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Physiological and Enzymatic Properties of the Ram Epididymal Soluble Form of Germinal Angiotensin I-Converting Enzyme[1]

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ABSTRACT

The 94-kDa ram epididymal fluid form of the sperm membrane-derived germinal angiotensin I-converting enzyme (ACE) was purified by chromatography, and some of its enzymatic properties were studied. For the artificial substrate furanacryloyl-L-phenylalanylglycylglycine (FAPGG), the enzyme exhibited a Michaelis constant (K_m) of 0.18 mM and a V_{max} of 34 μmoles/(min·mg) and for hippuryl-L-histidyl-L-leucine a K_m of 2.65 mM and a V_{max} of 163 μmoles/(min·mg) under the defined standard conditions (300 mM NaCl and 50 mM Tris; pH 7.5 and 8.3, respectively). The FAPGG hydrolysis was decreased by 82.5% and 67.5% by EDTA and dithioerythritol, respectively, and was totally inhibited by specific ACE inhibitors such as captopril, P-Glu-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro, and lisinopril. Optimum activity for FAPGG was with pH 6.0, 50 mM chloride, and 500 μM zinc. Under the various conditions tested, bradykinin, angiotensin (Ang) I, Ang II, and LHRH were competitors for FAPGG. Bradykinin and angiotensin I were the best competitors. The enzyme cleaved Ang I into Ang II, and the optimal conditions were with pH 7.5 and 300 mM chloride. The relationship between the carboxypeptidase activity in seminal plasma and the prediction of fertility of young rams was also studied. These results indicated a correlation between sperm concentration and ACE activity in semen but showed no statistically significant correlation between such activity and fertility of the animal. Finally, we tested the role of ACE in fertilization; no difference in the in vitro fertilization rate was observed in the presence of 10^{-4} M captopril.

epididymis, fertilization, male reproductive tract, male sexual function, sperm maturation

INTRODUCTION

Male mammals produce two isoforms of angiotensin-converting enzyme (ACE), a somatic isoform and a germinal isoform (also known as testicular ACE), both of which are coded by the same gene. The somatic ACE (sACE) plays an important role in the control of blood pressure by acting on the bradykinin and angiotensin peptides. The sACE isoform has a molecular weight of approximately 140–180 kDa and is expressed in many tissues (e.g., lung, kidney, brain) [1–3]. Its sequence is composed of two redundant domains (N-terminal and C-terminal domains), each having a zinc-binding site and a functional catalytic site [4, 5].

The germinal ACE (gACE) isoform is exclusively expressed in the male haploid germ cells [6]. The gACE-specific sequence results from a testis-specific promoter within the 12th intron of the *ACE* gene [7, 8]. This germinal isoform of 90–110 kDa is restricted to the C-terminal domain of the sACE, but it has a specific N-terminal sequence [3, 9]. The gACE has only one catalytic and one zinc-binding site [3, 6].

Both sACE and gACE are type I ectoproteins anchored in the plasma membrane through a hydrophobic region near the carboxyl terminus [2, 10]. Blood-soluble, circulating sACE is produced by a specific membrane-associated cleavage secretion process that releases the extracellular domain of this enzyme [11]. Soluble gACE has recently been found at high concentrations in the epididymal fluid of several mammals. This 94- to 105-kDa protein is derived from the sperm plasma membrane during passage of spermatozoa through the anterior caput of the epididymis [12]. In rams, all seminal ACE is exclusively a gACE isoform carried by the epididymal fluid [12]. In the genital tract, this soluble epididymal gACE represents the counterpart of the blood-circulating isoform derived from sACE by a proteolytic process.

The *ACE* gene knockout (*ACE* KO) mice have shown that the germinal enzyme is essential for male fertility [13–15]. Impairment of male fertility was reversed when the part of the gene coding for gACE was reintroduced into *ACE* mutant mice, although this new mutant retained all other physical defects [16]. Moreover, replacement of gACE by sACE in the germinal cells did not restore the fertility of *ACE* KO mice, indicating the physiological non-equivalence of the two ACE isoforms [17].

The function of gACE in the male tract and in sperm fertility is unknown. It has been suggested that sperm lacking gACE are deficient in transport within the oviduct and in binding to the zona pellucida [13].

In this study, the enzymatic properties of purified, soluble gACE from the ram epididymal fluid were established and compared to those of the sACE isoforms reported in the literature. The relationship between gACE activity and fertility was also assessed by in vivo and in vitro studies.

MATERIALS AND METHODS

Chemicals

Furanacryloyl-L-phenylalanylglycylglycine (FAPGG), hippuryl-L-histidyl-L-leucine (HHL), dithioerythritol (DTE), bradykinin, LHRH, angiotensin II, angiotensin I, captopril, lisinopril, and the peptide inhibitor P-Glu-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro (derived from the venom of *Bothrops jararaca* [18]) were purchased from Sigma (Saint Quentin Fallavier, France). Molecular weight standards used for electrophoresis (14.4, 20.1, 30, 43, 67, and 94 kDa) were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). All other reagents were of the best available grade.

