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Synergistic modulation of cystinyl aminopeptidase by divalent cation chelators

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Abstract

Membranes of Chinese hamster ovary (CHO-K1) cells were used to study the opposite modulation of enzyme activity and [^{125}I]Ang IV binding to cystinyl aminopeptidase (EC 3.4.11.3) by divalent cation chelators. Whereas ethylene diamine tetraacetic acid (EDTA) or ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) alone only slightly affected the enzyme activity, 1,10-phenanthrolin (1,10-PHE) produced a complete and concentration-dependent inhibition. Interestingly EDTA ($\geq$0.05 mM) or EGTA ($\geq$0.15 mM) enhanced the inhibitory effect of 1,10-PHE. Two-site analysis of the corresponding inhibition curves revealed that EDTA and EGTA converted enzymes with low sensitivity towards 1,10-PHE into enzymes with high sensitivity. The combined inhibition by EDTA (0.1 mM) and 1,10-PHE (0.1 mM) could be prevented and reversed by addition of Zn^{2+} (at about 0.04–0.1 mM). In contrast, specific binding of [^{125}I]Ang IV was enhanced in the presence of 1,10-PHE. Binding was only slightly affected by EDTA or EGTA alone. Furthermore, the stimulatory effect of 1,10-PHE was potentiated by EDTA ($\geq$0.05 mM) as well as EGTA ($\geq$0.15 mM). In the presence of EDTA (0.1 mM) and 1,10-PHE (0.1 mM), specific [^{125}I]Ang IV binding was completely inhibited by Zn^{2+} ($IC_{50} = 39.7 \pm 6.2$ μM). The present data show that divalent cations such as Zn^{2+} are essential for the enzyme activity of cystinyl aminopeptidase and inhibitory for [^{125}I]Ang IV binding. Modulation of the effects of 1,10-PHE by other chelators such as EDTA or EGTA, suggests that, in addition to the binding site for zinc in the catalytic site, cystinyl aminopeptidase also bears a regulatory divalent cation binding site.

Keywords: Cystinyl aminopeptidase; EDTA; EGTA; 1,10-Phenanthrolin; Chinese hamster ovary cells

1. Introduction

Cystinyl aminopeptidase (EC 3.4.11.3) is a member of the M1 family of gluzincin metallopeptidases [1,2]. It is a type II integral membrane protein of 1024 amino acid residues, consisting of an acidic, N-terminal intracellular region (109 AA) followed by a hydrophobic transmembrane segment (22 AA α-helix) and a large extracellular C-terminal domain (893 AA) bearing the enzymatic activity [3,4]. Its catalytic site contains a zinc atom which is tetracoordinated by two histidine residues, a glutamic acid residue and a water molecule. These three protein ligands (His^{464}, His^{468} and Glu^{487}) are part of a $HEXXH(X)_{18}$-E zinc-binding motif, which is common to the M1 family of metallopeptidases [5]. In addition, this family is also characterized by the presence of a GXMEN motif located N-terminally to the zinc site and whose glutamate side-chain is proposed to be involved in the recognition of the free α-amino group of substrates or inhibitors and in the

Abbreviations: Ang, angiotensin; CHO-K1, Chinese hamster ovary cells; IRAP, insulin regulated aminopeptidase; EDTA, ethylene diamine tetraacetic acid; 1,10-PHE, 1,10-phenanthrolin; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEK293, human embryonal kidney cells; L-Leu-pNA, L-leucine-*p*-nitroanilide

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stabilisation of the transition state of the enzyme complex [5–7].

Cystinyl aminopeptidase received no less than 14 different denominations. Most of them are related to the alleged substrate specificity and to the physiological context in which this enzyme has been investigated. The denominations ''vasopressinase'' and ''oxytocinase'' refer to the fact that vasopressin and oxytocin peptides were initially thought to be the preferential substrates [8–10]. Although both possess an N-terminal cysteine residue, it is now well established that this property is not sufficient, nor even necessary for a peptide to be hydrolysed by this enzyme in vitro. Some peptide targets do not contain cysteine residues and are cleaved by cystinyl aminopeptidase, while some peptides are endowed with an N-terminal cysteine but not cleaved [9,10].

The tissue distribution of cystinyl aminopeptidase appears to be widespread. Besides its presence in fat, muscle and placenta, it is also expressed in several regions of the CNS like hippocampus, cerebral cortex, basal ganglia, cerebellar cortex and medulla oblongata [11,12] as well as in various other peripheral tissues like kidney, bladder, heart, spleen, prostate, adrenals and colon [3]. In the preceding paper [13], we presented evidence that this enzyme is also endogenously expressed in Chinese hamster ovary (CHO-K1) cells.

