A comparison of the zinc contents and substrate specificities of the endothelial and testicular forms of porcine angiotensin converting enzyme and the preparation of isoenzyme-specific antisera

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Angiotensin converting enzyme (ACE; EC 3.4.15.1) was purified from porcine kidney and lung (endothelial isoenzyme) and testis (testicular isoenzyme) by affinity chromatography on lisinopril-2.8 nm-Sepharose. Atomic-absorption spectroscopy revealed that ACE purified from kidney and lung contained 2.58 and 2.35 atoms of zinc per molecule of enzyme (Mr, 147000) respectively. In contrast, ACE purified from testis contained only 1.58 atoms of zinc per molecule of enzyme (Mr, 80000). Thus it would appear that both putative zinc-binding sites in endothelial ACE contain zinc and may therefore be catalytically active. No differences were observed in the pattern of products generated on hydrolysis of benzoyl (Bz)-Gly-His-Leu, substance P, luteinizing-hormone-releasing hormone (LH-RH) and its analogue, des-Gly10-LH-RH-ethylamide, by kidney and testicular ACE. There was also no difference in the initial rates of hydrolysis of Bz-Gly-His-Leu or substance P by the two isoenzymes, although LH-RH and its analogue were hydrolysed twice as rapidly by kidney ACE. It is therefore unlikely that the N-terminal catalytic site in porcine endothelial ACE is predominantly responsible for the atypical cleavage of LH-RH generating the N-terminal tripeptide. Two polyclonal antisera were raised to the affinity-purified forms of pig kidney and testicular ACE. Isoenzyme-specific antisera were then isolated from these by absorbing out those antibodies recognizing determinants on the other isoenzyme. Immunoelectrophoretic blot analyses and immunofluorescent staining of sections of pig kidney were used to demonstrate the specificity of the antisera. Immunofluorescent staining of sections of pig testis with the antiserum specific to testicular ACE localized testicular ACE solely to the lumen of the seminiferous tubules, whereas the antiserum specific to endothelial ACE revealed the presence of this isoenzyme only in blood vessels. The antiserum to endothelial ACE, which recognizes determinants in the unique N-terminal domain, was investigated as a possible specific inhibitor of the N-terminal catalytic site. Although this antiserum failed to inhibit testicular ACE, the effect on the activity of endothelial ACE appeared to be due to inhibition of both the N- and C-terminal catalytic sites.

INTRODUCTION

Angiotensin converting enzyme (ACE; peptidyl dipeptidase A, EC 3.4.15.1) is a zinc metallopeptidase that displays a broad distribution in mammalian tissues. The classical physiological substrates of ACE are angiotensin I, which is activated to the potent vasopressor peptide angiotensin II, and the vasodilatory peptide bradykinin, which is inactivated (Soffer, 1976). As a consequence, ACE has become a crucial enzyme target in the treatment of hypertension and congestive heart failure, leading to the development of selective inhibitors, e.g. captopril and enalaprilat, to control these conditions (Cushman & Ondetti, 1980; Abrams et al., 1984; Gavras & Gavras, 1988; Mancia, 1991). ACE has been purified from a number of different mammalian tissues and appears to display some degree of heterogeneity with respect to molecular size. However, there are only two distinct isoenzymes. The larger form (Mr, 140000–180000, depending on species) is present throughout the body on both endothelial and epithelial cell surfaces, with the highest concentrations found in lung and kidney [for reviews, see Patchett & Cordes (1985), Ehlers & Riordan (1989) and Hooper (1991)]. This form of ACE is referred to here as ‘endothelial ACE’. A distinct and smaller isoenzyme of ACE (Mr, 90000–110000, depending on species) is found exclusively in the testes (El-Dorry et al., 1982; Lanzillo et al., 1985) and is referred to here as ‘testicular ACE’.

