A secretory phospholipase C-like enzyme in the bovine epididymal caput

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An enzyme hydrolysing the synthetic substrate *p*-nitrophenylphosphorylcholine was highly active in the epididymal caput and very low or absent in other parts of the bovine reproductive organs. This enzyme was also found in the secretory fluid obtained from the epididymal caput but much lower activities were encountered in cauda epididymis and seminal plasma. The enzyme displayed an optimum at pH 6.5, an M_r of about 66000, a p*I* value of 5.0 and was suppressed by chelating agents and reactivated by Co^{2+} and Zn^{2+}. After partial purification the enzyme also showed hydrolysis of *p*-nitrophenyl phenylphosphonate, bis-*p*-nitrophenyl phosphate and hexadecanoyl-*p*-nitrophenylphosphorylcholine with slightly different pH optima and modifier characteristics. It is concluded that this secretory enzyme is distinct from the membrane-bound phospholipase C and may play a role in processing of the spermatozoan phospholipids during the epididymal maturation.

Phospholipase C *(Bovine epididymis)* *Enzyme characterization*

1. INTRODUCTION

In the bull [1], ram [2–4] and boar [5] the passage of spermatozoa in the epididymis is accompanied by a progressive fall in the concentration of several phospholipids including phosphatidylcholine, phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylserine and sphingomyelin. The studies of Bjerve and Reitan [6] have indicated that the epididymal cauda in the rat contains a membrane-bound epithelial phospholipase A and lysophospholipase, which are possibly responsible for the accumulation of glycerylphosphorylcholine in the epididymal plasma. No proof, however, is available that the same enzymes are involved in the processing of the spermatozoan phospholipids during maturation. Here, we present evidence for a phospholipase C-type enzyme secreted by the epididymal caput and active on various synthetic phospholipase substrates.

2. MATERIALS AND METHODS

Samples of reproductive organs from immature (1 month), juvenile (5–6 months) and adult (1 year) Ayrshire bulls were obtained from a local slaughterhouse and bovine seminal samples from the AI Station for East and Middle Finland. The epididymides were divided into six segments (1–3 in caput, 4 in corpus, 5–6 in cauda) and all tissues were homogenized in 0.025 M imidazole-HCl buffer, pH 7.4, and 0.1% Triton X-100 with a glass-teflon homogenizer and centrifuged at 105000 × *g* for 1 h. The epididymal secretion and spermatozoa were collected from segments E_2, E_4 and E_6 as indicated previously [7].

The hydrolysis of the synthetic substrate *p*-nitrophenylphosphorylcholine (Sigma, St. Louis, MO) was measured in the supernatant of tissue homogenates and seminal fluid (after centrifugation at 50000 × *g* for 30 min) with and without sorbitol (60%, v/v) in 0.1 M acetate buffer, pH 6.5

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[8]. The active samples (segments E_2–E_3) of the adult bovine epididymis were applied on a column (1.6 × 89 cm) of Sephacryl S-300 superfine (Pharmacia, Uppsala) and eluted with 0.025 M imidazole-HCl buffer, pH 7.4, containing 0.15 M NaCl. The active fractions were pooled, concentrated and dialyzed with an Amicon Diaflo concentrator and PM-30 filter. The sample was applied on a Mono P chromatofocusing column (Pharmacia) and eluted with a pH gradient of 7–4 developed with Polybuffer 74. The active fractions were pooled and used for studies on pH optima, modifiers and K_m values with *p*-nitrophenylphosphorylcholine (p-NPPC), *p*-nitrophenyl phenylphosphonate (p-NP-PheP), bis-*p*-nitrophenylphosphate (bis-p-NPP) and hexadecanoyl-*p*-nitrophenylphosphorylcholine (HD-p-NPPC) as substrates. All substrates were from Sigma and the modifier reagents from Merck (Darmstadt). The enzyme activities in the tissue samples and pooled fractions are given in units of nmol·min^{-1}·mg protein^{-1} and in fractionations as absorbances at 410 nm after incubation at 37°C for a specified time [7].