A major advance in our knowledge about the physiological role of this enzyme was provided by Albiston et al. [11] who discovered that it binds with high affinity to Ang IV, a hexapeptide that forms part of the renin angiotensin system. High affinity [^{125}I]Ang IV binding sites were first reported by Harding et al. [14] and they have subsequently been detected in membrane preparations from various tissues and cell types. They have been denominated as ''AT_4 receptors'' [15] and, recently they have been shown to represent cystinyl aminopeptidase [11,16]. Ang IV and LVV-hemorphin-7 (a decapeptide which is abundant in the CNS) inhibit the cystinyl aminopeptidase activity [11] and it has therefore been suggested that both peptides exert physiological effects by reducing the processing of other peptide hormones and neurotransmitters [10,11,16]. In the CNS, these ligands could extend the half-life of endogenous neuropeptides like vasopressin, substance P, and somatostatin which are known to play important roles in cognitive function [17–19] and, by this way, improve memory and learning [11,16].

Until recently, the cystinyl aminopeptidase could be detected semi-quantitatively by measuring its cleaving activity, by detecting its mRNA [3,4,11] and by the use of specific antibodies [3,10,20,21]. Quantitative determinations should now be greatly facilitated by binding studies with [^{125}I]Ang IV and by the availability of metabolically even more stable analogs like [^{125}I]divalinal^{1}-Ang IV or [^{125}I]norleucine^{1}-Ang IV [11,22–24]. The interaction between cystinyl aminopeptidase and Ang IV, LVV-hemorphin-7 and related peptides can now be compared by measuring its catalytic activity as well as by radioligand binding. Studies in this respect have already been carried out with the recombinant enzyme expressed in HEK293T and with the endogenous enzyme expressed in CHO-K1 cells. The results were remarkably similar for both cell systems; it was shown that Ang IV and LVV-hemorphin-7 act as competitive inhibitors of the enzyme activity and that the affinity of these peptides is much lower in the enzyme activity assays as compared to the [^{125}I]Ang IV binding studies on the same membrane preparation [13,24,25]. The discrepant behaviour of these peptides was not ascribed to differences in their degradation in both assays [24,25]. Instead, as only the binding assays were carried out in the presence of the divalent cation chelators ethylene diamine tetraacetic acid (EDTA) and 1,10-phenanthrolin (1,10-PHE), the presence or absence of zinc in the catalytic site (respectively in the enzyme activity assays and the binding studies) was put forward as the most likely explanation [13,24,25]. To get a better insight in this phenomenon, we have compared the effects of the divalent cation chelators EDTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 1,10-PHE on the enzymatic and [^{125}I]Ang IV binding properties of cystinyl aminopeptidase in CHO-K1 cell membranes. Here, we show that both types of chelators act in concert to remove zinc from the catalytic site and that this process is necessary for the enzyme to acquire high affinity for Ang IV and related peptides.

2. Materials and methods

2.1. Materials

Ang IV or Ang II (3–8) was obtained from Neosystem. EDTA, EGTA, 1,10-phenantrolin (1,10-PHE), 1,7-PHE and ascorbic acid were obtained from Sigma. L-Leucine-*p*-nitroanilide (L-Leu-pNA) 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. 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 and membranes were prepared as described previously [13]. In short, cells were grown in 5% CO_2 at 37 °C in Dulbecco's modified essential medium (DMEM), supplemented with L-glutamine, penicillin, streptomycine, non-essential amino-acids, sodium pyruvate and foetal bovine serum (Invitrogen), until confluent. The cells were harvested with 0.2% EDTA (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 and Potter homogenizer and 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 supernatans was removed. The resulting pellets were stored at −20 °C until use.

2.3. Enzyme assay

As described previously [13], the enzyme assays were performed using L-leucine-*p*-nitroanilide (L-Leu-pNA). In short, the pellets were thawed and resuspended using a Polytron homogenizer in enzyme buffer containing 50 mM Tris–Cl (pH 7.4), 140 mM NaCl, 0.1% (w/v) BSA and 100 μM PMSF. The incubation mixture comprised of 50 μl membrane homogenate (at a concentration corresponding to 4×10^5 cells/incubation, 200 μl L-Leu-pNA (1.5 mM or at the concentrations indicated) and 50 μl enzyme buffer, test compound (EDTA, EGTA, 1,10-PHE, 1,7-PHE and ascorbic acid) or 10 μM Ang IV. The membrane homogenate was incubated at 37 °C in 96 well plates (Medisch Labo Service) and the formation of the cleavage product *p*-nitroaniline was followed by measuring the absorption at 405 nm between 10 and 50 min in a Bio-Whittaker ELISA reader. The corresponding rate constants (further denoted as enzymatic activities) were calculated by linear regression analysis of these time curves.