The cDNAs for both endothelial (Soubrier et al., 1988; Bernstein et al., 1989) and testicular (Ehlers et al., 1989; Kumar et al., 1989; Lattion et al., 1989) ACE have now been sequenced and their corresponding amino acid sequences deduced. Endothelial ACE is composed of two highly similar domains, referred to as the N- and C-terminal domains, each bearing a putative zinc-binding motif (His-Glu-Met-Gly-His), implying the presence of two catalytic sites. In contrast, the cDNA-deduced amino acid sequence of testicular ACE, with the exception of 67 amino acids at the N-terminus, is identical with the C-terminal domain of endothelial ACE and contains only a single zinc-binding motif. For both putative catalytic sites in endothelial ACE to be active, an atom of zinc would need to be bound at each zinc-binding motif. Earlier experiments, though, predicted a single atom of zinc to be bound per molecule of endothelial ACE (Das & Soffer, 1975; Bunning et al., 1983; Bunning & Riordan, 1985), thus implying that only one of the two putative catalytic sites is active. However, the construction of mutants of endothelial ACE containing only one intact zinc-binding site demonstrated that both domains were catalytically active, each able to cleave the C-terminal dipeptide from benzoyl (Bz)-Gly-His-Leu and angiotensin I (Wei et al., 1991). One of the groups which performed some of the original zinc analyses has recently re-analysed the zinc content of ACE and obtained values of approx. 2 and 1 atoms of zinc per molecule of enzyme for the

Abbreviations used: ACE, angiotensin converting enzyme; FITC, fluorescein isothiocyanate; LH-RH, luteinizing-hormone-releasing hormone (luteinizing); Bz, benzoyl.

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endothelial and testicular isoenzymes respectively (Ehlers & Riordan, 1991).

In addition to its classical specificity to act as a peptidyl dipeptidase, ACE can act in vitro as an endopeptidase releasing C-terminal tripeptides and or N-terminal tripeptides from certain terminally blocked peptides, e.g. substance P and luteinizing-hormone-releasing hormone (LH-RH) (Yokosawa et al., 1983; Skidgel & Erdös, 1985). It is conceivable that the N- and C-terminal catalytic sites of endothelial ACE may be involved differently in these atypical cleavages. In support of this, Ehlers & Riordan (1991) have reported that, of a range of peptides examined, only LH-RH and its analogue, des-Gly10-LH-RH-ethylamide, were hydrolysed at significantly higher rates by endothelial ACE compared with testicular ACE, and that the patterns of product peaks produced were different for the two isoenzymes. These observations suggest that the two catalytic sites may have different substrate specificities.

ACE has been mapped in most tissues by immunohistochemistry employing either monoclonal or polyclonal antibodies by both optical and electron microscopy (e.g. Defendini et al., 1983; Danilov et al., 1987; Barnes et al., 1988; Schulz et al., 1988). In addition, radiolabelled inhibitors (e.g. [3H]captopril, [125I]MK351A) have also been used to localize ACE by autoradiography (e.g. Mendelsohn, 1984; Strittmatter & Snyder, 1984). However, these approaches have limitations when attempting to define the distribution of testicular ACE, owing to the immunological and catalytic similarities between the two isoenzymes.

In the present study we have analysed the zinc content of porcine endothelial and testicular ACE and compared the patterns and initial rates of hydrolysis by the two isoenzymes of certain substrates which are cleaved atypically. Furthermore, we have prepared antibodies specific for each isoenzyme from polyclonal antisera generated to each isoenzyme by absorbing out the cross-reacting antibodies on a column to which the other isoenzyme had been immobilized. These isoenzyme-specific antisera have then been employed to map the two forms by immunohistochemistry. In addition, we have explored the possibility of one of these antisera acting as a selective inhibitor of the N-terminal catalytic site of endothelial ACE.

**EXPERIMENTAL**

**Materials**

Pig kidneys, lungs and testes were kindly provided by ASDA Farmstores, Lofthousegate, W. Yorkshire, U.K., and were obtained within 10 min of death. Testes were dissected from the epididymis, surrounding capsule and other associated tissues and stored at -70 °C until required. Kidney cortex was dissected out and stored similarly. Adult male New Zealand White rabbits (2-3 kg) were used to raise polyclonal antibodies and were provided and maintained by the University of Leeds Animal Service. LH-RH, des-Gly10-LH-RH-ethylamide and substance P were purchased from BACHEM (U.K.) Ltd., Saffron Walden, Essex, U.K., and were >99% pure as determined by h.p.l.c. analysis. Bz-Gly-His-Leu and the donkey anti-rabbit IgG [fluorescein isothiocyanate (FITC)-conjugated] were from Sigma Chemical Co. Lisinopril [MK251, N-[S-(1-carboxy-3-phenylpropyl)-L-lysyl-L-proline] and enalaprilat [MK422, N-[S-(1-carboxy-3-phenylpropyl)-L-alaninyl-L-proline)] were gifts from Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A. All other materials were from sources previously noted.

