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Endogenous cystinyl aminopeptidase in Chinese hamster ovary cells: characterization by [^{125}I]Ang IV binding and catalytic activity

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Abstract

The angiotensin II C-terminal hexapeptide fragment angiotensin IV (Ang IV) exerts central and cardiovascular effects. Cystinyl aminopeptidase (EC 3.4.11.3), a membrane-associated zinc-dependent metallopeptidase of the M1 family, has recently been found to display high affinity for Ang IV and it was proposed to represent the AT_4 receptor. We present evidence for the presence of endogenous cystinyl aminopeptidase in membranes from Chinese hamster ovary (CHO-K1) cells by binding studies with [^{125}I]Ang IV and by measuring the cleavage of L-leucine-*p*-nitroanilide. The equilibrium dissociation constant of [^{125}I]Ang IV in saturation binding studies ($K_D = 0.90$ nM) was similar to the value ($K_D = 0.70$ nM) calculated from the association and dissociation rates. Binding was displaced with high potency by the "AT_4 receptor" ligands (Ang IV > divalinal^1-Ang IV ~ LVV-hemorphin-7 ~ LVV-hemorphin-6 > Ang (3–7) > Ang III > Ang (4–8)) but not by AT_1/AT_2 receptor antagonists. Enzymatic activity in CHO-K1 cell membranes was competitively inhibited upto 94% by Ang IV and other "AT_4 receptor" ligands (Ang IV > Ang III ~ divalinal^1-Ang IV ~ Ang (3–7) ~ LVV-hemorphin-7 > Ang (4–8) ~ LVV-hemorphin-6). High affinity binding of [^{125}I]Ang IV required the presence of metal chelators and the ligands such as Ang IV and LVV-hemorphin-7 displayed higher potency in the binding studies as in the enzyme assay. This difference in potency varied from one peptide to another. These pharmacological properties match those previously reported for the recombinantly-expressed human cystinyl aminopeptidase in embryonal kidney cells.

Keywords: Cystinyl aminopeptidase; Angiotensin IV; AT_4 receptor; Chinese hamster ovary cells

1. Introduction

Angiotensin II (Ang II) is well-known to cause hypertension and to stimulate cardiac remodelling. G-protein coupled receptors of the AT_1 subtype play a major role in these processes while AT_2 receptors, the other major subtype, are primordially expressed in foetal tissues and involved in the inhibition of cell proliferation, apoptosis and neuronal differentiation [1–3]. Ang II has long been considered to represent the end product of the renin–angiotensin system, but there is accumulating evidence that some of its fragments such as Ang III (deletion of the N-terminal amino acid) and Ang IV (deletion of the two N-terminal amino acids) have central, cardiovascular and renal actions as well [4–9]. Ang III is still a potent agonist for AT_1 and AT_2 receptors so that there is no need to invoke alternative receptors to explain its biological actions. On the other hand, Ang IV possesses only very low potency for these receptors [10,11]. Yet, most of its biological effects are already observed at nanomolar concentrations and are not blocked by the classical non-peptide AT_1 and AT_2

Abbreviations: Ang, Angiotensin; CHO-K1, Chinese hamster ovary cells; IRAP, Insulin regulated aminopeptidase; EDTA, Ethylene diamine tetraacetic acid; 1,10-PHE, 1,10-phenanthrolin; HEK293, Human embryonal kidney cells; L-Leu-*p*NA, L-Leucine-*p*-nitroanilide; GLUT4, Insulin-dependent glucose transporter

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receptor antagonists losartan and PD123177. The deviant pharmacological properties led to the concept of a novel angiotensin receptor subtype: the "AT_4 receptor" [3,12].

High affinity [^{125}I]Ang IV binding sites have been shown to be present on membrane preparations from various tissues and cell types [10,13,14]. Their pharmacological profile corresponds with that of AT_4 receptors, namely they display low affinity for Ang II and the classical AT_1 and AT_2 receptor antagonists but high affinity for Ang IV, synthetic peptide analogues like divalinal^1-Ang IV as well as for endogenous peptides like LVV-hemorphin-7 [10,15–18]. This latter peptide has been discovered in brain extracts and, as the concentration of Ang IV in brain is very low, it has been suggested to constitute a native ligand for the AT_4 receptor [19,20]. Considerable progress in the understanding about the nature of Ang IV binding sites was provided by a recent study of Albiston et al. [21]. Based on purification and partial sequencing, these authors convincingly identified these binding sites as cystinyl aminopeptidase (EC 3.4.11.3), a membrane-associated zinc-dependent metallopeptidase of the M1 family. This enzyme bears a short intracellular domain, a single transmembrane-spanning domain and a large extracellular domain containing the catalytic site which is able to cleave the N-terminal amino acid from bioactive peptides such as oxytocin, vasopressin and somatostatin in vitro [22,23]. Ang IV and LVV-hemorphin-7 have been shown to inhibit this catalytic activity and, by this way, they may extend the half-life of endogenous neuropeptides like vasopressin and somatostatin that potentiate memory [24]. Currently, this explanation [21,25] provides a very attractive rationale for the observed propensity of Ang IV to improve memory and learning.