2.4. Radioligand binding

Radioligand binding was performed as described previously [13]. Briefly, the membrane pellets were thawed and resuspended in 50 mM Tris–HCl (pH 7.4) binding buffer containing 140 mM NaCl, 5 mM EDTA, 0.1% (w/v) BSA, 100 μM PMSF and 100 μM 1,10-PHE. All binding experiments were performed by incubating the membrane homogenate at a concentration corresponding to 4×10^5 cells/incubation. The incubations were carried out in a final volume of 250 μl per well consisting of 200 μl membrane homogenate, 25 μl binding buffer (for total binding), test compound (EDTA, EGTA, 1,10-PHE, 1,7-PHE and ascorbic acid) or 10 μM unlabeled Ang IV for measuring non-specific binding. Subsequently 25 μl [^{125}I]Ang IV was added at a final concentration of 1 nM for all other experiments. After incubation at 37 °C for 60 min the mixture was vacuum filtered 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.

2.5. Data analysis

All experiments were performed at least three times, with each determination being the average of triplicate and duplicate determinations for the radioligand binding and enzyme assay experiments respectively. The calculation of IC_{50} values from binding studies and enzyme inhibitory experiments were performed by non-linear regression analysis using GraphPad Prism 4.0.

3. Results

3.1. Enzyme activity

Cleavage of the synthetic substrate L-Leu-pNA in the presence of CHO-K1 cell membranes can be inhibited by up to 94% with Ang IV (IC_{50} is about 0.72 μM for 1.5 mM substrate). This Ang IV-sensitive enzyme activity has been attributed to cystinyl aminopeptidase (EC 3.4.11.3) endogenously expressed in these cells [13].

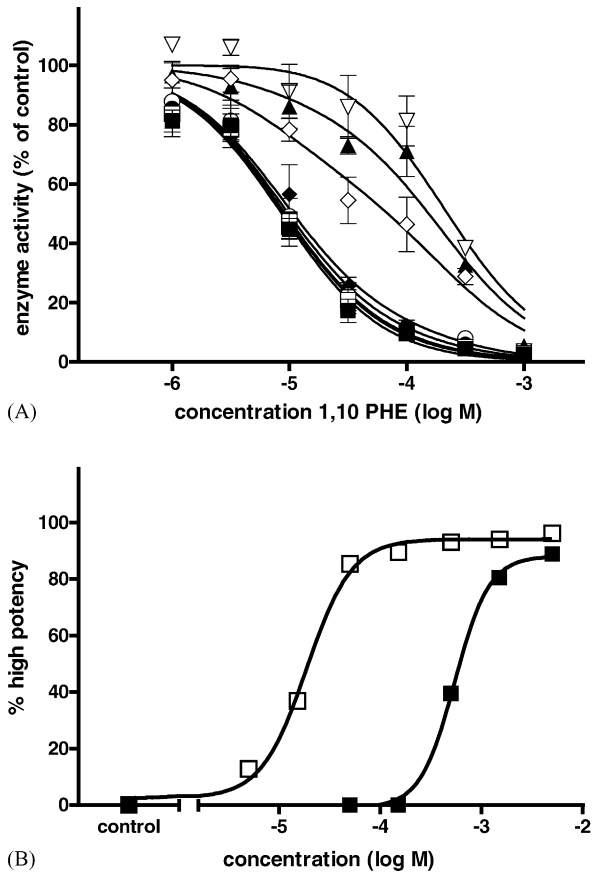

Fig. 1. (A) 1,10-PHE concentration-inhibition curves of enzymatic activity in CHO-K1 cell membranes in the absence (▽) or presence of increasing concentrations (mM) of EDTA: 0.005 (▲), 0.015 (◇), 0.05 (◆), 0.15 (○), 0.5 (●), 1.5 (□) and 5 (■). Values are the average ± S.E.M. of three independent experiments performed in duplicate and are normalized according to the control activity in the absence of chelator. (B) Effect of EDTA (□) or EGTA (■) on the enzyme activity that is inhibited with high potency by 1,10-PHE (IC_{50} values of 8.9 ± 0.6 μM and 9.9 ± 0.8 μM, respectively). The relative contribution of this component is calculated by non-linear regression analysis of 1,10-PHE concentration-inhibition curves (shown in Panel A for EDTA and data not shown for EGTA) and expressed as percent of total activity.

