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Angiotensin AT_4 receptor ligand interaction with cystinyl aminopeptidase and aminopeptidase N: [^{125}I]Angiotensin IV only binds to the cystinyl aminopeptidase apo-enzyme

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Abstract

Due to its high affinity for [^{125}I]Angiotensin IV, cystinyl aminopeptidase (CAP) has recently been assigned as the 'angiotensin AT_4 receptor'. Since the aminopeptidase N (AP-N) activity is also susceptible to inhibition by Angiotensin IV, it might represent an additional target for this peptide. Based on [^{125}I]Angiotensin IV binding and catalytic activity measurements, we compared the ligand interaction properties of recombinant human CAP and human AP-N. Both enzymes displayed distinct pharmacological profiles. Although their activity is inhibited by Angiotensin IV and LVV-hemorphin 7, both peptides are more potent CAP-inhibitors. On the other hand, substance P and L-methionine have a higher potency for AP-N. High affinity binding of [^{125}I]Angiotensin IV to CAP occurs in the presence of chelators but not to AP-N in either the absence or presence of chelators. These differences were exploited to determine whether CAP and/or AP-N are present in different cell lines (CHO-K1, COS-7, HEK293, SK-N-MC and MDBK). We provide evidence that CAP predominates in these cell lines and that, comparatively, CHO-K1 cells display the highest level of this enzyme.

Keywords: Angiotensin IV; Angiotensin AT_4 receptor; Cystinyl aminopeptidase; Aminopeptidase N

1. Introduction

Cystinyl aminopeptidase (CAP, EC 3.4.11.3), is a membrane bound ectoenzyme of the M1 metallopeptidase family and is most often referred to as the insulin regulated aminopeptidase (i.e. IRAP). It occurs in intracellular endosome-derived vesicles along with the glucose transporter GLUT4 (Keller et al., 1995). Translocation of these vesicles to the plasma membrane is greatly enhanced by insulin in healthy subjects (Keller et al., 1995; Ross et al., 1997) but not in type II diabetic patients (Garvey et al., 1998). CAP is also known as oxytocinase, vasopressinase and placental leucine aminopeptidase. The CAP ectodomain can be cleaved and, since its serum level increases dramatically with gestation, it has been proposed to contribute to the maintenance of normal pregnancy by hydrolysing oxytocin and vasopressin (Rogi et al., 1996; Mizutani, 1998). Since [^{125}I]Angiotensin IV displays high affinity for CAP, it has recently been assigned as the 'angiotensin AT_4 receptor', a member of the angiotensin receptor family (Albiston et al., 2001). These binding sites have been shown to occur in various tissues and cell types (Zhang et al., 1999; Miller-Wing et al., 1993; Hanesworth et al., 1993; Chai

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et al., 2000). The pharmacological profile of the 'angiotensin AT_4 receptor' differs markedly from either the angiotensin AT_1 or AT_2 receptors. It displays a low affinity for Angiotensin II and the classical angiotensin AT_1 and AT_2 receptor antagonists, but high affinity for Angiotensin IV (Miller-Wing et al., 1993; Hanesworth et al., 1993), synthetic peptide analogues like divalinal-Angiotensin IV and endogenous peptides like LVV-hemorphin 7 (Krebs et al., 1996; Moeller et al., 1997). The interaction between 'angiotensin AT_4 receptor' ligands and human CAP in recombinant HEK293 cells or CAP endogenously expressed in CHO-K1 cells has also been extensively characterized by [^{125}I]Angiotensin IV binding and by catalytic activity measurements (Albiston et al., 2001; Lew et al., 2003; Demaegdt et al., 2004a).

Angiotensin IV (Angiotensin (3–8)) is a naturally occurring amino-terminal deleted fragment of Angiotensin II. This peptide mediates a range of physiological effects, including facilitation of memory retention and retrieval in rats as well as hemodynamic actions in the renal and vascular system (Handa et al., 1998; Coleman et al., 1998; Kramar et al., 1997; Braszko et al., 1988; Wright et al., 1999; Pederson et al., 1998; Lee et al., 2004). Since the major substrates of CAP; oxytocine, vasopressin and somatostatin, are known to play an important role in cognitive function (Alescio-Lautier et al., 2000; Matsuoka et al., 1995; Kovacs and De Wied, 1994), it was proposed that Angiotensin IV mediates its physiological effects through inhibition of CAP catalytic activity (Albiston et al., 2001). However, such mechanism does not explain the many effects of Angiotensin IV and related peptides in isolated cell systems. These include the induction of cytosolic Ca^{2+} transients, increased cGMP and nitric oxide production, DNA- and RNA synthesis and nuclear factor κB activation (Coleman et al., 1998; Patel et al., 1998; Mustafa et al., 2001; Wang et al., 1995; Esteban et al., 2005). To explain these latter findings, it has been proposed that CAP may also act as a classical receptor or, alternatively, that some recognition sites for Angiotensin IV still need to be disclosed (Vauquelin et al., 2002; Albiston et al., 2003; Wright and Harding, 2004). In this respect, the catalytic activity of soluble aminopeptidase N (i.e. the cleaved, AP-N ectodomain, M1, EC 3.4.11.2) has also been found to be susceptible to inhibition by LVV-hemorphin 7 and Angiotensin IV (Garreau et al., 1998). Interestingly, it has also been documented that AP-N, which is closely related and structurally similar to CAP, is able to induce a variety of responses in immune cells in the presence of monoclonal antibodies (Santos et al., 2000). These findings spark the possibility for AP-N to constitute an alternative receptor for Angiotensin IV and related ligands.