Fluid and Sperm Collection

Epididymides were surgically removed by castration or obtained from freshly killed, adult Ile de France rams. Caudal epididymal fluid was collected by retroperfusion [19] from the vas deferens with either PBS or

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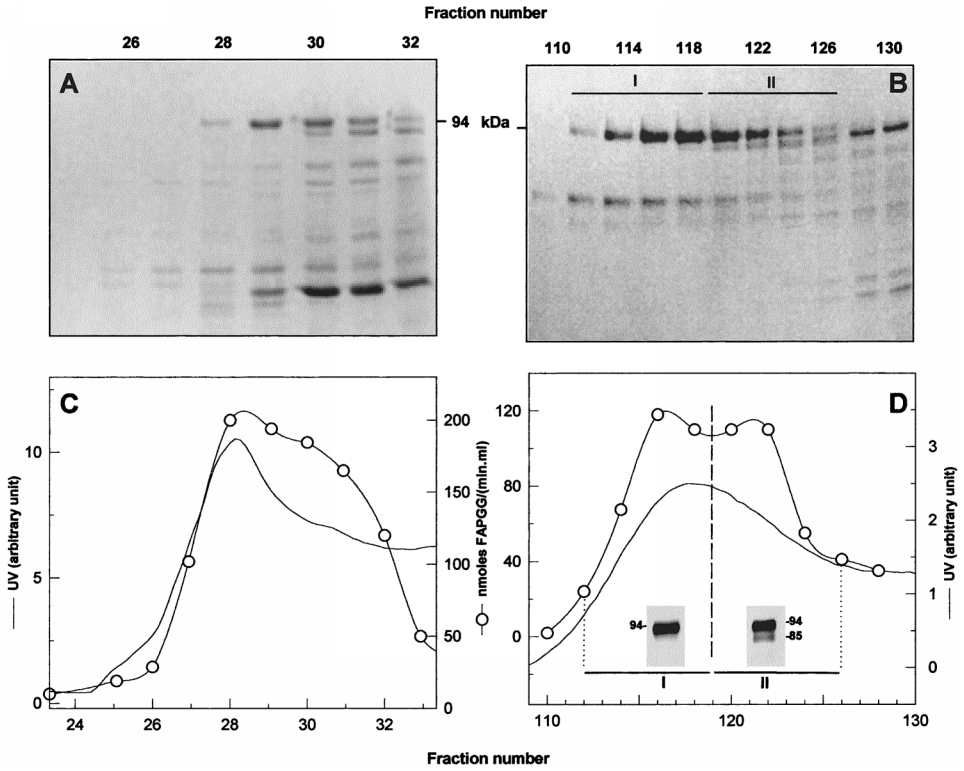

FIG. 1. Purification of soluble gACE from the CEF. The SDS-PAGE gel electrophoresis (6–16%) of fractions from anion-exchange (**A**) and from gel filtration (**B**) chromatography were colored with Coomassie blue. The corresponding protein elution profile (arbitrary units) and ACE activity measured under standard conditions with FAPGG (expressed in nmoles FAPGG/[min·ml]) are also shown (**C** and **D**). Inserts in **D** show the immunoreaction of the pooled fractions I and II (as indicated in **B** and **D**) when probed with a polyclonal antibody made against the epididymal ram gACE [12].

mineral oil. Ejaculates from 2-yr-old animals (Ile de France) and 6- to 8-mo-old rams (Lacaune) were collected using an artificial vagina. Cauda epididymal fluid (CEF) and seminal plasma were separated from sperm by two centrifugations (15 min, 15 000 × *g*, 4°C). The samples were kept at −20°C until use.

Purification of the CEF Form of gACE

The soluble form of gACE from the CEF was purified by high-performance liquid chromatography (HPLC) as previously described [12]. Briefly, dialyzed CEF (173 mg of proteins) with a carboxypeptidase activity of 0.096 μmoles/(min·mg) of FAPGG was loaded on an anion-exchange column (Q-Hyper-D 10; Biosepra, Cergy-Saint-Christophe, France) and eluted with an NaCl gradient. The fractions containing the 94-kDa protein were pooled and further separated on a gel filtration column (Hiload 16/60, Superdex 200; Pharmacia). Each step of protein purification was controlled by electrophoresis separation on 6–16% (w/v) SDS-PAGE followed by Coomassie blue staining and measurement of carboxypeptidase activity. The fraction pools I and II were separated by SDS-PAGE and Western blotted. The blot was probed with the rabbit polyclonal antibody obtained against the ram gACE [12]. Protein concentrations of pooled, active fractions were estimated by the Bradford method (Biorad, Ivry-sur Seine, France) with bovine serum albumin as reference.

Measurement of Carboxypeptidase Activity

Carboxypeptidase activity was estimated by spectrophotometry in 300 mM NaCl and 50 mM Tris, either at pH 7.5 for FAPGG [20] or at pH 8.3 for HHL [21]. These standard ionic conditions were first used to compare the effects of varying H^+, Cl^-, and Zn^{2+} concentrations. From then on, a medium containing 50 mM NaCl and 0.5 mM zinc at pH 6, which was found to give the optimum activity of epididymal ACE, was used in most of our assays. A ''CEF-like'' medium (34 mM Tris-HCl [pH 6.8], 120 mM $NaHCO_3$, 25 μM Zn^{2+}) was also used to mimic CEF ionic conditions for zinc, chloride ion concentration, and pH [22, 23].