Whereas the divalent cation chelators EDTA and EGTA only had a weak inhibitory effect on the enzyme activity at ≤1 mM, 1,10-PHE produced a full and concentration-dependent inhibition with an IC_{50} of 200 ± 33 μM (Fig. 1A). However, the inhibitory effect of 1,10-PHE was substantially potentiated by both EDTA and EGTA. At low concentrations (5 and 15 μM) EDTA produced a leftward shift and flattened the 1,10-PHE concentration-inhibition curve (Fig. 1A). At 0.05 mM EDTA, the 1,10-PHE concentration-inhibition curve was even further shifted to the left but it became steep again. The potency (IC_{50} of 8.9 ± 0.6 μM) was no longer affected by further increasing the EDTA concentration up to 5 mM (Fig. 1A). One- and two-site analysis of these inhibition data are compatible with a model in which EDTA concentration-dependently converts enzymes with low sensitivity for 1,10-PHE into enzymes with high sensitivity. This conversion is nearly complete at 0.05 mM EDTA (Fig. 1B). The 1,10-PHE concentration-inhibition curve is similarly affected by increasing concentrations of EGTA (IC_{50} decreases to 9.9 ± 0.8 μM, data not shown), except that EGTA was about 30 times less potent than EDTA (Fig. 1B).

The combined and complete inhibition of the enzyme activity by 0.1 mM EDTA and 0.1 mM 1,10-PHE could be prevented in the presence of 0.04–0.1 mM $ZnCl_2$ (Fig. 2A). A further increase in the $ZnCl_2$ concentration resulted in a gradual decline in the activity. A similar biphasic effect seems to be common for most metallopeptidases including the human analogue of placental leucine aminopeptidase and aminopeptidase N from *Bombix mori* [26,27]. When membranes were preincubated with 0.1 mM EDTA and

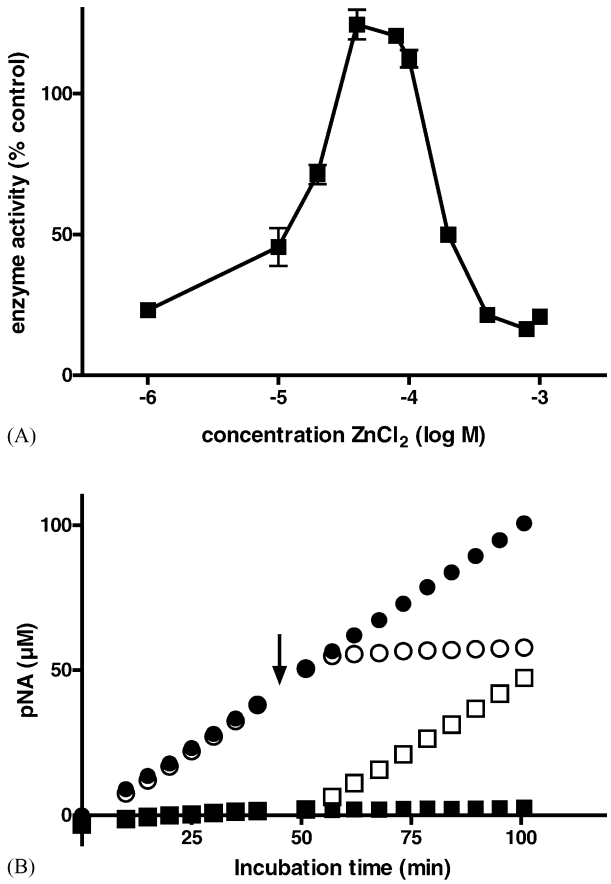

Fig. 2. Prevention of 1,10-PHE/EDTA-mediated enzyme inhibition by $ZnCl_2$. (A) CHO-K1 cell membranes were incubated in buffer containing 0.1 mM 1,10-PHE and 0.1 mM EDTA in the presence of increasing concentrations of $ZnCl_2$. Values are the average ± S.E.M. of one representative experiment performed in triplicate and are normalized according to control activity in the absence of chelators (i.e., 100%). (B) Reversal of 1,10-PHE/EDTA-mediated enzyme inhibition by $ZnCl_2$. CHO-K1 cell membranes were incubated at 37 °C in buffer without (●) or containing 0.1 mM 1,10-PHE and 0.1 mM EDTA (■). Formation of the product *p*-nitroanilide was measured as a function of time. After 45 min (arrow) 10 μl of either buffer (● and ■) or a solution of $ZnCl_2$ (○ and □, giving a final concentration of 0.1 mM) was added, after which the incubation was continued.