In the present study, catalytic activity measurements and [^{125}I]Angiotensin IV binding studies were used to compare the ligand interaction properties of human CAP and human AP-N in both recombinant HEK293 cells and soluble porcine AP-N. Both enzymes displayed distinct pharmacological profiles and, most importantly, no high affinity binding of [^{125}I]Angiotensin IV to AP-N could be detected under the conditions most favourable for CAP detection. These properties were used to examine the presence of CAP and/or AP-N in different cell lines (CHO-K1, COS-7, HEK293, SK-N-MC and MDBK). Most of these cell lines were earlier described to contain 'angiotensin AT4 receptors' (SK-N-MC and MDBK cells, based solely on binding studies, Mustafa et al., 2001; Handa et al., 1999) and/or CAP activity (CHOK1 and HEK293 cells, Demaegdt et al., 2004a; Lew et al., 2003). The remaining COS-7 cell line was also studied because of its wide use in transfection studies.

2. Materials and methods

2.1. Materials

Angiotensin II, Angiotensin III or Angiotensin II (2–8), Angiotensin IV or Angiotensin II (3–8) and substance P were obtained from NeoMPS (Strasbourg, France), Angiotensin II (4–8) from Bachem (Bubendorf, Switzerland), LVV-hemorphin 7 from Invitrogen (Merelbeke, Belgium) and L-methionine from Sigma-Aldrich (Bornem, Belgium). Divalinal-Angiotensin IV was synthesized by Dr. G. Munske (Washington State University, USA). L-leucine-*p*-nitroanilide (L-Leu-*p*NA) and L-alanine-*p*-nitroanilide were obtained from Sigma-Aldrich (Bornem, Belgium) and *p*-nitroaniline from VWR International (Leuven, Belgium). Tyr^4 of Angiotensin IV was iodinated using the Iodogen iodination reagent from Pierce (Erembodegem, Belgium) as described by Lahoutte et al. (2003). ^{125}I was obtained from MP Biomedicals (Asse, Belgium). Monoiodinated Angiotensin IV was isolated on a GraceVydac C18 monomeric 120A reverse-phase HPLC column and stored at −20 °C in 10 mM KH_2PO_4, pH 6.5 containing 45% ethanol. All other reagents were of the highest grade available commercially. CHO-K1 cells (Chinese hamster ovary cells) were kindly provided by the Pasteur Institute (Brussels, Belgium), HEK293 (human embryonal kidney cells) and SK-N-MC cells (human neuroblastoma cells) from AstraZeneca (Mölndal, Sweden), COS-7 cells (African green monkey kidney cells) from Prof. B. Goddeeris (Department of Animal Sciences, Leuven, Belgium) and MDBK cells (Madin-Darby bovine kidney cells) from Prof. R. Kettmann (Faculty of Agromomy, Gembloux, Belgium). Soluble AP-N from porcine kidney was provided by Roche Diagnostics (Vilvoorde, Belgium).

2.2. Cell culture, transient transfection and membrane preparation

All cell lines were cultured in 75 and 500 cm^2 culture flasks and cell factories (Sanbio, Uden, Nederland) in Dulbecco's modified essential medium (DMEM) supplemented with L-glutamine (2 mM), 2% (v/v) of a stock solution containing 5000 IU/ml penicillin and 5000 μg/ml streptomycin (Invitrogen, Merelbeke, Belgium), 1% (v/v) of a stock solution containing non-essential amino-acids, 1 mM sodium pyruvate and 10% (v/v) foetal bovine serum (Invitrogen, Merelbeke, Belgium). The cells were grown in 5% CO_2 at 37 °C until confluent.

HEK293 cells were transiently transfected with plasmid DNA, pCIneo containing the gene of human cystinyl aminopeptidase (kindly provided by Prof. M. Tsujimoto, Lab. Of Cellular Biochemistry, Saitama, Japan) or pTEJ4 (Johansen et al., 1990) carrying the complete human aminopeptidase N cDNA (Olsen et al., 1988). Transient transfections were performed as described previously with 8 μl/ml LipofectAMINE (Invitrogen, Merelbeke,

Belgium) and 1 μg/ml plasmid DNA (Le et al., 2005). After transfection, the cells were cultured for 2 more days. CAP and AP-N transfected HEK293 cells displayed a 10 and 8 times higher enzyme activity than non-transfected cells.

Membranes were prepared as described previously (Demaegdt et al., 2004a). In short, the cells were harvested with 0.2% ethylene diamine tetraacetic acid (EDTA) (w/v) (in PBS, pH 7.4) and centrifuged for 5 min at 500 *g* at room temperature. After resuspending in PBS, the number of cells were counted and washed. The cells were then homogenized in 50 mM Tris–HCl (at pH 7.4) using a Polytron (10 s at maximum speed) and Potter homogenizer (30 strokes at 1000 rpm) and then centrifuged for 30 min (30,000 *g* at 4 °C). The pellet was resuspended in 50 mM Tris–HCl, centrifuged (30 min 30,000 *g* at 4 °C) and the supernatant was removed. The resulting pellets were stored at −20 °C until use.