**Purification of ACE**

ACE was purified from pig kidney, lung and testis by a modification of the method described by Hooper et al. (1987), using lisinopril-2.8 nm-SePro5ase as the affinity resin. The major difference was that bound enzyme was eluted with 50 mM-Na2B407/0.1% (w/v) Triton X-100, pH 9.5 (Ehlers et al., 1986) instead of with free lisinopril.

**Zinc analyses**

All buffers were made up with deionized water. Dialysis tubing was boiled in the presence of EDTA and then washed extensively with deionized water. All glassware was acid-washed in 6 M HCl and rinsed thoroughly with deionized water before use. Enzyme samples (0.2 mg of protein/ml) were dialysed against three changes of 5 mM-Tris/HCl/0.1% (w/v) Triton X-100, pH 7.9 at 4 °C to remove any non-specifically bound zinc. Protein was determined by the method of Bensadoun & Weinstein (1976) and by using biocinchonic acid in a microtitre plate assay; both methods gave results which did not differ by more than 10%. Duplicate 0.4 ml aliquots of each sample were analysed on an Instrumental Laboratories IL 157 atomic-absorption spectrometer (Thermo Electron Ltd., Birchwood, Warrington, Cheshire, U.K.). ZnSO4 (0-6 μm) was used as standard.

**Hydrolysis of peptides**

Equimolar amounts (1.12 pmol) of affinity-purified pig kidney ACE (M180000) or pig testicular ACE (M110000) were incubated with LH-RH (0.1 nm) or des-Gly10-LH-RH-ethylamide (0.1 mm) in 50 mM-Hepes/0.3 M-NaCl, pH 7.5, for 18 h at 37 °C. With substance P (0.1 mm), equimolar amounts (0.56 pmol) of kidney or testicular ACE were incubated in 50 mM-Hepes/0.3 M-NaCl, pH 7.5, for 90 min at 37 °C. Routine assays with Bz-Gly-His-Leu (5 mm) were carried out in 0.1 M-Tris/HCl/0.3 M-NaCl/10 μM-ZnCl2, pH 8.3. Reactions were terminated by heating at 100 °C for 4 min, and the substrate and reaction products resolved and quantified by reverse phase h.p.l.c. as described in Hooper & Turner (1987).

**Production and purification of antibodies**

Antibodies were raised against purified kidney and testicular ACE in New Zealand White rabbits. An IgG fraction was prepared from the serum by affinity chromatography on a column of Protein A-Sepharose. Antibodies cross-reacting with the other isoenzyme were removed from the IgG fraction by affinity chromatography on a column of the appropriate isoenzyme immobilized on CNBr-activated Sepharose as described in Matsas et al. (1986) for endopeptidase-24.11. RP127, which was raised to pig kidney ACE, was passed down a column of immobilized pig testicular ACE. The population of antibodies that did not bind to the immobilized testicular ACE should therefore be specific for kidney ACE and are referred to as RP127k (see Table 1). Likewise, RP175, which was raised to pig testicular ACE, was passed down a column of immobilized pig kidney ACE. The population of antibodies that did not bind to the immobilized kidney ACE should therefore be specific for testicular ACE and are referred to as RP175t (see Table 1).

**Immunoelectrophoretic-blot analysis**

SDS/PAGE was performed by the system of Laemmli (1970), with a 7-17% (w/v) polyacrylamide gradient as described previously (Relton et al., 1983). Immunoelectrophoretic-blot analysis was performed as described by Hooper & Turner (1987), except that poly(vinylidene difluoride) transfer membranes were used together with a [125I]labelled anti-rabbit IgG second antibody as described by Hooper et al. (1990).

**Immunohistochemistry**

Specimens of kidney cortex from 2-4-week-old piglets and testis from adult pigs were obtained immediately after death and...
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Table 1. Properties of the antibodies used in the present study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Immobilized ligand used to purify antibody</th>
<th>Cross-reactivity on immunoelectrophoretic-blot analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP127</td>
<td>Kidney ACE</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>RP127₇₅</td>
<td>Kidney ACE</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>RP175</td>
<td>Testicular ACE</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>RP175₇₅</td>
<td>Testicular ACE</td>
<td>–</td>
<td>Yes</td>
</tr>
</tbody>
</table>

blocks frozen and stored as described by Matsas et al. (1986). Cryostat sections (8 μm) were cut, and immunofluorescent staining was performed by the method of Gee et al. (1983). Primary antibodies (approx. 2 μg of IgG protein/ml final concentration) were applied for 2 h at 22 °C, followed by a second antibody, donkey anti-rabbit IgG (FITC-conjugated) (dilution 1 in 50). All antibodies were diluted in 0.2% (w/v) gelatin in 50 mM-Tris/HCl/0.1% NaCl, pH 7.4, containing a pig IgG fraction (1 mg/ml). Controls were prepared by replacing the first antibody with either 0.2% (w/v) gelatin in 50 mM-Tris/HCl/0.1% NaCl, pH 7.4, or a rabbit preimmune IgG. In both cases no specific immunofluorescence was observed.