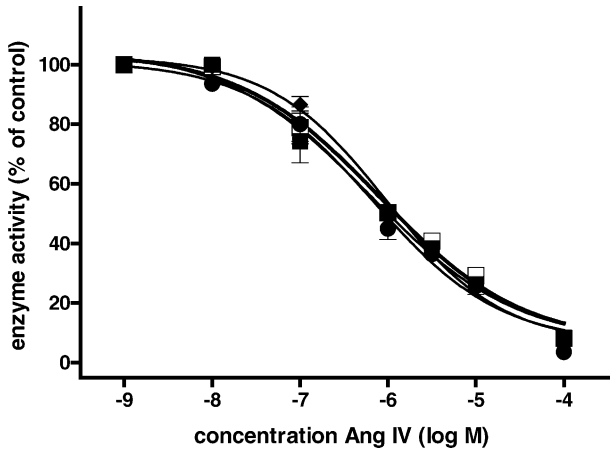

Fig. 3. Ang IV concentration-inhibition curves of enzymatic activity in CHO-K1 cell membranes in the absence (■) or presence of EDTA: 0.05 (◆), 0.5 (●) and 5 mM (□). Values are the average ± S.E.M. of three independent experiments performed in duplicate and are normalized according to control activity in the absence of Ang IV.

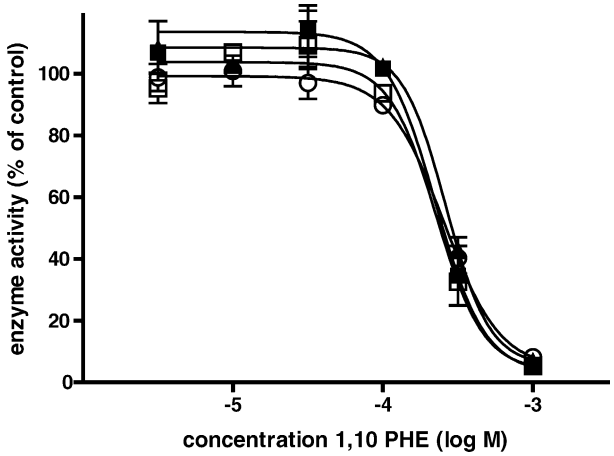

Fig. 4. 1,10-PHE concentration-inhibition curves of enzymatic activity in CHO-K1 cell membranes in the absence (○) or presence of ascorbic acid: 0.5 (▲), 1.5 (□) and 5 mM (■).Values are the average ± S.E.M. of three independent experiments performed in duplicate and normalized as in Fig. 1A.

0.1 mM 1,10-PHE, addition of 0.1 mM $ZnCl_2$ produced very fast and full recovery of the activity (Fig. 2B).

The cystinyl aminopeptidase activity was also concentration-dependently inhibited by Ang IV (IC_{50} of 0.72 ± 0.06 μM) but, in contrast to 1,10-PHE, the Ang IV inhibition-curves were not affected by EDTA at concentrations up to 5 mM (Fig. 3). Also, 0.1 mM $ZnCl_2$ was unable to restore the inhibitory effect of 100 μM Ang IV (data not shown).

Control experiments revealed that ascorbic acid (antioxidant) failed to inhibit the activity by itself at concentrations up to 5 mM and that it did not affect the concentration-inhibition curve of 1,10-PHE either (Fig. 4). 1,7-PHE (a non-ion chelating isomer of 1,10-PHE) also inhibited the activity with very low potency and its effect was not potentiated by 0.05–5 mM EDTA (Fig. 5).

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

[^{125}I]Ang IV binding was inversely regulated by the investigated divalent cation chelators. No specific (i.e., Ang IV-displaceable) binding of this radioligand was observed in the absence of chelator. EDTA (between 0 and 1.5 mM) only caused a modest increase in binding whereas 1,10-PHE produced a substantial and concentration-dependent increase at 1 mM, the highest concentration tested (Fig. 6A). No effect thereon was observed upon co-addition of ≤0.015 mM EDTA, but there was a marked potentiation at higher concentrations of EDTA. In the presence of 0.05–1.5 mM EDTA, 1,10-PHE increased the [^{125}I]Ang IV binding activity half-maximally at 6.7 ± 0.7 μM. EGTA produced a modest, concentration-dependent increase in [^{125}I]Ang IV binding (up to 30% of maximal binding at 5 mM EGTA) and it potentiated the effect of 1,10-PHE as well (Fig. 6B). However, the potentiation by EGTA took place at a higher concentration range as for EDTA.