2.3. Enzyme assay

Determination of the aminopeptidase catalytic activity was based on the cleavage of the substrate L-leucine-*p*-nitroanilide (L-Leu-*p*NA) (Demaegdt et al., 2004a) or L-alanine-*p*-nitroanilide (L-Ala-*p*NA) into L-leucine or L-alanine and *p*-nitroaniline. This latter compound displays a characteristic light absorption maximum at 405 nm. Pellets, prepared as described above, were thawed and resuspended using a Polytron homogenizer in enzyme assay buffer containing 50 mM Tris–HCl (pH 7.4), 140 mM NaCl, 0.1% (w/v) bovine serum albumin (BSA) and 100 μM phenylmethylsulphonyl fluoride (PMSF). The incubation mixture comprised 50 μl membrane homogenate, 200 μl L-Leu-*p*NA or L-Ala-*p*NA (1.5 mM for the enzyme inhibition curves or a range of 0.09 to 3 mM for the Michaelis–Menten curves) and 50 μl enzyme assay buffer alone or with test compound or 10 μM Angiotensin IV. The amount of membrane homogenate corresponded to 4×10^5 CHO-K1 cells, 7×10^5 COS-7 cells, 1×10^6 native HEK293 cells, 1.5×10^5 transfected HEK293 cells, 1×10^6 SK-N-MC cells and 1×10^6 MDBK cells in each well. For soluble porcine AP-N, 0.8 mU of this enzyme was added in stead of cell membranes. Assays were carried out at 37 °C in 96 well plates (Medisch Labo Service, Menen, Belgium) and the formation of *p*-nitroaniline was followed by measuring the absorption at 405 nm every 5 min between 10 and 50 min in a Bio-Whittaker 'Enzyme-Linked Immunosorbent Assay' (ELISA) reader. The enzymatic activities were calculated by linear regression analysis of the time-wise increase of the absorption. The enzymatic activities were always proportional to the membrane concentration or amount of enzyme added (data not shown).

2.4. Radioligand binding

Radioligand binding was performed as described previously (Demaegdt et al., 2004a). Briefly, the membrane pellets were thawed and resuspended using a Polytron homogenizer in 50 mM Tris–HCl (pH 7.4) binding buffer containing 140 mM NaCl, 0.1% (w/v) BSA and 100 μM PMSF. All binding experiments were performed with membrane homogenates at a concentration corresponding to the one used for the enzyme assay. The incubations were carried out in polyethylene 24 well plates (Elscolab, Kruibeke, Belgium) in a final volume of 300 μl per well consisting of 150 μl membrane homogenate, 50 μl of an EDTA/1,10-phenanthrolin mixture with a final concentration of 5 mM EDTA and 100 μM 1,10-phenanthrolin, 50 μl binding buffer (for total binding), test compound (for the competition binding assays) or 10 μM unlabeled Angiotensin IV (for non-specific binding). Subsequently 50 μl [^{125}I]Angiotensin IV was added at final concentrations ranging between 0.2 and 4 nM for the saturation binding experiments and at 1 nM for all other experiments. After incubation, at 37 °C for 60 min, the mixture was vacuum filtered using a Inotech 24 well cell-harvester through glass fibre filters (Whatman) pre-soaked for 30 min in 1% (w/v) BSA. After drying, the radioactivity retained in the filters was measured using a Perkin-Elmer γ-counter.

2.5. Data analysis

All experiments were performed at least 3 times with duplicate determinations each. The calculation of IC_{50} values from competition binding (or enzyme inhibition) experiments and of K_D (or K_m) and B_{max} (or V_{max}) values from the saturation binding (or Michaelis–Menten) curves were performed by non-linear regression analysis using GraphPad Prism 4.0. The pK_i values of the tested compounds in the binding and enzyme assays were calculated using the equation $pK_i = -\log\{IC_{50}/(1+[L]/K)\}$ in which $[L]$ is the concentration of free radioligand (binding) or free substrate concentration (enzyme assay) and K the equilibrium dissociation constant (K_D) of [^{125}I]Angiotensin IV (from saturation binding experiments) or the Michaelis–Menten constant (K_m) for substrate cleavage (Cheng and Prusoff, 1973).

3. Results

3.1. Enzyme activity

As described previously for CHO-K1 cell membranes, the enzymatic activity of CAP was assessed spectrophotometrically by measuring the absorption of *p*-nitroaniline (i.e. the cleavage product of the synthetic substrate L-Leu-*p*NA) at 405 nm over time at 37 °C (Demaegdt et al., 2004a). The corresponding rate constants (further denoted as enzymatic activities) were calculated by linear regression analysis of the time-wise increase of absorption.

