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Selective labeling of IRAP by the tritiated AT₄ receptor ligands [³H]Angiotensin IV and its stable analog [³H]AL-11

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Abstract

'AT₄ receptors' through which angiotensin IV (Ang IV) improves memory acquisition, were recently identified as insulin regulated aminopeptidase (IRAP). Radioligand binding studies have hitherto been performed with iodinated Ang IV in the presence of divalent cation chelators EDTA and 1,10-phenanthroline. Hence, they referred to the apo-form of IRAP. Presently, binding of [³H]Ang IV and [³H]AL-11, a stable Ang IV analog, was compared on Chinese hamster ovary (CHO-K1) and mouse hippocampal (P40H1) cell membranes. With chelators, their high affinity sites showed the same pharmacological profile as for [¹²⁵I]Ang IV binding. Without chelators, only high affinity binding was perceived for [³H]AL-11. The same pharmacological profile was recorded in both membrane preparations; it was different from the one in presence of chelators and corresponded to catalytically active IRAP (despite the concurrent presence aminopeptidase N (APN) in P40H1 cell membranes). This confirms that the active and apo-forms of IRAP have a distinct pharmacological profile.

Keywords: IRAP, Ang IV, AL-11

Abbreviations: Ang, Angiotensin; APN, Aminopeptidase N; CHO-K1, Chinese hamster ovary cells; EDTA, Ethylene diamine tetra-acetic acid; GLUT4, Glucose transporter member 4; HEK, human embryonal kidney cells; IRAP, Insulin regulated aminopeptidase; L-Leu-pNA, L-Leucine-p-nitroanilide; LVV-H7, LVV-hemorphin 7 1,10-Phe, 1,10-phenanthroline; P40H1, mouse hippocampal cell line;

1. Introduction

Angiotensin IV (Ang IV: H-Val-Tyr-Ile-His-Pro-Phe-OH) is a physiologically active metabolite of Angiotensin II (Ang II). It improves memory acquisition and has vascular and renal actions (Chai et al., 2004; Wright et al., 1995). It was proposed that Ang IV may exert its effects by binding to 'AT₄ receptors' which were recently identified as cystinyl aminopeptidase (EC 3.4.11.3, also denoted as insulin regulated aminopeptidase or IRAP), a membrane-associated zinc-dependent metallopeptidase of the M1 family (Albiston et al., 2001). It is still not clear how Ang IV exerts its biological effects but a number of potential mechanisms have been proposed over the years. The earliest explanation deals with the propensity of Ang IV to inhibit the IRAP catalytic activity, resulting in a delayed catabolism of other bioactive peptides (e.g. vasopressin, oxytocin). Alternative explanations include the activation of one or more intracellular signaling cascades in the event that IRAP also acts as a receptor and the interference of Ang IV in the recycling of IRAP and the glucose transporter GLUT4, leading to an increased glucose uptake and to an improvement in memory and learning (Albiston et al., 2001; Vauquelin et al., 2002; Lew et al., 2003; Matsumoto et al., 2001, Chai et al., 2004; Fernando et al., 2008).

The pharmacological profile of recombinant IRAP has already been studied in great detail both by radioligand competition binding and enzyme activity inhibition experiments (Lee et al., 2003, Lew et al., 2003; Demaegdt et al., 2004, 2006). Without exception, the radioligand binding studies have hitherto been performed with iodinated Ang IV or its analogs and it is of notice that the so-obtained pharmacological profile of human IRAP in recombinant cell systems is the same as the one of the 'AT₄ receptors' in endogenously expressed in cell lines as well as in tissue homogenates of different animal species (Harding et al., 1994; Handa et al., 1999). This points at the pronounced selectivity of those radioligands for IRAP and also at the limited inter-species variability of the binding profile of this enzyme. Despite of this, the already published binding studies suffer from a number of handicaps. Most importantly, there is a pronounced difference in the pharmacological profile of IRAP when comparing the binding studies with the enzyme activity measurements. This difference can be imputed to the fact that the radioligand binding studies have routinely been carried out in the combined presence of the divalent cation chelators EDTA and 1,10-phenanthroline (Harding et al., 1994; Handa et al., 1999; Lee et al., 2003, Lew et al., 2003). This procedure likely emanated from a concern to prevent the proteolytic degradation of Ang IV and other alleged AT₄ ligands (Krebs et al., 1996; Chansel et al., 1998). Yet, it was recently found that, as these chelators work in concert to remove the catalytic zinc of IRAP, the radioligand binding studies only provide information about the IRAP apo-enzyme (Demaegdt et al., 2004b, 2006). This is well illustrated by the observation that high affinity [¹²⁵I]Ang IV binding to membranes of recombinant human IRAP expressing human embryonal kidney (HEK) cells and of native IRAP expressing Chinese hamster ovary (CHO-K1) cells only takes place in the presence of those chelators and that, when working with different chelator concentrations, the binding activity is inversely proportional to the catalytic activity. These studies further established that the lack of correspondence between the ligand affinities obtained in radioligand competition binding and enzyme activity inhibition

experiments can, at least in part, be attributed to the distinct pharmacological profiles of the apo- and the catalytically active forms of IRAP.

Besides its ability to inhibit the catalytic activity of IRAP and its degradation by different proteases, Ang IV is also capable to interact with non-IRAP targets such as the related aminopeptidase N (EC3.4.11.2, APN) and aminopeptidase A (EC 3.4.11.7, APA) metallopeptidases and with AT₁-type angiotensin II receptors, albeit with a more than 10-, 100 and about 100-fold lower potency respectively (Garreau et al., 1998; Goto et al., 2006; Stragier et al., 2006; Le et al., 2003). Based on the ability of sartans to block the AT₁ receptors with high affinity and selectivity, it has already been well-established that these receptors intervene in a number of physiological actions of Ang IV both *in vivo* and *in vitro* (Yang et al., 2008; Le et al., 2003). Of particular interest is that Ang IV inhibits the catalytic activity of APN (Garreau et al., 1998) and, although this protein is best known for its enzymatic properties, it has also been reported to undergo homodimerisation and to trigger intracellular responses after the binding of antibodies (Santos et al., 2000). Hence, APN could also play the role of a potential Ang IV receptor.

To examine IRAP by radioligand binding under physiologically relevant conditions, it is imperative for those experiments to be performed in the absence of chelators. This should not only allow a more objective comparison with enzyme activity and functional assays but eventually also unveil alternative chelator-sensitive 'AT₄ receptor' sites. Such binding studies should ideally be carried out with a metabolically stable radioligand whose structure is as close as possible to that of Ang IV. In this respect, recent structure-activity relationship studies with Ang IV analogs pointed at AL-11 as the most suitable candidate (Axen et al. 2006, 2007, compound 13 in Lukaszuk et al., 2008). AL-11 (H-(R)-β²hVal-Tyr-Ile-His-Pro-β³hPhe-OH) is an Ang IV analog containing two beta amino acids. It is highly stable under IRAP enzyme assay conditions, it inhibits the activity of this enzyme with nanomolar affinity and, as a bonus, it displays marked selectivity towards IRAP when compared to APN and AT₁ receptors (Lukaszuk et al., 2008).

