Key amino acids of vasopressin V1a receptor responsible for the species difference in the affinity of OPC-21268

Hitomi Shinoura[a,1], Hitoshi Take[a,1], Akira Hirasawa[a], Kazuhide Inoue[b], Yasuo Ohno[b], Keitaro Hashimoto[c], Gozoh Tsujimoto[a,*]

[a] *Department of Molecular, Cell Pharmacology, National Children's Medical Research Center, 3-35-31 Taishido, Setagaya-Ku, Tokyo 154-8509, Japan*
[b] *Division of Pharmacology, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-Ku, Tokyo 158-8501, Japan*
[c] *Department of Pharmacology, Yamanashi Medical College, Yamanashi 409-38, Japan*

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Abstract A non-peptide, vasopressin V1a receptor-selective antagonist, OPC-21268, exhibited a markedly higher affinity for the rat V1a receptor (K_i = 380 nM) than for the human V1a receptor (K_i = 140 μM). To delineate the region responsible for the high affinity binding of OPC-21268 for the rat V1a receptor, we have constructed a series of chimeric human and rat V1a receptors, and examined the chimeric and point-mutated receptors by competitive radioligand binding analysis. The results showed that the transmembrane domain (TMD) VI–VII of the vasopressin V1a receptor, in particular the amino acid residue Ala-342 in TMD VII, is the major component conferring the rat-selective binding of OPC-21268 to the V1a receptor.

Key words: OPC-21268; Vasopressin V1a receptor; Species difference; Chimeric receptor

1. Introduction

The neurohypophysal hormone, arginine vasopressin (AVP), induces diverse actions, including the inhibition of diuresis, the stimulation of hepatic glycogenolysis, the contraction of smooth muscle cells, the modulation of adrenocorticotropic hormone release from the pituitary and the aggregation of platelets [1]. AVP exerts its actions through binding to G protein-coupled specific membrane receptors linked to distinct second messengers [2–4]. AVP receptors can be divided pharmacologically into at least three subtypes: the V1a, V1b and V2 receptors. The V1a receptors are involved in vasoconstriction and hepatic glycogenolysis, while the V1b receptors mediate the stimulation of corticotropin secretion by the adenohypophysis. The V2 receptors are responsible for the antidiuretic effect of AVP [5,6]. Among the receptor subtypes, the V1a receptor is considered to play an important role in the pathogenesis of human diseases, such as hypertension and congestive heart failure. Although many vasopressin antagonists have been developed as potential therapeutic agents for these disorders, they are all peptide analogs that do not have sufficient oral bioavailability.

OPC-21268 (1-{1-[4-(3-acetylaminopropoxy)benzoyl]-4-piperidyl}-3,4-dihydro-2-(1*H*)-quinolinone) was recently developed as the first orally effective, non-peptide V1 vasopressin receptor antagonist [7,8]. Previously, using cloned rat and human V1a receptors, we reported that OPC-21268 exhibited a markedly higher affinity for the rat V1a receptor than for the human V1a receptor [9]. In the present study, in order to delineate the responsible region for the species-dependent difference in the affinity of OPC-21268 for the V1a receptor, we have constructed a series of several chimeric human and rat V1a receptors, and examined their affinities by competitive radioligand binding analysis. The results demonstrate that the transmembrane domain (TMD) VI–VII portion is critical for the observed species difference in binding of OPC-21268 for the V1a receptor.

2. Materials and methods

2.1. Materials

The following drugs were used: [Arg[8]]vasopressin (AVP) (Sigma, St. Louis, MO, USA); OPC-21268 (Otsuka Pharmaceutical, Tokushima, Japan); [phenylalanyl-3,4,5,-^{3}H(*N*)]-8-L-AVP ([^{3}H]AVP) (specific activity, 64.2 Ci/mmol; New England Nuclear, Boston, MA, USA). All other chemicals were of reagent grade.

2.2. Construction of chimeric human/rat V1a receptors and amino acid-substituted human V1a receptor mutants

The cDNA constructs encoding chimeric human and rat V1a receptors were constructed by digestion at common restriction sites within the wild-type human and rat V1a receptor cDNAs [3,9,10] and exchanging the corresponding regions of each. The restriction sites and their positions in the deduced amino acid sequence of the rat/human V1a receptors, respectively, were as follows: *Bss*HIII, at Ala-163/Ala-164 in the intracellular loop (ICL) II; *Xho*I, at Ser-213/Thr-214 in the extracellular loop (ECL) II; *Dra*III, at Cys-275/Cys-280 in ICL III; *Aor*51HI, at Ala-334/Ala-339 in ECL III; *Afl*II, at Ala-348/Ala-353 in TMD VII. Although *Bss*HIII, *Dra*III and *Afl*II sites are commonly present within the wild-type human and rat V1a receptors, the *Xho*I and *Aor*51HI sites were created by oligonucleotide-directed mutagenesis. The structures of the chimeras are shown in Fig. 1. The positions of the junctions for individual chimeric human/rat V1a receptors and their component amino acids are as follows: chimeric receptor 1 (CR1), rat 1–163/human 164–418; CR2, rat 1–214/human 213–418; CR3, rat 1–280/human 275–418; CR4, rat 1–353/human 349–418; CR5, human 1–164/rat 163–394; CR6, human 1–213/rat 215–394; CR7, human 1–275/rat 280–394; CR8, human 1–

*Corresponding author. Fax: (81)-3-3419 1252.
E-mail: gtsujimoto@nch.go.jp

[1] These authors contributed equally to this work.