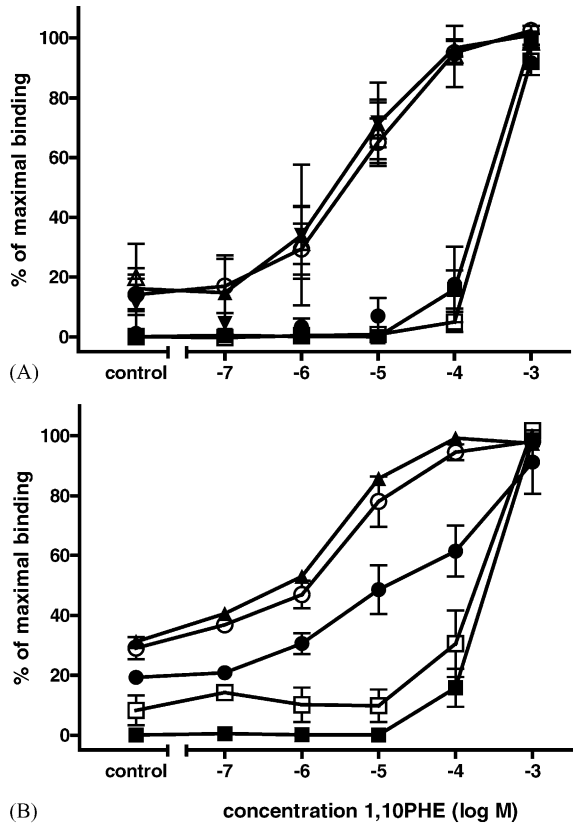

Fig. 6. [^{125}I]Ang IV binding to CHO-K1 cell membranes in presence of increasing concentrations of 1,10-PHE in the absence (■) or presence of: (A) EDTA (mM): 0.005 (□), 0.015 (●), 0.05 (○), 0.15 (▲), 0.5 (△) and 1.5 (▼) or (B) EGTA (mM): 0.05 (□), 0.15 (●), 0.5 (○) and 5 (▲). Specific binding represents the average ± S.E.M. of three independent experiments performed in duplicate. Control binding (i.e., 0%) was obtained in the absence of chelators and maximal binding (i.e., 100%) in the presence of 1.5 mM EDTA and 1 mM 1,10-PHE.

Binding of [^{125}I]Ang IV in the presence of 0.1 mM EDTA and 0.1 mM 1,10-PHE was concentration-dependently inhibited by $ZnCl_2$ (IC_{50} of 39.7 ± 6.2 μM, Fig. 7). Inhibition was complete at 0.1 mM $ZnCl_2$. At higher

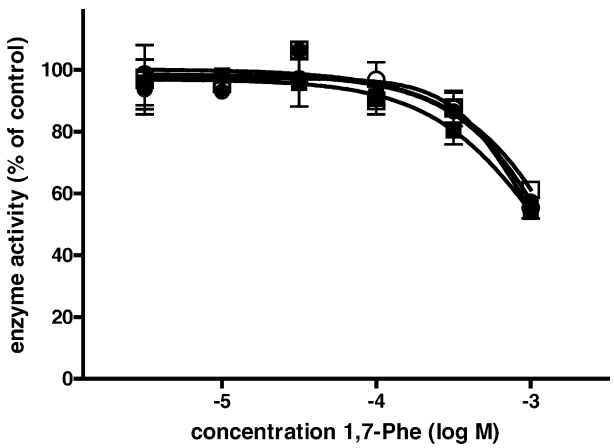

Fig. 5. 1,7-PHE concentration-inhibition curves of enzymatic activity in CHO-K1 cell membranes in the absence (○) or presence of EDTA: 0.05 (●), 0.5 (□) and 5 mM (●).Values are the average ± S.E.M. of three independent experiments performed in duplicate and normalized as in Fig. 1A.

