Interactions of Ligands at Angiotensin II-Receptors and Imidazoline Receptors

Uta Wethmar, Walter Raasch, Andreas Dendorfer and Peter Dominiak*

*Institute of Experimental and Clinical Pharmacology and Toxicology, Medical University of Lübeck,
Ratzeburger Allee 160, 23538 Lübeck, Germany*

Received August 22, 2000 Accepted November 13, 2000

ABSTRACT—Ligands for angiotensin II-(AT)-receptors and imidazoline receptors have structural similarities and influence blood pressure via various mechanisms. The goal of this study was to study the specificity of various ligands by displacement experiments. Antazoline, cimetidine, clonidine, efaroxan, guanabenz, guanethidine, idazoxan, moxonidine and rilmenidine up to a concentration of 100 μ M failed to displace the specific binding of [125 I]Sar¹,Ile⁸ angiotensin II at the AT₁-receptor characterized by losartan (IC_{50} = 26 $\pm$ 12 nM) in liver homogenate. The same substances up to 100 μ M produced no reduction of specific [125 I]Sar¹,Ile⁸ angiotensin II binding to the AT₂-receptor of pheochromocytoma cell membranes characterized by PD123319 (IC_{50} = 20 $\pm$ 5 nM). Displacement experiments at the imidazoline I₁-receptors were performed on bovine adrenal medulla membranes using [3 H]clonidine after characterization by the I₁-ligand clonidine (IC_{50} = 459 $\pm$ 13 nM) and the I₂-ligand idazoxan (IC_{50} = 3.29 $\pm$ 0.88 μ M). The investigated AT-receptor ligands angiotensin II, losartan, EXP 3174 and PD123319 revealed no displacement of [3 H]clonidine up to a concentration of 100 μ M. The I₂-receptor in liver homogenate was characterized by displacement of [3 H]idazoxan by cold idazoxan and clonidine (IC_{50} = 0.37 $\pm$ 0.17 and 68 $\pm$ 31 μ M, respectively). The investigated AT-receptor ligands angiotensin II, losartan and PD123319 failed to displace [3 H]idazoxan specifically up to 100 μ M. Hence, the tested substances showed no cross-reactivity at the corresponding AT- and I-receptors up to 100 μ M, a concentration markedly higher than the plasma concentrations achieved after therapeutic application.

Keywords: Angiotensin II-receptor, Imidazoline receptor, AT₁- and AT₂-antagonists, Clonidine, Losartan

Ligands at angiotensin II AT-receptors and imidazoline I-receptors show structural similarities concerning imidazole (e.g., losartan, PD123319), imidazoline (e.g., clonidine, idazoxan) or guanidine (e.g., guanabenz, guanethidine) residues (Fig. 1). Substances binding to AT-receptors, such as losartan, its metabolite EXP 3174, PD123319 and angiotensin II, contain an imidazole moiety in their structure (1), but not all AT-receptor antagonists possess an imidazole structure. Ligands at I-receptors possess an imidazoline, oxazoline or guanidine structure (2). Blood pressure is influenced by ligands at AT₁-receptors or I-receptors, such as the AT₁-antagonist losartan, the I₁-ligands clonidine or moxonidine, and the guanidine derivatives guanabenz and guanethidine, via various mechanisms. The question arises as to whether the blood pressure effect occurs due to normally described mechanisms, or whether

several receptor types with chemically similar structures participate.

For the octapeptide angiotensin II, two human receptor subtypes have been cloned and sequenced until now: the AT₁-receptor and the AT₂-receptor. While most of the established angiotensin II effects are mediated via the AT₁-receptor (vasoconstriction, aldosterone release, gluconeogenesis, drinking behavior and tissue growth), less is known about the function of the AT₂-receptor (3, 4). Most effects ascribed to the AT₂-receptor concern inhibition of growth and promotion of apoptosis, effects which counteract the function of the AT₁-receptor (5–7). There is conflicting evidence concerning the participation of AT₂-receptors in blood pressure regulation (8–10). Binding studies have allowed the I-receptors to be differentiated amongst two subtypes. The I₁-receptors characterized in the rostral ventrolateral medulla amongst other locations have been considered to be responsible for the blood-pressure reducing effect of the imidazolines clonidine, moxonidine

*Corresponding author. FAX: +49-451-5003327
E-mail: dominiak@medinf.mu-luebeck.de

Fig. 1. Chemical structure of various imidazoline- (clonidine, idazoxan), imidazole- (losartan, PD123319) and guanidine- (guanabenz, guanethidine) derivatives used in this study.