Membrane preparations of all native cell lines contained aminopeptidase catalytic activity, as judged by their ability to cleave the substrates L-Leu-*p*NA (into L-leucine and *p*-nitroaniline) and L-Ala-*p*NA (into L-alanine and *p*-nitroaniline). As shown in Table 1, each individual cell line displayed comparable V_{max} values for both substrates and K_m values were consistently lower for L-Leu-*p*NA. To establish the potential contribution of CAP and AP-N in the aminopeptidase activity in different cell lines, the catalytic and pharmacological properties of both recombinant and/or purified enzymes were investigated.

Table 1
K_m and V_{max} values of L-Leu-*p*NA and L-Ala-*p*NA for different cell lines

	K_m (mM)		V_{max} (μM/min (10^6 cells))	
	L-Leu-*p*NA	L-Ala-*p*NA	L-Leu-*p*NA	L-Ala-*p*NA
CHO-K1	0.39±0.04	1.87±0.42	3.59±0.14	3.53±0.71
COS-7	0.34±0.03	2.88±0.67	1.99±0.13	3.29±0.22
SK-N-MC	0.65±0.16	3.35±0.49	1.57±0.67	3.24±0.69
MDBK	0.04±0.01	4.10±0.29	0.66±0.07	1.53±0.06
HEK293	0.22±0.03	1.43±0.12	1.63±0.14	2.30±0.27
HEK293+CAP	0.25±0.05	4.50±0.10	17.34±3.66	27.43±4.30
HEK293+AP-N	0.41±0.02	0.31±0.03	11.15±0.48	11.89±0.40
AP-N (ectodomain)	0.26±0.03	0.06±0.02	/	/

The K_m and V_{max} values are calculated through non-linear regression analysis of Michaelis–Menten curves. The values are the mean±S.E.M. of three independent experiments. V_{max} values of soluble porcine AP-N were not included in the table, because they cannot be compared to the other cell lines.

Transient transfection of HEK293 cells with the gene coding for human CAP had little effect on the K_m for both substrates but resulted in a >10-fold increase in their V_{max} (Table 1). Similarly, transfection with the gene coding for human AP-N increased the V_{max} of both substrates >5-fold. While the K_m for L-Leu-*p*NA was little affected, there was a net decrease in the K_m for L-Ala-*p*NA (Table 1). Based on the K_m ratios, CAP appears to be selective for L-Leu-*p*NA while AP-N is non-selective (for recombinant human AP-N) to moderately selective for L-Ala-*p*NA (for soluble porcine AP-N, Table 1).

Next, a number of ligands were tested for their ability to inhibit the catalytic activity of recombinant human CAP and AP-N and of soluble porcine AP-N. All of them displayed monophasic inhibition curves. Their pK_i values, as calculated by the Cheng and Prusoff (1973) equation from their IC_{50} values and K_m values of L-Leu-*p*NA (Table 1), are listed in Table 2. The potency order of the angiotensins and related peptides for recombinant human CAP was Angiotensin IV>Angiotensin III ~ divalinal-Angiotensin IV ~ LVV-hemorphin 7>Angiotensin 4–8 ~ Angiotensin II. Substance P displayed a slightly lower and L-methionine a very low potency compared to the 'angiotensin AT_4 receptor' ligands. The potency order for recombinant human AP-N and soluble porcine AP-N showed some differences with that for CAP: Angiotensin IV ~ Angiotensin III ~ substance P>LVV-hemorphin 7 ~ divalinal-Angiotensin IV>Angiotensin 4–8 ~ Angiotensin II ~ L-methionine (Table 2). Compared to CAP, AP-N was characterized by an overall reduction in potency of the angiotensins and related peptides, though their potency order was preserved. Conversely, substance P and L-methionine gained in potency and, as a result, substance P now ranked amongst the most potent ligands. Thus, even by using the non-selective substrate L-Leu-*p*NA, CAP and AP-N can easily be distinguished by comparing the inhibition profiles of Angiotensin IV, substance P and L-methionine (Fig. 1).

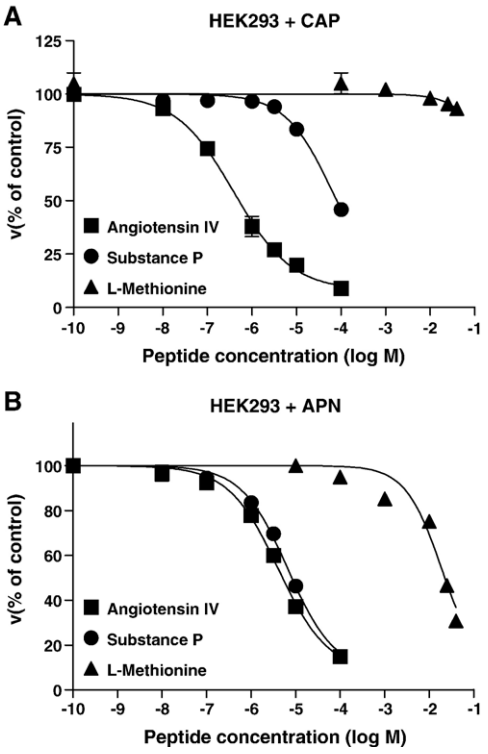

Fig. 1. Inhibition of the enzymatic activity found in HEK293 transfected with human CAP (A) or human AP-N (B). Membranes (corresponding to 1.5×10^5 cells/incubation) were incubated at 37 °C with 1.5 mM L-Leu-*p*NA in the absence (control activity) or the presence of increasing concentrations of Angiotensin IV (■), Substance P (●) and L-methionine (▲). The rate constants of L-Leu-*p*NA cleavage (v, corresponding to enzyme activity and expressed as a percentage of control) were calculated by linear regression analysis of the absorption (at 405 nm) vs. time curves with measurements made every 5 min (between 10 and 50 min). The pK_i values of these peptides are given in Table 2.