With one exception (Demaegdt et al., 2008), all the past radioligand studies have been performed with Tyr²-radioiodinated Ang IV or analogues (Lahoutte et al., 2003). Yet, tritiated radioligands should be preferred to avoid the potential interference of iodine in binding studies with the catalytically active form of IRAP. AL-11 has now been tritiated and, in the present study we compare its binding properties with those of [³H]Ang IV in the absence and presence of chelators and, under the latter conditions, also with those earlier reported for [¹²⁵I]Ang IV (Demaegdt et al., 2004; 2006). Binding of these tritiated ligands was performed on Chinese hamster ovary (CHO-K1) and hippocampal (P40H1) cell membranes and the pharmacological profile of the detected high affinity sites was also compared to that of the Ang IV-sensitive metalloproteases on the same membrane preparations. CHO-K1 cell membranes have previously been used as a model system to characterize IRAP both enzymatically and by [¹²⁵I]Ang IV binding (Demaegdt et al., 2004a,b, 2006). The investigation of IRAP as well as the search for alternative 'AT₄ receptor' sites in membranes of the P40H1 hippocampal neuronal cell line is also of special interest because of the alleged role of the hippocampus in the beneficial effects of Ang IV in memory and learning. Taken together, the present study reveals that [³H]AL-

11 represents the first exemplar of a class of radioligands that display high potency and selectivity for both the apo- and native (i.e. catalytically active) forms of IRAP.

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2. Methods

2.1 Materials

Angiotensin II, Angiotensin IV or Angiotensin II (3-8) were obtained from NeoMPS (Strasbourg, France), LVV-hemorphin 7 (LVV-H7) from Invitrogen (Merelbeke, Belgium) and L-methionine from Sigma-Aldrich (Bornem, Belgium). Divalinal-Angiotensin IV and AL-11 (Lukaszuk et al., 2008) were synthesized at the Department of Organic Chemistry (Vrije Universiteit Brussel, Brussels). The APN inhibitor 7B (Chen et al., 1999), was a gift from Prof. Yiotakis (Athens, Greece). L-leucine-p-nitroanilide (L-Leu-pNA) was purchased from Sigma-Aldrich (Bornem, Belgium) and p-nitroaniline from VWR International (Leuven, Belgium). CHO-K1 cells (Chinese hamster ovary cells) were kindly obtained from the Pasteur Institute (Brussels, Belgium). P40H1 cells (obtained in collaboration with Dr. Vanderheyden, VUB, Brussels), a mouse hippocampal cell line, were derived by Sridhara Chakravarthy and M.M. Panicker (NCBS, Bangalore, India). P40H1 is a conditionally-immortal (SV40Tts) cell line derived from the hippocampus of a 40 day old adult "Immortomouse" (Charles River, USA). It expresses neuronal markers but not glial markers. All other reagents were of the highest grade commercially available.

$^{3,4}\Delta\text{Pro}^5\text{-Ang IV}$ and $^{3,4}\Delta\text{Pro}^5\text{-AL-11}$ (i.e. the precursor peptides for [^3H]Ang IV and [^3H]AL-11) were synthesized by solid phase peptide synthesis method using Fmoc-chemistry at the Department of Organic Chemistry (Vrije Universiteit Brussel, Brussels). The precursors were tritiated at the Institute of Biochemistry (Biological Research Centre, Szeged, Hungary) in a self-designed vacuum manifold by the catalytic saturation as described earlier (Tóth et al., 1997). $^3\text{H}_2$ gas was purchased from Technobexport, Russia, and contained $\geq 98\%$ tritium. The total radioactivity of the crude [^3H]Pro 5 -Ang IV and [^3H]Pro 5 -AL-11 was measured with a TRI-CARB 2100TR liquid scintillation counter in a toluene-Triton X-100 cocktail and amounted 2.79 GBq (75.5 mCi) and 3.7 GBq (100 mCi), respectively. [^3H]Pro 5 -Ang IV and [^3H]Pro 5 -AL-11 (further denoted as [^3H]Ang IV and [^3H]AL-11) were purified by HPLC using a Grace Vydac 218TP54 C18 column on a Jasco Radio-HPLC instrument, and liquid scintillation detection was performed on a Canberra Packard Radiomatic 505TR Flow Radiochromatography Detector with the Ultima-Flo M scintillation cocktail. The specific activity of the pure, tritiated peptides was determined by HPLC using standard peptide calibration curve. It amounted 1.5 TBq mmol $^{-1}$ (40.8 Ci mmol $^{-1}$) and 1.29 TBq mmol $^{-1}$ (35.0 Ci mmol $^{-1}$) for [^3H]Ang IV and [^3H]AL-11, respectively.

2.2 Cell culture and membrane preparation

CHO-K1 and P40H1 cells 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 $\mu\text{g/ml}$ 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 confluency.

Membranes were prepared as described previously (Demaegdt et al., 2004a). In short, the cells were harvested with 0.2 % ethylene diamine tetra-acetic 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 (30000 g at 4 °C). The pellet was resuspended in 50 mM Tris-HCl, centrifuged (30 min 30000 g at 4 °C) and the supernatant was removed. The resulting pellets were stored at – 20 °C until use.

2.3 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. All binding experiments were performed with membrane homogenates corresponding to 4×10^5 CHO-K1 cells or 3×10^5 P40H1 cells per assay. The incubations were carried out in polyethylene 24 well plates (Elscolab, Kruibeke, Belgium) in a final volume of 300 μ l per well consisting of 150 μ l membrane homogenate, 50 μ l of an EDTA/1,10-phenanthroline mixture with a final concentration of 500 μ M EDTA and 100 μ M 1,10-phenanthroline, 50 μ l binding buffer either alone (for total binding) or containing unlabeled ligands at the indicated final concentrations (for the competition binding assays) or 10 μ M unlabeled Ang IV or AL-11 (for non-specific binding). Subsequently 50 μ l [3 H]Ang IV or [3 H]AL-11 was added at final concentrations ranging between 0.5 and 8 nM for the saturation binding experiments and at 3 nM for all other experiments. After incubation, at 37 °C for the indicated time periods (for kinetic experiments) or 60 min (for all other experiments), 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 (after adding 3 ml scintillation liquid (Optiphase Hisafe)) in a beta counter (Perkin Elmer, Boston, USA).

