcholine</th>
<th>Snake venom</th>
<th>Caput spermatozoa</th>
<th>Caput spermatozoa + 5 mM-deoxycholate</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>41:20</td>
<td>8:74</td>
<td>33:50</td>
<td>45:45</td>
</tr>
<tr>
<td>18:1</td>
<td>2:37</td>
<td>0:00</td>
<td>1:53</td>
<td>3:03</td>
</tr>
<tr>
<td>18:2</td>
<td>14:13</td>
<td>3:89</td>
<td>20:30</td>
<td>15:15</td>
</tr>
<tr>
<td>18:2</td>
<td>12:49</td>
<td>9:14</td>
<td>9:14</td>
<td>8:08</td>
</tr>
</tbody>
</table>

* The standard assay system was scaled up 5-fold and hydrolysis of phosphatidylcholine was carried out in Tris-HCl buffer in the presence of 5 mM-CaCl₂ with snake venom (0.5 mg/ml) and spermatozoa without and with sodium deoxycholate (5 mM, final conc.). The fatty acids released were converted to methyl esters and analysed by gas-liquid chromatography. The results presented are of a typical experiment but the fatty acids were analysed 5 times in different experiments. The fatty acids of phosphatidylcholine were also analysed for comparison.

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References


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