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SHORT COMMUNICATION

Angiotensin IV displays only low affinity for native insulin-regulated aminopeptidase (IRAP)

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ABSTRACT

Radioligand binding studies revealed that Ang IV binds to insulin-regulated aminopeptidase (IRAP)/'AT_4 receptors' with high affinity. Yet, as these experiments were routinely carried out in the presence of chelators, only the catalytic zinc-depleted apo-form of IRAP was labelled. While the chelators remove the catalytic zinc from IRAP and protect Ang IV from proteolytic degradation, the aminopeptidase N selective inhibitor '7B' only exerts the latter effect. By using 7B along with the new stable Ang IV-analog [^{3}H]AL-11, we here show that the native enzyme is only a low-affinity target for Ang IV.

INTRODUCTION

'AT_4 receptors', the putative cellular targets through which angiotensin IV (Ang IV) improves memory acquisition, were identified as the insulin-regulated aminopeptidase (IRAP) enzyme [1]. Interestingly, the affinity of Ang IV in binding studies with [^{125}I]Ang IV is markedly higher than the potency by which it inhibits the enzymatic activity of IRAP [2–5]. High-affinity [^{125}I]Ang IV binding requires the combined presence of divalent cation chelators like EDTA and 1,10-phenanthrolin, and studies with increasing chelator concentrations shed light on the inverse proportionality between the amount of binding sites and the catalytic activity [3,5]. This suggests that Ang IV only displays high affinity for the catalytic zinc-depleted apo-form of IRAP. Alternatively, both forms of the enzyme could display similar Ang IV binding properties, but chelators could be needed to prevent Ang IV degradation by aminopeptidase-N (AP-N) and other metalloproteases in cell membranes [6–8].

At concentrations only moderately affecting the catalytic activity of IRAP, the AP-N-selective inhibitor, compound 7B, has recently also been shown to almost completely prevent the degradation of Ang IV in vivo and by CHO-K1 cell membranes [9,10]. By making use of 7B and the radioligands [^{3}H]Ang IV and its stable analogue [^{3}H]AL-11 [5], we here confirm that both forms of IRAP display different Ang IV binding affinity.

MATERIAL AND METHODS

Materials, cell culture, and membrane preparation

Angiotensin IV was obtained from NeoMPS (Strasbourg, France). Synthesis and labelling of [^{3}H]Ang IV (40.8 Ci mmol^{-1}) and [^{3}H]AL-11 (35.0 Ci mmol^{-1}), their precursor peptides and AL-11 were described earlier [5,11]. The AP-N inhibitor 7B [12] was a gift from Prof. Yiotakis

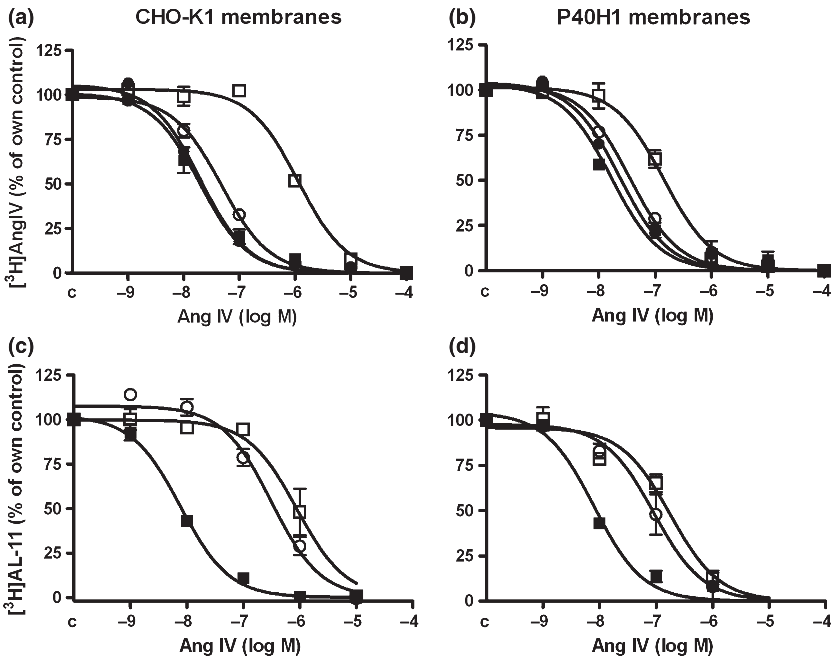

Figure 1 (a, b) Stability experiments performed in membrane homogenates of CHO-K1 and P40H1 cells. Membranes were pre-incubated for 40 min at 37 °C with increasing concentrations of compounds in the presence (■ and ●) or absence (□ and ○) of chelators (EDTA and 1,10-phenanthrolin) and in the presence (● and ○) or absence (■ and □) of 3.10^{-7} M 7B. Then, 5 nM [^{3}H]Ang IV and (if necessary) chelators were added, and the incubation was continued for 30 min. Results were expressed as a percentage of the control binding in the absence of Ang IV. The corresponding pK_i values for (a) are 8.13 ± 0.07 nM (■), 6.25 ± 0.09 nM (□), 8.08 ± 0.07 nM (●) and 7.69 ± 0.04 nM (○) and for (b) are 8.10 ± 0.09 nM (■), 7.03 ± 0.07 nM (□), 7.92 ± 0.12 nM (●) and 7.72 ± 0.12 nM (○) (the K_D value from [5] was used). (c, d) [^{3}H]AL-11 competition binding in CHO-K1 and P40H1 cell membranes. Membranes were incubated for 40 min at 37 °C with increasing concentrations of Ang IV in the presence (■) or absence of the chelators with (○) or without (□) 3.10^{-7} M 7B. Results were expressed as a percentage of the control binding in the absence of Ang IV. The corresponding pKi values for (c) are 8.34 ± 0.04, 6.70 ± 0.10, and 6.16 ± 0.13 nM, respectively and for (d): 8.30 ± 0.06, 7.16 ± 0.11, and 6.84 ± 0.11 nM, respectively (the K_D values from [5] were used).