Inhibitor studies using anti-ACE antibodies

Equivalent activities of affinity purified kidney or testicular ACE (8.3 nmol of Bz-Gly produced/min) were incubated in the absence and presence of RP127₇₅ in 0.1 mM-Tris/HCl/0.3% NaCl/10 μM-ZnCl₂, pH 8.3, for 72 h at 4 °C. Control incubations replaced RP127₇₅ with equivalent concentrations of a rabbit preimmune IgG. Aliquots (90 μl) of the enzyme/antibody mixture were incubated with Bz-Gly-His-Leu (5 mM) for 45 min at 37 °C or with LH-RH (0.1 mM) for 18 h at 37 °C. After terminating the reaction by heating at 100 °C for 4 min, the substrate and reaction products were resolved and quantified by reverse-phase h.p.l.c. as described by Hooper & Turner (1987).

RESULTS AND DISCUSSION

Purification and zinc analyses of endothelial and testicular ACE

ACE from pig kidney, lung and testes was purified by affinity chromatography on lisinopril-2.8 nm-Sepharose to apparent homogeneity as determined by SDS/PAGE (results not shown). ACE purified from kidney or lung migrated as a single polypeptide band with an apparent Mₐ of 180000. In contrast, ACE purified from testis migrated as a single polypeptide with an apparent Mₐ of 110000. The zinc content of endothelial ACE was determined by atomic-absorption spectroscopy and compared with that of the testicular enzyme (Table 2). ACE purified from pig kidney or lung had a similar zinc content of 2.58 and 2.35 atoms of zinc per molecule of enzyme respectively. In contrast, analysis of testicular ACE revealed a lower zinc content compared with the kidney or lung enzymes of 1.58 atoms of zinc per molecule of enzyme. Membrane dipeptidase, another zinc-metallopeptidase, contained 0.92 atom of zinc per molecule of enzyme subunit, in agreement with previous results (Armstrong et al., 1974). Thus these results indicate the presence of one atom of zinc at each of the two putative zinc-binding sites predicted from the CDNA sequence of endothelial ACE, in agreement with the more recent results of Ehlers & Riordan (1991). The slightly higher values observed in the present study may be caused by using Mₐ values predicted from the human CDNA-derived sequences.

Hydrolysis of peptides by endothelial and testicular ACE

ACE acts as an endopeptidase on, among other substrates, substance P and LH-RH. Substance P is hydrolysed by ACE by one of two pathways: either the C-terminal dipeptide amide is removed, followed by the subsequent release of dipeptides from the newly formed C-terminus, or the C-terminal dipeptide amide is removed initially, with subsequent dipeptides released (Yokosawa et al., 1983). In the case of LH-RH, ACE releases tripeptide fragments from both the N- and C-termini [LH-RH-(1–3)-peptide; pGlu-His-Trp and LH-RH-(8–10)-peptide; Arg-Pro-GlyNH₂] (Skidgel & Erdös, 1985). At high chloride-ion concentrations, however, release of the N-terminal tripeptide predominates producing LH-RH-(1–3)-peptide and LH-RH-(4–10)-peptide. We therefore investigated the hydrolysis of substance P, LH-RH and the LH-RH analogue des-Gly⁶⁸-LH-RH-ethylamide by endothelial and testicular ACE in order to examine whether these atypical cleavages can be attributed to one or other of the catalytic sites in endothelial ACE. Equimolar amounts of affinity purified porcine kidney or testicular ACE were incubated with various peptide substrates (Table 3). Bz-Gly-His-Leu and substance P were hydrolysed at comparable initial rates by both kidney and testicular ACE. However, LH-RH and des-Gly⁶⁸-LH-RH-ethylamide were hydrolysed more rapidly (2.3- and 1.9-fold respectively) by kidney ACE than by testicular ACE. The patterns of hydrolysis of substance P and des-Gly⁶⁸-LH-RH-ethylamide by kidney and testicular ACE were identical (results not shown). With LH-RH the ratio of the peak areas, the N-terminal tripeptide LH-RH-(1–3)-peptide and the C-terminal heptapeptide LH-RH-(4–10)-peptide, were identical with both kidney and testicular ACE (Fig. 1). These results