2.4 Enzyme assay

Determination of the aminopeptidase catalytic activity in P40H1 cell membranes was based on the cleavage of the substrate L-leucine-p-nitroanilide (L-Leu-pNA) (Demaegdt et al., 2004a) into L-leucine and p-nitroaniline. This latter product 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) and 140 mM NaCl. All catalytic activity measurements were performed with membrane homogenates corresponding to 2.5×10^5 P40H1 cells per assay. The incubation mixture comprised 50 μ l membrane homogenate, 200 μ l L-Leu-pNA (1.5 mM for the enzyme inhibition curves or a range of 0.09 to 3 mM for the Michaelis-Menten curves) and 50 μ l enzyme assay buffer either alone or with test compound. The amount of membrane homogenate corresponded to 2.5×10^5 P40H1 cells in each well. 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 (rate constant v). Fixed P-nitroaniline concentrations were used as standard to convert absorption into

concentration. The enzymatic activities were always proportional to the membrane concentration or amount of enzyme added (data not shown).

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 obtained from saturation binding experiments (K_D) of [3H]Ang IV or [3H]AL-11 or the Michaelis-Menten constant (K_m) for substrate cleavage (Cheng and Prusoff, 1973).

3. Results

Radioligand binding

Under steady-state conditions (i.e. after an incubation of 60 min at 37 °C, see kinetic experiments below), specific binding of [³H]Ang IV and of [³H]AL-11 to the CHO-K1 and P40H1 cell membrane homogenates was a saturable process in the presence of divalent cation chelators EDTA and 1,10-phenanthroline (+E/P) (Figure 1). In agreement with a simple bimolecular interaction between the radioligands and a single class of high affinity binding sites, specific binding increased with the free radioligand concentration according to a hyperbolic function. Single-site analysis of these curves yielded the density of binding sites (B_{\max}) as well as the equilibrium dissociation constants (K_D) for each radioligand/cell type combination (Table 1). When the same experiments were performed in the absence of chelators (-E/P), the specific binding of [³H]AL-11 to the CHO-K1 and P40H1 cell membrane homogenates was saturable as well (Figure 1). The B_{\max} and K_D values, obtained by a single-site analysis, were very similar to those obtained in the presence of chelators (Table 1). The B_{\max} values of [³H]AL-11 (+E/P and -E/P) were in the same range as for [³H]Ang IV (Table 1). This suggests that the same binding sites are involved.

The non-specific binding of 3 nM [³H]Ang IV (+E/P) and [³H]AL-11 (+E/P and -E/P) to each membrane preparation was less than 20 % of the total binding (data not shown). In the absence of chelators, specific binding of [³H]Ang IV was only minimal (especially when compared to [³H]Ang IV binding in the presence of chelators) and no high affinity sites could be discerned (Figure 1). The same difference was noticed earlier when comparing the binding of [¹²⁵I]Ang IV on CHO-K1 and recombinant IRAP HEK293 cell membranes in the presence and absence of chelators (Demaegdt et al., 2004a, 2006).

Only the specific binding of [³H]Ang IV (+E/P) and of [³H]AL-11 (+E/P and -E/P) to both membrane preparations was further studied. Ang IV, AL-11, LVV-H7, Divalinal-Ang IV, 7B, Ang II and L-methionine inhibited the specific binding of [³H]Ang IV and [³H]AL-11 in a concentration-dependent fashion in both CHO-K1 and P40H1 cell membranes (data not shown). In accordance with the premise that specific binding takes place to a single class of sites, the competition binding curves were monophasic for all these ligands. Their pK_i values are given in Table 2. For both membrane preparations and both radioligands, their (+E/P) potency order was: Ang IV > AL-11 > LVV-H7 > Divalinal-Ang IV > Ang II > 7B >> L-methionine. For both membrane preparations and [³H]AL-11, their (-E/P) potency order was quite distinct: AL-11 > Ang IV ~ 7B > Ang II > LVV-H7 > Divalinal-Ang IV >> L-methionine.

Specific binding of 3nM of [³H]Ang IV and [³H]AL-11 increased rapidly with time and reached equilibrium within less than 30 min at 37 °C (Figure 2). The binding was reversible since addition of an excess (10 μM) of unlabeled Ang IV or AL-11 resulted in an almost complete dissociation of the specifically bound radioligand. Association and dissociation of [³H]Ang IV (+E/P) or [³H]AL-11 (+E/P) from both membrane preparations was very rapid (Figure 2A and B, 2C and D) but association and dissociation of [³H]AL-11 (-E/P) proceeded sufficiently slowly (Figure 2E and F) to calculate the

pseudo first-order association (k_{obs}) and first-order dissociation rate constants (k_{-1}). These values allowed the calculation of the second-order association rate constants (k_{+1}) and the corresponding 'kinetic' equilibrium dissociation constant (K_D). Values are given in Table 3.

Enzyme assays

The aminopeptidase activity of IRAP in P40H1 cell membranes (CHO-K1 cells were previously studied, Demaegdts et al., 2004a) was assessed spectrophotometrically by measuring the absorption of p-nitroaniline (i.e. the cleavage product of the synthetic substrate L-Leu-pNA) at 405 nm over time at 37 °C. Combining the membranes and the substrate resulted in a steady, time-wise increase in absorption for at least 50 min (data not shown). The corresponding rate constants (v , further denoted as catalytic activity) were calculated by linear regression analysis of such absorption vs. time curves. Figure 3 shows the enzymatic activity as a function of the substrate concentration for P40H1 cell membranes. The activity increased with the free substrate concentration according to a hyperbolic function, suggesting the presence of a single class of catalytic sites with calculated K_m and V_{max} values of 0.49 ± 0.14 mM and 4.83 ± 1.80 $\mu\text{M}/\text{min}$, respectively.

The enzymatic activity (measured with a substrate concentration of 1.5 mM) was inhibited by Ang IV and the other unlabeled ligands in a concentration-dependent fashion. The inhibition curves for LVV-H7, Divalinal-Ang IV, Ang II and L-methionine were monophasic (data not shown) but biphasic curves were clearly observed in case of Ang IV, 7B and AL-11 (Figure 4A). High- and low affinity sites were each responsible for about half of the inhibitory effect of Ang IV, 7B and AL-11 (Figure 4A). This constancy in the high-/low affinity site ratio suggests that at least two different enzymes with similar K_m for the substrate are operational in the P40H1 cell membranes. In this respect it was previously observed that L-Leu-pNA is a non-selective substrate for IRAP and the related APN enzyme but that they display distinct affinity for Ang IV, 7B and AL-11 (Garreau et al., 1998; Lew et al., 2003; Demaegdts et al., 2006; Stragier et al., 2006; Lukaszuk et al., 2008). From these compounds, and in agreement with its alleged selectivity for APN, only 7B was found to display higher affinity for inhibiting the APN activity (Stragier et al., 2006). When in the present studies on P40H1 cell membranes, the high affinity component of the 7B inhibition curve was occluded in the presence of 10^{-6} M of this ligand, the remaining enzymatic activity could still be inhibited concentration-dependently by Ang IV and AL-11 (Figure 4B). Yet, the such-obtained inhibition curves were monophasic and displayed high affinity. This implies that the low affinity site of 7B corresponds to the high affinity site of Ang IV and AL-11. As a precaution, activity inhibition experiments with LVV-H7, Divalinal-Ang IV, Ang II and L-methionine were repeated in the presence of 10^{-6} M 7B but, with the exception of L-methionine, 7B had no impact on the IC_{50} values (Table 4).