Captopril, lisinopril, and peptide P-Glu-Trp-Pro-Arg-Pro-Glu-Ile-Pro-Pro were used in ACE inhibition analysis. They were prepared as 10^{-3} M stock solutions and diluted at concentrations ranging from 10^{-6} to 10^{-11} M in the presence of FAPGG (5×10^{-5} M). The inhibitory effect was measured during the first minute (initial rate) of FAPGG hydrolysis. Substrate competition analysis was also studied between FAPGG (5×10^{-5} M) and angiotensin II or native substrates of the ACE such as bradykinin,

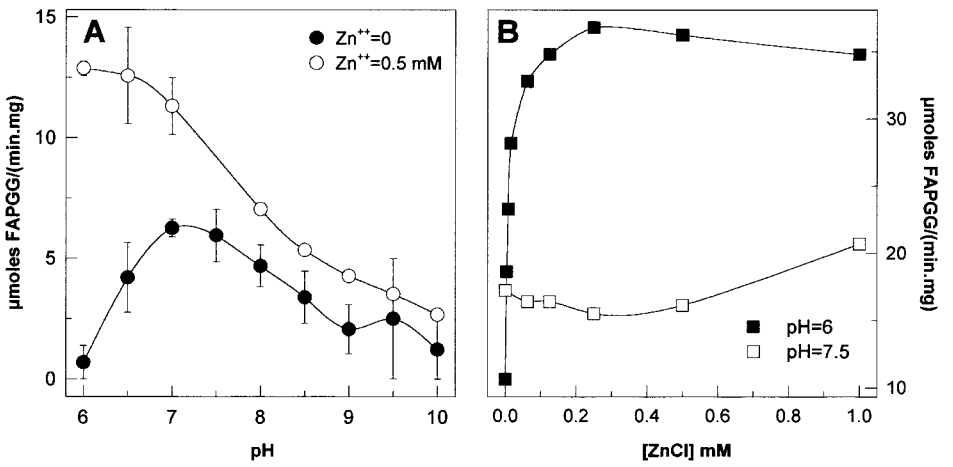

FIG. 2. Effect of pH and zinc. **A**) Activity of the purified enzyme measured with FAPPG in the presence of 300 mM NaCl at pH varying between 6 and 10 and in the absence (●) or presence (○) of 0.5 mM zinc (mean ± SD for three experiments). **B**) Effect of increasing the zinc concentration at pH 6 (■) and at pH 7.5 (□).

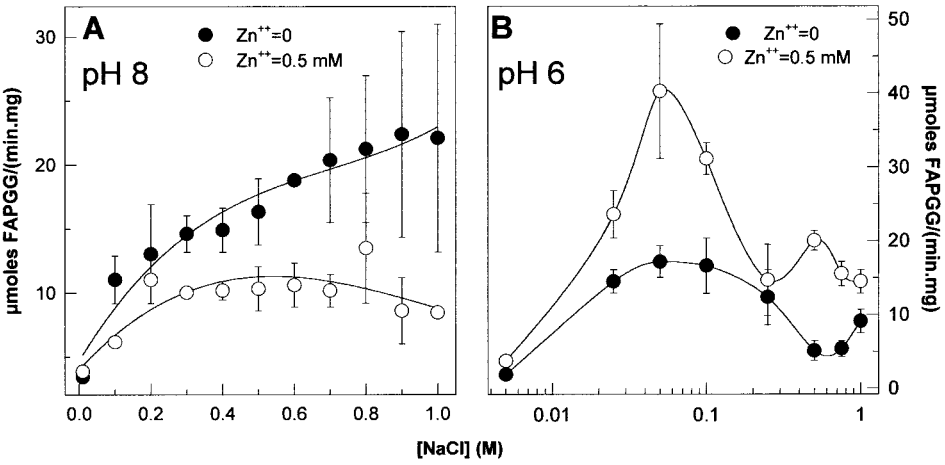

FIG. 3. Effect of Cl^- and zinc concentrations at pH 8 and pH 6. The FAPGG hydrolysis by the purified enzyme was measured at varying Cl^- concentrations in the absence (●) or presence (○) of 0.5 mM zinc at pH 8 (**A**) or pH 6 (**B**) (mean ± SD for three experiments). Note that the scale is logarithmic for **B**.

angiotensin I, and LHRH at concentrations ranging from 10^{-4} to 10^{-8} M. Results are presented as the mean of three experiments.

Angiotensin I hydrolysis was estimated by HPLC using a C-3 reverse-phase column (Beckman, Gagny, France) and an acetonitrile-water mobile phase (acetonitrile:water:TFA, 10:89.9:0.1 [v/v]). The elution times of angiotensin I and II were determined at 214 nm using synthetic human angiotensin I and II. For the assay, human angiotensin I (sequence similar to sheep angiotensin I [24, 25]) was diluted at a concentration of 5×10^{-5} M in different media in the presence of either purified ACE or crude CEF. The rate of hydrolysis was assessed at different times by measuring the surface of the angiotensin I and II peaks. No other angiotensinase activity was present in our preparations, because no extra peaks other than angiotensin I or II were detected.