Table 2. Zinc analyses of ACE purified from pig kidney, lung and testes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity</th>
<th>Enzyme</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mₐ (μmol of Bz-Gly/min per mg)</td>
<td></td>
<td>Mₐ (μmol of Bz-Gly/min per μmol of protein)</td>
</tr>
<tr>
<td>Kidney ACE</td>
<td>146.6*</td>
<td>6.20</td>
<td>1.05</td>
</tr>
<tr>
<td>Lung ACE</td>
<td>146.6*</td>
<td>6.90</td>
<td>1.17</td>
</tr>
<tr>
<td>Testicular ACE</td>
<td>80†</td>
<td>11.00</td>
<td>1.21</td>
</tr>
<tr>
<td>Membrane dipeptidase</td>
<td>44.7‡</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Data from Soubrier et al. (1988).
† Data from Ehlers et al. (1989).
‡ Data from Rachef et al. (1990).
Equimolar amounts of kidney or testicular ACE were incubated with the indicated substrate at 37 °C in 50 mM-Hepes/0.3 mM- NaCl, pH 7.5, and the substrate and reaction products resolved and quantified by h.p.l.c. as described in the Experimental section. The extent of Bz-Gly-His-Leu hydrolysis was calculated by the amount of Bz-Gly produced. The extent of hydrolysis of the other peptides was calculated by comparison of the peak area of remaining substrate with the peak area of the unchanged substrate. Results are means (±S.E.M.) for four separate determinations. The inclusion of 10 μM-enalaprilat in control incubations completely inhibited the hydrolysis of all substrates by both kidney and testicular ACE.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Extent of hydrolysis (%)</th>
<th>Ratio (kidney/testicular)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bz-Gly-His-Leu</td>
<td>8.6±0.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Substance P</td>
<td>22.3±0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>LH-RH</td>
<td>14.4±1.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Des-Gly₁⁰-LH-RH-ethylamide</td>
<td>9.1±0.7</td>
<td>1.9</td>
</tr>
</tbody>
</table>

suggest that the N-terminal catalytic site in endothelial ACE may play a greater role than the C-terminal site in the cleavage of the Trp³-Ser⁴ bond of LH-RH, but that there are no qualitative differences in specificity between the two sites, at least in regards to the peptides examined here. This is in contrast with the recent report indicating that LH-RH and des-Gly₁⁰-LH-RH-ethylamide are hydrolysed > 300-fold faster by human endothelial ACE than by the testicular isoenzyme, and that the pattern of hydrolysis of LH-RH was different for the two isoenzymes (Ehlers & Riordan, 1991). The reasons for the apparent differences in the rate and pattern of hydrolysis of LH-RH by the two isoenzymes of ACE between these two studies are unclear, but may reflect species differences. However, from the data of Ehlers & Riordan (1991) it is not clear whether the initial rates of hydrolysis were being compared, as in the present study.

Preparation of antibodies specific to either endothelial or testicular ACE

A polyclonal antibody raised to pig kidney ACE (RP127) was used in immunoelectrophoretic blot analysis of affinity-purified kidney and testicular ACE (Fig. 2, lanes 1 and 2). RP127 recognized kidney ACE, Mᵣ 180000, and the lower-Mᵣ, 110000 testicular isoenzyme, owing to their common determinants, in agreement with previous results (El-Dorry et al., 1982). Antibodies specific to kidney ACE were prepared by absorption of the antibody RP127 on a column of testicular-ACE-Sepharose to remove cross-reacting antibodies. The population of antibodies recovered in the unbound fraction were termed RP127ₓ (see Table 1) and were then used in immunoelectrophoretic-blot analysis of affinity-purified kidney and testicular ACE (Fig. 2, lanes 3 and 4). RP127ₓ recognized only kidney ACE and not testicular ACE. A polyclonal antibody (RP175) was also raised to purified testicular ACE. Affinity-purified testicular and kidney ACE were then subjected to immunoelectrophoretic blot analysis using RP175 (Fig. 2, lanes 5 and 6). RP175 recognized the Mᵣ-110000 form of testicular ACE and cross-reacted to a lesser extent with the higher-Mᵣ, 180000 form of kidney ACE. Cross-reacting antibodies were removed from the antibody RP175 by absorption on a column of kidney-ACE-Sepharose. The population of antibodies recovered in the unbound fraction were termed RP175ᵧ (see Table 1). Immunoelectrophoretic blot analysis of affinity-purified testicular and kidney ACE (Fig. 2, lanes 7 and 8) showed that RP175ᵧ recognized only testicular ACE and not kidney ACE. Thus these results are consistent with RP175ᵧ recognizing determinants present on the unique N-terminal sequence of testicular ACE and with RP127ₓ recognizing determinants on the unique N-terminal domain of endothelial ACE (see Fig. 3).