(Athens, Greece). All other reagents were of the highest grade commercially available.

Chinese hamster ovary (CHO-K1) cells were obtained from the Pasteur Institute (Brussels, Belgium). Mouse hippocampal P40H1 cells (obtained in collaboration with Dr. Vanderheyden, VUB, Brussels) were derived by Sridhara Chakravarthy and M.M. Panicker (NCBS, Bangalore, India). Both cell lines were cultured, and membranes were prepared as described previously [3].

Radioligand binding

Radioligand binding was performed as described previously [3]. All experiments were performed with membrane homogenates corresponding to 4×10^5 CHO-K1 cells or 3×10^5 P40H1 cells per assay. [^{3}H]Ang IV or [^{3}H]AL-11 was added at a final concentration of 5 nM for all experiments.

Data analysis

Values refer to specific (i.e. displaceable by 10 μM of the same unlabelled ligand) radioligand binding (expressed as percentage of control binding, i.e. without other addition) and are the mean ± SEM of three independent experiments performed in duplicate. Nonlinear regression analysis of the data was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS AND DISCUSSION

While it is not possible to distinguish between the ability of chelators to remove the catalytic zinc from IRAP and to protect Ang IV against metabolic breakdown, this is now possible with 7B. This paves the way to finding out whether or not native IRAP displays high affinity for Ang IV.

Table I Specific [^{3}H]Ang IV binding in CHO-K1 and P40H1 cell membranes in the presence or absence of chelators (EDTA and 1,10-phenanthrolin) and 7B. Cells were incubated for 40 min at 37 °C with 5 nM [^{3}H]Ang IV. Results were expressed as a percentage of the specific control binding in the presence of chelators (corresponding to 698 ± 27 and 384 ± 11 cpm for CHO-K1 and P40H1 membranes, respectively).

	Control		+0.3 μM 7B	
Origin of membranes	With chelators (%)	No chelators (%)	With chelators (%)	No chelators (%)
CHO-K1 cells	100	15.6 ± 3.6*	103.6 ± 6.6†	14.8 ± 2.3*
P40H1 cells	100	14.4 ± 4.9*	94.3 ± 8.8†	12.5 ± 2.5*

The differences between control with chelators and the other conditions were determined by using one-way ANOVA and Dunnett's post hoc test: *$P < 0.01$; †$P > 0.05$, not significantly different.

In co-incubation experiments (40 min, 37 °C) in the presence of chelators, up to 1 μM 7B did not affect the specific binding of 5 nM [^{3}H]Ang IV to CHO-K1 and P40H1 cell membranes (698 ± 27 vs. 705 ± 13 cpm and 384 ± 11 vs. 384 ± 8 cpm in presence or absence of 1 μM 7B, respectively). By contrast to this very low affinity for the apo-form of IRAP, earlier catalytic activity and [^{3}H]AL-11 binding inhibition experiments revealed that 7B displays moderate affinity for naïve IRAP (pKi of 7.04 and 6.75–6.91, respectively) [5,9]. Yet, no inhibitory effect was perceived in a two-step binding paradigm in where membranes are first pretreated with 0.3 μM 7B alone and then in combination with 5 nM [^{3}H]Ang IV and chelators ('c' in *Figure 1a,b*).

The ability of 7B to protect Ang IV against proteolytic breakdown was investigated by a two-step binding paradigm in where the membranes are pretreated with increasing concentrations of Ang IV with or without 0.3 μM 7B and then subjected to binding of [^{3}H]Ang IV in the presence of chelators. A leftward shift of the resulting Ang IV competition curves, indicative for a protecting effect of 7B, clearly takes place with both membrane preparations (*Figure 1a,b*). An equally pronounced shift also takes place when including the chelators in the preincubation step, and the effects of the chelators and 7B are not additive. Hence, 7B fully protects Ang IV from degradation by metalloproteases. The exact nature of these proteases is still undisclosed. Although it is known that AP-N displays very high affinity for 7B, it cannot be excluded that other metalloproteases, including IRAP itself, are involved as well.

While the chelators markedly increase the specific [^{3}H]Ang IV binding to both membrane preparations, 0.3 μM 7B is unable to do so (*Table I*). Even by assuming that 7B occupies 63–77% of naïve IRAP under those conditions (based on the pKi values mentioned above), a perceptible increase should have been expected in case of high-affinity binding of Ang IV to naïve IRAP. Further support for a low-affinity Ang IV–naïve IRAP interaction is provided by experiments with the stable Ang IV-analogue [^{3}H]AL-11. [^{3}H]AL-11 binds selectively and with high affinity to IRAP both in the absence and in the presence of chelators [5]. At 5 nM, specific binding of [^{3}H]AL-11 was 967 ± 82 and 506 ± 5 cpm for CHO-K1 and P40H1 membranes, respectively. For both membrane preparations, co-incubation experiments with increasing concentrations of Ang IV only reveal high potency competition in presence of the chelators but not in presence of 0.3 μM 7B (*Figure 1c,d*).

Contrary to the tenacious belief based on the earlier binding experiments in the presence of chelators, the present data indicate that the physiologically relevant, native form of IRAP only possesses low affinity for Ang IV. Unless binding is performed with radioligands like [^{3}H]AL-11 that effectively target naïve IRAP, enzyme activity measurements offer the best approach to determine physiologically relevant affinities of this enzyme for its ligands.

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