Based on this complementary information, the potency order of these inhibitors could be established for the two enzymes. The potency for the enzyme with high affinity for Ang IV and AL-11 decreased as follows: Ang IV > AL-11 > 7B > Ang II ~ LVV-H7 > Divalinal-Ang IV. This is in agreement with the enzyme profile of IRAP. The potency order for the enzyme with high affinity for 7B was: 7B > Ang IV > AL-11 >> L-

methionine, corresponding to the profile of APN (Demaegdt et al., 2004a; 2006; Stragier et al., 2006; Lukaszuk et al., 2008). The pK_i values of these ligands for each enzyme, calculated according to the 'Cheng and Prusoff' equation (Cheng and Prusoff, 1973) on basis of their IC_{50} values obtained either by single- or two-site analysis of the inhibition curves and the K_m value of the substrate, are given in Table 4.

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4. Discussion

The interaction of two new radioligands, [³H]Ang IV and its stable analog [³H]AL-11 (compound 13 in Lukaszuk et al., 2008), with the AT₄/IRAP receptor was studied in membrane preparations of both Chinese hamster ovary (CHO-K1) cells and a hippocampal cell line (P40H1). Despite the low specific radioactivity of such tritiated radioligands when compared to radioiodinated analogs (28.7 Ci/mmol versus 2190 Ci/mmol for ¹²⁵I), [³H]Ang IV and [³H]AL-11 were well suited for the labeling of IRAP apo-enzyme (i.e. in the presence of the chelators EDTA and 1,10-phenanthroline) in both membrane preparations (Demaegdt et al., 2004, 2006 and unpublished results). Most importantly, [³H]AL-11 was also capable to specifically label the catalytically active form of IRAP (i.e. in the absence of chelators) while [³H]Ang IV and [¹²⁵I]Ang IV (Demaegdt et al., 2004a, 2006) failed to do so.

CHO-K1 cell membranes

CHO-K1 cells contain a fair number of endogenous IRAP proteins (Demaegdt et al., 2004a; 2006) and their characterization, both by enzymatic activity measurements and by binding experiments with [¹²⁵I]Ang IV on membrane preparations, revealed that they display the same ligand interaction (i.e. pharmacological) profile as human IRAP expressed in recombinant HEK cells (Demaegdt et al., 2006). Enzyme activity studies with L-Leu-pNA, a non-selective substrate for enzymes like IRAP and the related APN, only revealed the presence of IRAP in CHO-K1 cells whereas several other cell types also contained APN (unpublished results). APN has been proposed to constitute an alternative cellular recognition site for Ang IV (Garreau et al., 1998) and, in support of this proposal, the APN catalytic activity can also be inhibited by Ang IV, albeit with about 10 fold lower potency as for IRAP (Demaegdt et al., 2006). As CHO-K1 cell membranes lack detectable APN activity, they constitute a preeminent starting point for comparing the binding properties of [³H]Ang IV and [³H]AL-11 with those earlier found for [¹²⁵I]Ang IV.

Similar to [¹²⁵I]Ang IV, [³H]Ang IV binding was only minor in medium only, but a substantial amount of high affinity binding readily appeared in presence of the chelators EDTA and 1,10-phenanthroline. As extensively examined in previous studies (Lew et al., 2003; Demaegdt et al. 2004b, Laeremans et al., 2005) these chelators work in concert to remove the catalytic zinc of IRAP to yield the apo-enzyme. [³H]AL-11 also displayed high affinity binding in the presence of chelators and competition binding experiments revealed that the high affinity binding of both tritiated ligands could be fully, dose-dependently displaced by the unlabeled Ang IV and related ‘AT₄-receptor’ ligands. In agreement with a single class of sites, all these competition curves were steep and, in agreement with the selective labeling of the IRAP apo-enzyme, the pharmacological profile of the high affinity [³H]Ang IV and [³H]AL-11 binding sites was identical to that of the IRAP apo-enzyme obtained by [¹²⁵I]Ang IV competition binding assays in membrane preparations of CHO-K1 and other cell lines (Figure 5A, Demaegdt et al., 2004a; 2006, Lew et al., 2003, Lukaszuk et al., 2008, Stragier et al., 2006; Handa et al., 1999). Hence, ‘specific’/ high affinity binding of both tritiated ligands is likely to involve the IRAP apo-enzyme only. Moreover, tritiated and radioiodinated Ang IV (and analogs)

are all suitable for labeling apo-IRAP. The choice of the radiolabel should only be dictated by the concentration of binding sites in the cell material. In the present study on CHO-K1 cell membranes and P40H1 cell membranes (with about twice the density of IRAP), tritiated ligands were found to be perfectly suitable.

The lack of high affinity [³H]Ang IV in medium only, fits with the relatively low potency by which this peptide inhibits the catalytic activity of IRAP. Whether this low potency is related to the rapid degradation of Ang IV by metalloproteases or whether it constitutes a genuine property of the catalytic zinc-containing IRAP enzyme remains to be established. Saturation curves (Figure 1A) of specific [³H]Ang IV binding revealed that this radioligand displays a somewhat lower affinity for apo-IRAP ($K_D = 4.1 \pm 0.5$ nM) than earlier found for [¹²⁵I]Ang IV on the same membrane preparation ($K_D = 0.90 \pm 0.12$ nM, Demaegdts et al., 2004a). In fact, this difference is rather modest if one takes into account that:

- a) the attachment of iodine, a large and hydrophobic atom, could alter the pharmacological and physicochemical properties of a ligand and that
- b) in the case of [¹²⁵I]Ang IV, this attachment takes place at the tyrosine residue (Lahoutte et al., 2003) which is known for its important role in the binding to the ‘AT₄ receptor’/IRAP apo- enzyme (Sardinia et al. 1993, 1994).