3. RESULTS

The hydrolysis of p-NPPC in the bovine reproductive tissues with and without sorbitol at pH 6.0 is shown in fig.1. The highest hydrolysis rates were found in segments E_2 and E_3 of the epididymis with subsequent rapid decline in the more caudal segments. The testicular homogenate contained a moderate activity, while the enzyme levels in vas deferens and the accessory sex glands were rather low or negligible. The seminal vesicle secretion (not shown) displayed no activity and also the seminal plasma gave low specific activities. In the presence of sorbitol the hydrolysis rates were 30–50% higher in all specimens.

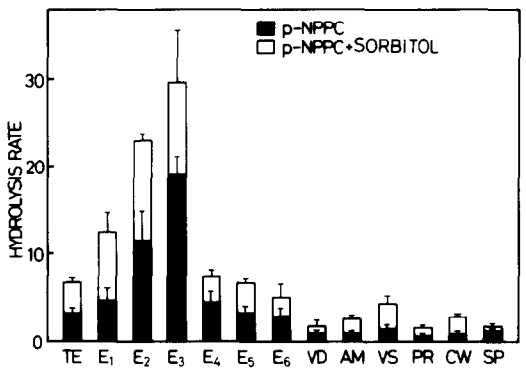

Fig.1. Hydrolysis rate (nmol·min^{-1}·mg protein^{-1}) of p-NPPC at pH 6.5 without and with sorbitol (60%, v/v) by homogenates of bovine testis (TE), six segments (E_1–E_6) of the epididymis, vas deferens (VD), ampulla (AM), seminal vesicle (VS), prostate (PR), Cowper's gland (CW) and seminal plasma (SP). The columns indicate the mean (± SD) of 3 duplicate measurements.

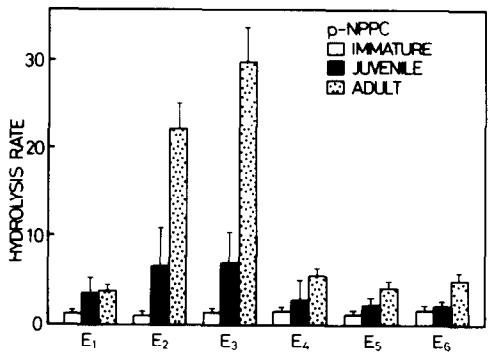

Fig.2. Hydrolysis rate (nmol·min^{-1}·mg protein^{-1}) of p-NPPC at pH 6.5 by homogenates of six epididymal segments (E_1–E_6) from immature (1 month), juvenile (5–6 months) and adult (1 year) bulls. The columns indicate the mean (± SD) of 3 duplicate measurements.

The activity levels in the immature bulls were very low in all tissues including the epididymis (fig.2). In the juvenile bulls the hydrolysis rates showed some elevation in the epididymal caput segments E_2 and E_3, where the most obvious increase was apparent in the adult bulls. The rates of hydrolysis of p-NPPC by the epididymal secretion, epididymal spermatozoa and epididymal tissue in segments E_2, E_4 and E_6 are shown in table 1. The highest levels were encountered in the epididymal secretion and tissue of segment E_2 with ensuing decline in the lower parts. The spermatozoa (washed 3 times with saline) gave more equal activity levels in all epididymal segments.

Fractionation of the homogenate of the epididymal caput (spec. act. 15 units) resulted in a single hydrolysis peak in gel filtration (fig.3A). With standard proteins (aldolase, M_r 158000; bovine serum albumin, M_r 67000; chymotrypsinogen A, M_r 25000) a tentative M_r of 66000 was obtained for the enzyme. The active fractions after concentration and dialysis (spec. act. 214 units)

Table 1

Hydrolysis rate (nmol · min^{-1} · mg protein^{-1}) of p-NPPC by secretion and homogenates of spermatozoa and tissue obtained from bovine epididymal segments E_2, E_4 and E_6