In Vitro Fertilization with Sheep Oocytes

Oocytes were matured in vitro as previously described [26]. For each experiment, freshly ejaculated spermatozoa from two adult rams were mixed and diluted at 8×10^8 spermatozoa/ml in Dulbecco modified Hepes (DMH) at pH 7 before a final wash through two layers of Percoll (45% and 90%; 15 min, 800 × *g*). The washed sperm were diluted at a concentration of 10^7 spermatozoa/ml and capacitated by 2-h incubation at 38°C in DMH (pH 7.3) containing 20% serum from ewes in estrus. Capacitated spermatozoa were diluted to 10^6 spermatozoa/ml in the fertilization medium (DMH [pH 7.7]) and maintained with oocytes at 38°C. For the treated group, the ACE-inhibitor captopril (10^{-4} M) was added to all media. The fertilization rate was assessed after 17 h using an inverted microscope, and degenerated oocytes were eliminated. Fertilized oocytes were cultured under 5% CO_2 and 5% O_2 at 38°C. After 7 days, they were stained with Hoechst 33342, and the developed blastocysts were counted.

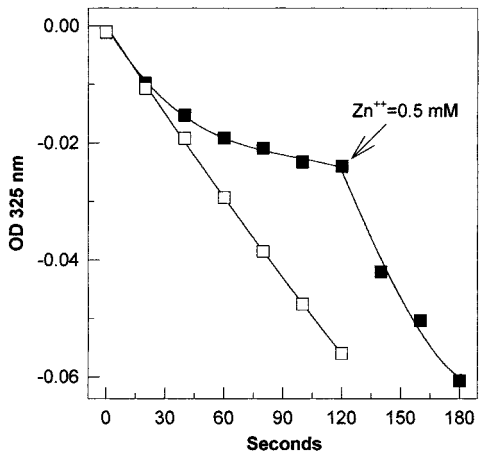

FIG. 4. Effect of the addition of zinc at pH 6 and 50 mM chloride ion. Spectrophotometric kinetics of FAPGG hydrolysis were measured in 50 mM NaCl medium at pH 6 in the absence (■) or presence (□) of 0.5 mM zinc. Addition of zinc (arrow) in the assay medium returned the rate of hydrolysis to its initial value.

The experiment was performed with four different batches of oocytes (treated group, 274 oocytes; control group, 283 oocytes).

Collection of Ejaculate Samples from Rams with Known Fertility

Seminal plasma of young rams (n = 43) was collected at the beginning of the fertility evaluation period. The samples were centrifuged at 15 000 × *g* for 15 min, and the supernatant was stored at −20°C until analysis. Fertility for each ram was assessed by intracervical insemination with 12–147 females (mean, 100) with 3×10^8 spermatozoa stored for less than 8 h at 15°C. The fertility index was established as the ratio between the number of ewes giving lambs to the total number of ewes inseminated. Each seminal plasma sample was probed by Western blot analysis with the rabbit polyclonal antibody made against the gACE [12] and showed only the 94- and 85-kDa immunoreactive compounds.

RESULTS

Purification of Soluble gACE from Ram CEF

Using anion-exchange chromatography, the soluble isoform of gACE, detected by its carboxypeptidase activity, was desorbed from the column with 50–75 mM NaCl (Fig. 1, A and C, fractions 26–33). After separation of these active pooled fractions by gel filtration, the 80- to 95-kDa fractions (Fig. 1, B and D) contained the main peak of carboxypeptidase activity, which corresponded to the 94-kDa protein expected (fractions 112–126) and also to an 85- to 90-kDa protein (fractions 120–126) (Fig. 1D) previously described as a low molecular weight form of the epididymal gACE in ram cauda fluid [12]. The pooled fractions 112–119 (pool I) contained mainly the 94-kDa immunoreactive gACE form, whereas the pooled fractions 120–126 (pool II) showed both the 94- and 85-kDa gACE forms.

Only pool I, which corresponded to a 133-fold ACE enrichment of the original cauda fluid and represented 0.43% (w/w) of the starting material was used for enzymatic studies.

Enzymatic Properties of the CEF gACE

The K_m and V_{max} of the purified 94-kDa epididymal gACE were determined under standard buffer conditions according to the method described by Holmquist et al. [20] for FAPGG, and by Cushman and Cheung [21] for HHL, respectively. Under these conditions, a linear relationship between the reciprocal substrate concentration (1/[S]) and the reciprocal rate (1/v) was obtained using Lineweaver-Burk graphical determination. A K_m of 0.18 mM and V_{max}

TABLE 1. Effects of specific inhibitors on epididymal ACE carboxypeptidase activity.

	Captopril (nM)		Lisinopril (nM)		ACE inhibitor[a] (nM)	
	50%[b]	100%	50%	100%	50%	100%
pH 6 (50 mM NaCl, 0.5 mM zinc)	15	100	200	1000	200	10 000
pH 6 (300 mM NaCl)	0.5	10	20	100	30	500
pH 7.5 (300 mM NaCl)	3	50	30	500	3	50

[a] P-Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro [18].
[b] Percentage inhibitions.

of 34 μmoles/(min·mg) were measured with FAPGG and a K_m of 2.6 mM and V_{max} of 163 μmoles/(min·mg) of hippuric acid with HHL.

Optimal Conditions for the Carboxypeptidase Activity of Soluble Epididymal gACE

Because carboxypeptidase activity of the sACE isoform is dependent on the pH and on zinc and chloride ions, the effects of these three parameters on the activity of soluble epididymal gACE were analyzed using FAPGG as substrate.