[³H]AL-11 bound with about two-fold lower affinity to apo-IRAP than [³H]Ang IV (Table 1) when comparing K_D 's obtained from saturation binding experiments (association and dissociation occurred too fast to determine accurate “kinetic” K_d 's). This fits with the comparable pK_i values of both unlabeled ligands in competition binding studies with both tritiated ligands (Table 2) as well as in earlier studies with [¹²⁵I]Ang IV (Lukaszuk et al., 2008). This implies that substitution of Val¹ and Phe⁶ by (R)-β²-homo-Val and β³-homo-Phe, respectively, has only little effect on the affinity of Ang IV for apo-IRAP. Yet, these substitutions have been reported to profoundly and positively affect other properties of this ligand. Indeed, (R)-β²-homo-Val¹ substitution in Ang IV is likely to confer metabolic stability, β³-homo-Phe⁶ substitution abolishes AT₁ receptor affinity and both substitutions confer more pronounced (i.e. more than 100 fold) IRAP- versus APN-selectivity. Besides its resistance to aminopeptidase degradation, AL-11 also inhibited the catalytic activity of IRAP in recombinant HEK and in CHO-K1 cell membranes with high potency (Lukaszuk et al., 2008, unpublished results). This made us presuppose that native IRAP could be selectively labeled by high affinity binding of tritiated AL-11, as earlier attempts with tritiated and radioiodinated Ang IV failed to achieve this. As shown in Figure 1C, this is indeed the case. In contrast to the modest, low affinity binding of [³H]Ang IV in medium only, a substantial amount of high affinity [³H]AL-11 binding took place under this condition (Figure 1A and 1C). The K_D values for [³H]AL-11, calculated from saturation binding experiments (Table 1) and kinetic experiments (Table 3) were in the same range as the ones for [³H]AL-11 in the presence of chelators. Competition binding experiments with unlabeled Ang IV and related ‘AT₄-receptor’ ligands suggested that this high affinity binding involved a single class of sites. It could be fully, dose-dependently displaced by these ligands and the competition curves were steep. However, the pharmacological profile of these sites was different from those responsible for high affinity binding of [³H]AL-11 in the presence of chelators (i.e. to the

apo-form of IRAP) but identical to that obtained in IRAP activity inhibition studies in membrane preparations of CHO-K1 (Figure 5B) and other cell lines (Demaegdt et al., 2004a, 2006; Stragier et al., 2006, Lukaszuk et al., 2008). Large differences in both binding profiles (in the presence or absence of chelators) and the enzyme profile could have given an indication of possible alternative binding sites. This is clearly not the case here and as a consequence [³H]AL-11 binding can be used for binding to the catalytic active IRAP (in the absence of chelators) or for binding to apo-IRAP (in the presence of chelators).

The susceptibility of Ang IV and LVV-H7 to aminopeptidase-degradation could at least in part be responsible for their lower potency in [³H]AL-11 competition binding studies in medium only (as well as in IRAP activity studies) when compared with those in the presence of chelators. At first sight, this interpretation could also explain why the metabolically stable Ang IV analog AL-11 displays the same affinity for IRAP in the presence and absence of chelators. Indeed, the chelators did not affect the K_D of [³H]AL-11 in saturation binding experiments (Table 1) and, in the same line, it was earlier shown that AL-11 displays the same potency in competition binding studies with [¹²⁵I]Ang IV (i.e. for the apo-enzyme) as in enzyme activity inhibition studies (i.e. pK_i of 7.70 ± 0.32 versus 7.56 ± 0.21 , Lukaszuk et al., 2008). However, differences in the pharmacological profiles of both forms of IRAP are also at stake since the equally stable divalinal-Ang IV showed about 100-fold higher potency in competition binding studies with [¹²⁵I]Ang IV as in enzyme activity inhibition studies (i.e. pK_i of 7.10 ± 0.1 versus 5.22 ± 0.06 , Demaegdt et al., 2008). Moreover, compound 7B displayed higher potency for the native enzyme as seen in [³H]AL-11 competition binding and enzyme activity inhibition studies as for the apo-form of IRAP as seen in competition binding studies with all radioligands in the presence of chelators (Table 2, Demaegdt 2008). These latter findings strongly support the hypothesis that the catalytic Zn^{2+} is also able to influence the binding affinity of IRAP for some of its ligands (Demaegdt et al., 2004b; Lew et al 2003).

P40H1 cell membranes

Whereas the observed L-Leu-pNA cleaving activity in CHO-K1 cell membranes could be attributed to IRAP, the hippocampal P40H1 cell line offers a more complex situation for the present study since its membrane preparations contain about equi-active amounts of IRAP and APN. In this respect, using recombinant HEK cells expressing human IRAP or human APN, it has been shown earlier that both enzymes cleave this substrate with comparable K_m (Demaegdt et al., 2006). This explains why, when measuring the enzymatic activity as a function of the substrate concentration, similar K_m values were obtained with P40H1- and CHO-K1 cell membranes: i.e. 0.49 ± 0.14 and 0.39 ± 0.04 mM respectively (Fig. 3) (Demaegdt et al., 2006). Yet, the inhibition curves for Ang IV, 7B and AL-11 were clearly biphasic with each portion being responsible for about half of the cleaving activity (Figure 4A). From these ligands, 7B has been reported to represent an APN-selective inhibitor and, in studies with the recombinant cell lines, it was confirmed that this compound exhibits a 30 fold APN vs. IRAP selectivity (Stragier et al., 2006). Masking experiments, where the high affinity component of the 7B inhibition curve was occluded in the presence of 10^{-6} M of this ligand, revealed that Ang IV and AL-11 inhibited the remaining activity uniformly and with high potency (Figure 4B). Based on

its pharmacological profile (Figure 5C), the enzyme that was responsible for this remaining activity was identified as IRAP. On the other hand, the pharmacological profile of the enzyme with high affinity for 7B and low affinity for Ang IV and AL-11 corresponded to that of APN (Figure 5D). In this respect, it should be noted that the inhibition curves of LVV-H7, Divalinal-Ang IV, Ang II and L-methionine were apparently monophasic. However, as the inhibition by these ligands was not complete at the highest concentration tested, it cannot be ascertained whether they display similar affinity for the two L-Leu-pNA cleaving enzymes present in the P40H1 cell membranes or whether they show preference for one of both (such as in the case of L-methionine).

Despite of this added complexity, binding studies with [^3H]Ang IV and [^3H]AL-11 on P40H1 cell membranes offered a simple picture, quite consistent with their ability to selectively label IRAP. In the presence of chelators, both radioligands displayed high affinity binding to a single class of sites. Although the calculated K_D values of these radioligands in these cells were 1.5 to 2-fold higher than those obtained from saturation binding studies with CHO-K1 cell membranes (Table 1), the overall pharmacological profile of these high affinity sites was very similar to the profiles obtained by competition binding experiments with [^{125}I]Ang IV, [^3H]Ang IV and [^3H]AL-11 with CHO-K1 cell membranes (Figure 5A and 5E). These high affinity sites are therefore likely to represent the apo-form of IRAP.