Cystinyl aminopeptidase received no less than 14 different denominations. This is related to its independent identification and characterization by different research teams as well as to differences in the physiological context in which it has been investigated. Being the major human placental protease, it is often denominated as oxytocinase and placental-leucine aminopeptidase (P-LAP). In this context, it was found that its large extracellular domain can be cleaved off proteolytically and that its enzymatic activity in serum increases with gestation. Since two of its major substrates, oxytocin and vasopressin, have uterine contractile and vasoconstrictive activities, it has been put forth that cystinyl aminopeptidase contributes to the maintenance of normal pregnancy and suppresses labor pain by regulating the plasma level of these hormones [26–29]. This enzyme (here denominated as insulin regulated aminopeptidase or (IRAP)) has also been identified in intracellular endosome-derived vesicles along with GLUT4, an insulin-regulated glucose transporter that is abundantly expressed in muscle and adipose cells [30,31]. In these cells, insulin was found to increase the translocation rate of these vesicles to the plasma membrane, resulting in an increased surface expression of GLUT4 and cystinyl aminopeptidase in healthy subjects [30,32–39]. Yet, both appear to exhibit trafficking/targeting defects in muscle and adipose cells of type II diabetic patients [37,40].

The ability to directly identify cystinyl aminopeptidase by binding studies with [^{125}I]Ang IV and related peptides opens new possibilities for the study of its structural and functional properties under normal and pathophysiological conditions. Recent studies involving the transfection of HEK293T cells with the cDNA of human cystinyl aminopeptidase have compared the binding and catalytic properties of this enzyme on the same cell membrane preparation. Unexpectedly, these studies revealed major discrepancies when comparing the potency of certain peptides to inhibit the binding of [^{125}I]Ang IV and to inhibit the hydrolysis of a fluorescent substrate [25,41]. Also, the competitive nature of Ang IV with the substrate in these experiments is at odds with a recent report dealing with the ability of Ang IV to interact with a juxtamembrane site of cystinyl aminopeptidase and the thereon derived conclusion that Ang IV modulates the enzyme's activity by an allosteric mechanism [42]. By studying the expression of recombinant human cystinyl aminopeptidase in Chinese hamster ovary (CHO-K1) cells, we were struck by the high background levels of [^{125}I]Ang IV binding in membranes from the non-transfected cells. This led us to ascertain the presence of high levels of endogenous cystinyl aminopeptidase in this cell line.

2. Materials and methods

2.1. Materials

Angiotensin II (Ang II), Ang II (3–7), Ang III or Ang II (2–8), Ang IV or Ang II (3–8), oxytocin and vasopressin were obtained from Neosystem, Ang II (5–8), Ang II (4–8) and LVV-hemorphin-6 from Bachem, Ang II (1–7) from Sigma, LVV-hemorphin-7 or LVV-H7 from Invitrogen. Divalinal^1-Ang IV was synthesized by Dr. G. Munske (Washington State University). L-Leucine-*p*-nitroanilide (L-Leu-*p*NA) was obtained from Sigma and *p*-nitroaniline from VWR International. Tyr^4 of Ang IV was iodinated using the Iodogen® iodination reagent from Pierce and ^{125}I from ICN. Monoiodinated Ang IV was isolated on a Hypersil BDS C18 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 commercially available.

2.2. Cell culture and membrane preparation

CHO-K1 cells (kindly obtained from the Pasteur Institute) were cultured in 75 and 500 cm^2 culture flasks and cell factories (Nunc) 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 streptomycine (Invitrogen), 1% (v/v) of a stock solution containing non-essential amino-acids, 1 mM sodium pyruvate and 10% (v/v) foetal bovine serum (Invitrogen). The cells were grown in 5% CO_2 at 37 °C until confluent. Before harvesting, the cells were washed with PBS and then briefly treated with PBS (pH 7.4) containing 0.2% EDTA (w/v). The cell suspension was centrifuged for 5 min at 500 × *g* at room temperature and resuspended in PBS and then, the number of cells was counted. After one more wash-step the cells were 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 CHO-K1 cell membrane-containing pellets were stored at −20 °C until use.

