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Binding of “AT₄ receptor” ligands to insulin regulated aminopeptidase (IRAP) in intact Chinese hamster ovary cells.

By

Heidi Demaegdt^a, Paul Gard^b, Jean-Paul De Backer^a, Aneta Lukaszuk^c, Erzsébet Szemenyei^d, Géza Tóth^d, Dirk Tourwé^c, Georges Vauquelin^{a*}.

^a Department of Molecular and Biochemical Pharmacology, Research group of Experimental Pharmacology, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium.

^b School of Pharmacy and Biomolecular Sciences, University of Brighton, Moulsecoomb, Brighton, UK.

^c Department of Organic Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussel, Belgium.

^d Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, Temesvari krt. 62, 6726 Szeged, Hungary.

* corresponding author: Vauquelin Georges
Department of Molecular and Biochemical Pharmacology,
Vrije Universiteit Brussel,
Pleinlaan 2, 1050 Brussels, Belgium.
Tel: (+32) 2 629 19 55;
Fax: (+32) 2 629 13 58;
email: gvauquel@vub.ac.be

Abstract.

Insulin regulated aminopeptidase (IRAP) recognises “AT₄-receptor” ligands like angiotensin IV (Ang IV) and peptidomimetics like AL-11. The metabolic stability and high affinity of [³H]AL-11 for catalytically active IRAP allowed its detection in Chinese hamster ovary (CHO-K1) cell membranes in the absence of chelators (Demaegdt et al., 2009). Here, we show that, contrary to [³H]Ang IV, [³H]AL-11 displays high affinity and specificity for IRAP in intact CHO-K1 cells as well. After binding to IRAP at the surface, [³H]AL-11 is effectively internalized by an endocytotic process. Unexpectedly, surface binding and internalization of [³H]AL-11 was not affected by pretreating the cells with Ang IV but declined with AL-11. In the latter case surface expression of IRAP even increased. After elimination of simpler explanations, it is proposed that metabolically stable “AT₄-receptor” ligands undergo semi-continuous cycling between the cell surface and endosomal compartments. The *in vivo* efficacy of stable and unstable “AT₄-receptor” ligands could therefore differ.

Keywords.

IRAP, CHO cells, internalization, radioligand binding, Ang IV, AL-11

Abbreviations.

AL-11, H-(R)-β²hVal-Tyr-Ile-His-Pro-β³hPhe-OH

AL-40, (R)-β²hVal-Tyr-Ile-Aia-Gly-Phe

Ang, Angiotensin;

AT₄ receptor, type 4 angiotensin receptor

AP-N, Aminopeptidase N;

CHO-K1 cells, Chinese hamster ovary cells;

EDTA, Ethylene diamine tetra-acetic acid;

GLUT4, Glucose transporter member 4;

HEK293 cells, human embryonal kidney cells;

IRAP, Insulin regulated aminopeptidase;

LVV-H7, LVV-hemorphin 7;

P40H1, mouse hippocampal cell line

1,10-Phe, 1,10-phenanthroline

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 insulin regulated aminopeptidase (IRAP, EC 3.4.11.3), 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., 2002a; Lew et al., 2003; Matsumoto et al., 2001; Chai et al., 2004; Fernando et al., 2008). A mechanistic rationale for this latter explanation stems from studies on adipose tissue and skeletal muscle. Indeed, these studies have shed light on the co-localisation of IRAP and GLUT4 in specific intracellular vesicles (i.e. GLUT4 vesicles). While, these vesicles move slowly to the cell surface, this translocation is markedly enhanced by insulin, resulting in a higher glucose uptake (Mastick et al., 1994; Kandror and Pilch, 1994; Keller et al., 1995; Ross et al., 1996). Evidence is now also accumulating for IRAP to play an important role in the sorting and trafficking of GLUT4 insulin-dependent glucose transporter (Waters et al., 1997; Johnson et al., 2001; Katagiri et al., 2002; Tojo et al., 2003; Larance et al., 2005; Hosaka et al., 2005; Peck et al., 2006; Yeh et al., 2007). Of note is that the initial studies dealing with the translocation of IRAP to the cell surface have been hindered by the laborious multistep detection of this enzyme (Johnson et al., 1998; Nakamura et al., 2000; Garza and Birnbaum, 2000).

IRAP is a type II integral membrane protein and, when this enzyme is present at the cell surface, its catalytic domain is exposed at the extracellular face thereof. This allows the IRAP molecules to interact with its substrates as well as with other 'AT₄ receptor' ligands like Ang IV and LVV-H7 (Stragier et al., 2008). Based on this principle and on the ability of IRAP to bind radiolabeled Ang IV with high-affinity and selectivity, we recently developed a

technically much simpler method to detect the enzyme at the surface of intact cells of different origin: i.e. Chinese hamster ovary (CHO-K1) cells, recombinant human embryonic kidney (HEK293) cells and mouse 3T3-L1 adipocytes (Demaegdt et al., 2008). Based on the destabilization of IRAP-Ang IV complexes by a weak acidic environment, these studies also revealed that [^3H]Ang IV and [^{125}I]Ang IV undergo marked IRAP-mediated internalization. Relying on earlier experience (Demaegdt et al., 2004b), the divalent cation chelators EDTA and 1,10-phenanthroline were also included during the incubation steps with radioligand.

Past binding experiments with radiolabelled Ang IV and analogs thereof were nearly invariably carried out on cell membrane preparations. In those studies the combined presence of EDTA and 1,10-phenanthroline was imperative for the detection of IRAP (Demaegdt et al., 2004b, 2009). One reason for this requirement is that Ang IV is highly susceptible to enzymatic degradation when exposed to membrane preparations and that these metal chelators impede the catalytic activity of many peptidases. Yet, a major drawback of this procedure is that the chelators also remove the catalytic zinc from IRAP (as well as from related Zn^{2+} -dependent enzymes of the gluzincin aminopeptidase family). This implies that the engendered high affinity binding of radiolabelled Ang IV does not take place to catalytically active IRAP but to its inactive apo-form.

To examine IRAP by radioligand binding under physiologically relevant conditions, it is imperative for those experiments to be performed in the absence of chelators. 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. To this end, AL-11 (H-(R)- $\beta^2\text{hVal-Tyr-Ile-His-Pro-}\beta^3\text{hPhe-OH}$) (compound 13 in Lukaszuk et al., 2008), a stable Ang IV analog containing two beta amino acids, was recently tritiated and its binding properties were compared with those of [^3H]Ang IV and [^{125}I]Ang IV in the absence and presence of chelators on Chinese hamster ovary (CHO-K1) and hippocampal (P40H1) cell membranes (Demaegdt et al., 2009). Interestingly, it was found that [^3H]AL-11 displays similar high affinity for both forms of IRAP and, in both instances, it also only exhibits a very low level of non-specific binding. Hence, [^3H]AL-11 represents a prototype of a class of metabolically stable radioligands that display high potency and selectivity for native IRAP. In intact cell binding studies, an additional interest in using this radioligand is that the presence of EDTA (which weakens cell adherence to the bottom of microwell plates) can be avoided.