In the absence of chelators, [^3H]AL-11 showed again high affinity binding to P40H1 cell membranes whereas similar sites failed to show up with [^3H]Ang IV (Figure 1). The K_D value obtained from [^3H]AL-11 saturation binding was also about twice the value obtained for similar experiments with CHO-K1 cell membranes. Yet, this difference should be put in perspective as the opposite relationship showed up when the K_D value-calculation was based on the [^3H]AL-11 association and dissociation rate constants (Table 3). Moreover, similar to the results from the competition binding studies with CHO-K1 cell membranes, the [^3H]AL-11 high affinity sites on P40H1 cell membranes behaved as a homogenous population for all the competitors and the pharmacological profile of these sites was very similar to that of native IRAP based on catalytic activity studies (Figure 5C). The pharmacological profile of these high affinity sites was clearly distinct from that of APN (Figure 5D), indicating that this enzyme fails to experience significant labeling by [^3H]AL-11. This deduction is in line with:

- a) the lack of high affinity [^3H]AL-11 binding to membrane preparations of recombinant HEK cells expressing human APN, both in the absence and presence of chelators (data not shown) and
- b) the previous demonstration that AL-11 inhibits the catalytic activity of IRAP with > 100-fold higher potency than the activity of APN (Lukaszuk et al., 2008).

Concluding remarks

Taken together, the present study reveals that [^3H]AL-11 displays high potency and selectivity for both the apo- and native (i.e. catalytically active) forms of IRAP. These distinctive properties, along with the metabolic stability of AL-11, make [^3H]AL-11 the first radioligand that is suitable for the labeling of native IRAP under physiologically relevant conditions (i.e. in the absence of metal chelators such as in all previous binding

studies with labeled Ang IV or analogs). Comparison of [³H]AL-11 binding in the presence and absence of chelators confirms the previous suggestion that the active and apo-forms of IRAP have a distinct pharmacological profile.

Despite the outspoken selectivity of AL-11 for IRAP versus other well-documented targets for Ang IV like AT₁ angiotensin II receptors and the APN enzyme, and the fact that only IRAP could be detected by binding of [³H]AL-11 to CHO-K1 and P40H1 cell membranes, it cannot be excluded that alternative high affinity cellular targets might be unveiled in ensuing experiments with other cell systems. Indeed, if the low potency by which Ang IV antagonizes the catalytic activity of IRAP reflects a genuine property of this enzyme (i.e. if the low potency of Ang IV is not merely related to its fast metabolic breakdown), the occurrence of alternative sites with high affinity for Ang IV might be expected based on its capability to trigger intracellular events such as calcium transients with nanomolar potency (Dulin et al., 1995; Chansel et al., 2001; Handa et al., 2001). In this respect, the existence of such sites has recently been hinted at (Wright et al., 2008) but the experimental data have not yet been published in full detail. The fact that such non-IRAP, high affinity 'AT₄-receptor' targets have not been unambiguously identified by binding studies with [¹²⁵I]Ang IV could, among others, be related to artifacts including their alteration by the divalent cation chelators, their masking by an excess of IRAP apo-enzymes or even by the fact that Ang IV is radioiodinated. We hope that further binding studies with [3H]AL-11, similar stable tritiated Ang IV analogs, or even [³H]Ang IV (provided that its degradation can be prevented without using chelators) on a wide variety of cellular material will be of help to resolve this puzzling problem.

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Legends of the figures

Figure 1

Saturation binding of [³H]Ang IV and [³H]AL-11. Membranes of CHO-K1 (A, C) and P40H1 (B, D) cells were incubated with increasing concentrations of radioligand for 60 min at 37°C either in the presence (■) or absence (▲) of 500 μM EDTA and 100 μM 1,10-Phe (E/P). Data refer to specific binding (expressed as fmol/10⁶ cells), calculated by subtracting non-specific binding in the presence of 10 μM Ang IV or AL-11 respectively, from total binding. Values are the mean ± SEM of three independent experiments performed in duplicate. Corresponding K_D and B_{max} values are given in Table 1.

Figure 2

Association and dissociation of [³H]Ang IV and [³H]AL-11 either in the presence or absence of 500 μM EDTA and 100 μM 1,10-Phe (E/P). Membranes of CHO-K1 (A, C and E) and P40H1 (B, D and F) cells were incubated with 3 nM of radioligand at 37°C for increasing periods of time (■). Dissociation was initiated after 60 min with 10 μM Ang IV or AL-11 respectively (▲). Values are the mean ± SEM of three independent experiments performed in duplicate.

Figure 3

Enzymatic cleavage of L-Leu-pNA as a function of the substrate concentration (Michaelis Menten curve). Membranes of P40H1 cells were incubated with increasing concentrations of L-Leu-pNA (S) and the corresponding rate constants (v) were determined. A representative of three independent experiments is illustrated. The mean ± SEM corresponding K_m and V_{max} values of all three experiments are 0.49 ± 0.14 mM and 4.83 ± 1.80 μM/min.

Figure 4

Inhibition of L-Leu-pNA cleaving activity in P40H1 cell membranes. Membranes were incubated at 37°C with 1.5 mM L-Leu-pNA in the absence (control activity) or presence of:

A) increasing concentrations of Ang IV (□), 7B (▲) and AL-11 (●)

B) increasing concentrations of Ang IV (□) and AL-11 (●) together with 10⁻⁶ M 7B

Values (rate constants v, expressed as percentage of control) are the mean ± SEM of three independent experiments performed in duplicate.

Figure 5

Radar diagrams of pharmacological profiles of [³H]Ang IV and [³H]AL-11 competition binding (in the presence (+E/P) or absence of chelators (-E/P)) in CHO-K1 (CHO) and P40H1 (P40) cell membranes compared to the ones of [¹²⁵I]Ang IV binding and IRAP and APN activity inhibition in CHO-K1, P40H1 and recombinant HEK293 cell membranes. The axes of the diagrams represent pK_i values.

Tables

Table 1: Saturation binding properties of [³H]Ang IV and [³H]AL-11 to CHO-K1 and P40H1 cell membranes in the presence and absence of 500 μM EDTA and 100 μM 1,10-Phe (E/P). Values are the mean ± SEM of three independent experiments performed in duplicate.

	CHO-K1 cells		P40H1 cells	
	K _D (nM)	Bmax (fmol/10 ⁶ cells)	K _D (nM)	Bmax (fmol/10 ⁶ cells)
[³ H]Ang IV (+ E/P)	4.1 ± 0.5	98.4 ± 13.0	8.1 ± 1.6	154.0 ± 14.5
[³ H]AL-11 (+ E/P)	7.7 ± 1.1	107.2 ± 22.2	11.8 ± 1.1	209.8 ± 23.3
[³ H]AL-11 (- E/P)	7.3 ± 1.2	114.9 ± 31.5	14.7 ± 0.5	179.1 ± 66.5

Table 2: [^3H]Ang IV and [^3H]AL-11 competition binding in CHO-K1 and P40H1 cell membranes in the presence and absence of 500 μM EDTA and 100 μM 1,10-Phe (E/P) by putative AT₄ receptor ligands, Ang II and APN inhibitors. pK_i values are the mean \pm SEM of three independent experiments performed in duplicate.