2.3. Radioligand binding

Just before use, pellets were thawed and resuspended using a Polytron homogenizer in 50 mM Tris–HCl (pH 7.4) binding buffer containing 140 mM NaCl, 5 mM ethylene diamine tetraacetic acid (EDTA), 0.1% (w/v) bovine serum albumin (BSA), 100 μM phenyl methyl sulforyl fluoride (PMSF) and 100 μM 1,10-phenanthrolin (1,10-PHE). Except for the experiment shown in Fig. 1, all binding experiments were performed with membrane homogenates at a concentration corresponding to 4 × 10^5 cells/incubation. The incubations were carried out in polyethylene 24-well plates (Elscolab) in a final volume of 250 μl per well consisting of 200 μl membrane homogenate, 25 μl binding buffer (for total binding), test compound (for the competition binding assays) or 10 μM unlabeled Ang IV (for non-specific binding). Subsequently 25 μl [^{125}I]Ang IV was added at final concentrations ranging between 0.05 and 6 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 an Inotech 24-well cell-harvester through GF/B 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.

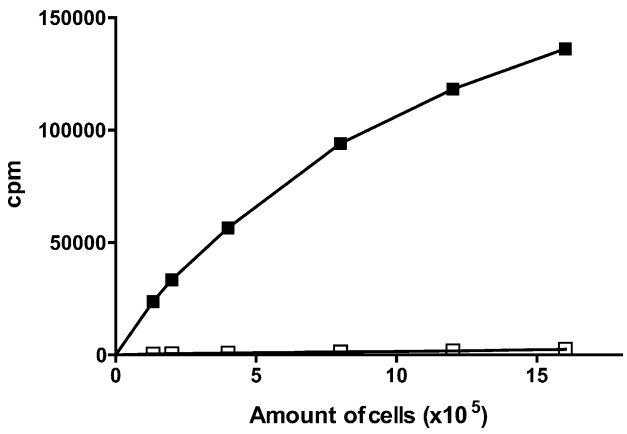

Fig. 1. Binding of 1 nM [^{125}I]Ang IV (expressed as cpm) in the absence (total binding, ■) or presence of 10 μM Ang IV (non-specific binding, □) was measured as a function of the membrane concentration (expressed as the corresponding amount of CHO-K1 cells/incubation). Incubations were carried out at 37 °C for 60 min. A representative of three independent experiments is illustrated. Error bars are shown, but are smaller than the symbols.

2.4. Enzyme assay

Determination of the cystinyl aminopeptidase catalytic activity was based on the cleavage of the substrate L-leucine-*p*-nitroanilide (L-Leu-*p*NA) into L-leucine and *p*-nitroaniline. This latter compound displays a characteristic light absorption maximum at 405 nm. To this end, pellets were thawed and resuspended using a Polytron homogenizer in enzyme buffer containing 50 mM Tris–HCl (pH 7.4), 140 mM NaCl, 0.1% (w/v) BSA and 100 μM PMSF. The incubation mixture comprised 50 μl membrane homogenate (at a concentration corresponding to 4 × 10^5 cells/incubation, except for the experiment shown in Fig. 5), 200 μl L-Leu-*p*NA (1.5 mM or at the concentrations indicated) and 50 μl enzyme buffer alone or with test compound or 100 μM Ang IV. The membrane homogenate was incubated at 37 °C in 96-well plates (Medisch Labo Service) and the formation of *p*-nitroaniline was followed by measuring the absorption at 405 nm between 10 and 50 min in a Bio-Whittaker ELISA reader.

2.5. Data analysis

All experiments were performed at least 3 times with triplicate (for radioligand binding) or duplicate determinations (for enzyme assays) each. The calculation of IC_{50} values from competition binding and enzyme inhibitory experiments and of K_D and B_{max} values from the saturation binding 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]Ang IV (from kinetic experiments) or the Michaelis–Menten constant (K_m) for substrate cleavage [43].