These arguments prompted us now to, first, compare the binding properties of [^3H]Ang IV and [^3H]AL-11 to intact CHO-K1 cells in the absence of chelators. While these initial experiments were aimed at comparing the pharmacological properties of the 'AT₄ receptor' ligand- displaceable binding sites, subsequent research was focused on the kinetic properties of [^3H]AL-11 binding, the ensuing sub-cellular localization of this radioligand and the repercussion of pretreating the cells with distinct 'AT₄ receptor' ligands thereon. In this respect, earlier work with chimeric proteins containing the cytoplasmic domain of IRAP revealed that CHO cells also have a *bona fide* insulin-regulated recycling mechanism (Johnson et al., 1998; Subtil et al., 2000; Lampson et al., 2000). In the basal state, and similar as in adipocytes, these chimeras underwent rapid internalization from the plasma membrane and slow exocytosis from endosomal recycling compartments. The dileucine motif in the cytoplasmic domain of IRAP provided the necessary information thereto. Morphological studies further suggested that the internalized chimeric proteins were trafficked through the general endosomal recycling pathway, involving their transit through early endosomes and the pericentriolar endocytic recycling endosomes.

A most striking observation was that pretreating the cells with AL-11 and AL-40 ((*R*)- $\beta^2\text{hVal-Tyr-Ile-Aia-Gly-Phe}$, another stable Ang IV- analog (Lukaszuk et al., 2009)) decreased the ensuing surface binding and resulting intracellular accumulation of [^3H]AL-11 while Ang IV and LVV-H7 were unable to produce this effect. Although the mechanistic basis for this difference still remains to be elucidated, it could already be of interest to find out whether metabolically stable and unstable Ang IV analogs produce the same *in vivo* effects or not.

2. Methods

2.1 Materials

Angiotensin II, Angiotensin IV or Angiotensin II (3-8) were obtained from NeoMPS (Strasbourg, France), LVV-hemorphin 7 from Invitrogen (Merelbeke, Belgium). AL-11 (H-(*R*)- β^2 hVal-Tyr-Ile-His-Pro- β^3 hPhe-OH, compound 13 in Lukaszuk et al., 2008), AL-40 ((*R*)- β^2 hVal-Tyr-Ile-Aia-Gly-Phe, Lukaszuk et al., 2009), and the precursor peptides for [3 H]Ang IV and [3 H]AL-11 were synthesized at the Department of Organic Chemistry (Vrije Universiteit Brussel, Brussels). [3 H]Ang IV and [3 H]AL-11 were prepared therefrom at the Institute of Biochemistry (Biological Research Centre, Szeged, Hungary) as described earlier (Tóth et al., 1997, Demaegdt et al., 2009). The specific activity of the pure, tritiated peptides amounted 1.5 TBq mmol⁻¹ (40.8 Ci mmol⁻¹) and 1.29 TBq mmol⁻¹ (35.0 Ci mmol⁻¹) for [3 H]Ang IV and [3 H]AL-11, respectively. The APN inhibitor compound 7B (2(S)-benzyl-3-[hydroxy(1'R-aminoethyl)phospinyl]propanoyl-L-tyrosine, Chen et al., 1999), was a gift from Prof. Yiotakis (Athens, Greece). V5-HRP antibody was purchased from Invitrogen (Merelbeke, Belgium). All other reagents were of the highest grade commercially available. CHO-K1 cells (Chinese hamster ovary cells) were kindly obtained from the Pasteur Institute (Brussels, Belgium). HEK293 (human embryonal kidney) cells were obtained from Astra Zeneca (Mölndal, Sweden).

2.2 Cell culture and transient transfection

CHO-K1 and HEK293 cells were cultured in 75 cm² culture flasks (Sanbio, Uden, The Netherlands) in Dulbecco's modified essential medium (DMEM) supplemented with L-glutamine (2 mM), 2 % (v/v) of a stock solution containing 5000 IU/ml penicillin and 5000 µg/ml streptomycin (Invitrogen, Merelbeke, Belgium), 1 % (v/v) of a stock solution containing non-essential amino-acids, 1 mM sodium pyruvate and 10 % (v/v) foetal bovine serum (FBS, Invitrogen, Merelbeke, Belgium). The cells were grown in 5 % CO₂ at 37 °C until confluency.

HEK293 cells at 75% confluency in 24 well plates coated with poly-L-lysine (Greiner, Wemmel, Belgium) were transiently transfected with plasmid DNA, pCIneo containing the gene of human IRAP (kindly obtained from Prof. M. Tsujimoto, Lab. Of Cellular Biochemistry, Saitama, Japan). Transfections were performed as described previously with 8

$\mu\text{l/ml}$ LipofectAMINE (Invitrogen, Merelbeke, Belgium) and $1\ \mu\text{g/ml}$ plasmid DNA (Le et al., 2005). After transfection, the cells were cultured for 2 more days.

For the Elisa experiments, CHO-K1 cells were transiently transfected in $75\ \text{cm}^2$ culture flasks with plasmid DNA, pCIneo containing the gene of human IRAP C-terminally tagged to the V5 epitope. The transfections were performed as mentioned above with $1.5\ \mu\text{g/ml}$ plasmid DNA. One day after transfection, the cells were plated at 10^5 cells/well in 96-well plates coated with poly-L-lysine and cultured for one more day.

2.3 Radioligand binding:

2.3.1 General principles.

CHO-K1 cells were plated in 24-well plates (Becton Dickinson, Erembodegem, Belgium) and cultured until confluence. Native and recombinant HEK293 cells were plated as mentioned above. Before the experiment, cells were washed two times with $500\ \mu\text{l}$ per well of Krebs Ringer binding medium ($20\ \text{mM}$, CaCl_2 , $2.7\ \text{mM}$ KCl , $2.1\ \text{mM}$ MgCl_2 , $137\ \text{mM}$ NaCl , and $20\ \text{mM}$ HEPES , $\text{pH } 7.4$) at room temperature. Incubations were carried out at 37°C in a final volume of $500\ \mu\text{l}$ per well of binding medium. At the end of all experiments, the plates were placed on ice and washed three times with ice-cold PBS. For whole-cell binding (i.e. without distinction between surface-binding and intracellular accumulation of the radioligand) cells were then solubilized by addition of $300\ \mu\text{l}$ $1\ \text{M}$ NaOH and $200\ \mu\text{l}$ H_2O per well. After 60 min treatment at room temperature, solutes were transferred in scintillation vials. $3\ \text{ml}$ of scintillation liquid (Optiphase Hisafe, Perkin Elmer) was added and the remaining radioactivity was measured in a β -counter (Perkin Elmer, Boston, USA). In some experiments, cell surface binding and intracellular accumulation of $[^3\text{H}]\text{AL-11}$ was differentiated by mild acid treatment (Fierens et al., 1999; Demaegdt et al., 2009). To this end, cells were treated twice for 5 min with $500\ \mu\text{l}$ of glycine buffer ($30\ \text{mM}$ glycine, $125\ \text{mM}$ NaCl , $\text{pH } 3$) on ice. The radioactivity present in the combined supernatants accounted for cell-surface binding. The remaining radioactivity in the cells accounted for internalized radioligand. This fraction was dissolved in NaOH as mentioned above.

2.3.2 $[^3\text{H}]\text{Ang IV}$ binding.

The incubations were carried out with [^3H]Ang IV at a final concentration of 5 nM. For the competition binding assays (Figure 1), CHO-K1 cells were co-incubated with increasing concentrations of unlabelled ligands and [^3H]Ang IV for 60 min at 37°C.

2.3.3 [^3H]AL-11 binding.