and rilmenidine (11–13). Ligands at I_2 -receptors should influence monoamine oxidase (MAO) activity amongst other processes (14–17), but the exact function of these receptors has not been decisively determined. There is evidence that ligands at I_1 -receptors, such as [125 I]-*p*-iodo-clonidine, are displaced dose-dependently by imidazoles such as cimetidine and imidazole-4-acetic acid between 10 nM and 1 μ M (18), and that the imidazoles detomidine and medetomidine can displace the imidazoline [3 H]idazoxan from the I_2 -receptor on guinea pig ileum at high affinities (60–400 nM) (19). Furthermore, according to ref. 20, the entire antihypertensive effect of the AT_1 -antagonist losartan can not be explained just by AT_1 -receptor blockade, so that other receptors or binding sites might also be involved. Binding studies on the liver (21) and kidney (22, 23) show that losartan binds at significantly larger numbers at high affinity sites than are allowed by the AT -receptor density.

The goal of our investigation was to study the specificity of various ligands at AT -receptors and I -receptors. To this end, we performed binding studies at these receptors using substances containing an imidazoline, imidazole or guanidine structure.

MATERIALS AND METHODS

All binding studies were performed as displacement studies. For investigations on interactions at I -receptors

and AT -receptors, homogenized liver served as the model for the AT_1 -receptor and the I_2 -receptor, the phaeochromocytoma cell line PC12W served as the AT_2 -receptor model, and the adrenal medulla served as the model for the I_1 -receptor.

Substances

Angiotensin II, antipain, antazolin, bacitracin, bestatin, bovine serum albumin (protease-free), cimetidine, fetal calf serum, guanabenz, guanethidine, idazoxan, leupeptin, penicillin-streptomycin solution (10.000 I.E./ml – 10 mg/ml), pepstatin A, horse serum and Sar¹Ile⁸ angiotensin II were all acquired from Sigma (Deisenhofen, Germany). [3 H]Clonidine (24 Ci/mmol), [3 H]idazoxan (43 Ci/mmol) and 125 iodide (2180 Ci/mmol) in NaOH for iodination were obtained from Amersham (Brunswick, Germany). Efaroxan was from RBI (Cologne, Germany). Clonidine (Boehringer, Ingelheim, Germany); losartan and EXP 3174 (MSD Sharp&Dohme, Haar, Germany); moxonidine (Beiersdorf-Lilly, Hamburg, Germany); PD123319 (Parke Davis, Morris Plains, NJ, USA); and rilmenidine (Servier Itherapia, München, Germany) were generously provided as gifts. All other reagents were supplied by Sigma and were also of the highest grade available.

Protein preparation

Liver preparation: Rat livers (11–15 g) from Wistar-Kyoto rats (Charles River, Sulzfeld, Germany) were

minced with a scalpel and homogenized in 100 ml saccharose buffer (1665 mM saccharose, 6.6 mM tris(hydroxymethyl)aminomethane (TRIS), 6.6 mM ethylenediaminetetraacetic acid (EDTA)) for 5 min with an Ultra-Turrax (Jahnke & Kunkel, Staufen, Germany). The homogenate was centrifuged for 20 min at 4°C at $100 \times g$ and the pellet was discarded. The supernatant was centrifuged for 10 min (4°C) at $20,000 \times g$, and the pellet was resuspended in 50 ml phosphate buffer (50 mM NaH_2PO_4 , set to pH 7.4) before use in binding studies.

Adrenal medulla membranes: Adrenal glands were removed directly after the cattle were slaughtered and the abdomens were opened up. The glands were transported from the slaughterhouse in 4°C cold Locke's solution (7.7 mM NaCl, 0.28 mM KCl, 0.25 mM hydroxymethylpiperazine *N*-2 ethanesulfonic acid (HEPES®) (pH 7.88), 0.25 mM glucose, 100 I.E./ml penicillin G, 10 µg/l gentamicin). All membrane preparation steps were performed at 4°C. The adrenal medulla was separated on ice-cooled petri dishes, weighed and homogenized in 30 vol HEPES-saccharose buffer (330 mM saccharose, 10 mM HEPES®, 1 mM phenylmethylsulfonyl fluoride (PMSF) from a stock solution of 1 M PMSF in dimethylsulfoxide, adjusted to pH 7.4 with NaOH) in a teflon glass potter (20 times at 500 U/min). The homogenate was centrifuged at $600 \times g$ for 10 min, after which the supernatant from this step was centrifuged at $30,000 \times g$ for 20 min. The resulting pellet was resuspended in 24 vol TRIS-EDTA buffer (relative to the wet weight, TRIS-EDTA buffer: 50 mM TRIS, 5 mM EDTA, 1 mM PMSF, 1 mM 2-mercaptoethanol, adjusted to pH 7.4 with HCl) and centrifuged for 30 min at $40,000 \times g$. The pellet was then washed twice with 16 vol TRIS-EDTA buffer before it was stored in incubation buffer 1 (50 mM TRIS, 1 mM PMSF, adjusted to pH 7.4 with HCl) at 80°C as a crude membrane suspension.