3. Results

3.1. Binding of AT_4 receptor ligands to CHO-K1 cell membranes

Binding of 1 nM [^{125}I]Ang IV to the CHO-K1 cell membrane homogenates was almost completely inhibited

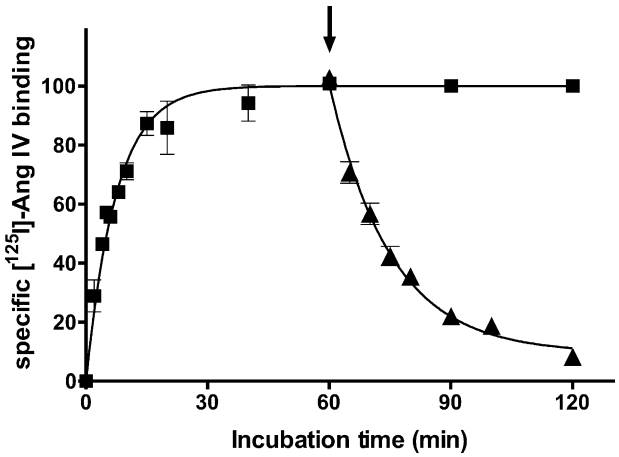

Fig. 2. Association and dissociation of [^{125}I]Ang IV. Membranes were incubated with 1 nM [^{125}I]Ang IV at 37 °C for increasing periods of time (■). The corresponding pseudo first-order rate constant for assoaciation (k_{obs}) was 0.16 ± 0.01 min^{-1}. Dissociation was initiated after 60 min (arrow) upon isotopic dilution with 10 μM Ang IV (▲). The corresponding dissociation rate constant (k_{-1}) was 0.065 ± 0.006 min^{-1}. Binding is expressed as percentage of steady-state binding after 120 min.

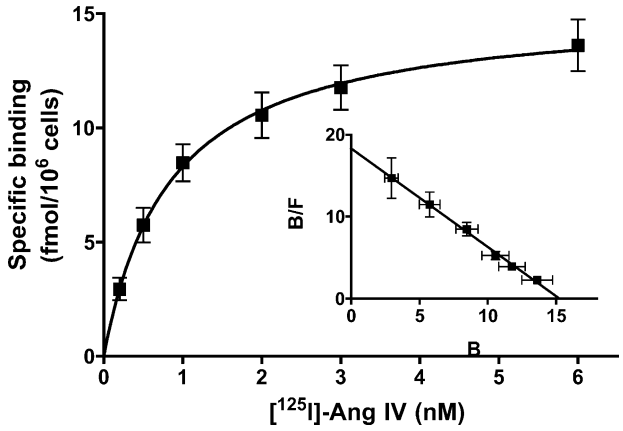

Fig. 3. Saturation binding of [^{125}I]Ang IV. Membranes were incubated for 60 min at 37 °C with increasing concentrations of [^{125}I]Ang IV. Data refer to specific binding (expressed as fmol/10^6 cells), calculated by subtracting non-specific binding in the presence of 10 μM unlabeled Ang IV from total binding. The corresponding K_D and B_{max} value were 0.90 ± 0.12 nM and 15.5 ± 1.2 fmol/10^6 cells, respectively. Insert: Scatchard plot of the saturation binding data with B = specific binding (in fmol/10^6 cells) and F = free concentration (in nM).

in the presence of 10 μM unlabeled Ang IV. This displaceable (i.e. specific) binding increased with the cell membrane concentration (Fig. 1). Ensuing binding experiments were carried out with a membrane concentration corresponding to 4 × 10^5 cells/incubation. Specific binding of [^{125}I]Ang IV was time-dependent and reached equilibrium within 30 min at 37 °C. The binding was reversible since addition of an excess of unlabeled Ang IV (10 μM) resulted in the complete dissociation of the specifically bound radioligand. The pseudo first order association (k_{obs}) and dissociation (k_{-1}) rate constants were respectively 0.16 ± 0.01 and 0.065 ± 0.006 min^{-1} (Fig. 2). The corresponding "kinetic" equilibrium dissociation constant (K_D) was 0.70 nM. Under steady-state conditions (i.e. after an incubation of 60 min at 37 °C), the binding of [^{125}I]Ang IV was saturable. Binding occurred to a single class of sites with a corresponding K_D of 0.90 ± 0.12 nM and a B_{max} of 15.5 ± 1.2 fmol/10^6 cells (Fig. 3).

The specific binding of [^{125}I]Ang IV was inhibited by Ang IV, LVV-H7, Ang II (5–8), Ang II (Fig. 4) and several other putative AT_4 receptor ligands in a concentration-dependent fashion. The competition binding curves were monophasic for all compounds and the potency order was: Ang IV > divalinal[1]- Ang IV ~ LVV-H7 ~ LVV-H6 > Ang (3–7) > Ang III > Ang (5–8) > vasopressin ~ Ang II ~ Ang (4–8). The pK_i values of these peptides are summarized in Table 1. pK_i values of Ang (1–7), oxytocin, the AT_1 receptor antagonist losartan and the AT_2 receptor antagonist PD 123319 were too low to be accurately determined.