The incubations were carried out with [^3H]AL-11 at a final concentration ranging between 0.5 and 10 nM for saturation binding and 5 nM for all other experiments. Non-specific binding (when relevant) was always determined in the presence of 10 μM of unlabeled Ang IV.

For the competition binding assays (Figure 1), CHO-K1 cells were co-incubated with increasing concentrations of unlabelled ligands and the radioligand for 60 min at 37°C. Association binding assays (Figure 2) were performed by incubating the cells with [^3H]AL-11 at 37°C for the times indicated either alone or in the presence of 0.1 μM insulin or 10 μM phenyl arsine oxide (PAO). For dissociation binding assays (Figure 3), CHO-K1 cells were incubated with [^3H]AL-11 with or without 10 μM PAO for 60 min at 37°C and further incubated with 10^{-5} M Ang IV for the indicated time periods. For two-step binding assays (Figures 4, 6, 7 and 8), CHO-K1 cells were pre-incubated at 37°C for the indicated periods of time with unlabelled ligands at the indicated concentrations, washed twice (briefly) with binding buffer or not and further incubated at 37°C for the indicated periods of time with [^3H]AL-11 at the indicated concentrations. To study the time dependency of the pre-incubation, as a variation, cells were pre-incubated for the indicated time periods at 37 °C with a single concentration of unlabelled ligand, washed twice with binding buffer and further incubated for 30 min at 37°C with [^3H]AL-11. Efflux experiments (Figure 5) were performed by pre-incubating CHO-K1 cells for 30 min at 37°C with a single concentration of unlabelled ligand, washed twice with binding buffer and further incubated for the indicated time periods with binding medium alone. At the “wash-out” times indicated 400 μl of this medium was removed and added along with 100 μl [^3H]AL-10 (5 nM final concentration) to a second plate containing naïve CHO-K1 cells. [^3H]AL-11 binding to these new cells was measured after 60 min incubation at 37°C.

2.4 Whole-cell ELISA.

CHO-K1 cells transiently transfected with V5IRAP were washed two times with serum free DMEM and incubated with unlabelled ligands for 30 min at 37°C in the same medium. Cells

were then washed with PBS on ice and fixated with 4% formaldehyde for 30 min on ice. After washing two times with PBS, cells were blocked with DMEM with 10% fetal bovine serum (FBS) for 1 hour at 37°C. The cells were then incubated with antibody V5-HRP (1/1000) for 2 hours at 37°C, washed 4 times with PBS, and further incubated for 20 min with 150 μ l of TMB reaction solution (prepared by adding 1 mg 3,3',5,5'-tetramethylbenzidine (solubilised in 1 ml DMSO) to 9 ml phosphate-citrate buffer (0.1 M, pH 5) and 2 μ l of fresh 30% hydrogen peroxide). The plate was then read spectrophotometrically at 370 nm.

2.5 Data analysis.

All experimental values are shown as the mean $\pm$ SEM of at least 3 independent experiments (each performed in duplicate or triplicate). The IC₅₀ values for the competition binding experiments were calculated by non-linear regression analysis using GraphPad Prism 5 according to a one-site (or two-site when relevant) model. Radioligand saturation, association and dissociation curves were obtained by analyzing the data according to a one-site model. For the ELISA experiment, a one-way ANOVA was performed to determine the significance with GraphPad Prism 5.0. A statistically significant difference was detected at < 0.01 , and therefore, Dunnetts *post hoc* test was performed to compare each condition with the control.

3. Results

Whole-cell competition binding with [³H]AL-11 and [³H]Ang IV.

Intact CHO-K1 cells were co-incubated for 60 min at 37°C with increasing concentrations of unlabelled “AT₄ receptor” ligands and 5 nM of either [³H]AL-11 or [³H]Ang IV. Competition binding curves for [³H]AL-11 are monophasic for all compounds and almost 90 % of the total binding could be displaced (Figure 1A). As illustrated in Table 1, the unlabelled ligand's potency order fits with the one earlier found in comparable [³H]AL-11 binding studies on CHO-K1 cell membrane preparations (Demaegdt et al., 2009). Since the binding experiments were carried out in the absence of divalent cation chelators EDTA and 1,10-phenanthroline, this binding profile is likely to correspond to that of active IRAP. In agreement therewith, the same order of “AT₄ receptor” ligand potencies was found with IRAP catalytic activity inhibition studies on the same membrane preparations (Demaegdt et al., 2004a, 2006, Lukaszuk et al., 2009). While, at 5 nM, [³H]Ang IV barely displayed specific binding to CHO-K1 cell membranes in the absence of chelators (Demaegdt et al., 2009), “AT₄ receptor” ligand- displaceable binding of this radioligand was readily observed in intact cells under the same conditions (Figure 1B). Yet, the competition binding curves with [³H]Ang IV are more complex than those with [³H]AL-11. Only about 60 % of the total [³H]Ang IV binding can be displaced and, while the curves for Ang IV and LVV-H7 appear to be monophasic, those for AL-11 and compound 7B show outspoken biphasicity (Table 1). This suggests that [³H]Ang IV binds with similar affinity to two distinct sites and that each of them constitutes about half of the displaceable binding. As the concentration of [³H]Ang IV in those experiments is well below the IC₅₀ of Ang IV (i.e. 0.21 μM), the Cheng and Prusoff equation (1973) reveals that this IC₅₀ closely matches the K_D of [³H]Ang IV. Further comparison of the IC₅₀ values with those obtained with [³H]AL-11 suggests that one of the displacelable [³H]Ang IV binding sites (marked with an asterisk in Table 1) correspond to IRAP. To evaluate the soundness of this assertion, we compared the binding of [³H]AL-11 and [³H]Ang IV to intact native and human IRAP-expressing HEK293 cells. As depicted in Table 2, both radioligands exhibited appreciably more Ang IV and AL-11- displaceable binding to the recombinant cells.

[³H]AL-11 binding kinetics.

[³H]Ang IV binding has recently been utilized as a tool to study the uptake/internalization of IRAP in intact CHO-K1 and other cell lines (Demaegdt et al., 2008). In those studies, cell surface-associated [³H]Ang IV molecules could be discriminated from the internalized ones

by virtue of their fast dissociation (and dispersion in the fluid phase) in a mild acidic environment. Similarly, fast dissociation of [^3H]AL-11-IRAP complexes also took place when subjecting pre-incubated CHO-K1 cell membranes to glycine buffer at pH 3 on ice (data not shown). Banking on this property, we checked for the subcellular location of “specifically bound” [^3H]AL-11 molecules after exposing intact CHO-K1 cells for increasing periods of time with 5 nM of this radioligand. These as well as all ensuing experiments were carried out at 37°C. As shown in Figure 2A, acid-sensitive binding increased with time and only reached a plateau after about 45 min. Acid-resistant binding (Figure 2B) was comparatively much more pronounced and still increased with time at 60 min. Due to the IRAP-matching profile of the specific whole-cell [^3H]AL-11 binding (Figure 1A, Table 1), the acid-resistant fraction is likely to result from the co-internalisation of IRAP and bound radioligand molecules. To verify this assertion, we also exposed the CHO-K1 cells to [^3H]AL-11 in combination with 10 μM phenyl arsine oxide (PAO, a substance widely used to block the endocytosis of cell-surface proteins) or 0.1 μM insulin. Similar as in earlier studies with [^3H]Ang IV (Demaegdt et al., 2008), PAO prevented the emergence of acid-resistant [^3H]AL-11 binding (Figure 2A). Concomitantly, the acid-sensitive binding was about 5-fold higher (compared to exposing the cells to [^3H]AL-11 only) and reached equilibrium after a shorter time lapse (Figure 2B). The k_{obs} of this association is in good agreement with the value obtained for specific [^3H]AL-11 binding to CHO-K1 cell membranes (0.33 ± 0.08 vs. $0.16 \pm 0.02 \text{ min}^{-1}$, Demaegdt et al., 2009). On the other hand, insulin promotes the translocation of IRAP molecules to the cell surface and this steps up their internalization. In agreement therewith, the acid-sensitive and -resistant [^3H]AL-11 binding fractions were about 2-fold higher in the presence of insulin (Figure 2A and B). Yet, the time-dependent increase in binding was not substantially affected by insulin.