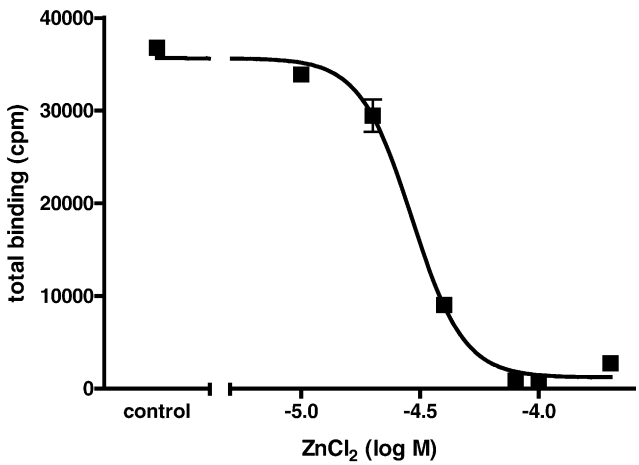

Fig. 7. Total binding of [^{125}I]Ang IV to CHO-K1 cell membranes in the presence of increasing concentrations of $ZnCl_2$ in buffer containing 0.1 mM 1,10-PHE and 0.1 mM EDTA. Values are the average ± S.E.M. of one representative experiment performed in triplicate.

concentrations of $ZnCl_2$, total binding started to rise again but this effect could be completely attributed to non-specific, Ang IV-resistant binding.

Control experiments with ascorbic acid revealed that the antioxidant was unable to stimulate [^{125}I]Ang IV binding or to potentiate the effect of 1,10-PHE (data not shown). 1,7-PHE did not increase [^{125}I]Ang IV binding either alone or in the presence of up to 1.5 mM EDTA (data not shown).

4. Discussion

Cystinyl aminopeptidase (EC 3.4.11.3) was found to be endogenously expressed in CHO-K1 cells [13,20,28]. This enzyme can be investigated by the high affinity binding of [^{125}I]Ang IV as well as by the Ang IV-sensitive enzymatic cleavage of L-Leu-pNA in CHO-K1 cell membrane preparations [13]. Major findings of the present study are that [^{125}I]Ang IV only binds with high affinity to the Zn^{2+}-depleted apo-enzyme and that divalent cation chelators like EDTA and 1,10-PHE act in a synergistic fashion to produce this apo-enzyme.

[^{125}I]Ang IV binding assays, 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 chelators have also been shown to facilitate the high affinity binding of [^{125}I]Ang IV to the endogenous recombinant human cystinyl aminopeptidase in HEK293T cell membranes but this issue was not addressed in detail in those studies [11,24,25]. The present experiments on CHO-K1 cell membranes consistently reveal that depletion of the enzymatic activity by divalent cation chelators, either alone or in combination, goes along with the appearance of high affinity [^{125}I]Ang IV binding sites. The lack of measurable [^{125}I]Ang IV binding in the absence of divalent ion chelators such as EDTA and 1,10-PHE could be attributed to its low affinity for the holoenzyme or to degradation of the radioligand. In favour of the first possibility, Lew et al. [24] did not find any degradation of Ang IV in HEK293 cells transfected with human cystinyl aminopeptidase.

Chelators like EDTA and EGTA only had a minimal effect on the enzymatic activity and, conversely, they induced only little [^{125}I]Ang IV binding at the concentrations used (0.01–5 mM). On the other hand, 1,10-PHE inhibited the activity completely and it induced [^{125}I]Ang IV binding with comparable potency (half-maximal effects at about 200 and 222 μM, respectively). Interestingly, 1,10-PHE produced both effects with about a 35-fold higher potency when EDTA was also present and this potentiating effect of EDTA was observed at concentrations at which it had no measurable effect on its own. A similar synergistic effect took place when combining 1,10-PHE and EGTA but this latter chelator was less potent than EDTA.

1,10-PHE is known to be a predominant Zn^{2+}-specific chelator [29] and its effects on cystinyl aminopeptidase are therefore most likely related to the depletion of Zn^{2+} in its active site. The results of two different experimental strategies are in agreement with this assumption. First, Zn^{2+} was shown to decrease the [^{125}I]Ang IV binding activity and to rapidly restore the catalytic activity in the presence of EDTA (0.1 mM) and 1,10-PHE (0.1 mM) (Figs. 2 and 7). Under these conditions, Zn^{2+} was equipotent for both effects and these were maximal around 0.1 mM. In the same line, Zn^{2+} has also been shown by Jarvis and Gessner [30] to inhibit the binding of [^{125}I]ANG IV to bovine adrenal cortex membranes with high potency. Second, 1,7-PHE, a non-chelating isomer of 1,10-PHE [31], was unable to induce [^{125}I]Ang IV binding. It only partially inhibited the enzyme activity and this was not affected by the presence of EDTA.