Based on the K_m values of both substrates and K_i values of the other ligands, the catalytic activities present in membranes from native CHO-K1, COS-7, HEK293, MDBK and SK-N-MC cells could be largely attributed to CAP. Indeed, for recombinant human CAP and for each of the native cells, the K_m for L-Leu-

Table 2
Inhibition of enzyme activity by putative 'angiotensin AT_4 receptor' ligands in different cell lines

	CHO-K1	COS-7	SK-N-MC	MDBK	HEK293	HEK293+CAP	HEK293+AP-N	AP-N (ectodomain)
Angiotensin II	5.33±0.02	5.34±0.10	5.51±0.10	6.52±0.03	5.30±0.16	5.34±0.19	<4	<4
Angiotensin III	6.41±0.08	6.34±0.14	6.38±0.33	7.28±0.11	5.72±0.16	6.86±0.10	5.77±0.03	5.72±0.05
Angiotensin IV	6.90±0.14	6.64±0.15	6.70±0.13	7.15±0.02	6.31±0.17	7.25±0.14	6.08±0.02	5.72±0.02
Angiotensin 4–8	5.74±0.06	5.60±0.09	5.43±0.23	6.23±0.03	5.29±0.09	6.05±0.02	<4	<4
LVV-hemorphin 7	6.29±0.07	6.16±0.03	6.33±0.09	7.13±0.03	6.39±0.22	6.59±0.11	5.22±0.01	5.35±0.08
Divalinal-Angiotensin IV	6.39±0.06	6.09±0.18	6.38±0.23	6.97±0.04	6.11±0.11	6.71±0.04	5.52±0.01	5.11±0.05
Substance P	<4	<4	5.06±0.10	5.95±0.14	<4	5.11±0.07	5.83±0.01	5.82±0.04
L-methionine	2.17±0.02	1.76±0.1	2.52±0.09	2.79±0.04	1.98±0.06	2.08±0.14	3.17±0.04	3.52±0.01

pK_i values are calculated according to the Cheng and Prusoff equation using IC_{50} values and the K_m values for L-Leu-*p*NA from Table 1. Data are the mean±S.E.M. of three independent experiments (illustrated in Fig. 1).

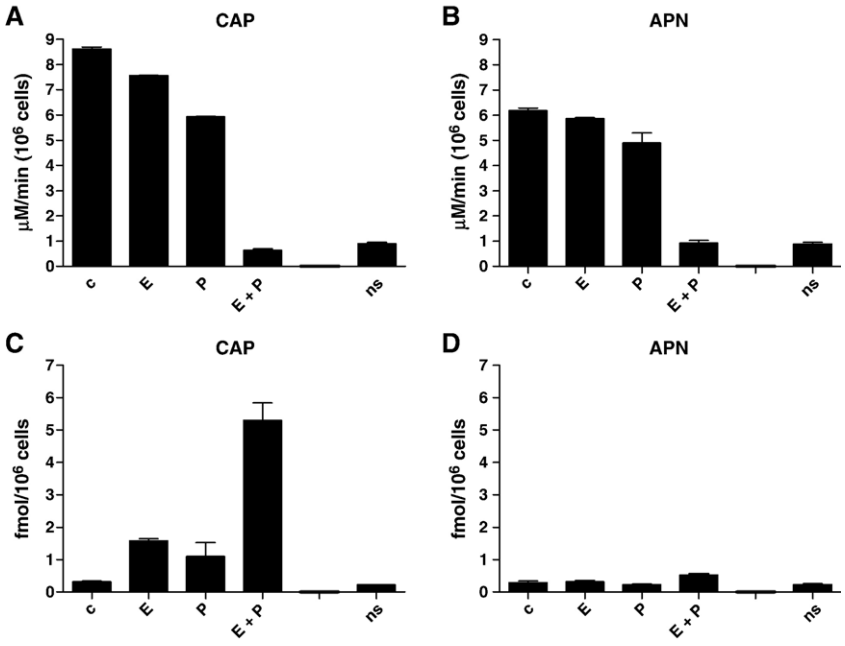

Fig. 2. Synergistic effect of EDTA and 1,10-phenanthrolin on the enzyme activity and [^{125}I]Angiotensin IV binding of CAP and AP-N. 1) Enzyme activity. Membranes (corresponding to 1.5×10^5 cells/incubation) with human CAP (A) or AP-N (B) transfected cells were incubated at 37 °C with 1.5 mM L-Leu-*p*NA in the absence (C) or presence of 150 µM EDTA (E) and/or 100 µM 1,10-phenanthrolin (P). The rate constants (expressed in µM/min for 10^6 cells) were obtained as in Fig. 1. Non-specific (ns) enzyme activity was measured in the presence of 100 µM Angiotensin IV. 2) [^{125}I]Angiotensin IV Binding. Membranes of with human CAP (C) or AP-N (D) transfected cells were incubated for 60 min at 37 °C with 1 nM [^{125}I]Angiotensin IV in the absence (C) or presence of 150 µM EDTA (E) and/or 100 µM 1,10-phenanthrolin (P). Non-specific (ns) [^{125}I]Angiotensin IV binding was measured in the presence of 10 µM unlabelled Angiotensin IV.