PC12W cell membranes: The PC12W cells were cultured at 37°C in 100% saturated air and 5% CO_2 in Dulbecco's MEM (1×) with 3.7 g/l NaHCO_3 , 4.5 g/l D-glucose, 1.028 g/l *N*-acetyl-L-alanyl-L-glutamine (Biochrom KG, Berlin, Germany), supplemented with 10% heat inactivated horse serum, 5% fetal calf serum and 1% penicillin-streptomycin solution. They were washed with 1×6 ml ice cold phosphate-buffered salt solution (137 mM NaCl, 1.5 mM KH_2PO_4 , 2.7 mM KCl, 6.5 mM Na_2HPO_4 , pH 7.2), removed from the bottom of the culture dishes with a rubber policeman in another 6 ml volume, and transferred to ultracentrifuge tubes before centrifugation at $30,000 \times g$ for 30 min. The pellets were suspended using an ultrasonic probe for 20 s at 50 Watts in TRIS-buffer 2 (125 mM NaCl, 6.5 mM MgCl_2 , 1 mM EDTA, 50 mM TRIS, pH 7.4 (HCl), 1 mg/ml bovine serum albumin (BSA) and 1.25 µg/ml antipain, leupeptin, bestatin, pepstatin A) before recentrifugation, resuspension and storage at -80°C.

Displacement experiments

Binding studies at the AT_1 -receptor were performed as described elsewhere (24, 25). An incubation sample (200 µl) contained 130–150 µg protein, approximately 0.2–0.5 nM [^{125}I]Sar¹,Ile⁸ angiotensin II and displacing compounds in 50 mM TRIS, 5 mM MgCl_2 , 1.2 mg/ml BSA and 1.2 mg/ml bacitracin (pH 7.4). The incubation (60 min, 20°C) was halted by addition of 2 ml ice-cold buffer (50 mM TRIS, 5 mM MgCl_2 , pH 7.4), immediate filtration, and washing with 3×2 ml buffer. For AT_2 -receptor binding studies, the incubations (250 µl) contained 40–60 µg protein, approximately 0.2–0.5 nM [^{125}I]Sar¹,Ile⁸ angiotensin II and displacing substances in TRIS-buffer 2. Sar¹,Ile⁸ angiotensin II was iodinated using the chloramine-T method (26). Incubations (90 min, 20°C) were terminated by filtration and washing with 3×2 ml phosphate buffer (137 mM NaCl, 1.5 mM KH_2PO_4 , 3.2 mM KCl, 8.1 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, pH 7.2). The radioactivity on the filter (Whatman GF/B Paper, Maidstone, UK) was measured in a gamma counter (70% recovery).

Displacement studies with the I_1 -receptor were based on methods described elsewhere (27). Incubations (250 µl) contained 60–90 µg protein, 2 nM [^3H]clonidine and displacing substances in incubation buffer 1. The stability of angiotensin II in the incubations was tested by HPLC. The α_2 -adrenoceptors were masked using 100 µM adrenaline, stabilized in 0.001% ascorbic acid. The incubation was terminated after 40 min by filtration and washing with 5×2 ml buffer (50 mM TRIS (HCl), pH 7.4).

Incubations for investigating the I_2 -receptor were carried out according to the refs. 28 and 29. Incubations (200 µl) contained 150 µg protein, 10 nM [^3H]idazoxan and displacing substances in incubation buffer (50 mM TRIS, 3 mM MgCl_2 , 0.6 mM EDTA, 0.01 mM PMSF, 1.2 mg/ml BSA and bacitracin, 3 µM rauwolscine for masking the α_2 -adrenoceptors, pH 7.4). Incubations were stopped after 60 min by adding 4 ml buffer (100 mM TRIS (HCl), pH 7.4), filtration and washing with 3×4 ml buffer. Filters (Whatman GF/B Paper) from experiments with tritiated clonidine and idazoxan were added to vials containing 7 ml Hydroluma® (Baker, Deventer, Netherlands) scintillation fluid and shaken for 24 h before they were measured in a liquid scintillation counter for 10 min. Protein levels in incubation experiments were photometrically determined according to the ref. 30.

Calculation of displacement curves

For AT-receptor displacement studies, non-specific binding was accounted for by determining the number of counts measured in the presence of 1 µM Sar¹,Ile⁸ angiotensin II; for I_1 -receptors, 100 µM naphazoline was used, while for I_2 -receptors, 100 µM idazoxan was used. The values obtained were deducted from the total counts

measured to obtain specific binding. Basal values (dpm, mean $\pm$ S.E.M., $n = 12 - 19$) of 100% binding were 1639 ± 136 (AT₁-receptor), 2819 ± 289 (AT₂-receptor), 312 ± 89 (I₁-receptor) and 477 ± 47 (I₂-receptor). Specific binding in the sole presence of radioligands was normalized to 100%, and the remaining measurement values were corrected to this figure. IC₅₀-values were calculated using a non-linear curve fitting function (Prism®; Graph Pad, San Diego, CA, USA).