EDTA is a potent chelator for a variety of divalent cations, including Zn^{2+}. It is therefore surprising that, on its own, it produced so little effect on the binding and the catalytic activity. A possible explanation is that, for steric or electrostatic reasons, this chelator has only limited access to the catalytic site of cystinyl aminopeptidase. On the other hand, the ability of EDTA (and to a lesser extend EGTA) to potentiate the effect of 1,10-PHE constitutes a new finding and it should be of great interest to unveil the molecular mechanism of this synergistic action. The most simple interpretation of the present data is that EDTA facilitates the accessibility of the catalytic site of cystinyl aminopeptidase to 1,10-PHE. An interesting observation in this respect is that the effects of all tested 1,10-PHE/EDTA combinations are very rapid and constant with time. The inhibition curves shown in Fig. 1A, therefore represent steady-state situations. In addition, the shallow 1,10-PHE inhibition curves of the enzyme activity in the presence of intermediate concentrations of EDTA (5 and 15 μM) can be explained by assuming that: (i) the enzyme only adopts two states, one with low accessibility to 1,10-PHE and the other with high accessibility to 1,10-PHE (denoted as L and H states, respectively), (ii) both states may coexist and that (iii) EDTA shifts the equilibrium between them towards H in a concentration-dependent fashion. At 0.05 mM EDTA and above, nearly all the enzyme molecules reside in the H state.

Cystinyl aminopeptidase only bears a single, catalytic Zn^{2+} ion [9]. Based on sequence homologies among the members of the M1 family of gluzincin metallopeptidases and on the X-ray crystal structure of one of them, leukotriene A_4 hydrolase [32], this Zn^{2+} is likely to be located at the bottom of an active site cleft of cystinyl aminopeptidase. Here, it should be tetrahedrically bound by two histidine residues (His^{464} and His^{468}), a glutamic acid residue (Glu^{487}) and a water molecule [5]. In this context, the ability of EDTA to potentiate the effect of 1,10-PHE suggests the presence of one or more additional divalent cation binding sites on cystinyl aminopeptidase. In fact,

this is quite common for zinc metalloproteinases and, for some of them, it has been established that Ca^{2+} ions modulate their catalytic activity and/or structural stability [33]. Closest to cystinyl aminopeptidase, it has been demonstrated that the M1 gluzincin metallopeptidase aminopeptidase A (EC 3.4.11.7) contains a Ca^{2+} ion that increases the enzymatic activity and the affinity of synthetic substrates [34]. For *Streptomyces griseus* aminopeptidase (Family M28), a more distantly related zinc metalloproteinase, it has even been shown that Ca^{2+} modulates the affinity of antagonists like bestatin and amastatin [35,36]. In line with these previous studies, it is tempting to suggest that cystinyl aminopeptidase also bears a regulatory divalent cation binding site at its surface and that removal of this cation produces structural changes of the enzyme. Based on the results obtained with EDTA (Fig. 1A), these structural changes appear to go along with an increased accessibility of the catalytic Zn^{2+} to more hydrophobic chelators like 1,10-PHE, but without affecting the K_m of the synthetic substrate L-Leu-pNA (data not shown) and the K_i of the competitive antagonist Ang IV (Fig. 3). The X-ray crystal structure of cystinyl aminopeptidase and of nearly all other members of the M1 family of gluzincin metallopeptidases (with the exception of leukotriene A_4 hydrolase [32]) has not been determined yet. When finally performed, such experiments will provide the necessary structural information to evaluate the present assumption that cystinyl aminopeptidase possesses an additional binding site for a regulatory divalent cation. Such structural information, along with the identification of the ion's nature might also provide an explanation for the difference in potency between EGTA and EDTA to potentiate the effect of 1,10-PHE (Fig. 1B).

Finally, the fact that Ang IV and LVV-H7 only display high affinity for the apo-enzyme is difficult to reconciliate with the idea that the physiological effects of such peptides is related to their ability to reduce the processing of other peptide messengers [10,11,37]. Additional research will hopefully clarify how Ang IV is able to trigger biochemical events in a number of cellular systems. These events include, among others, a transient increase in the cytosolic calcium concentration in cells of pulmonary, cardiac and renal origin [38–42], DNA synthesis in the human neuroblastoma SK-N-MC cells and in coronary venular endothelial cells [43,44] and stimulation of mitogen-activated protein kinases in human proximal tubule epithelial cells [23]. As already shown for the related M1 metallopeptidases aminopeptidase-*N* (EC 3.4.11.2) and dipeptidylpeptidase IV (EC 3.4.14.3), the possibility arises that the dimeric form of cystinyl aminopeptidase conveys information across the cell membrane by itself [45]. Like RAMPs [46], monomeric cystinyl aminopeptidase could also modify the activity of more established receptors and, last but not least, doubt may be raised whether this enzyme represents the only high affinity recognition site for Ang IV.

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