*p*NA was appreciably lower as compared to L-Ala-*p*NA (Table 1). Moreover, 'angiotensin AT_4 receptor' ligands as well as substance P and L-methionine displayed a similar potency as for recombinant CAP (Table 2).

The divalent cation chelators EDTA and 1,10-phenanthrolin have previously been shown to inhibit the CAP activity in CHO-K1 cell membranes in a synergistic fashion (Demaegdt et al., 2004b; Laeremans et al., 2005). As shown in Fig. 2(A and B), both chelators display the same synergy for inhibiting the activity of recombinant human AP-N. The presence of 150 µM EDTA or 100 µM 1,10-phenanthrolin only minimally affected the activity of both enzymes when added alone, whereas, their combined addition inhibited activity completely.

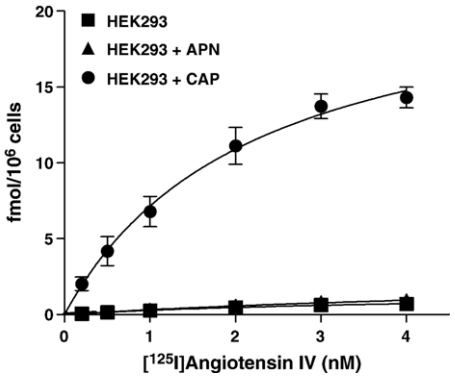

Fig. 3. Saturation binding of [^{125}I]Angiotensin IV. Membranes of HEK293 cells (■), HEK293 cells transfected with human CAP (●) and human AP-N (▲) were incubated for 60 min at 37 °C with increasing concentrations of [^{125}I] Angiotensin IV. Data refer to specific binding (expressed as fmol/10^6 cells), calculated by subtracting non-specific binding in the presence of 10 µM unlabelled Angiotensin IV from total binding. The corresponding K_D and B_{max} values are given in Table 3.

3.2. [^{125}I]Angiotensin IV binding

Specific (i.e. Angiotensin IV-displaceable) binding of 1 nM [^{125}I]Angiotensin IV to membranes from human CAP-expressing HEK293 cells was minimal in the absence of chelators, moderately increased in the presence of 150 µM EDTA or 100 µM 1,10-phenanthrolin and was maximal in the presence of both chelators (Fig. 2(C and D)). Under these latter conditions, binding was appreciably higher than in membranes from the non-transfected HEK293 cells (Fig. 3). In both instances, [^{125}I] Angiotensin IV binding was potently and almost completely

Table 3
K_D and B_{max} values from [^{125}I]Angiotensin IV saturation binding curves for different cell lines

	K_D (nM)	B_{max} (fmol/10^6 cells)
CHO-K1	0.9±0.12	15.5±1.2
COS-7	0.55±0.12	3.13±0.3
SK-N-MC	2.47±0.19	0.8±0.2
MDBK	0.30±0.02	4.52±0.77
HEK293	2.54±0.44	0.8±0.2
HEK293+CAP	1.56±0.38	19.0±1.9

The K_D and B_{max} values are calculated through non-linear regression analysis of the saturation binding curves. The values are the mean±S.E.M. of three independent experiments. Recombinant and soluble AP-N are not included in the table, because they show no detectable binding.

Table 4
[^{125}I] Angiotensin IV competition binding by putative 'angiotensin AT_4 receptor' ligands in different cell lines

	CHO-K1	COS-7	SK-N-MC	MDBK	HEK293	HEK293+CAP
Angiotensin II	4.95±0.32	5.71±0.08	5.59±0.02	5.96±0.17	5.13±0.20	5.03±0.13
Angiotensin III	6.59±0.10	7.13±0.16	6.24±0.32	8.08±0.20	6.47±0.11	6.95±0.03
Angiotensin IV	8.13±0.09	8.25±0.14	7.97±0.07	9.20±0.36	7.53±0.07	7.98±0.02
Angiotensin 4–8	4.69±0.18	5.14±0.10	4.96±0.01	6.60±0.09	4.43±0.08	5.03±0.13
LVV-hemorphin 7	7.71±0.07	7.62±0.07	7.48±0.20	8.38±0.17	7.33±0.07	7.87±0.12
Divalinal-Angiotensin IV	7.78±0.10	7.72±0.16	7.11±0.12	8.89±0.16	7.10±0.09	7.51±0.25
Substance P	<4	<4	<4	3.71±0.57	<4	<4
L-methionine	<1.4	1.65±0.13	1.96±0.16	2.04±0.78	2.04±0.29	1.89±0.13

pK_i values are calculated according to the Cheng and Prusoff equation using IC_{50} values and the K_D values from Table 3. Data are the mean±S.E.M. of three independent experiments (illustrated in Fig. 3).

displaced by 10 μM Angiotensin IV (data not shown). In contrast, specific binding of [^{125}I]Angiotensin IV to membranes from human AP-N-expressing HEK293 cells remained minimal in the absence or presence of both chelators, either alone or in combination (Figs. 2 (B) and 3). As a control, it was ascertained that the recombinant AP-N- and CAP- expressing cells used in the binding study displayed comparable levels of catalytic activity. [^{125}I]Angiotensin IV binding could not be performed on soluble AP-N since it was not retained by the glass fibre filters during filtration.