Effects of pH and zinc. Epididymal gACE activity was analyzed in the presence of 300 mM NaCl with a pH varying from 6 to 10 in the presence and absence of zinc (Fig. 2A). Without zinc in the buffer, the activity showed a bell-shaped curve, with a maximum between pH 7 and 7.5. In the presence of zinc, no difference was observed at a pH greater than 7.5, but the carboxypeptidase activity gradually increased for pH $<$ 7.5, reaching a maximum at pH 6. In a more focused analysis, we observed that the activity remained maximal between pH 6 and 5.8 but decreased sharply below this value (data not shown).

At pH 6, changes in zinc concentration strongly affected carboxypeptidase activity, and maximum activity was reached in the presence of 250 μM zinc (Fig. 2B). At pH 7.5, variations in the Zn^{2+} concentration produced no effect on the enzymatic activity of ACE (Fig. 2B). Other divalent cations, such as Mg^{2+} and Mn^{2+}, did not change the enzymatic activity regardless of the concentration used (data not shown).

Effects of chloride and zinc. The effects of different concentrations of chloride were analyzed at pH 8 (Fig. 3A) and pH 6 (Fig. 3B) in the presence and absence of zinc.

At pH 8 in the absence of zinc (Fig. 3A), carboxypeptidase activity increased gradually from 3.8 to 22.4 μmoles/(min·mg) of FAPGG between 0 and 1 M chloride. In the presence of 0.5 mM zinc, the activity increased more slowly between 0 and 200 mM chloride from 3.5 to 11 μmoles/(min·mg) of FAPGG and reached a plateau at 200 mM chloride (Fig. 3A).

At pH 6 (Fig. 3B), carboxypeptidase activity was maximal with 50 mM chloride. Under these conditions, the FAPGG hydrolysis rate in the absence of zinc was 17 μmoles/(min·mg), whereas in the presence of zinc, the activity was strongly increased, reaching a maximum of 40 μmoles/(min·mg). In the absence of zinc, the initial rate of hydrolysis decreased very rapidly, and the activity stopped after 1 min (Fig. 4). However, adding 0.5 mM of zinc immediately restored the enzyme activity to its initial rate.

Inhibitory Characteristics of Soluble gACE

Because ACE is a zinc metalloprotease, EDTA was tested on FAPGG hydrolysis by epididymal gACE. Under standard buffer conditions, EDTA decreased the activity in a dose-dependent manner, with a maximum of 82.5% inhibition with 5 mM. In the presence of DTE (a reducing compound shown to inhibit the enzyme [21]), a maximum of 67.5% inhibition was observed with 5 mM, and no further effect was obtained with higher DTE concentrations.

The effects of specific inhibitors of sACE (captopril, lisinopril, and the peptide inhibitor P-Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) were measured on soluble gACE. Concentrations of inhibitors necessary to reduce the activity by 50% (IC_{50}) and 100% were determined in the presence of zinc at pH 6 with either 50 or 300 mM chloride ion and at pH 7.5 with 300 mM chloride ion (Table 1). Under all conditions, captopril was the most powerful inhibitor. The three inhibitors were less effective at low pH and low chloride concentrations. Captopril was also tested with preincubation and with two different quantities of enzyme, but no difference was observed in either IC_{50} or 100% inhibition.

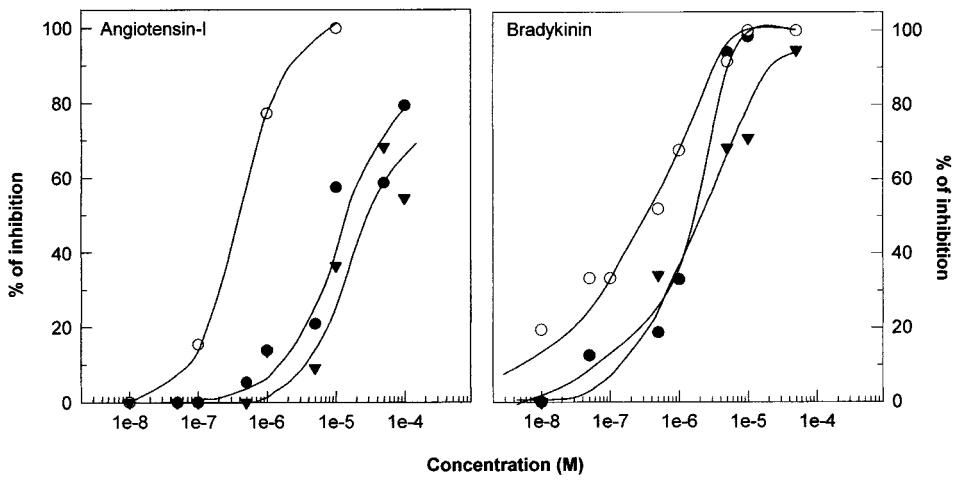

FIG. 5. Competition between angiotensin I or bradykinin and FAPGG. Inhibition of FAPGG hydrolysis was measured in presence of increasing doses of angiotensin I (**A**) or bradykinin (**B**). Competition was performed in three different ionic conditions: 300 mM NaCl, pH 6 (○); 50 mM NaCl, 0.5 mM zinc, pH 6 (▼); and 300 mM NaCl, pH 7.5 (●).