To investigate the dissociation of [^3H]AL-11 from IRAP at the cell surface, CHO-K1 cells were exposed to [^3H]AL-11 and 10 μM PAO for 60 min at 37°C and, after a brief wash step, further exposed for increasing time periods to a large excess of Ang IV for different time periods. The presence of PAO allowed the specific binding of [^3H]AL-11 to be comfortably high at the onset of the wash-out and the radioligand's dissociation was not perturbed by internalization phenomena. As depicted in Figure 3, the binding decreased with a $t_{1/2}$ of less than 10 min. Similar, relatively fast dissociation ($t_{1/2} = 6 \text{ min}$) was earlier reported to take place for [^3H]AL-11-IRAP complexes in CHO-K1 cell membranes (Demaegdt et al., 2009). When similar experiments were performed in the absence of PAO, the acid-resistant [^3H]AL-

11 binding declined much more slowly (Figure 3). These findings suggest that most of the internalized [^3H]AL-11 molecules are only slowly, if at all, recycled to the cell surface.

Unlabelled ligand pre-incubation: effect on [^3H]AL-11 surface binding and intracellular accumulation.

The slow release of internalized [^3H]AL-11 molecules (Figure 3) prompted us to find out whether pretreatment of intact CHO-K1 cells with distinct “AT₄ receptor” ligands could affect the subsequent surface binding and intracellular accumulation of [^3H]AL-11. To this end, CHO-K1 cells were pre-incubated for 30 min at 37°C with increasing concentrations of Ang IV, LVV-H7 and AL-11, briefly washed or not, and then exposed for 30 min to 5 nM [^3H]AL-11. The unlabelled ligands were still present in the latter (i.e. reference) condition and, as expected, they caused a concentration-wise decline in the surface binding and intracellular accumulation of [^3H]AL-11 (Figure 4A to C). All three compounds produced a similar maximal decrease in [^3H]AL-11 surface binding and accumulation and their pIC₅₀ values (Table 3) were very similar to those obtained by the single-step whole-cell [^3H]AL-11 competition binding experiments shown in Figure 1.

[^3H]AL-11 surface binding and intracellular accumulation no longer declined when the cells were pretreated with different concentrations of Ang IV and LVV-H7 up to 10 μM and then briefly washed to remove remaining free ligand molecules (Figure 4A and B). These data suggest that IRAP-associated and therewith internalized Ang IV and LVV-H7 molecules do not have the capacity to affect subsequent surface binding and intracellular accumulation of [^3H]AL-11. It is only at the highest concentrations of these unlabelled ligands that the subsequent accumulation of [^3H]AL-11 started to decline. This is likely to result from the incomplete removal of free Ang IV and LVV-H7 molecules during the intermediate wash step or to their slow release from the cells and/or the plastic walls of the wells or during the subsequent challenge with [^3H]AL-11 (Packeu et al., 2010a).

By contrast, [^3H]AL-11 surface binding and intracellular accumulation did only partially recover when the cells were pretreated with AL-11 and then briefly washed (Figure 4C). Indeed, [^3H]AL-11 accumulation still decreased in a AL-11 concentration- dependent fashion and this decrease took even place with closely the same pIC₅₀ as in the reference experiment (i.e. without the intermediary wash step) (Table 3). This close correspondence argues against a mechanism involving the incomplete removal of free AL-11 (Packeu et al., 2010a). Release

of AL-11 from the plastic or the cells is an unlikely cause either, at least if one assumes that such released molecules only interfere with [^3H]AL-11 binding by a mass-action type equilibrium process (or, in other words, that released AL-11 molecules are immediately homogeneously dispersed in the medium). The following experiments support this refutation. In the first experiment, CHO-K1 cells were pretreated for 30 min at 37°C with a maximally effective concentration of Ang IV (10 μM) or AL-11 (1 μM), briefly washed and finally incubated with fresh medium only for different time intervals for up to 60 min. The presence of released Ang IV or AL-11 in the resulting wash-out medium was then evaluated by monitoring their ability to decrease [^3H]AL-11 binding to fresh CHO-K1 cells in a co-incubation experiment. As shown in Figure 5, no inhibitory effect could be perceived for any wash-out time period. In the second experiment, cells were pre-incubated with medium only or with a maximally effective concentration of AL-11 (1 μM), briefly washed and finally incubated with increasing concentrations of [^3H]AL-11. A concentration-wise increase in specific whole cell binding (i.e. surface binding + intracellular accumulation) was observed in both cases but it was always lower for the AL-11- pretreated cells (Figure 6). Analysis of both curves according to a single-site saturation binding paradigm yielded quite distinct B_{max} values (385 ± 58 vs. 657 ± 148 fmol/mg protein for AL-11- and medium only- pretreated cells, respectively) but similar K_D values (9.8 ± 2 nM vs. 10.4 ± 4 nM). Hence, increasing the radioligand's concentration did not surmount the inhibitory effect of pre-exposing the cells to AL-11.

Alternatively, it is also possible to explain the remnant inhibition curve in AL-11-pretreated and washed cells (Figure 5C) by the ability of this ligand to bring about long-lasting occupancy of IRAP (Packeu et al., 2010a). In such eventuality, the inhibitory effect of AL-11 should wane gradually when the ensuing incubation with [^3H]AL-11 is prolonged (Malany et al., 2009; Packeu et al., 2010a). To get more insight into this issue, CHO-K1 cells were pre-incubated for 30 min at 37°C either with medium alone (as reference), with 10 μM Ang IV or with 1 μM AL-11, briefly washed and finally incubated for different time intervals for up to 60 min with 5 nM [^3H]AL-11. Under reference conditions, the specific cell surface binding of [^3H]AL-11 increased swiftly until an equilibrium was reached (Figure 7A). The association curve for Ang IV- pretreated cells is undistinguishable from the reference one. The fact that Ang IV pretreatment not even affected the binding after as little as 5 min (Figure 7A) indicates that its inhibitory effect is only short-lived. Similar association behavior was also observed for LVV-H7- pretreated cells (data not shown). Compared to the

reference curve, the association curve for AL-11 pretreated cells remained clearly depressed at all time points (Figure 7A). The ratio between the binding values at corresponding time points no longer changed after 20 min incubation (i.e. 64, 66 and 63 % of reference binding after AL-11 pretreatment at 20, 30 and 60 min, respectively) (Figure 7A). This suggests that, after exposing the cells to AL-11, there is a decline of the IRAP population that is normally present at the surface and/or that part of this population becomes enduringly refractory to subsequent [3 H]AL-11 binding.