CHO-K1 cells	[^3H]Ang IV (+ E/P)	[^3H]AL-11 (+ E/P)	[^3H]AL-11 (- E/P)
Ang IV	7.83 \pm 0.34	8.34 \pm 0.13	6.76 \pm 0.18
AL-11	7.72 \pm 0.05	7.80 \pm 0.14	8.09 \pm 0.14
LVV-H7	7.33 \pm 0.40	7.22 \pm 0.19	6.20 \pm 0.17
Divalinal-Ang IV	6.76 \pm 0.71	6.58 \pm 0.14	5.48 \pm 0.29
Ang II	5.38 \pm 0.21	5.49 \pm 0.03	6.25 \pm 0.21
7B	4.98 \pm 0.06	4.44 \pm 0.34	6.91 \pm 0.20
L-methionine	1.86 \pm 0.17	2.34 \pm 0.05	2.91 \pm 0.07
P40H1 cells	[^3H]Ang IV (+ E/P)	[^3H]AL-11 (+ E/P)	[^3H]AL-11 (- E/P)
Ang IV	7.35 \pm 0.08	8.17 \pm 0.07	6.78 \pm 0.13
AL-11	7.14 \pm 0.06	7.42 \pm 0.08	7.14 \pm 0.06
LVV-H7	6.80 \pm 0.13	7.22 \pm 0.11	5.67 \pm 0.12
Divalinal-Ang IV	6.78 \pm 0.19	6.96 \pm 0.09	4.75 \pm 0.06
Ang II	6.20 \pm 0.3	5.36 \pm 0.05	6.12 \pm 0.12
7B	4.43 \pm 0.24	3.93 \pm 0.05	6.75 \pm 0.05
L-methionine	1.34 \pm 0.18	1.30 \pm 0.14	1.72 \pm 0.09

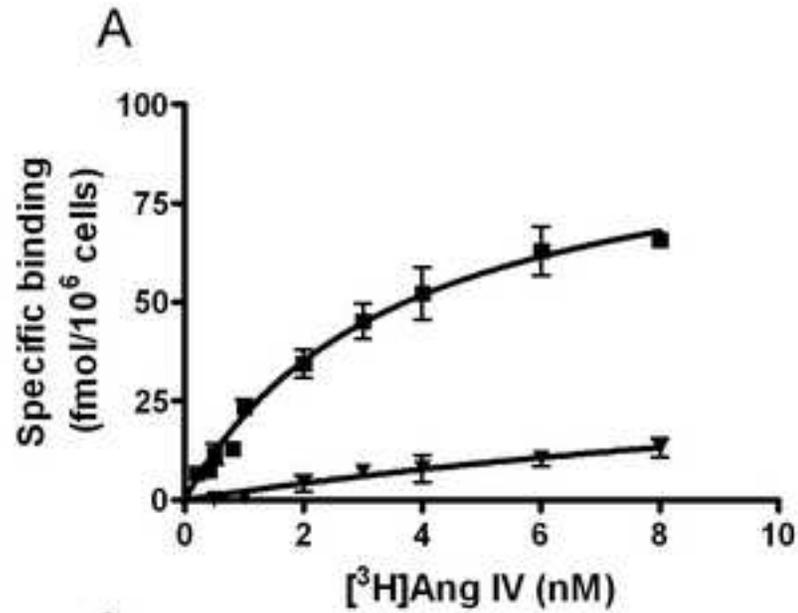
Table 3: Kinetic properties of [³H]AL-11 binding to CHO-K1 and P40H1 cell membranes in the absence of 500 μ M EDTA and 100 μ M 1,10-Phe. Values are the mean \pm SEM of three independent experiments performed in duplicate.

	CHO-K1 cells	P40H1 cells
k_{obs} (min^{-1})	0.16 ± 0.02	0.15 ± 0.05
k_{-1} (min^{-1})	0.12 ± 0.02	0.09 ± 0.03
k_1 (Mmin^{-1})	0.013	0.02
K_d (nM)	9.0	4.5

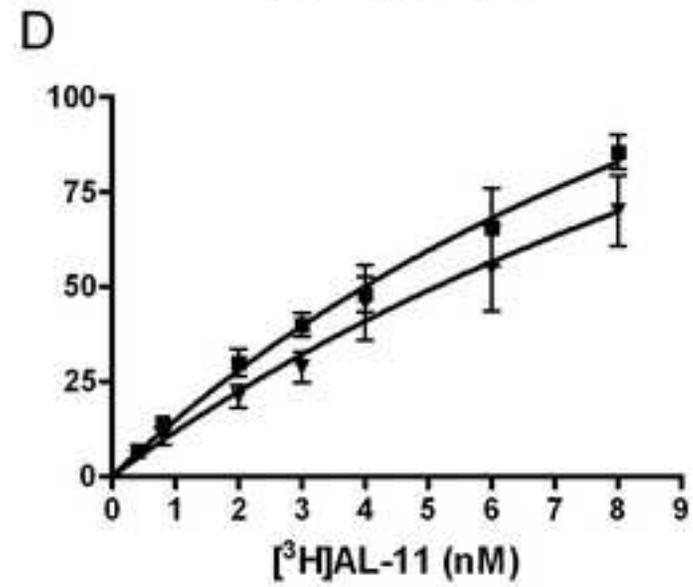
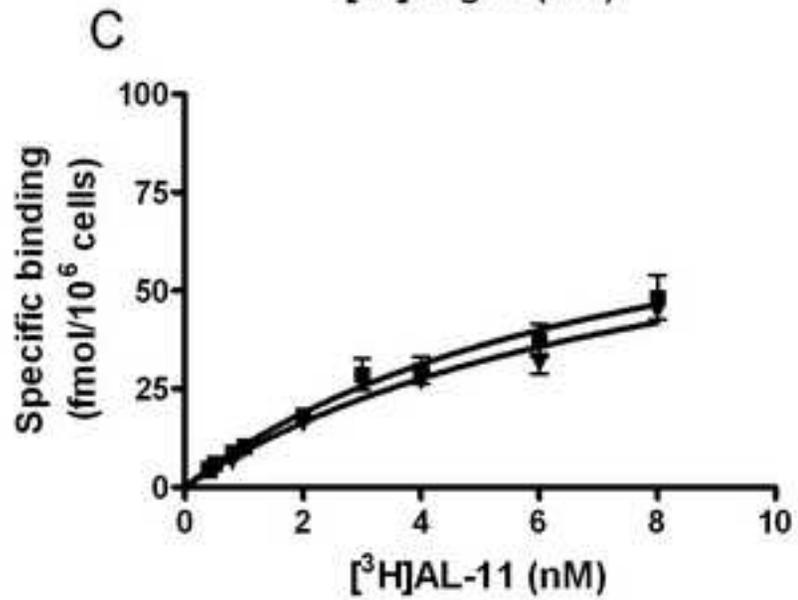
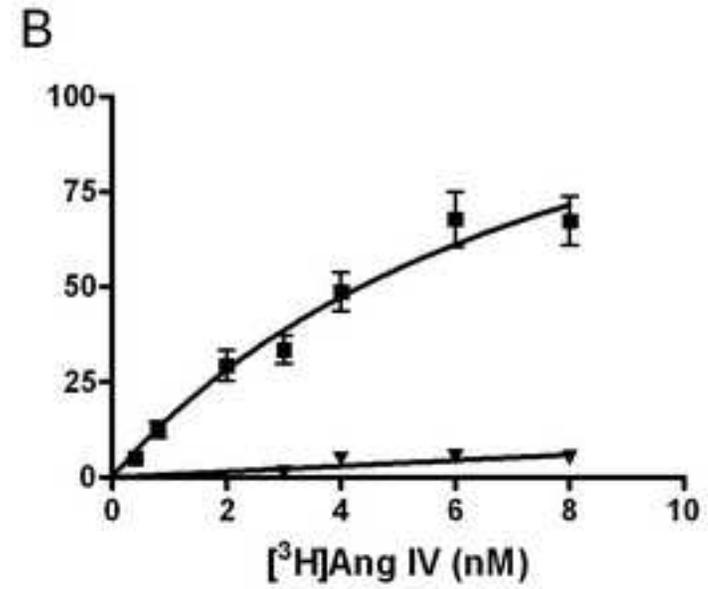
Table 4: Inhibition of enzyme activity in P40H1 cell membranes by AT₄ receptor ligands, Ang II and APN inhibitors (either analyzed by one or two site analysis). pK_i values are the mean ± SEM of three independent experiments performed in duplicate. H = high affinity site for Ang IV; L = low affinity site for Ang IV.