Segment	Secretion	Spermatozoa	Tissue
E_2	23.28 ± 2.81	11.47 ± 2.74	43.81 ± 11.86
E_4	14.11 ± 2.44	9.20 ± 2.04	21.15 ± 6.64
E_6	7.20 ± 1.22	8.41 ± 1.80	7.17 ± 3.66

Enzyme assays were as described in section 2. Results are means ± SD of 4 duplicate assays

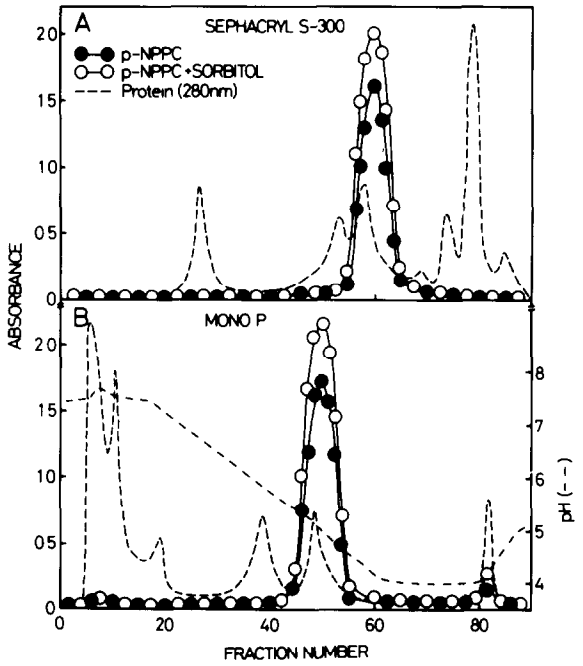

Fig.3. Hydrolysis of p-NPPC without and with sorbitol (60%, v/v) after fractionation of bovine epididymal homogenate from segments E_2–E_3 with gel filtration on Sephacryl S-300 (A). The active pool (fractions 55–65) after concentration and dialysis was fractionated with chromatofocusing on a Mono P column (B). The enzyme activity is given as the absorbance at 410 nm after 30 min incubation at 37°C. The protein in the fractions was monitored by the absorbance at 280 nm and the pH after chromatofocusing with a microelectrode.

also resulted in a single peak of p-NPPC hydrolysis on chromatofocusing (fig.3B) with a p*I* value of 5.0. The pH optimum of the pooled enzyme was analysed with four different synthetic substrates. The lowest optima were obtained for HD-p-NPPC (pH 5.5) and bis-p-NPP (pH 5.5). p-NPPC was optimally hydrolysed at pH 6.5 and p-NP-PheP at pH 7.0. The specific activities obtained for these four substrates by the pooled enzyme preparation were 24, 112, 770 and 1924 units, respectively, at the specified pH optima. With all substrates the enzyme activity was strongly suppressed by chelating agents (EDTA, *o*-phenanthroline) at 1 mM final concentration (fig.4). A partial or total reactivation of the hydrolysis was obtained with Co^{2+} and Zn^{2+} with some differences in the preference of these ions between the four substrates. Co^{2+} was also able to activate the hydrolysis of p-NPPC (fig.4), while Zn^{2+} was a more effective activator of p-NP-PheP hydrolysis (not shown). With all substrates Ca^{2+} had mainly a suppressive action. Thiol reagents (Hg^{2+}, *p*-chloromercuribenzoate) caused no effect on the