TABLE 2. Hydrolysis of angiotensin I into angiotensin II.

	nmoles of angiotensin I/(min·mg)
Undiluted CEF	47
Standard (300 mM NaCl, pH 7.5)	1730
"CEF-like conditions"	89
pH 6 (50 mM NaCl, 0.5 mM zinc)	81

TABLE 3. Influence of captopril on in vitro fertilization.

Group	No. of oocytes	% of fertilized oocytes	% of cleaved embryos	% of blastocysts
Treated	274	70.8	68.3	13.1
Control	283	66.1	56.2	8.8

Potential Natural Substrates for gACE

Bradykinin, angiotensin I, and LHRH are natural substrates hydrolyzed by sACE. The affinity of gACE for these potential substrates was studied by in vitro competition with FAPGG (Fig. 5). Concentrations of competitors necessary to obtain 50% (i.e., IC_{50}) and 100% inhibition of ACE activity toward FAPGG were determined in the presence of zinc at pH 6 either with 50 or 300 mM chloride ion and at pH 7.5 with 300 mM chloride ion.

Bradykinin and angiotensin I were the most potent competitors at pH 6 with 300 mM chloride (IC_{50} = 500 nM, 100% inhibition with 10 μM). Under the other ionic conditions, bradykinin was the best competitor and the only one to inhibit ACE activity totally (100% inhibition with 10–50 μM according to the ionic conditions). High concentrations of LHRH or angiotensin II (up to 100 μM) inhibited only 20–60% of FAPGG hydrolysis under the same ionic conditions.

Hydrolysis of angiotensin I by the purified enzyme diluted under different ionic conditions was measured and compared with the activity obtained when angiotensin I was added directly to crude CEF (Table 2). Maximal hydrolysis of angiotensin I was obtained when purified epididymal gACE was diluted in 300 mM NaCl at pH 7.5. Hydrolysis activity was approximately 20-fold higher under this condition than under other ionic conditions and when compared to undiluted CEF. Under all other conditions, angiotensin I was cleaved at approximately the same rate.

The ACE activity toward HHL, a substrate with the same C-terminal amino acids as angiotensin I, was also determined in the CEF from different adult rams by adding this substrate directly to the CEF, with the same CEF diluted under standard conditions [21], or under "CEF-like condition" medium. Wide differences in cauda fluid activity were observed between the different males (Fig. 6A), but in all cases, the artificial substrate was hydrolyzed three- to fourfold more rapidly under diluted standard condition than under CEF or CEF-like conditions (71 ± 36 vs. 16 ± 10 and 20 ± 13 nmole/[min·mg], mean ± SEM, respectively) (Fig. 6B).

Relationship Between ACE Activity in Seminal Plasma and Fertility

The ACE activity was further estimated with HHL in seminal plasma from 43 young rams. As expected, a significant correlation was found between sperm concentrations in ejaculates and ACE activity (Fig. 7A). The ACE activity, expressed as the number of sperm in the ejaculates of these young animals and of the older animals, was very similar (data not shown)

When the activity was plotted according to the fertility observed for each young ram during the reproductive season, the less fertile rams seemed to have lower activity than the most fertile animals, but the difference was not statistically significant (Fig. 7B). It is noteworthy that the animals with the lowest activity and fertility were not those with the lowest numbers of sperm.

Effect of Captopril on In Vitro Fertilization

Ram sperm did not loose all their gACE during transit in the epididymis, and part of the enzyme remained within the acrosome [12]. To study the potential role of this cellular gACE, we performed an in vitro fertilization experiment in the presence of 10^{-4} M captopril to prevent ACE activity. The presence of captopril during the fertilization process did not inhibit oocyte fertilization by spermatozoa (Table 3). We observed a higher percentage of cleaved oocytes (68.3% vs. 56.2%) and of blastocysts (13.1% vs. 8.8%) obtained after 7 days of culture in the treated group than in the control group.

DISCUSSION

The large amount of soluble gACE found in the epididymal fluid of mammals provides the possibility of purifying and studying the native soluble form of gACE. Such an opportunity does not arise for soluble sACE, and most studies of sACE have been performed with membrane-extracted sACE from lungs and kidneys or expressed in CHO cells. Moreover, the rare studies of gACE characteristics have