The effect of the pretreatments on the time-dependency on the accumulation of intracellular [3 H]AL-11 is shown in Figure 7B. Under reference conditions, specific acid-resistant [3 H]AL-11 binding keeps on increasing with time to exceed by about 8-fold the amount of specific cell surface binding after 60 min. Yet, this intracellular accumulation is most rapid at the shortest incubation times (insert) and this even though the surface binding is still submaximal under those conditions (Figure 7A). This suggests that the internalization of [3 H]AL-11-bound IRAP recedes with time and/or that this radioligand is slowly recycled back to the surface. Similar to the surface binding, the time-wise accumulation of [3 H]AL-11 is not affected by prior exposure of the cells to Ang IV (Figure 7B and insert) as well as to LVV-H7 (data not shown). Yet, compared to the reference curve, the time-wise accumulation of the radioligand is markedly depressed for AL-11 pretreated cells (Figure 7B). This accumulation remains most rapid early on but, at all times, it occurs at a smaller pace when compared to the reference condition (insert).

The influence of the pre-incubation time and “AT₄ receptor” ligand stability on their ability to affect the intracellular accumulation of [3 H]AL-11 was examined next. To this end, CHO-K1 cells were pre-incubated for different time periods with 10 μ M Ang IV, 10 μ M Ang IV + 0.1 μ M compound 7B, 10 μ M LVV-H7, 1 μ M AL-11 and 1 μ M AL-40, followed by a brief wash and final 30-min incubation with 5 nM [3 H]AL-11. The combination of Ang IV and compound 7B (a synthetic AP-N- selective antagonist, Chen et al., 1999) was included because of the ability of compound 7B to prevent the metabolic degradation of Ang IV in the presence of CHO-K1 cell membrane preparations (Stragier et al., 2007; Vanderheyden et al., 2008). AL-40 (a recently developed His-substituted analog of Ang IV, Lukaszuk et al., 2009) was also included because of its inherent refractoriness to such degradation. As shown in Figure 8, Ang IV, either in the absence or presence of compound 7B, did not affect the subsequent accumulation of [3 H]AL-11 at all pre-incubation times examined. A modest

inhibitory effect of LVV-H7 became apparent at the highest pre-incubation times examined. In contrast, both AL-11 and AL-40 produced an equally rapid and extensive decline (Figure 8). Yet, their inhibitory effect was incomplete as the accumulation of [³H]AL-11 only decreased to about 40 % of the extent observed for naïve cells.

Effect of Ang IV, LVV-H7 and AL-11 on the cell surface expression of V5IRAP.

To find out whether “AT₄ receptor” ligands do affect the cell surface expression of IRAP, CHO-K1 cells were transiently transfected with V5IRAP (i.e. human IRAP tagged at the C-terminus with the V5 epitope). These cells were incubated with medium alone or with Ang IV, LVV-H7 and AL-11 (2 concentrations each) for 30 min at 37°C. After fixation, a whole cell ELISA with anti-V5 was performed. As these V5 epitopes are only accessible to the antibodies when IRAP is present at the cell-surface, only this population of recombinant IRAP molecules will be detected. As shown in Table 4 , Ang IV and LVV-H7 did not significantly affect the expression of V5IRAP at the cell surface. Yet, a net increase was observed when the cells were pretreated with AL-11.

4. Discussion

In this study, we compared for the first time the binding properties of [^3H]Ang IV and its metabolically stable analogue [^3H]AL-11 (compound 13 in Lukaszuk et al., 2008) to intact cells in the absence of chelators. Under these conditions, IRAP (which is widely recognized to represent the “AT₄ receptor”) is present in its physiologically relevant, catalytically active form. Because of its appreciably higher affinity and IRAP- selectivity, [^3H]AL-11 was further used as a tool to investigate the dynamic translocation properties of this enzyme. It was found that the specific, “whole cell” binding of [^3H]AL-11 was mostly composed of molecules that had accumulated inside the cells and that this accumulation declined with time. This phenomenon was especially apparent following pre-treatment of the cells with an IRAP-saturating concentration of unlabelled AL-11 but, unexpectedly, it did not take place following pre-treatment with the most potent endogenous “AT₄ receptor” peptide ligands Ang IV and LVV-H7 (Lee et al., 2003).

CHO-K1 cells were chosen for this study because of their high endogenous expression level of IRAP and the apparent absence of the related Aminopeptidase-N (AP-N) enzymes, another alleged Ang IV-target. Earlier studies on membrane preparations thereof revealed that [^3H]AL-11 displays equally high potency and selectivity for both the apo- and native (i.e. catalytically active) forms of IRAP. By contrast, high affinity labeling of this enzyme by [^3H]Ang IV or [^{125}I]Ang IV (hitherto the most widely used radioligands for the purpose of “AT₄ receptor”/IRAP labeling) could only be achieved in the presence of chelators and, hence, only encompassed apo-IRAP (Demaegdt et al., 2004, 2006, 2009). The present findings on intact CHO-K1 cells further support the assertion [^3H]AL-11 is suitable for the labeling of native IRAP under physiologically relevant conditions. In the “whole cell”-competition binding experiments (Figure 1A), the curves were monophasic, all unlabelled “AT₄ receptor” ligands decreased [^3H]AL-11 binding to the same maximal extent (about 90 %) and with the same order of potencies as seen in related [^3H]AL-11 binding studies and IRAP catalytic activity inhibition studies on CHO-K1 cell membranes: i.e. AL-11 > Ang IV > LVV-H7 > Ang II (Demaegdt et al., 2009). The single-site K_D value obtained from “whole cell” [^3H]AL-11 saturation binding experiment (11.2 ± 2.8 nM, for reference curve in Figure 6) is also similar to the one (7.7 ± 1.1 nM) earlier found for CHO-K1 cell membrane preparations in the absence of chelators (Demaegdt et al., 2009). Additionally, Ang IV and

AL-11- displaceable [^3H]AL-11 binding was also much higher in human IRAP expressing recombinant HEK293 cells than in the native cells (Table 2).

However, notwithstanding that the “whole cell” [^3H]AL-11 saturation and competition binding data can be analyzed in terms of a single site, two binding fractions can be discriminated by subjecting the cells to a mild acidic environment. As shown in Figures 4 and 7, most of the specific (i.e. 10 μM Ang IV- displaceable) [^3H]AL-11 binding is refractory to mild acid treatment while, in membrane preparations all such binding is rapidly lost (not shown). This refractory binding fraction is thus likely to represent [^3H]AL-11 molecules that have accumulated inside the cells. Similar conclusions were previously also reached for “whole cell” binding studies with [^3H]Ang II and [^3H]Ang IV (Vanderheyden et al., 1999; Demaegdt et al. 2008). Several findings support the contention that this internalization process requires the initial binding of [^3H]AL-11 to IRAP molecules at the cell surface and the endocytosis of those complexes. Most importantly, specific acid-sensitive [^3H]AL-11 binding increased while the refractory binding was no longer be detectable when the cells were pretreated with 10 μM PAO (Figure 2). PAO is well known for its ability to block the endocytosis of cell-surface proteins by disrupting the structure of clathrin coated pits (Visser et al., 2004; Rückert et al., 2003). At the concentration used, PAO also considerably reduced the acid-resistant binding of [^3H]Ang IV in CHO-K1 cells and over 90 % of those cells remained alive (Demaegdt et al., 2008). Additionally, the unlabelled competitor pIC_{50} values are very similar for the acid-refractory and -sensitive binding fractions of [^3H]AL-11 (Table 3, for continuous presence of unlabelled ligand).