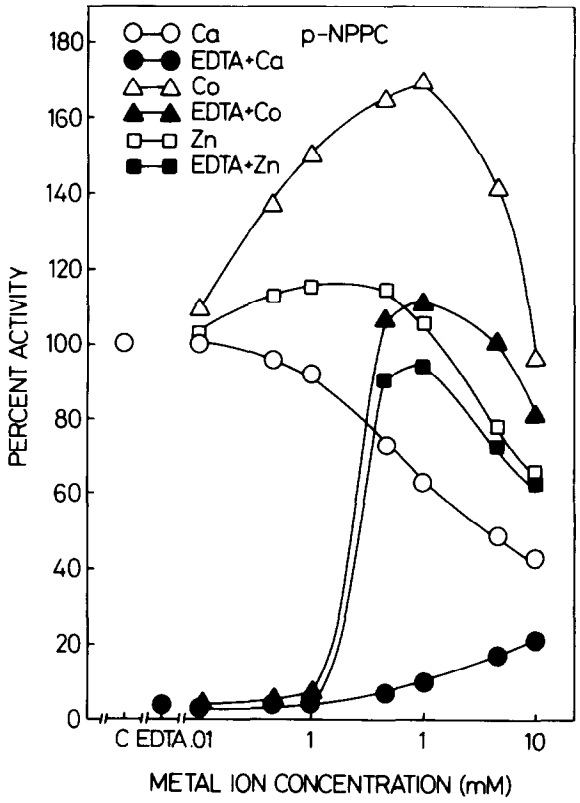

Fig.4. Percentage changes in hydrolysis rate of p-NPPC by the active chromatofocusing pool (fractions 55–65) after EDTA (1 mM) as well as in the presence of various concentrations of Ca^{2+}, Co^{2+} and Zn^{2+} without or with a prior addition of EDTA (1 mM) as indicated.

hydrolysis, while dithioerythritol was highly suppressive.

Tentative K_m values of 0.24, 2.10 and 22.76 mM were obtained with the enzyme preparation for p-NP-PheP at pH 7.0, p-NPPC at pH 6.5 and bis-p-NPP at pH 5.5, respectively. Due to weak solubility no reliable K_m estimate was obtained for HD-p-NPPC.

4. DISCUSSION

The present results provide the first evidence that the bovine caput epididymis contains an enzyme activity hydrolysing p-nitrophenylphosphorylcholine, a substrate developed for phospholipase C [8]. This enzyme appears to be secretory and associated with the sexual maturation. When mixed with the epididymal sperm the enzyme may have an effect on their plasma membrane and participate in epididymal sperm maturation. In the lower parts of the epididymis the enzyme activity rapidly declined and only low levels were detected in the seminal plasma. This would indicate that the enzyme is possibly absorbed or inactivated. This may be important in order to maintain the viability of sperm even during prolonged storage in the cauda epididymis.

Sequential fractionation of the homogenate of the epididymal caput with gel filtration and chromatofocusing resulted in a single activity peak for p-NPPC hydrolysis. The final purification was only about 500-fold. This preparation was also highly active for p-NP-PheP, a substrate developed for phosphonate esters [9] but able to hydrolyse phosphodiesters of nucleotides [10] as well. In addition, lower hydrolysis rates were obtained with bis-p-NPP and DC-p-NPPC. The former substrate has been used for the quantitation of phosphodiesterases and both may be split by sphingomyelinases [11,12]. In the bovine reproductive tissues including the epididymis, p-NP-PheP and bis-p-NPP are also hydrolysed by other enzymes, which are not associated with the p-NPPC peak after fractionations and have distinct biochemical properties (unpublished).

The studies indicated that the partially purified enzyme was dependent on divalent ions. Both Co^{2+} and Zn^{2+} were able to reactivate the hydrolysis after EDTA suppression. Some variation was observed in the preference of the activating ion when p-NPPC (Co_2^+) or the other substrates (Zn^{2+}) were used. The identification of the divalent metal ion in the active center of the enzyme would require a more extensively purified enzyme preparation for analyses. The results indicated, however, that the secretory epididymal enzyme was not identical with the membrane-bound Ca^{2+}-dependent phospholipase C [13], since Ca^{2+} had no or very slight effect in reactivation. However, this enzyme obviously can be classified as a phospholipase C-like enzyme. Detailed studies with a purified enzyme and natural phospholipid substrates would eventually disclose the specificity of the enzyme and its possible role in sperm maturation.

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