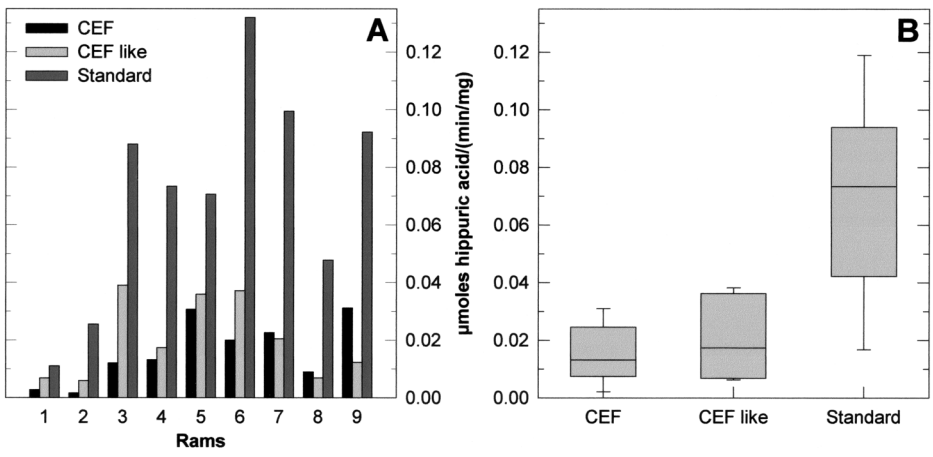

FIG. 6. Evaluation of ACE activity in the CEF from adult rams. The ACE variability in CEF between nine adult rams was measured in three different ionic conditions with HHL, CEF or "CEF-like," or standard medium. **A**) Individual results. **B**) Box plot graph of the previous results (the box boundary closest to zero indicates the 25th percentile, the upper boundary the 75th percentile, the bars above and below the 30th and 10th percentile, the line within the box median of the population distribution).

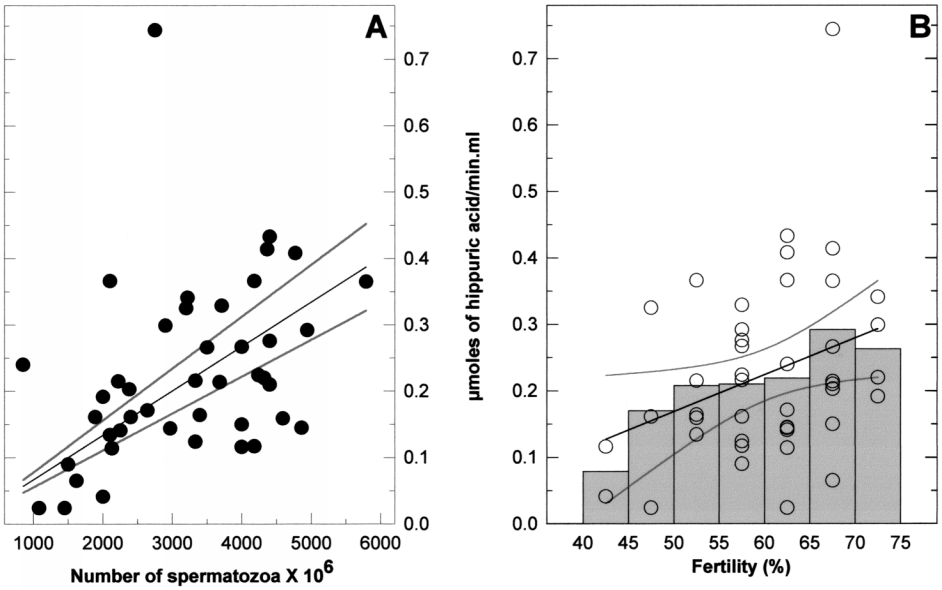

FIG. 7. Relationship between ACE activity and fertility in 43 young rams. **A**) Relationship between ACE activity and number of spermatozoa present in the ejaculates of young rams originating from an insemination center. **B**) Relationship between ACE activity measured with HHL and class of fertility of the same animals as estimated by artificial insemination ($r^2 = 0.11$).

been performed either with recombinant enzyme obtained by gene expression in a heterologous system [27] or with enzyme obtained from testicular or epididymal extracts [28, 29] that contained both sACE and gACE isoforms.

The principal characteristics of soluble epididymal gACE (similar to the C-domain of sACE) and those of sACE and its C- and N-domains are summarized in Table 4. Biochemical and enzymatic properties were very close for the different isoforms of ACE. The K_m and V_{max} values of epididymal gACE were similar to those published for somatic and purified germinal enzyme (Table 4). Epididymal ACE was inhibited by EDTA and DTE to the same extent as pulmonary and testicular isoforms [21]. However, a difference was found between ACE isoforms in their responses to specific inhibitors. Captopril was slightly more effective at inhibiting soluble epididymal gACE, whereas lisinopril was more effective at inhibiting sACE or its derived C-domain [2, 29–31].

As with other ACE isoforms, the soluble epididymal gACE was zinc, chloride, and pH dependent, but in a very complex relationship with the nature of the substrate. Acidic pH reduces enzyme activity, which is probably related to a loss of zinc bound to the active site, as has been previously reported for somatic isoforms [20]. This was verified in our study by restoration of enzyme activity after additional zinc was placed in the acidic buffer. This allowed us to demonstrate that the optimal conditions of activity toward FAPGG were low chloride ion concentration (50 mM), the presence of zinc (500 µM), and acidic pH. Although gACE is a zinc-dependent enzyme, the presence of zinc could be inhibitory when associated with a high chloride concentration or a basic pH (Fig. 3A). Moreover, acidic conditions were not the best for angiotensin I and HHL hydrolysis, because these two substrates need a higher concentration of chloride and a neutral pH. This chloride activation of angiotensin I cleavage is similar to that observed for the C-terminal site of sACE (for review, see [2]).

The chloride ion concentration decreases in vivo and the

TABLE 4. Biochemical and enzymatic properites of the different ACE isoforms.