Statistics

IC₅₀-values are given as means $\pm$ S.E.M. of the individual values. The Friedman Test was used to compare 100% corrected means of controls with the binding observed in the presence of different concentrations of displacing substances.

RESULTS

Interactions at the AT₁-receptor

The AT₁-receptor was characterized by displacement of the radioligand [¹²⁵I]Sar¹,Ile⁸ angiotensin II by unmarked Sar¹,Ile⁸ angiotensin II and the AT₁-antagonist losartan in rat liver homogenate (Fig. 2: A and B). Respective IC₅₀-values of 1.3 ± 0.6 and 26 ± 12 nM ($n = 5$) were obtained. The imidazole cimetidine; the imidazolines antazoline, clonidine, efaroxan, idazoxan and moxonidine; the oxazoline rilmenidine; and the guanidines guanabenz and guanethidine were tested at the AT₁-receptor. None of the substances showed any displacement of [¹²⁵I]Sar¹,Ile⁸ angiotensin II up to 100 μ M ($P > 0.05$, Fig. 2: A and B). At the highest tested concentration of 1 mM, guanabenz, guanethidine and antazoline reduced the specific bindings of [¹²⁵I]Sar¹,Ile⁸ angiotensin II significantly to $12 \pm 3\%$, $69 \pm 5\%$ and $70 \pm 1\%$, respectively (Fig. 2A); and the remaining substances showed no significant deviations from 100%, with reductions amounting to between 3% and 10% ($P > 0.05$, Fig. 2B). Non-specific binding was determined using 1 μ M Sar¹,Ile⁸ angiotensin II and amounted to $2.7 \pm 0.3\%$ ($n = 19$).

Interactions at the AT₂-receptor

The AT₂-receptor in PC12W membranes was characterized by displacement of the radioligand [¹²⁵I]Sar¹,Ile⁸ angiotensin II by unmarked Sar¹,Ile⁸ angiotensin II and the AT₂-receptor antagonist PD123319 (Fig. 3: A and B). The calculated IC₅₀-values were 1.2 ± 0.4 and 20 ± 5 nM ($n = 10$), respectively. The AT₁-receptor antagonist losartan did not reduce specific binding at the AT₂-receptor at both tested concentrations of 1 and 10 μ M ($P > 0.05$). The substances tested at the AT₂-receptor, i.e., the imidazole cimetidine; the imidazolines antazoline, clonidine, efaroxan, idazoxan and moxonidine; the oxazoline rilmenidine; and the guanidine guanethidine, revealed no displacement

Fig. 2. Displacement of [¹²⁵I]Sar¹,Ile⁸ angiotensin II by Sar¹,Ile⁸ angiotensin II (▲), losartan (◆) (A and B); guanabenz (*), guanethidine (○), antazoline (▼) (A); cimetidine (×), clonidine (○), moxonidine (■), efaroxan (△), rilmenidine (◇) and idazoxan (▼) (B); in rat liver homogenate. Means $\pm$ S.E.M., $n = 5$ in duplicate, * $P < 0.05$.

of [¹²⁵I]Sar¹,Ile⁸ angiotensin II ($P > 0.05$) up to 1 mM (Fig. 3: A and B). Only guanabenz reduced specific binding at this concentration by $49 \pm 10\%$ ($P < 0.05$) (Fig. 2A). The non-specific binding determined in the presence of 1 μ M Sar¹,Ile⁸ angiotensin II was $3.7 \pm 0.6\%$ ($n = 13$).

Interactions at the I₁-receptor

Displacement experiments at the I₁-receptor were performed on bovine adrenal medulla membranes in the pres-

Fig. 3. Displacement of $[^{125}\text{I}]\text{Sar}^1, \text{Ile}^8$ angiotensin II by $\text{Sar}^1, \text{Ile}^8$ angiotensin II (▲), PD123319 (●) (A and B); antazoline (▽), cimetidine (×), rilmenidine (◇), guanabenz (*), guanethidine (○) (A); clonidine (○), moxonidine (■), efaroxan (△) and idazoxan (▼) (B); from PC12W cell membranes. Means $\pm$ S.E.M., $n = 5$ in duplicate, * $P < 0.05$.