Although the “whole cell” [^3H]Ang IV competition binding curves were steep for Ang IV and LVV-H7 they were clearly biphasic with AL-11, compound 7B and Ang II (Figure 1B). A reasonable interpretation of these findings is that the displaceable [^3H]Ang IV binding can be divided in two distinct components, one of them being IRAP (and associated internalized [^3H]Ang IV molecules) and the other one representing a site of yet undisclosed identity. An IRAP-favourable argument is that the pIC_{50} -values for Ang IV and LVV-H7 as well as for one binding fraction in case of the other ligands closely matched their respective pIC_{50} -values for competing with [^3H]AL-11 (Table 1). The calculated affinity of [^3H]Ang IV for both sites combined (0.21 μM) fits also well with the K_i value of Ang IV (0.17 μM) in competition binding experiments with [^3H]AL-11 on CHO-K1 cell membranes in the absence of chelators (Demaegdt et al., 2009). Recent [^3H]AL-11 competition binding

experiments, in which chelating agents were substituted by compound 7B, suggest that naïve Ang IV also displays equally low affinity for the catalytically active form of IRAP on CHO cell membranes (Demaegdt et al., 2011). These experiments exploited the ability of compound 7B to fully protect Ang IV from degradation at concentrations only moderately affecting the catalytic and binding activity of IRAP.

The rather modest affinity of Ang IV for the catalytically active form of IRAP provides a rationale for the low extent of “specific” [^3H]Ang IV binding to cell membranes under those experimental conditions (Demaegdt et al., 2009). The ability to detect IRAP under the same conditions in intact cell experiments may therefore seem somewhat surprising. Yet, this could, among others, be attributed to IRAP-mediated internalization of [^3H]Ang IV (which exacerbates the IRAP-related binding component and only takes place in intact cells). With respect to the present undisclosed [^3H]Ang IV binding site, it does not seem to correspond to AP-N, another alleged Ang IV-recognition site that belongs to the M1 gluzincin aminopeptidase family (Garreau et al., 1998). Indeed, AP-N enzyme activity was not detectable in CHO-K1 cell membranes and the pharmacological profile of the present site does not fully fit with that of AP-N (Demaegdt et al., 2006, 2009). Yet, as Ang IV also interacts with the AT₁-type angiotensin II receptor and, conceivably, also with the hepatocyte growth factor binding tyrosine kinase receptor, c-Met (Le et al., 2002, Wright et al., 2008), it cannot be excluded that the structure of the additional target in CHO cells is totally different from that of IRAP.

Finally, it must be conceded that the preceding considerations are based on the premise that one of the [^3H]Ang IV-labeled sites in intact CHO cells corresponds to IRAP. Yet as both sites seem to represent about 50 % of the “specific” [^3H]Ang IV binding, it cannot be excluded that we “unscrambled” the affinity profile of both sites in an incorrect way so that, in reality, none of them corresponds to IRAP. Masking experiments to find out whether the component with high affinity for AL-11 also displays high affinity for compound 7B and low affinity for Ang II (such as in Demaegdt et al., 2009) could provide a more conclusive answer. However, seen the much more clear-cut results obtained with [^3H]AL-11, we opted for deploying our efforts to further study the kinetic properties of [^3H]AL-11 binding, the ensuing sub-cellular localization of this radioligand and the repercussion of pretreating the cells with distinct ‘AT₄ receptor’ ligands thereon.

A remarkable and unexpected finding was that pretreating the cells with AL-11 produced a sizable decline of the subsequent surface binding and intracellular accumulation of [3 H]AL-11 whereas pretreating the cells with the natural “AT $_4$ receptor” ligands Ang IV and LVV-H7 produced no such effect (Figures 4, 7 and 8). Some straightforward explanations do not seem to constitute an adequate rationale for this difference. First, it is of note that all experiments were performed according to a similar configuration, comprising a preincubation of the cells with the unlabelled ligand, a brief wash step and a final incubation with the radioligand alone. This opens the possibility for AL-11 molecules that were incompletely removed by the intermediate wash step or that were subsequently released from aspecific sites (on the walls of the wells, plasma membrane) to compete with [3 H]AL-11. This explanation is highly unlikely because of the very low non-specific binding of [3 H]AL-11 (Figure 1A) along with the lack of inhibitory effect when binding of [3 H]AL-11 to naïve cells was measured in presence of the supernatant of AL-11- pretreated cells (Figure 5). Moreover [3 H]AL-11 displayed similar K_D values when the cells were pretreated with AL-11 or with medium only (Figure 6). These results plead against a competition between the radioligand and potentially released AL-11 molecules according to a mass-action type process (implying that both are homogenously dispersed in the medium). Instead, the “two step” AL-11 competition binding experiment shown in Figure 4C rather suggests that the interaction between AL-11 and IRAP itself is responsible for the decline in the surface binding and intracellular accumulation of [3 H]AL-11. Indeed, the residual inhibitory effect of AL-11 increases concentration-wise till a limit is reached and the therefrom calculated pIC_{50} is very close to the values obtained from the reference experiment (i.e. without the intermediary wash step) and the single step whole-cell competition experiments (Tables 1 and 3).

It is well known that some GPCR agonists are capable to desensitize their cognate receptor, to internalize along with the receptor by an endocytotic process and to finally down-regulate the receptor concentration (Koenig and Edwardson, 1997; Luttrell et al., 2002). As antagonists are generally not considered to mediate such effect, it could be envisaged that AL-11 and Ang IV are able to trigger different conformational changes of IRAP, finally leading to differences in its cell surface expression. In agreement with the lack of effect of Ang IV and LVV-H7 on subsequent [3 H]AL-11 binding, ELISA experiments with recombinant V5IRAP- expressing CHO-K1 cells show that neither natural ligands affect the cell surface expression of IRAP (Table 4). As experiments with [125 I]Ang IV disclosed the propensity of this ligand to internalize in CHO-K1 cells (Demaegdt et al., 2008), it can be

deduced that the internalization process does not produce a fast drop in the surface expression of IRAP *per se*. Also, while the decreased binding of [³H]AL-11 to AL-11-pretreated cells prompted us to expect a decline in the surface expression of IRAP, rather the opposite was found to take place (Table 4). Along with the observation that this pretreatment does not affect the K_D of [³H]AL-11 (Figure 6), these findings suggest that the radioligand is only able to access part of the IRAP molecules that are present at the surface of the pretreated cells.

The inaccessibility of the remaining IRAP molecules to the radioligand suggests that they reside in particular state and/or conformation and, in this respect, the formation of irreversible AL-11- IRAP complexes (along with a potential continuous recycling thereof to the cell surface) represents maybe the most straightforward mechanism. Yet, this is quite unlikely to take place since the binding of [³H]AL-11 to cell surface-associated IRAP (Figure 3) as well as to IRAP in CHO-K1 cell membranes (Demaegdt et al., 2009) is a relatively fast reversible process. Yet, kinetics- based explanations cannot be completely revoked either. Indeed, the present observations are reminiscent of the notion of “insurmountable antagonism” in organ bath experiments (Leff et al., 1986). Those experiments also proceed in two steps, involving a preincubation of the tissue with a given concentration of antagonist and, subsequently (but still in the presence of the antagonist), with increasing concentrations of antagonist. Even for competitive antagonists, the maximal response may still be depressed provided that they dissociate sufficiently slowly and that the ensuing challenge with the agonist is too short to allow the a new mass-action equilibrium to be attained (Vauquelin et al., 2002b; Charlton and Vauquelin, 2010). According to the model proposed by Koenig et al. (1998) for sst₂ receptor agonists, such “insurmountable” shielding of IRAP at the cell surface by previously administered AL-11 could indeed take place provided that both are able to recycle back to the cell surface, that at least some of these pairs bind to each-other again and that the resulting complexes survive till the next internalization round. The same type of reasoning may also explain why, in “association experiments” with naïve cells (Figure 7), the intracellular accumulation of [³H]AL-11 is most rapid at the shortest incubation times despite the still submaximal surface binding.