In the presence of both chelators, membranes from all native cell lines contained Angiotensin IV-displaceable [^{125}I]Angiotensin IV binding activity. The K_D values from the saturation binding curves were in the same nanomolar range, while B_{max} values decreased in the order: CHO-K1 > COS-7 ~ MDBK > SK-N-MC ~ HEK293 (Table 3). Compared to the human CAP recombinant HEK293 cells, only CHO-K1 cells exhibited equivalent B_{max} values. Competition binding experiments with unlabelled ligands were performed to compare the pharmacological profile of the high affinity [^{125}I]Angiotensin IV binding sites in membranes from the native cell lines with those of CAP recombinant HEK293 cells. The competition binding curves were monophasic for all ligands and their pK_i values are given in Table 4. The potency order was very similar for all cell systems and typical for 'angiotensin AT_4 receptors': i.e. Angiotensin IV > divalinal-Angiotensin IV ~ LVV-hemorphin 7 > Angiotensin (3–7) > Angiotensin III > Angiotensin II > Angiotensin (4–8) > substance P and L-methionine.

4. Discussion

The present study compares the ligand interaction properties of human cystinyl aminopeptidase (CAP, EC 3.4.11.3) and human aminopeptidase N (AP-N, EC 3.4.11.2) expressed in HEK293 cells, in addition to soluble porcine AP-N. It was found that, although the AP-N catalytic activity was, like CAP, susceptible to inhibition by Angiotensin IV, both enzymes showed distinct pharmacological profiles. Moreover, no extra high affinity binding of [^{125}I]Angiotensin IV could be detected in HEK293 cells expressing AP-N under conditions most favourable for high affinity binding of this radioligand to CAP.

Based on binding studies with [^{125}I]Angiotensin IV, CAP was identified as the angiotensin AT_4 receptor by Albiston et al. (2001). The presence of endogenous CAP in membranes of CHO-K1 cells has been demonstrated previously (Demaegdt et al., 2004a) by the occurrence of high affinity binding sites for [^{125}I]Angiotensin IV and the Angiotensin IV-inhibitable cleavage of L-Leu-*p*NA.

Interestingly, Garreau et al. (1998) observed that LVV-hemorphin 7 and Angiotensin IV also inhibited the catalytic activity of AP-N. In agreement with these studies, we found that the enzyme activity of recombinant and soluble AP-N could also be inhibited by Angiotensin IV, LVV-hemorphin 7 as well as by other 'angiotensin AT_4 receptor' ligands, albeit with less potency when compared to recombinant CAP. Additional differences in the inhibition profiles of CAP and AP-N include greater sensitivity of AP-N to substance P and L-methionine (Fig. 1). In this respect, substance P was already known as an inhibitor of AP-N (Xu et al., 1995), while L-methionine, which inhibits most metallopeptidases, has little effect on CAP (Laustsen et al., 2001). CAP and AP-N can also be discriminated from each other based on their K_m ratios for L-Leu-*p*NA and L-Ala-*p*NA, while CAP showed a clear preference for L-Leu-*p*NA, AP-N is non-selective (for recombinant human AP-N) to moderately selective for L-Ala-*p*NA (for the soluble ectodomain of porcine AP-N, Table 1). L-Ala-*p*NA is a commonly used substrate to measure the enzyme activity of AP-N (Riemann et al., 1999). As its K_m value is 15 times higher for human CAP (Table 1), only moderate interference of CAP should be expected in studies focussed on AP-N provided that the concentration of this substrate is sufficiently low.

Based on the substrate preferences and inhibition profiles, the Angiotensin IV-displaceable aminopeptidase activities in membranes of CHO-K1, COS-7, HEK293, MDBK and SK-N-MC

Table 5
Difference between pK_i values of Angiotensin IV and substance P from Table 2

	Δ pK_i
CHO-K1	>2.90
COS-7	>2.64
SK-N-MC	1.64
MDBK	1.20
HEK293	>2.31
HEK293+CAP	2.14
HEK293+AP-N	0.25
AP-N (ectodomain)	−0.10

cells could be mainly attributed to CAP. Indeed, for recombinant human CAP and for each of the native cells, the K_m for L-Leu-*p*NA was appreciably lower as compared to L-Ala-*p*NA (Table 1). As with recombinant CAP, the aminopeptidase activity in these cells was inhibited with high potency by 'angiotensin AT_4 receptor' ligands and with low potency by substance P and L-methionine (Table 2). As shown in Table 5, the difference in the pK_i values of Angiotensin IV and substance P for inhibiting the activities of CAP and AP-N already constitutes a fair basis for their discrimination; i.e. Δ pK_i=2.14 for recombinant CAP while it is close to zero for recombinant as well as soluble AP-N. The native cell lines displayed a largely positive Δ pK_i ranging between 1.20 and 2.90. Potential explanations for this variation may include species-related differences of CAP in general and, especially when Δ pK_i values are less than 2, the potential contribution of AP-N or other enzymes to the overall Angiotensin IV-inhibitable aminopeptidase activity.