ence of $100 \mu\text{M}$ adrenaline to block the α -adrenoceptors. The I_1 -ligand clonidine already completely displaced the radioligand $[^3\text{H}]\text{clonidine}$ at low concentrations ($\text{IC}_{50} = 459 \pm 13 \text{ nM}$, $n = 5$), whereas higher concentrations of the I_2 -ligand idazoxan were needed ($\text{IC}_{50} = 3.29 \pm 0.88 \mu\text{M}$, $n = 5$) (Fig. 4). The investigated AT receptor ligands angiotensin II, losartan, its metabolite EXP 3174 and PD123319 revealed no displacement of $[^3\text{H}]\text{clonidine}$ up to $100 \mu\text{M}$ (Fig. 4). At a concentration of 1 mM PD123319, $44.8 \pm 2.5\%$ ($n = 5$) of specific binding was significantly displaced, while the other substances failed to reduce

Fig. 4. Displacement of $[^3\text{H}]\text{clonidine}$ by unmarked clonidine (○), idazoxan (▼), EXP 3174 (*), angiotensin II (□), losartan (◆) and PD123319 (●) in the crude membrane fraction of the bovine adrenal medulla. Means $\pm$ S.E.M., $n = 5$ in triplicate, * $P < 0.05$.

Fig. 5. Displacement of $[^3\text{H}]\text{idazoxan}$ by unmarked idazoxan (▼), clonidine (○), losartan (◆), angiotensin II (□) and PD123319 (●) in rat liver homogenate. Means $\pm$ S.E.M., $n = 5$ in duplicate, * $P < 0.05$.

specific binding at this concentration. Non-specific binding determined in the presence of $100 \mu\text{M}$ naphazoline was $33.4 \pm 1.5\%$ ($n = 12$).

Interactions at the I_2 -receptor

The I_2 -receptor in rat liver homogenate was determined by measuring the displacement of the I_2 -ligand $[^3\text{H}]\text{idazoxan}$ with unlabeled idazoxan and the I_1 -ligand clonidine (Fig. 5). For these substances, the respective IC_{50} -values were 0.37 ± 0.17 and $68 \pm 31 \mu\text{M}$ ($n = 5$). Idazoxan binding

showed a n_{Hill} of 0.48 by sigmoidal dose-response curve fitting. The substances tested at the I_2 -receptor, the AT_2 -antagonist PD123319, the AT_1 -antagonist losartan and angiotensin II, produced no displacement of [^3H]idazoxan up to 100 μM (Fig. 5). At the highest concentration of 1 mM, PD123319 significantly displaced specific [^3H]idazoxan binding by $66 \pm 3\%$ ($n = 5$), while angiotensin II and losartan were ineffective ($P > 0.05$). Non-specific binding determined in the presence of 100 μM idazoxan was $25.7 \pm 2.1\%$ ($n = 17$) of total binding.

DISCUSSION

The liver homogenate was utilized as a model for the AT_1 -receptor. The affinity of $\text{Sar}^1, \text{Ile}^8$ angiotensin found by us is consistent with the binding properties described in rat liver (31) ($\text{IC}_{50} = 1.22 \text{ nM}$ for angiotensin II and $\text{IC}_{50} = 41.4 \text{ nM}$ for losartan regarding the displacement of [^{125}I] $\text{Sar}^1, \text{Ile}^8$ angiotensin II) at the AT_1 -receptor. One has to assume that the majority of AT-receptors in our protein suspension arose from cell nuclear membranes, that these AT-receptors, just like plasma membrane receptors, are displaceable by losartan, and that the order of affinities for the peptides angiotensin III, angiotensin I, angiotensin (1–7) and angiotensin (3–8) are the same (32). The displacement experiments on PC12W-membranes confirmed the exclusive presence of AT_2 -receptors: the AT_2 -receptor antagonist PD123319 displaced the specific binding of [^{125}I] $\text{Sar}^1, \text{Ile}^8$ angiotensin II with an IC_{50} of $20 \pm 5 \text{ nM}$ (Fig. 2), while the AT_1 -receptor antagonist losartan was ineffective up to 10 μM . On bovine adrenal medulla membranes, the IC_{50} measured for clonidine (459 nM) at the [^3H]clonidine labeled I-receptor was markedly higher than the dissociation constant $K_D = 16 \text{ nM}$ found elsewhere (33). Clonidine revealed, however, a simultaneous blockade of α -adrenoceptors with a higher affinity than idazoxan at the [^3H]clonidine labeled I-receptor, consistent with literature reports (33), so that the membrane suspension from bovine adrenal medulla could be properly considered as a model for the I_1 -receptor. However, the shallow competition curves ($n_{\text{Hill}} = 0.46$) indicate more than one specific binding site. This may indicate the existence of I_2 -receptors in adrenal medulla as suggested by binding and autoradiographic studies (34, 35). Furthermore, clonidine was shown to bind at I_2 -receptors in bovine adrenal medullary membranes with a moderate affinity (34), which is somehow in contrast to the very weak inhibitory potency of clonidine on MAO activity ($\text{IC}_{50} 1.2 \text{ mM}$) (15), inasmuch as I_2 -receptors have modulatory properties on MAO (14, 15). Since clonidine interacts only partially with the I_2 -receptor (34) as was demonstrated by its only 50% inhibition of the idazoxan binding, it was suggested that imidazoline receptors may exist in two different interconvertible forms which are in-

duced in different proportions by the imidazolines.