Internalized GPCR agonists and their receptors accumulate in endosomes from where they can be directed to lysosomes for degradation or recycled back to the cell surface (Mellman, 1996; Koenig and Edwardson, 1997; Luttrell et al., 2002). In this respect, Koenig et al.

(1998) showed that, while internalization of the endogenous sst₂ receptor agonist somatostatin-14 prompts its degradation, the more peptidase-resistant analog BM-23027 remains largely intact. Interestingly, the authors further observed that BM-23027 undergoes continuous cycling between the cell surface and intracellular compartments and, based on wash-out experiments, it even turned out that freshly recycled BM-23027 molecules are able to re-activate the receptor despite of their only scanty accumulation in the extracellular medium. Although not explicitly demonstrated in their study, the authors reasoned that, even after wash-out, recycling of internalized ligand is likely to inhibit subsequent radioligand binding. The present radioligand binding experiments fully support this conjecture. In this respect, it is also particularly striking that whole-cell [³H]AL-11 binding was decreased when the cells were preincubated with the metabolically stable peptidomimetics AL-11 and AL-40 but not with the unstable natural “AT₄ receptor” ligands Ang IV and LVV-H7 (Figure 8) (Sanderson Nydahl et al., 2003; Axén et al., 2006; Johna et al., 2007; Lukaszuk et al., 2008, 2009). Yet, one may wonder why compound 7B, which effectively prevents the degradation of Ang IV in the presence of cell membranes (Stragier et al., 2007; Vanderheyden et al., 2008), failed to confer AL-11- like properties to Ang IV (Figure 8). Although this issue merits further dedicated investigation, a tentative explanation is that the internalization process brings about the physical segregation between compound 7B and IRAP- associated Ang IV so that metabolization of the latter within the cell takes place unabated. During the recycling process AL-11- IRAP complexes are expected to dissociate rapidly when exposed the acidic environment of the intervening endosomes. The same reasoning was also held for somatostatin agonist- receptor complexes (Koenig et al., 1998). This implies that those ligands should no longer be associated to their binding sites when they reappear at the cell surface. At first sight, the formation of new complexes constitute a weak link in the model. Koenig et al. (1998) overcame this chokepoint by suggesting that the freshly recycled ligand molecules reside in an “unstirred layer” near the cell surface. As commented upon in detail elsewhere (Vauquelin and Szczuka, 2007; Vauquelin and Charlton, 2010; Vauquelin, 2010), the ability of such “unstirred layers” in juxtaposition to receptor-bearing planar surfaces has been held responsible for the “rebinding” of dissociated ligand molecules. The increased accumulation of recycled [¹²⁵I]BM-23027 in the extracellular medium when the radioligand- pretreated cells are challenged with an excess of competing ligand, as observed by Koenig et al. (1998), is also compatible with the “rebinding” concept. Indeed, wash-out experiments with different GPCR- expressing recombinant CHO-K1 cell lines also revealed that radioligand rebinding can be prevented by unlabelled ligands but only when they are

present at very high concentrations (Fierens et al., 1999; Packeu et al., 2010b; Wennerberg et al., 2010). Similar observations were earlier also made in *ex vivo* wash-out experiments (Perry et al., 1980; Gifford et al., 1998). Additionally, it has also been argued that the occurrence of semi-confined spaces like synapses and the clefts separating the plated CHO cells could positively contribute to this rebinding process by further delaying the escape the recycled ligand molecules (Sadée et al., 1982; Coombs and Goldstein, 2004; Spivak et al., 2006). Although never performed before, even in case of well-documented situations of radioligand rebinding (Fierens et al., 1999; Packeu et al., 2010b; Wennerberg et al., 2010), it could be of interest to examine the substance of this additional claim by comparing ligand rebinding to plated and suspended cells of the same type.

In conclusion. The subcellular distribution of well established receptors and their (usually agonist) ligands depends on a complex interplay between their recycling to the plasma membrane and their transfer towards lysosomes. Earlier studies with chimeric proteins also shed light on the predisposition of CHO cells to traffic IRAP through the general endosomal recycling pathway under basal (i.e. non insulin-stimulated) conditions (Johnson et al., 1998; Subtil et al., 2000; Lampson et al., 2000) and we recently also reported that binding of [125 I]Ang IV to IRAP promotes the internalization of this radioligand in CHO-K1 and other cell lines (Demaegdt et al., 2008). The use of the metabolically stable Ang IV analog, [3 H]AL-11, now allows such intact cell binding studies to be performed under physiologically relevant conditions (i.e. in the absence of chelators). The differential effect of pretreating the CHO-K1 cells with metabolically stable and unstable “AT₄ receptor” ligands on the [3 H]AL-11- IRAP interaction came as a surprise to us. The mechanism that Koenig et al. (1998) advanced to explain comparable behaviour of sst₂ receptor agonists is, in our present opinion, the most relevant one to explain many of the experimental data that are reported here. Although this mechanism constitutes a credible starting hypothesis, the intracellular trafficking of IRAP and its associated ligands may, even in CHO cells (Lim et al., 2001) be of such complexity that additional or even alternative mechanisms need to be invoked. Detailed analysis of the repercussions of pretreating alternative IRAP-expressing cells (like hippocampal cells, adipocytes and skeletal muscle cells) with metabolically stable and unstable “AT₄ receptor” ligands could further contribute to a better understanding of the kinetics of “AT₄ receptor” ligand binding, trafficking and/or metabolism. Although the repercussions of such treatments could be distinct in different cell lines, the so gained information might provide helpful directions for future drug design. In the mean time, it

could already be of interest to find out whether Ang IV and its metabolically stable and unstable analogs produce the same *in vivo* effects or not.

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Table 1.

“AT₄ receptor” ligand pIC₅₀ values for whole-cell co-incubation competition binding studies with [³H]AL-11 and [³H]Ang IV shown in Figure 1. Biphasic [³H]Ang IV competition binding curves were subjected to a two-site analysis and the component of each curve that fits best with IRAP is denoted with an asterisk.

Radioligand (Figure)	[³H]AL-11 (1A)	[³H]Ang IV (1B)	
AL-11	-7.7 ± 0.1	-7.2 ± 0.2*	-4.5 ± 0.3
Ang IV	-6.9 ± 0.1	-6.7 ± 0.1	
7B	-6.7 ± 0.1	-6.4 ± 0.2*	-4.1 ± 1.1
LVV-H7	-6.3 ± 0.1	-6.5 ± 0.1	
Ang II	-5.9 ± 0.1	-6.9 ± 0.6	-5.8 ± 0.3*

Table 2.