Immunofluorescent staining of sections of pig kidney

The antibody preparations were then used for immunohistochemical studies on sections of pig kidney. The localization of ACE in sections of pig kidney was viewed by immunofluorescence microscopy (Fig. 4). Both RP127 and RP127ₓ recognize determinants located on kidney ACE (see above). Accordingly, the pattern of immunofluorescent staining of kidney sections produced by these two antibody preparations was very similar (Figs. 4a and 4b respectively). Intense staining of glomeruli and convoluted tubules was evident, in agreement with previous
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Fig. 2. Immunoelectrophoretic blot with anti-(kidney ACE) and anti-(testicular ACE) antibodies

Samples of affinity-purified pig kidney ACE (lanes 1, 3, 5 and 7; 1.0 μg of protein) or testicular ACE (lanes 2, 4, 6 and 8; 1.0 μg of protein) were prepared and analysed as described in the Experimental section. After electrophoretic transfer to poly(vinylidene difluoride) membranes, the tracks were blotted with either RP127 (lanes 1 and 2), RP127k (lanes 3 and 4), RP175 (lanes 5 and 6) or RP175k (lanes 7 and 8). Bound antiserum was detected with a 125I-labelled second antibody, followed by autoradiography.

Fig. 3. Schematic comparison of testicular and endothelial ACE showing the putative sites of binding of the antibodies used in the present study

The structure of testicular ACE is shown at the top of the Figure and that of endothelial ACE at the bottom. T, testicular; E, endothelial. The amino acids at the junction of the domains are numbered as for the human isoenzymes (Soubrier et al., 1988; Lattion et al., 1989). Domains T2 and E2 have 100% identity. T1 and E1 are unique to the respective isoenzymes. The zinc-binding sites are represented by HEMGH.

reports (e.g. Defendini et al., 1983). The antibody raised to pig testicular ACE (RP175) produced a pattern of fluorescent staining (Fig. 4c) that was similar to that produced by RP127 and RP127k. This is in agreement with the immunoelectrophoretic-blot analysis (Fig. 2), which showed that RP175 cross-reacted with both testicular ACE (M, 110000) and kidney ACE (M, 180000). In contrast, RP175k, which recognizes only specific determinants on testicular ACE, failed to produce fluorescent staining of sections of pig kidney (Fig. 4d), consistent with the absence of this isoenzyme in this tissue.

Immunofluorescent staining of sections of pig testis

ACE has been localized in the male reproductive tract both by immunohistochemistry using antibodies to endothelial ACE (Yotsumato et al., 1984; Berg et al., 1986; Brentjens et al., 1986) and by autoradiography using [3H]captopril (Strittmatter & Snyder, 1984; Strittmatter et al., 1985). The distribution of the two isoenzymes has been defined indirectly by comparing the pattern of staining either with [3H]captopril at different stages of development and after hypophysectomy (Strittmatter et al., 1985) or after preincubation of an anti-(endothelial ACE) antibody with either endothelial or testicular ACE (Berg et al., 1986). These studies have demonstrated that testicular ACE is present in the seminiferous tubules of the testis but not in the interstitial Leydig cells, and that endothelial ACE is present on the luminal surface of blood vessels. In the present study we have directly compared the localization of the endothelial and testicular forms of ACE using our isoenzyme-specific antibodies. Sexually mature pigs (12 weeks old) were used for this study, since it is established that the testicular form of ACE is developmentally controlled, being expressed at the onset of puberty (Velletri, 1985). Figs. 5 and 6 show phase-contrast and fluorescence micrographs of sections of pig testis with the different antibodies. The use of RP127 as the primary antibody produced an intense fluorescence of seminiferous tubules and blood vessels (Fig. 5b). This is in marked contrast with the pattern of immunofluorescence produced using the anti-(endothelial ACE) antibody, RP127k (Fig. 5d). In this instance the blood vessels were intensely fluorescent, whereas negligible fluorescence was observed in the seminiferous tubules (Fig. 5d). The polyclonal antibody raised to pig testicular
ACE (RP175) produced fluorescence of seminiferous tubules and blood vessels (Fig. 6b), a pattern similar to that produced by RP127 (Fig. 5b). With the anti-(testicular ACE) antibody, RP175	extsubscript{n}, only the seminiferous tubules were fluorescent, with the intensity of staining increasing towards the centre of the tubule (Fig. 6d), reflecting the increased expression of testicular ACE as spermatids mature (Strittmatter & Snyder, 1984: Berg et al., 1986). No fluorescence was observed in the blood vessels with RP175	extsubscript{n}. These antibody preparations specific to endothelial and testicular ACE may be useful tools for mapping the relative distribution of these two isoenzymes throughout the male genital tract during development and for confirming that it is a soluble form of testicular ACE which is present in mature spermatids (Strittmatter et al., 1985).