At the I_2 -receptor, similar affinities for idazoxan as reported by Brown et al. (36) with an IC_{50} of 100 nM in rat liver homogenate were described. In some cases, markedly higher affinities with K_D -values in the order of 10 nM have been found for idazoxan (37). Calculation of the K_D -value for idazoxan from our results revealed a value of 360 nM, assuming the same dissociation constants for labeled and unlabeled ligand. Since clonidine (consistent with ref. 37) showed a much lower affinity to the I_2 -receptor than idazoxan, we could perform investigations on the I_2 -receptor using the liver homogenate. Comparable to clonidine binding, idazoxan binding of liver homogenates yielded a shallow competition curve ($n_{\text{Hill}} = 0.48$), which indicates that there may be more than one binding-site. However, binding at I_1 -receptors could be excluded, since 1) clonidine does not show any displacement up to a concentration of 1 μM in our study (Fig. 4), and 2) no I_1 -receptors have been determined in rat liver (38) until now. As mentioned above, high and low affinity conformations of imidazoline receptors could be present (34), since competition curves fitted better to a two site model ($R^2 = 0.954$) than to a one site model ($R^2 = 0.923$).

Our results show that despite chemical similarities, no cross-reactions of specific AT_1/AT_2 -receptor ligands occurred at the I_1 - and I_2 -receptors and vice versa, since up to a concentration of 100 μM , there was no displacement of binding site specific radioligands. This concentration is 200–5000 times higher than the concentrations at which the specific substances had to be applied in order to bind to the corresponding I-receptors and AT-receptors. It is also 50–20,000 times higher than the plasma concentrations of the therapeutically applied substances clonidine (39), moxonidine (40), rilmenidine (41) and losartan (calculated from the bioavailability, dose and distribution volume; 42).

Hence, our results are consistent with the findings reported by Li et al. (43). They also found no interaction of AT-receptor ligands at I-receptors; however, they did describe a reduction of specific binding of the radioligand [^{125}I]AMIPI (2-(3-amino-4-[[^{125}I]iodophenoxy)methylimidazoline, a cirazoline derivative, by losartan at the high concentration of 100 μM , where we saw no displacement. This inconsistency may be related to differences in methodology (autoradiography and other radioligands) or the tissue investigated (subfornical organ, hypothalamic arcuate nucleus). In their paper, no concrete statements were made concerning the quantitative distribution of I_1/I_2 -receptors, and the binding of imidazolines to AT-receptors was not investigated.

In summary, one can assume that the results of our displacement experiment confirms the specificity of the imidazolines clonidine, moxonidine and rilmenidine and the AT_1 -receptor antagonist losartan used in the treatment of

hypertension. Even if the ligands for the AT-receptor and those for the I-receptor both share similar chemical structures, they interact solely with their specific receptor and do not bind at the other one. Therefore, they are incapable of weakening or strengthening their activity through such a mechanism.

Acknowledgments

The phaeochromocytoma cell line PC12W was generously provided by Prof. Dr. Th. Unger of the Department of Pharmacology, Christian Albrecht University Kiel, Germany. The authors would like to thank Dr. J.P. Keogh for his linguistic assistance in preparing the manuscript.