Total whole-cell binding (expressed in cpm/ μ g protein) of [3 H]AL-11 and [3 H]Ang IV to HEK293 control cells or recombinant HEK293 cells transiently expressing human IRAP in the absence (medium only) or presence of 10 μ M AL-11 and Ang IV.

Radioligand Cells	[^{3}H]AL-11		[^{3}H]Ang IV	
	control	recombinant	control	recombinant
Medium only	2.8 $\pm$ 0.3	9.5 $\pm$ 2.2	2.3 $\pm$ 0.1	9.8 $\pm$ 1.4
10 μ M AL-11	1.0 $\pm$ 0.2	1.2 $\pm$ 0.2	1.2 $\pm$ 0.3	3.3 $\pm$ 0.1
10 μ M Ang IV	1.1 $\pm$ 0.1	1.5 $\pm$ 0.3	0.9 $\pm$ 0.1	2.3 $\pm$ 0.3

Table 3.

“AT₄ receptor” ligand pIC₅₀ values for two-step competition binding studies with [³H]AL-11 shown in Figure 4. NA: not analysed.

Experiment type	ligand always present		ligand in pre-incubation only	
	acid-resistant	acid-sensitive	acid-resistant	acid-sensitive
AL-11	-8.1 ± 0.4	-7.8 ± 0.4	-8.0 ± 0.2	-8.6 ± 0.8
Ang IV	-6.5 ± 0.2	-6.5 ± 0.3	NA	NA
LVV-H7	-6.6 ± 0.1	-6.0 ± 0.8	NA	NA

Table 4.

Effect of Ang IV, LVV-H7 and AL-11 on the surface expression of V5IRAP. Recombinant CHO-K1 cells transiently expressing V5-tagged IRAP were incubated with these ligands at the concentrations indicated for 30 min at 37°C. After fixation, a whole cell ELISA with anti-V5 was performed as outlined in the methods section. Data refer to specific antibody binding, expressed as a percentage of control binding (cells pretreated with medium only) and N the number of measurements. $P < 0.05$ for 10^{-6} M AL-11. N = 3 to 4

Pretreatment	Surface V5IRAP expression (% control)
Angiotensin IV	
0.1 μ M	106 ± 7
10 μ M	117 ± 8
LVV-H7	
0.1 μ M	106 ± 1
10 μ M	111 ± 4
Al-11	
0.01 μ M	129 ± 5
1 μ M	147 ± 10

Legends to the figures.

Figure 1

Competition binding with [3 H]AL-11 and [3 H]Ang IV. Intact CHO-K1 cells were co-incubated for 60 min at 37°C with increasing concentrations of the following unlabelled ligands (AL-11 ■; Ang IV □; compound 7B ●, LVV-H7 ○; Ang II ▬) and 5 nM of [3 H]AL-11 (panel A) or 5 nM [3 H]Ang IV (panel B). Total binding is shown and is expressed as percentage of control binding (i.e. total binding in medium only). Single-site or two-site pIC₅₀ values of the competition curves are listed in Table 1.

Figure 2

Time-dependent [3 H]AL-11 binding to intact CHO-K1 cells. Cells were incubated at 37°C with 5 nM [3 H]AL-11 for different periods of time either in medium alone (■) or in the presence of 0.1 μM insulin (□) or 10 μM PAO (●). At the end of the experiment, cells were briefly washed and treated with mild acid to differentiate between surface binding (released in the medium, Panel A) and intracellular accumulation of the radioligand (acid-resistant binding fraction, Panel B). Data are given as specific binding (i.e. total binding minus non-specific binding in the presence of 10 μM Ang IV) and expressed as percent of control binding (i.e. specific surface binding determined after 60 min in the presence of 10 μM PAO).

Figure 3

Time dependent wash-out of [3 H]AL-11 surface binding (■) and internalized binding (□). For surface binding: intact CHO-K1 cells were pre-incubated with 5 nM [3 H]AL-11 and 10 μM PAO for 60 min at 37°C and further incubated with 10 μM Ang IV for the indicated time periods. For internalized binding: intact CHO-K1 cells were pre-incubated with 5 nM [3 H]AL-11 for 60 min at 37°C, incubated with 10 μM Ang IV for the indicated time periods, briefly washed and finely treated with mild acid to yield the resistant binding fraction. Data are expressed as total binding in fmol/mg protein.

Figure 4

“Two step competition” binding experiments. Intact CHO-K1 cells were pre-incubated for 30 min at 37°C with increasing concentrations of Ang IV (Panel A), LVV-H7 (Panel B) or AL-11 (Panel C) followed by a brief wash (□ and ○) or not (■ and ●). 5 nM [³H]AL-11 was then added and the incubation was continued for 30 min. At the end of the experiment, cells were briefly washed and treated with mild acid to differentiate between surface binding (■ and □) and internalized binding (● and ○). Data refer to total surface and internalized binding and are expressed as percentage of control binding (i.e. whole-cell binding in medium only). Single-site pIC₅₀ values of the competition curves are listed in Table 3.

Figure 5

Ang IV and AL-11 efflux experiments. Intact CHO-K1 cells were pre-incubated with 10 μM Ang IV (□) or 1 μM AL-11 (■) for 30 min at 37°C, briefly washed and further incubated with medium alone for the times indicated. Naïve CHO-K1 cells were incubated with the resulting supernatants along with 5 nM [³H]AL-11 (final concentration) without (for total binding) or with 10 μM Ang IV (non-specific binding) for 60 min at 37 °C. Data are given as specific whole-cell binding and expressed as percent of control binding (i.e. specific binding to naïve cells in fresh medium).

Figure 6

Effect of AL-11 pretreatment on [³H]AL-11 saturation binding. Intact CHO-K1 cells were pre-incubated for 30 min at 37°C with medium only (■) or with 1 μM AL-11 (□), briefly washed and further incubated for 60 min with the indicated concentrations of [³H]AL-11 without (for total binding) or with 10 μM Ang IV (non-specific binding). Data refer to the specific whole-cell binding and are expressed in fmol/mg protein.

Figure 7

Effect of “AT₄ receptor” ligand pretreatment on time dependent [³H]AL-11 surface binding (Panel A) and internalized binding (Panel B). Intact CHO-K1 cells were pre-incubated for 30 min at 37°C with medium only (■), with 10 μM Ang IV (□) or with 1 μM AL-11 (●), briefly washed and further incubated for the indicated periods of

time with 5 nM [3 H]AL-11 without (for total binding) or with 10 μ M Ang IV (non-specific binding). Cells were briefly washed again and treated with mild acid to differentiate between surface binding (Panel A) and internalized binding (Panel B). Data refer to specific binding and are expressed as percentage of control binding (i.e. specific whole-cell binding after 60 min in medium only).

Figure 8

Time-dependent effect of “AT₄ receptor” ligand pretreatment on [3 H]AL-11 binding. Intact CHO-K1 cells were pre-incubated for the indicated time periods at 37°C with 10 μ M Ang IV (■), 10 μ M Ang IV + 0.1 μ M compound 7B (□), 10 μ M LVV-H7 (○), 1 μ M AL-11 (●) or 1 μ M AL-40 (△), briefly washed and further incubated for 30 min with 5 nM [3 H]AL-11 without (for total binding) or with 10 μ M Ang IV (non-specific binding). Data refer to the specific whole-cell binding and are expressed as a percentage of control binding (i.e. specific binding to cells pretreated with medium only).

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.