Inhibition of kidney and testicular ACE activity by the anti-(endothelial ACE) antibody

A useful tool for elucidating the specificity and function of the N-terminal catalytic site of endothelial ACE would be an inhibitor selective for this site and not the C-terminal site. The specificity of RP127	extsubscript{k} for endothelial ACE was consistent with this antibody recognizing only epitopes specific to the N-terminal domain (see Fig. 3). The use of RP127	extsubscript{k} as a putative inhibitor of the catalytic site of the N-terminal domain was, therefore, investigated (Fig. 7). A ratio of antibody to enzyme of 15:1 and 20:1 (µg of IgG/µg of protein) resulted in a marked inhibition of both Bz-Gly-His-Leu and LH-RH hydrolysis (Figs. 7a and 7b respectively) by kidney ACE. In contrast, no inhibition of testicular ACE was observed at any of the concentrations of RP127	extsubscript{k} tested. Rabbit preimmune IgG also failed to inhibit either kidney or testicular ACE at the concentrations tested. However, it was apparent that the observed inhibition of kidney ACE was not specific for the N-terminal domain. The present data demonstrated that the hydrolysis of Bz-Gly-His-Leu was almost completely abolished (89% inhibition) by RP127	extsubscript{k}, which cannot be attributed to the selective inhibition of the N-terminal domain. Wei et al. (1991), using their mutant constructs in which either the N- or C-terminal domain was deleted or inactivated, showed that, at 300 mM-chloride, the C-terminal domain hydrolysed Bz-Gly-His-Leu 4-5-fold faster than the N-terminal domain. There-
Fig. 7. Inhibition of kidney and testicular ACE by RP127K

Antibody RP127K was incubated with equivalent activities, in terms of Bz-Gly-His-Leu hydrolysis, of either purified kidney or testicular ACE and enzyme activity then assayed with either (a) Bz-Gly-His-Leu or (b) LH-RH as described in the Experimental section. The relative activities (%R) were calculated from (a) the peak area of Bz-Gly compared with the corresponding peak from incubations performed in the absence of antibody or similarly from (b) the peak area of the N-terminal tripeptide (pGlu-His-Trp) of LH-RH. Each point is the mean of duplicate determinations which did not differ by more than 5%. ( ), Kidney ACE; ( ), testicular ACE. Closed symbols, preincubated with RP127K; open symbols, preincubated with rabbit preimmune IgG.

fore, approx. 20% inhibition of kidney ACE, with Bz-Gly-His-Leu as substrate, would be expected if the inhibition by RP127K was specific for the catalytic site in the N-terminal domain. Although the binding of RP127K to the N-terminal domain of endothelial ACE may have achieved partial, if not total, inhibition of this catalytic site, inhibition of the catalytic site in the C-terminal domain must also have occurred. One possibility is that binding of antibody to the N-terminal domain may induce a change in conformation of the enzyme which prevents access or perturbs binding of substrate to both catalytic sites. An alternative possibility may reflect the three-dimensional structure of endothelial ACE. If the enzyme is folded such that the catalytic sites are within close proximity of each other, then the binding of antibodies to epitopes located at or near the catalytic site of the N-terminal domain may also impede access of substrate to the C-terminal domain.

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