REFERENCES

- 1 Timmermans PB, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye JA and Smith RD: Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol Rev* **45**, 205–251 (1993)
- 2 Hieble JP and Ruffolo RR: Possible structural and functional relationships between imidazoline receptors and alpha 2-adrenoceptors. *Ann NY Acad Sci* **763**, 8–21 (1995)
- 3 Unger T, Chung O, Csikos T, Culman J, Gallinat S, Gohlke P, Höhle S, Meffert S, Stoll M, Stroth U and Zhu Y-Z: Angiotensin receptors. *J Hypertens* **14**, Suppl **5**, S95–S103 (1996)
- 4 Sayeski PP, Ali MS, Semeniuk DJ, Doan TN and Bernstein KE: Angiotensin II signal transduction pathways. *Regul Pept* **78**, 19–29 (1998)
- 5 Nakajima M, Hutchinson HG, Fujinaga M, Hayashida W, Morishita R, Zhang L, Horiuchi M, Pratt RE and Dzau VJ: The angiotensin II type 2 (AT₂) receptor antagonizes the growth effects of the AT₁ receptor: gain of function study using in vivo gene transfer. *Proc Natl Acad Sci USA* **92**, 10663–10667 (1995)
- 6 Stoll M, Steckelings UM, Paul M, Bottari SP, Metzger R and Unger T: The angiotensin AT₂-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J Clin Invest* **95**, 651–657 (1995)
- 7 Yamada T, Horiuchi M and Dzau VJ: Angiotensin II type 2 receptor mediates programmed cell death. *Proc Natl Acad Sci USA* **93**, 156–160 (1996)
- 8 Arima S, Endo Y, Yaota H, Omata K, Ogawa S, Tsunoda K, Abe M, Takeuchi K, Abe K and Ito S: Possible role of P-450 metabolite of arachidonic acid in vasodilator mechanism of angiotensin II type 2 receptor in the isolated microperfused rabbit afferent arteriole. *J Clin Invest* **100**, 2816–2823 (1997)
- 9 Munzenmaier DH and Greene AS: Opposing actions on microvascular growth and arterial blood pressure. *Hypertension* **27**, 760–765 (1996)
- 10 Liu Y-H, Yang X-P, Sharov VG, Nass O, Sabbah HN, Peterson E and Carretero OA: Effect of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. *J Clin Invest* **99**, 1926–1935 (1997)
- 11 Ernsberger PR, Giuliano R, Willette RN and Reis DJ: Role of imidazole receptors in the vasodepressor response to clonidine analogs in the rostral ventrolateral medulla. *J Pharmacol Exp Ther* **253**, 408–418 (1990)
- 12 Haxhiu MA, Dreshaj I, Schafer SG and Ernsberger P: Selective antihypertensive action of moxonidine is mediated mainly by I₁-imidazoline receptors in the rostral ventrolateral medulla. *J Cardiovasc Pharmacol* **24**, Suppl **1**, S1–S8 (1994)
- 13 Gomez RE, Ernsberger P, Feinland G and Reis DJ: Rilmenidine lowers arterial blood pressure via imidazole receptors in brainstem C1 area. *Eur J Pharmacol* **195**, 181–191 (1991)
- 14 Carpenne C, Collon P, Remaury A, Cordi A, Hudson A, Nutt D and Lafontan M: Inhibition of amine oxidase activity by derivatives that recognize imidazoline I₂ sites. *J Pharmacol Exp Ther* **272**, 681–688 (1995)
- 15 Raasch W, Muhle H and Dominiak P: Modulation of MAO activity by imidazoline and guanidine derivatives. *Ann NY Acad Sci* **881**, 313–331 (1999)
- 16 Regunathan S, Youngson C, Raasch W, Wang H and Reis DJ: Imidazoline receptors and agmatine in blood vessels: a novel system inhibiting vascular smooth muscle proliferation. *J Pharmacol Exp Ther* **276**, 1272–1282 (1996)
- 17 Regunathan S and Reis DJ: Stimulation of imidazoline receptors inhibits proliferation of human coronary artery vascular smooth muscle cells. *Hypertension* **30**, 295–300 (1997)
- 18 Ernsberger PR, Damon TH, Graff LM, Schäfer SG and Christen MO: Moxonidine, a centrally acting antihypertensive agent, is a selective ligand for I₁-imidazoline sites. *J Pharmacol Exp Ther* **264**, 172–182 (1993)
- 19 Wikberg JES, Uhlén S and Chhajlani V: Medetomidine stereoisomers delineate two closely related subtypes of imidazoline (imidazoline) I-receptors in the guinea-pig. *Eur J Pharmacol* **193**, 335–340 (1991)
- 20 Ohlstein EH, Gellai M, Brooks DP, Vickerey L, Jugus J, Sulpizio A, Ruffolo RR Jr, Weinstock J and Edwards RM: The antihypertensive effect of the angiotensin II receptor antagonist DuP753 may not be due solely to angiotensin II receptor antagonism. *J Pharmacol Exp Ther* **262**, 595–601 (1992)
- 21 Grove KL and Speth RC: Angiotensin II and non-angiotensin II displaceable binding sites for ³H-losartan in the rat liver. *Biochem Pharmacol* **46**, 1653–1660 (1993)
- 22 Chansel D, Vandermeersch S, Pham P and Ardaillou R: Characterization of ³H-losartan receptors in isolated rat glomeruli. *Eur J Pharmacol* **247**, 193–198 (1993)
- 23 Chansel D, Bizet T, Vandermeersch S, Pham P, Levy B and Ardaillou R: Differential regulation of angiotensin II and losartan binding sites in glomeruli and mesangial cells. *Am J Physiol* **266**, F384–F393 (1994)
- 24 Bonnafous JC, Tence M, Seyer R, Marie J, Aumelas A and Jard S: New probes for angiotensin II receptors. Synthesis, radioiodination and biological properties of biotinylated and haptenated angiotensin derivatives. *Biochem J* **251**, 873–880 (1988)
- 25 Jiménez E, Vinson GP and Montiel M: Angiotensin II (AII)-binding sites in nuclei from rat liver: partial characterization of the mechanism of AII accumulation in nuclei. *J Endocrinol* **143**, 449–453 (1994)
- 26 Greenwood FC, Hunter WM and Glover JS: The preparation of ¹³¹I-labeled human growth hormone of high specific radioactivity. *J Biochem* **89**, 114 (1963)
- 27 Steffen G, Dendorfer A and Dominiak P: Imidazoline binding sites on PC12 cells and bovine chromaffin cells. *Ann NY Acad Sci* **763**, 157–162 (1995)
- 28 Ivkovic B, Bakthavachalam V, Zhang W, Parini A, Diz D, Bosch S, Neumeyer JL and Lanier SM: Development of a high-affinity radioiodinated ligand for identification of imidazoline