	Soluble epididymal gACE[a]	Testicular gACE[b]	sACE
Molecular weight (kDa)	94	90–110 [1, 2, 54]	140–180 [1, 2, 50, 51] 135 (N-domain) 100 (C-domain) [1]
K_m HHL (mM)	2.6	1.7–3.5 [27, 28, 54]	1.2–2.6 [1, 21, 51] 2 (N-domain) 1.6 (C-domain) [53]
V_{max} HHL (µmoles/(min·mg))	163	85.5–222 [27, 54]	141 [51]
K_m FAPGG (mM)	0.18		0.136–0.7 [20, 50, 52, 53] 1.4 (N-domain) [52]
Inhibitors	C > L[c]	C > L, L > C (HHL) [28]	C > L (N-domain) L > C (C-domain) [30]
100% Inhibition	C = 50 nM, L = 100 nM		1 µM captopril (N- and C-domains) [1]
DTE	67.5% (5 mM)	70% (5 mM) [2]	65% (5 mM) [2]
EDTA	82.5% (5 mM)	90% (0.1 mM) [54]	100% (10 µM) [1] 99% (1 mM) [21]
Optimal conditions (FAPGG)	pH 7.5, 300 mM NaCl		pH 7.5–8.2, 300 mM NaCl [20, 50]

[a] This study.
[b] Extracted from epididymis [28], expressed in CHO cells [27], and purified enzyme [54].
[c] C, Captopril; L, lisinopril.

pH increases throughout the male genital tract (135 mM and pH 6.7 in the testicular fluid, 62 mM and pH 6.7 in the caput epididymis, 34 mM and pH 6.9 in the cauda epididymis, 24 mM and pH 6.9 in seminal plasma) [22, 23, 32]. Soluble gACE should, therefore, be active in the genital tract, but changes must occur due to these modifications in ionic conditions. For example, we observed that the caudal fluid or the ionic conditions of the caudal fluid were not adequate for the cleavage of angiotensin I and HHL. This was not due to the low zinc (25 μM) or chloride (34 mM) concentration in the caudal fluid, because their addition (0.5 mM zinc or 300 mM NaCl) had only a marginal effect on HHL hydrolysis (data not shown). Other modifications must, therefore, exist and remain to be clarified.

The in vivo substrates of gACE are not known. In our study, bradykinin was the best competitor for FAPGG under all the conditions tested, and this substrate is also reported to be hydrolyzed in a chloride-dependent manner by the C-domain [1]. However, this molecule has not been reported in the epididymal fluid, and male mice knocked out for the kinin receptors are apparently fertile [33, 34]. Angiotensin I is one of the principal substrates for the somatic isoform, with optimal hydrolysis in the presence of low chloride concentrations [35]. Angiotensinogen, angiotensin II, and angiotensin II receptors are principally localized in the caudal region [36–39]. Angiotensin I was hydrolyzed by soluble epididymal gACE and was a good competitor for FAPGG, but as we showed, the CEF conditions do not seem to be optimal for activity toward angiotensin I. Moreover, the presence of angiotensin I in the epididymal fluid was not found to be essential for male reproduction, because male mice knocked out for angiotensinogen [13, 40] or renin [41] (and, therefore, without angiotensin I) are fertile even though they show the same defects as those observed in mice without ACE. Moreover, male mice knocked out for the angiotensin II receptors are also fertile [42]. More studies will be needed to identify the substrate for gACE in the male genital tract involved in male fertility.

Significant heterogeneity was found in the levels of carboxypeptidase activity in the epididymal fluid and seminal plasma between rams, but the level of activity was correlated with the number of spermatozoa in these fluids. These results confirm that the presence of ACE in the ejaculate is highly correlated with the spermatozoa concentration [12]. No statistically significant relationship was found between seminal plasma ACE activity and fertility, although the animals with the lowest fertility also had the lowest seminal activity (but not the lowest numbers of sperm). However, few animals with low fertility were in the assay group, and a larger group of animals will be required to confirm the tendency we observed in this study.

To explain the infertility of male *ACE* KO mice, it was previously suggested that fewer sperm were able to go through the female genital tract and bind to the oocyte [13]. Ram sperm, like horse and human sperm, retain part of the gACE after epididymal maturation, and this ACE is localized on the acrosome [12, 43]. Angiotensin II has been reported to activate sperm motility and to induce sperm acrosome reaction [44] (for review, see [45]). The remaining sperm gACE could, therefore, play a role in capacitation/acrosomal reaction and, thus, recognition of the oocyte zona pellucida. Our in vitro fertilization experiments showed that inhibition of ACE did not change the number of fertilized oocytes or blastocysts. We even observed that the presence of captopril was advantageous for fertilization and blastocyst development, perhaps because of its antioxidizing effect [46]. This result confirmed those obtained using human sperm incubated with oocytes and captopril showing no difference in acrosome reaction or oocyte binding [43] and those obtained using ejaculated horse sperm that were able to bind to the zona pellucida after incubation with IgG or Fab against the ACE [47]. All these results indicate that inhibition of gACE does not impair binding or fertilization of oocytes in these different species.

The question regarding the exact role of gACE on the sperm and epididymal function, however, remains. The sperm enzyme could act on the female genital tract to improve sperm transport, but female mice without angiotensinogen or bradykinin receptors are apparently fertile. Another explanation is that gACE plays a role during epididymal sperm maturation, which can affect their capacity to bind to the oocyte. Epididymal maturation is recognized as a critical step in the acquisition of this capacity by sperm, and subtle changes in one stage of this process can result in male infertility without affecting other important factors, such as sperm motility [48, 49].

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