3.2. Inhibition of enzymatic activity in CHO-K1 cell membranes by AT_4 receptor ligands

The enzymatic activity of IRAP 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. While the absorption of a 1.5 mM L-Leu-*p*NA solution remained steady for at least 50 min, addition of CHO-K1 cell membrane homogenates induced a time-wise increase in the absorption. The corresponding rate constants (further denoted as enzymatic activities) were calculated by linear regression analysis of these time curves. As shown in Fig. 5, the enzymatic activity was proportional to the membrane concentration. Further enzyme assays were carried out with a membrane concentration corresponding to 4 × 10^5 cells/incubation and, under this condition, the enzymatic activity was substantially inhibited (upto 94%) in the presence of 100 μM Ang IV.

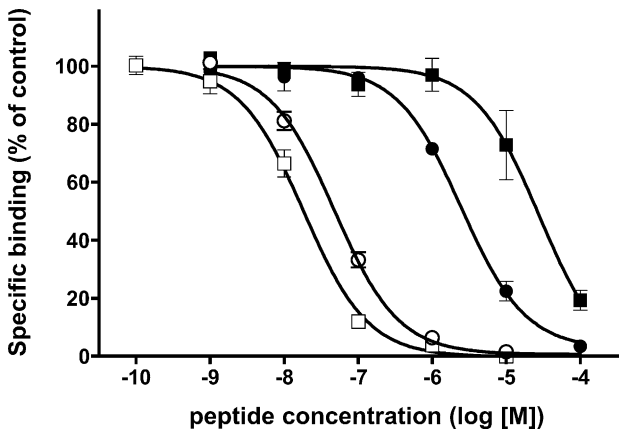

Fig. 4. [^{125}I]Ang IV competition binding. Membranes were incubated with 1 nM [^{125}I]-Ang IV for 60 min at 37 °C in the absence (control binding) or presence of increasing concentrations of Ang II (■), Ang IV (□), Ang II (5–8) (●) and LVV-H7 (○). Binding is expressed as percentage of specific control binding. The pK_i values of the unlabeled peptides are given in Table 1.

Table 1
[^{125}I]Ang IV competition binding and inhibition of cystinyl aminopeptidase activity in CHO-K1 cell membranes by putative AT_4 receptor ligands, natural substrates and AT_1 and AT_2 receptor antagonists

Ligands		Competition binding $pK_i \pm$ S.E.M.	Enzyme inhibition $pK_i \pm$ S.E.M.
AT_4 receptor ligands	Ang II	4.95 ± 0.32	5.33 ± 0.02
	Ang III	6.59 ± 0.10	6.41 ± 0.08
	Ang IV	8.13 ± 0.09	6.90 ± 0.14
	Ang (4–8)	4.69 ± 0.18	5.74 ± 0.06
	Ang (5–8)	6.00 ± 0.05	4.80 ± 0.16
	Ang (1–7)	N.M.	N.M.
	Ang (3–7)	6.99 ± 0.08	6.37 ± 0.09
	LVV-H7	7.71 ± 0.07	6.29 ± 0.07
	LVV-H6	7.61 ± 0.11	5.58 ± 0.05
	Divalinal[1]-Ang IV	7.78 ± 0.10	6.39 ± 0.06
Natural substrates	Oxytocin	N.M.	N.M.
	Vasopressin	4.98 ± 0.16	N.M.
AT_1 antagonist	Losartan	N.M.	N.M.
AT_2 antagonist	PD 123319	N.M.	N.M.

N.M. means not measurable (<4). pK_i values are calculated using the Cheng and Prusoff equation from the IC_{50} values. Data are the mean ± S.E.M. of three independent experiments illustrated in Figs. 4 and 7.

Fig. 6A illustrates the enzymatic activity as a function of the substrate concentration in the absence or presence of Ang IV. The K_m and V_{max} values were calculated by linear regression of the corresponding Hanes plots are shown in Fig. 6B. Whereas the K_m for L-Leu-*p*NA increased significantly ($P < 0.05$) from 0.429 ± 0.014 upto 1.010 ± 0.134 and 1.454 ± 0.326 mM in the presence of 0.1 and