Previous studies revealed that, at low concentrations, the chelators EDTA and 1,10-phenanthrolin have little effect on the catalytic activity of CAP when added alone but produce almost full inhibition when combined (Demaegdt et al., 2004b; Laeremans et al., 2005). Conversely, Angiotensin IV-displaceable binding of [^{125}I]Angiotensin IV only appeared in the presence of both chelators (Lew et al., 2003; Demaegdt et al., 2004b; Laeremans et al., 2005). The opposite effect of the chelators on [^{125}I]Angiotensin IV binding and on enzyme activity for CAP is also observed for recombinant human CAP in the present study (Fig. 2(A) and (C)). These observations have been explained by the ability of 1,10-phenanthrolin to remove the catalytic Zn^{2+} and by the ability of EDTA to potentiate this process by removing a modulatory ion from an allosteric site at the enzyme (Demaegdt et al., 2004b; Laeremans et al., 2005). This implies that high affinity [^{125}I]Angiotensin IV binding only takes place to the CAP apo-enzyme. EDTA and 1,10-phenanthrolin produced a similar effect on the AP-N catalytic activity (Fig. 2(B)). However, in contrast to CAP, no Angiotensin IV-displaceable [^{125}I]Angiotensin IV binding could be observed in membranes from recombinant human AP-N-expressing HEK293 cells, even in the presence of EDTA and 1,10-phenanthrolin (Figs. 2(D) and 3). This indicates that, while a high affinity binding of [^{125}I]Angiotensin IV only occurs to the apo-enzyme of CAP, no such binding occurs to native AP-N nor to the derived apo-enzyme. This lack of AP-N interference in [^{125}I]Angiotensin IV binding studies may explain why all the previously published 'angiotensin AT_4 receptor' pharmacological profiles are very similar to the one of CAP. Moreover, the 'angiotensin AT_4 receptor' purification experiments reported by Albiston et al. (2001), were based on the binding of a radiolabelled photoactivatable analogue of Ang IV for detection. This may explain the assignment of CAP as the 'angiotensin AT_4 receptor' by these authors.

The present findings challenge the suggestion of Garreau et al. (1998) that AP-N also constitutes a target for [^{125}I]Angiotensin IV. This was based on the detection of LVV-hemorphin 7-displaceable high affinity binding of [^{125}I]Angiotensin IV in membranes from rabbit collecting duct cells and the finding that the kidney is a rich source of AP-N. However, since the ligand-specificity of these binding sites were not characterized and CAP is also present in the kidney (Masuda et al., 2003); it cannot be excluded that [^{125}I]Angiotensin IV binding sites in the collecting duct cells correspond to CAP instead of AP-N.

Angiotensin IV-displaceable [^{125}I]Angiotensin IV binding activity was found to be present in all native cell lines tested. Overall, the inhibition profiles of [^{125}I]Ang IV binding (Table 4) were very similar amongst these native cell lines. Small differences in the pK_i values are likely to be attributed to minor species and/or tissue differences. Similarly, there are also only limited differences between the pK_i values derived from enzyme inhibition experiments among the investigated native cell lines (Table 2). On the other hand, substantial differences were observed when comparing the pK_i values derived from these [^{125}I]Angiotensin IV binding competition curves (Table 4) with those obtained from enzyme inhibition experiments (Table 2). These differences have already been noticed previously and extensively discussed (Lew et al., 2003; Demaegdt et al., 2004a). It was concluded in these studies that the presence or absence of Zn^{2+} in the catalytic site influences the affinity of the ligands. Taken together, the radioligand binding and enzyme activity measurements in the present reveal the presence of a similar 'angiotensin AT_4 receptor'/CAP profile in the tested native cell lines. As illustrated in Tables 1 and 3, CHO-K1 cells appear to contain the largest amount of CAP (Demaegdt et al., 2004a). Thus, CHO-K1 cells have the potential to constitute a suitable model for future functional assays.

In conclusion, although catalytic activity of CAP and AP-N can be inhibited by Angiotensin IV and other 'angiotensin AT_4 receptor' ligands, both enzymes show distinct ligand interaction profiles. More importantly, while high affinity binding of [^{125}I]Angiotensin IV occurs only to the CAP apo-enzyme, no such binding can be detected in the case of AP-N. Although high affinity [^{125}I]AngiotensinIV binding to CAP does not occur under physiological conditions, the selectivity of the process greatly facilitates the detection and quantitation of this protein and this opens new possibilities for the study of its structural and functional properties under normal and pathophysiological conditions. In this respect, we were able to detect the presence of CAP in the presently tested cell lines. This supports the idea that CAP plays an important physiological role.

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