- /guanidinium receptive sites (IGRS): intratissue distribution of IGRS in liver, forebrain and kidney. *Mol Pharmacol* **46**, 15 – 23 (1994)
- 29 Rangel N, Uhlén S and Wikberg JE: Detomidine binding to guinea pig liver imidazoline receptors (I₂-receptors) shows marked positive cooperativity. *Pharmacol Toxicol* **73**, 86 – 90 (1993)
 - 30 Lowry OH, Rosebrough NH, Farr AL and Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265 – 275 (1951)
 - 31 Speth RC and Kim KH: Discrimination of two angiotensin II receptor subtypes with a selective agonist analogue of angiotensin II, *p*-aminophenylalanine⁶ angiotensin II. *Biochem Biophys Res Commun* **169**, 997 – 1006 (1990)
 - 32 Booz GW, Conrad KM, Hess AL, Singer HA and Baker KM: Angiotensin-II-binding sites on hepatocyte nuclei. *Endocrinology* **130**, 3641 – 3649 (1992)
 - 33 Molderings GJ, Moura D, Fink K, Bönisch H and Göthert M: Binding of ³H-clonidine to I₁-imidazoline sites in bovine adrenal medullary membranes. *Naunyn Schmiedeberg Arch Pharmacol* **348**, 70 – 76 (1993)
 - 34 Molderings GJ, Kundt L and Göthert M: [³H]Idazoxan binding to bovine adrenal medullary membranes: identification and pharmacological characterization of I₂-imidazoline sites. *Naunyn Schmiedeberg Arch Pharmacol* **350**, 252 – 257 (1994)
 - 35 King PR, Gundlach AL and Louis WJ: Identification of imidazoline-receptor binding sites in cortex and medulla of the bovine adrenal gland. Colocalization with MAO-A and MAO-B. *Ann NY Acad Sci* **881**, 161 – 170 (1999)
 - 36 Brown C, Loweth AC, Smith SA and Morgan NG: Stimulation of insulin secretion by imidazoline compounds is not due to interaction with non-adrenoceptor idazoxan binding sites. *Br J Pharmacol* **108**, 312 – 317 (1993)
 - 37 Zonnenschein R, Diamant S and Atlas D: Imidazoline receptors in rat liver cells: a novel receptor or a subtype of α_2 -adrenoceptors? *Eur J Pharmacol* **190**, 203 – 215 (1990)
 - 38 Atlas D: Molecular and physiological properties of clonidine-displacing substance. *Ann NY Acad Sci* **763**, 314 – 324 (1995)
 - 39 Keranen A, Nykanen S and Taskinen J: Pharmacokinetics and side-effects of clonidine. *Eur J Clin Pharmacol* **13**, 97 – 101 (1978)
 - 40 Theodor R, Weimann HJ, Weber W and Michaelis K: Absolute bioavailability of moxonidine. *Eur J Drug Metab Pharmacokinet* **16**, 153 – 159 (1991)
 - 41 Genissel P and Bromet N: Pharmacokinetics of rilmenidine. *Am J Med* **87**, 18S – 23S (1989)
 - 42 Lo MW, Goldberg MR, McCrea JB, Lu H, Furtek CI and Björnsson TD: Pharmacokinetics of losartan, an Ang II receptor antagonist, and its active metabolite EXP3174 in humans. *Clin Pharmacol Ther* **58**, 641 – 649 (1995)
 - 43 Li Z, Bosch SM, Smith TL and Diz DI: Interactions of nonpeptide angiotensin II receptor antagonists at imidazoline/guanidinium receptor sites in rat forebrain. *J Cardiovasc Pharmacol* **28**, 425 – 431 (1996)