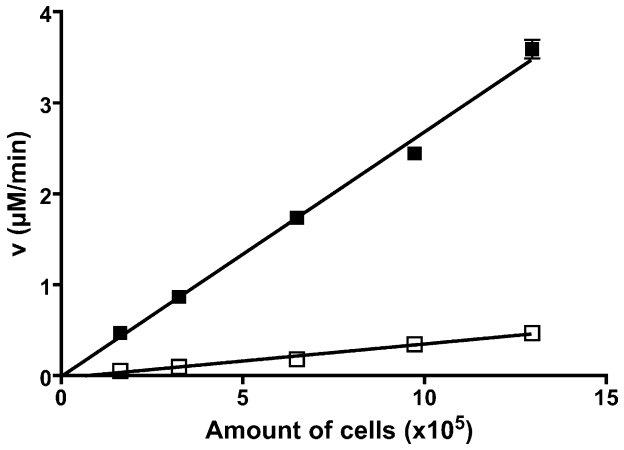

Fig. 5. Enzymatic cleavage of L-Leu-*p*NA as a function of the membrane concentration. 1.5 mM L-Leu-*p*NA was incubated at 37 °C with increasing membrane concentrations (expressed as the corresponding amount of CHO-K1 cells/incubation) in the absence (■) or presence of 100 µM Ang IV (□). Rate constants for L-Leu-*p*NA cleavage (*v*, expressed as µM/min/incubation) 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). Fixed *p*-nitroaniline concentrations were used as standard to convert absorption into concentration. A representative of three independent experiments is illustrated. Error bars are shown, but are smaller than the symbols.

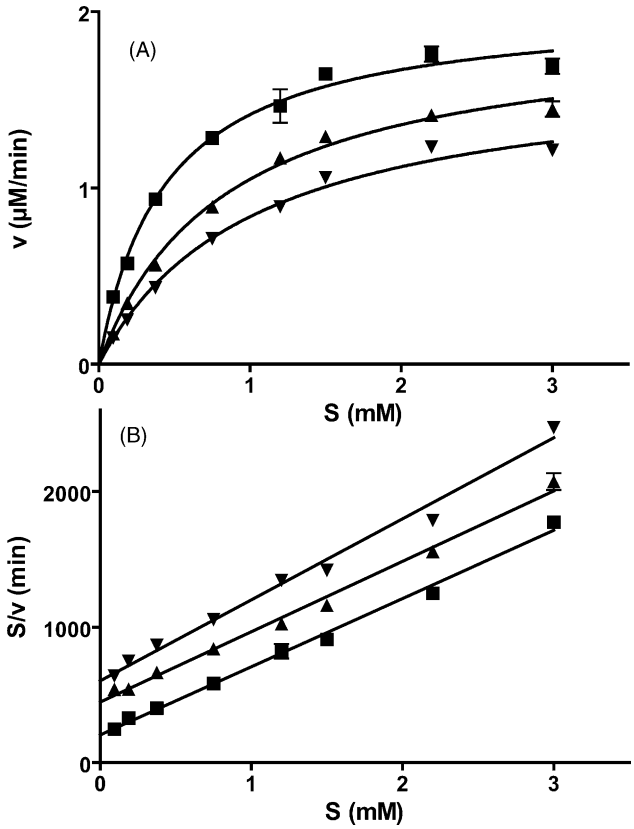

Fig. 6. Enzymatic cleavage of L-Leu-*p*NA as a function of the substrate concentation. (A) Membranes (corresponding to 4 × 10^5 cells/incubation) were incubated at 37 °C with increasing concentrations of L-Leu-*p*NA (*S*, in mM) in the absence (■) or presence of 0.1 (▲) or 0.3 (▼) µM Ang IV. The rate constants of L-Leu-*p*NA cleavage were determined as in Fig. 5. (B) The corresponding K_m and V_{max} values were calculated by linear regression analysis of the Hanes-plots (i.e. *S*/*v* vs. *S*). A representative of three independent experiments is illustrated. Error bars are shown, but are smaller than the symbols. Average values and S.E.M. of the three experiments are given in the Section 3.

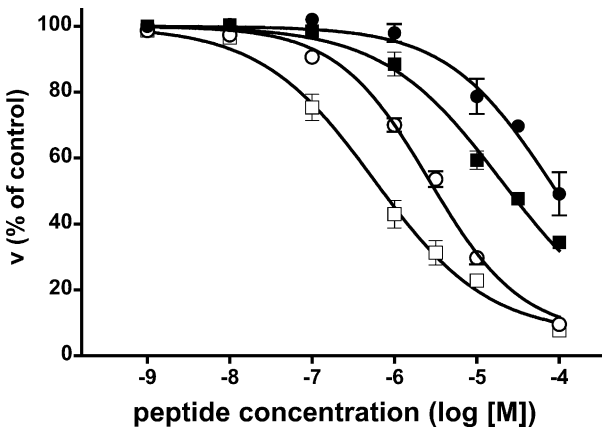

Fig. 7. Inhibition of the enzymatic activity by putative AT_4 receptor ligands. Membranes (corresponding to 4 × 10^5 cells/incubation) were incubated at 37 °C with 1.5 mM L-Leu-*p*NA in the absence (control activity) or presence of increasing concentrations of Ang II (■), Ang IV (□), Ang II (5–8) (●) and LVV-H7 (○). The rate constants of L-Leu-*p*NA cleavage (*v*, corresponding to specific activity and expressed as a percentage of control) were obtained as in Fig. 6. The pK_i values of the peptides are given in Table 1.