	1 site	2 sites H	2 sites L	+ 7B (-6) (1 site)
Ang IV	/	7.75 ± 0.09	5.62 ± 0.06	7.72 ± 0.30
AL-11	/	7.37 ± 0.10	5.16 ± 0.11	7.21 ± 0.09
LVV-H7	5.70 ± 0.01	/	/	5.85 ± 0.02
Divalinal-Ang IV	4.74 ± 0.13	/	/	4.91 ± 0.03
Ang II	5.79 ± 0.07	/	/	5.23 ± 0.06
7B	/	6.70 ± 0.10	8.39 ± 0.13	6.69 ± 0.04
L-methionine	3.12 ± 0.25	/	/	1.02 ± 0.10

CHO-K1



P40H1



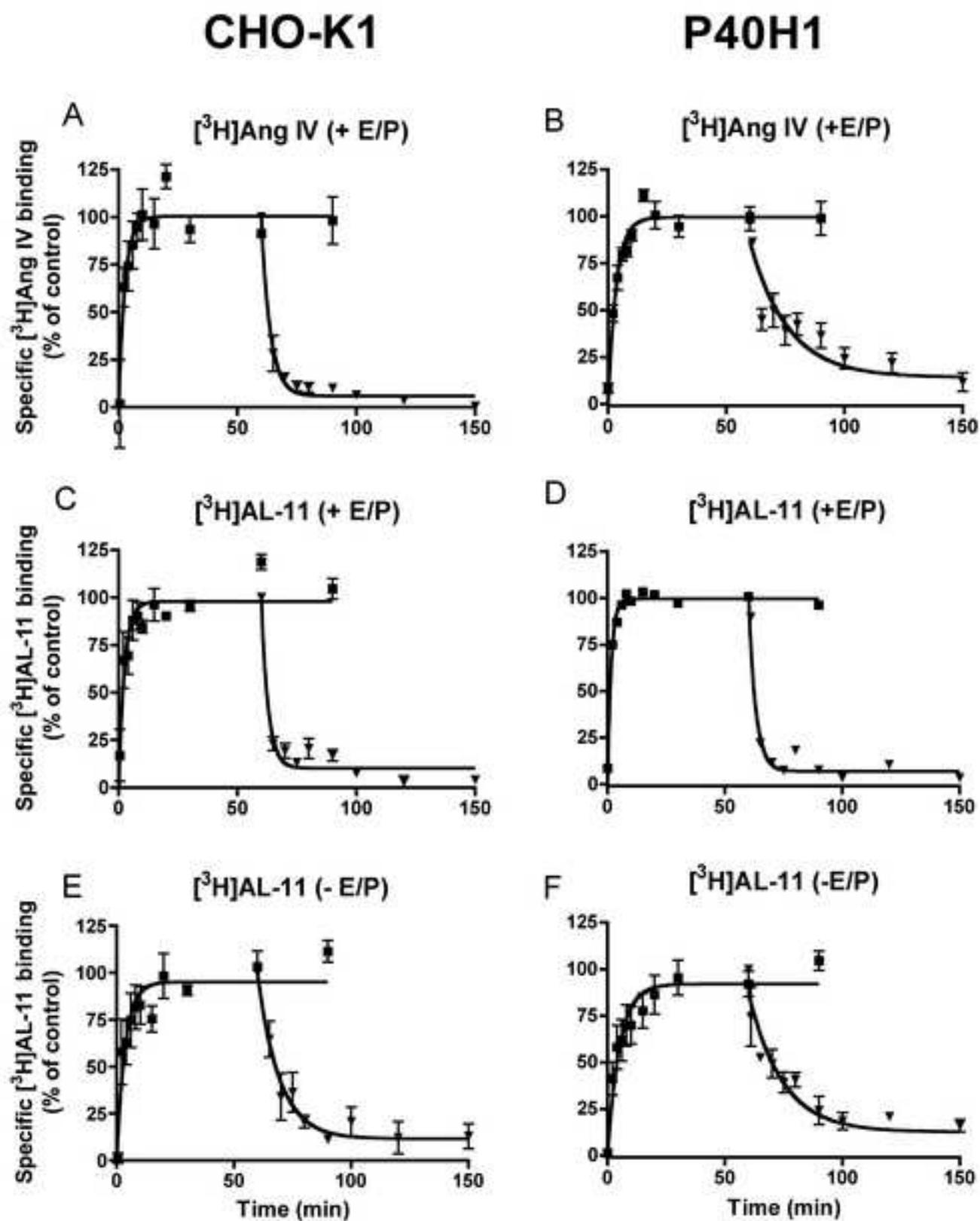


Figure 3