0.3 μM Ang IV, the corresponding V_{max} values were not significantly different ($P > 0.55$) from the control value (i.e. 1.62 ± 0.06 μM min^{-1}). This behaviour is typical for a competitive interaction between the substrate and an inhibitor. The enzymatic activity was inhibited by Ang IV, LVV-H7, Ang II (5–8), Ang II (Fig. 7) and several other putative AT_4 receptor ligands in a concentration-dependent fashion. The potency order was Ang IV > Ang III ∼ Divalinal-Ang IV ∼ Ang (3–7) ∼ LVV-H7 > Ang (4–8) ∼ LVV-H6 ∼ Ang II > Ang (5–8). Based on the assumption that all peptides compete with the substrate, their pK_i values were calculated from the IC_{50} values according to the ''Cheng and Prusoff'' equation [43] and summarized in Table 1. The potency of the natural substrates vasopressin and oxytocin and the AT_1 and AT_2 receptor antagonists was too low to be determined.

4. Discussion

The presence of endogenous cystinyl aminopeptidase (EC 3.4.11.3) in membranes from Chinese hamster ovary cells is demonstrated by the occurrence of high affinity binding sites for [^{125}I]Ang IV and by the Ang IV-sensitive cleavage of L-leucine-*p*-nitroanilide. The [^{125}I]Ang IV binding and catalytic properties in these cells match with those recently reported for the recombinant human enzyme (denoted as IRAP) in HEK293T cells [25,41].

Binding of [^{125}I]Ang IV occurred with high affinity to a single class of sites to CHO-K1 cell membranes and its K_D-value obtained from saturation binding experiments (0.90 nM) was in good agreement with the value (0.70 nM) calculated from its association and dissociation rate. Similar K_D values have been reported, not only for the recombinant cystinyl aminopeptidase in HEK293T cell membranes [25], but also for the alleged AT_4 receptors in earlier studies on membrane preparations of species like guinea pig, rabbit, rat, bovine, monkey and human and tissues as diverse as brain, adrenal cortex, heart, vascular smooth muscle, colon and prostate [44]. Specific binding of [^{125}I]Ang IV to the CHO-K1 cell membranes increased as a function of time until steady-state was reached after approximately 30 min. This steady-state was maintained for at least 90 min thereafter, suggesting that only minimal metabolism of the radiolabeled ligand took place during the period of incubation. In the same line, degradation of Ang IV was very low in HEK293T cell membranes and, as it was similar for control and transfected cells, it was unrelated to the catalytic activity of cystinyl aminopeptidase [25]. This contrasts with the relatively rapid metabolism of Ang IV when exposed to Madin-Darby bovine kidney cell membranes, as evidenced by the time-wise rightward shift of the competition binding curves of this peptide [45]. Hence, the necessity to recruit metabolically more stable radioligands like [^{125}I]divalinal^1-Ang IV or [^{125}I]norleucine^1-Ang IV for binding studies [21,25,45–47] is not dictated by the presence of cystinyl aminopeptidase itself but rather by other peptidases. Therefore, the choice of an appropriate radioligand largely depends on the cell type used.

The potency with which various peptides compete with the binding of [^{125}I]Ang IV to CHO-K1 cell membranes closely agrees with the binding profile of recombinant human cystinyl aminopeptidase and of ''AT_4 receptors'' in general [25,41,48–51]. When N-terminal amino acids were removed one by one from Ang II, there was a gradual increase in their potency till Ang IV. Further removal of even only one N-terminal amino acid produced a sharp drop in potency while removal of the C-terminal Phe had relatively less effect. This fits with previous studies in which the N-terminal amino acids were found to be the most important determinators for high affinity binding [3,52]. This rule also explains the high potency of divalinal^1-Ang IV and of LVV-H6, LVV-H7 in the present and previous binding studies [25,41]. In line with the distinct pharmacology of ''AT_4 receptors'', the binding of [^{125}I]Ang IV to CHO-K1 cell membranes was not perceptibly inhibited by the AT_1 receptor antagonist losartan nor by the AT_2 receptor antagonist PD 123319. As in previous binding and functional studies [16,49,53], oxytocin and vasopressin also displayed rather low affinity (Table 1).

The synthetic aminopeptidase substrate L-Leu-*p*NA was cleaved in the presence of CHO-K1 cell membranes. Up to 94% of this catalytic activity was inhibited by Ang IV and related AT_4 ligands. Kinetic experiments performed in the presence of increasing concentrations of substrate and Ang IV revealed that Ang IV produces a concentration-wise increase in the K_m of the substrate but that the maximal activity (V_{max}) remains the same. This behaviour is typical for a competitive inhibition and, by using a similar experimental approach, it was also observed by Lew et al. [25] for the recombinant cystinyl aminopeptidase in HEK293T cell membranes. At first glance, these findings are at odds with the recent claim by Caron et al. that Ang IV interacts with a juxtamembrane site on this enzyme and, accordingly, that Ang IV is unlikely to bind at its active zinc-binding site. These considerations were based on the covalent binding of the Ang IV-derived photoaffinity-label [^{125}I]-[N_3-Phe^6]Ang IV to an extracellular region of the enzyme that is in close proximity of the membrane (i.e. not beyond Lys^{159}) while the zinc coordinating residues are located between the His^{464} and Glu^{488} residues [42]. Until now, this issue cannot be resolved since the 3D structure of cystinyl aminopeptidase has not yet been elucidated. The same applies to nearly all other members of the M1 family of gluzincin metalloproteases except for the bestatin–leukotriene A_4 hydrolase complex, whose X-ray crystal structure was recently determined [54]. It was shown that the enzyme is highly folded and that, although the Zn^{2+}

binding site is formed by residues from the catalytic domain only, bestatin is able to make interactions with residues from different other domains of the enzyme. If a similar molecular organisation also applies to the Ang IV-cystinyl aminopeptidase complex, it is quite conceivable for its N-terminal amino acids to be close to the catalytic site while the more distant C-terminal N_3-Phe^6 residue makes contact with juxtamembrane regions of the enzyme. Hence, the inhibitory effect of Ang IV does not necessarily imply an allosteric interaction that causes a conformational change on the enzyme.

The K_i values of the investigated peptides in the enzyme assay correspond to those reported for enzyme inhibition studies with recombinantly-expressed cystinyl aminopeptidase in HEK293 cells [25]. On the other hand, when compared to the competition binding studies, Ang IV, divalinal^1-Ang IV, Ang (5–8), LVV-H6 and LVV-H7 were appreciably less potent in producing enzyme inhibition. The K_i values of these peptides were between 10- and 90-fold higher in the substrate cleavage assay. In contrast Ang II, Ang III and Ang (3–7) had about equal affinity in both assays while Ang (4–8) even displayed preference for enzyme inhibition. A similar, peptide-dependent disparity in potency in these two assays was also reported by Lew et al. for the recombinant human cystinyl aminopeptidase in HEK293T cell membranes [25]. These authors presented experimental evidence suggesting that peptide degradation is not responsible for the deviant behaviour of Ang IV and related peptides in both assays. Instead, the presence or absence of zinc in the catalytic site was put forward as the most likely explanation. It is of notice that the binding assays with Chinese hamster (present study) and recombinant human cystinyl aminopeptidase [21,25,41] and, in fact, with nearly all previous "AT_4 receptor"-containing membrane preparations have been carried out in the presence of the divalent cation chelators EDTA and 1,10-PHE. These findings therefore suggest that the presence of zinc in the catalytic site of cystinyl aminopeptidase alters its affinity of Ang IV and related peptides. The following study provides further support for the idea that Ang IV and LVV-H7 only display high affinity for the apo-enzyme and it also unveils that EDTA and 1,10-PHE act in concert to generate the apo-enzyme.

Previously, the presence of cystinyl aminopeptidase in the peri-centriolar endocytic recycling compartment of CHO-K1 cells was only alluded by immunofluorescence and Western blot studies [55–57]. Radioligand binding and enzyme activity measurements in this report provide the first pharmacological characterization of this enzyme in this cell line. The effect of Ang IV and related "AT_4 receptor" ligands are closely similar to those recently reported for the recombinant human enzyme (denoted as IRAP) in HEK293T cell membranes [25,41]. As both assays displayed a high signal-to-noise ratio, CHO-K1 cells represent a convenient source for the investigation of cystinyl aminopeptidase in a single cell line.

Acknowledgments

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