Abbreviations: AVP, arginine vasopressin; TMD, transmembrane domain; ICL, intracellular loop; ECL, extracellular loop; CR, chimeric receptor

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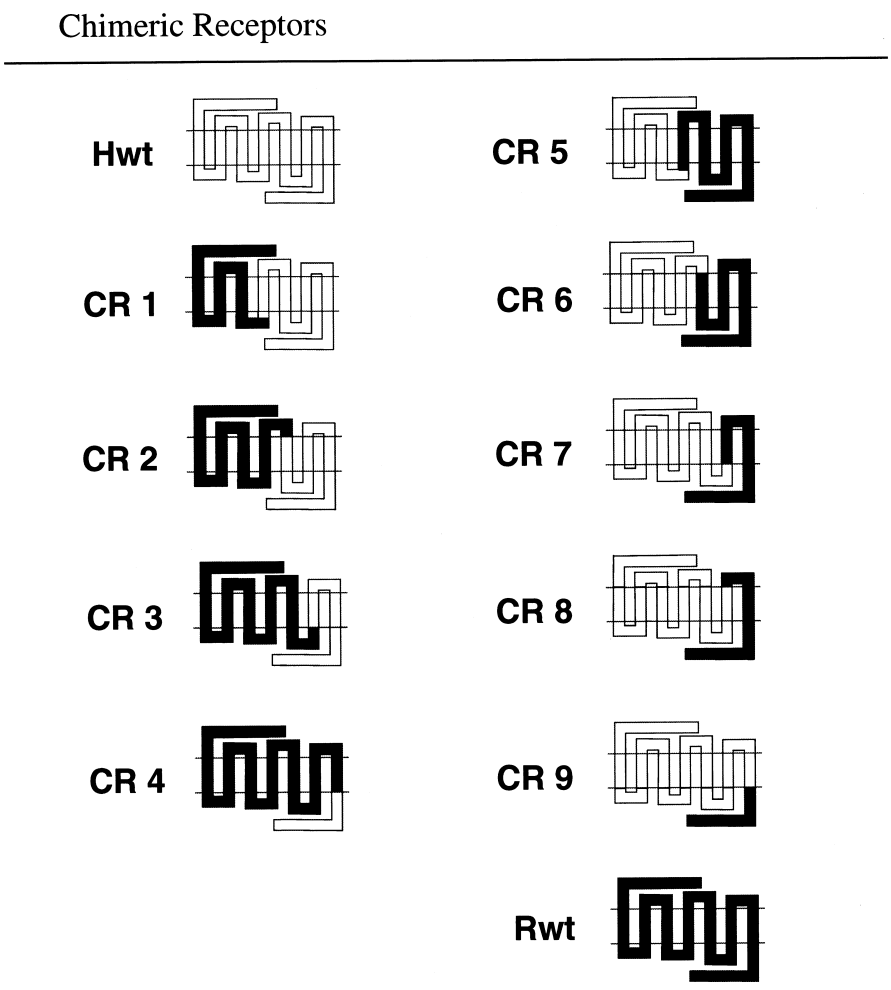

Fig. 1. Structure of chimeric human/rat V1a receptors (CRs 1–9). Filled bars, sequences of the wild-type rat V1a receptor (Rwt); open bars, sequences of the wild-type human V1a receptor (Hwt). The position of junctional amino acids in these CRs and the methods used for their constructions are described in Section 2.

317/rat 323–394; CR9, human 1–348/rat 354–394. Amino acid-substituted mutants of the human V1a receptor were constructed by PCR using site-directed mutagenesis. Four amino acids, Thr-298, Val-302, Ile-310 and Gly-337, in TMD VI–VII of the human V1a receptor differ from those of the rat V1a receptor. These four amino acids were changed individually to the corresponding amino acids of the rat V1a receptor, Ser-303, Leu-307, Val-315 and Ala-342, respectively. Each mutation was confirmed by sequencing. Finally, the wild-type human V1a receptor, the wild-type rat V1a receptor and all of the mutant receptors were inserted into *Eco*RI and *Xho*I sites of a mammalian expression vector pME 18S [11], respectively.

2.3. Cell transfection

The wild-type human V1a receptor, the wild-type rat V1a receptor and mutant receptors were transiently transfected into COS-7 cells by electroporation (240 V, 975 μF, Bio-Rad gene Pulser II electoroporator, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Cells were harvested 48–72 h after transfection.

2.4. Membrane preparations

The transfected cells were collected and homogenized by a Branson sonicator (model SONIFIER 250, Branson, Danbury, CT, USA; setting 5 for 8 s) in 2 ml of ice-cold buffer A (250 mM sucrose, 10 mM

Table 1
Binding affinities of AVP and OPC-21268 for the wild-type human, the wild-type rat and the chimeric human/rat V1a receptors

	OPC-21268 K_i (nM)	AVP K_i (nM)	OPC/AVP
Hwt	140000	1.4 ± 0.2	100000
CR1	190000 ± 25000	0.60 ± 0.01	320000
CR2	150000 ± 38000	1.0 ± 0.2	150000
CR3	29000 ± 1900	0.80 ± 0.10	36000
CR4	240 ± 11	1.7 ± 0.5	140
Rwt	380 ± 7	1.4 ± 0.6	271

Cell membranes from COS-7 cells, transfected with the pME 18S expression vector containing the wild-type human, the wild-type rat and chimeric human/rat V1a receptors (CRs 1–4), were incubated with ligand, [^{3}H]AVP, in the absence or presence of increasing concentration of AVP and OPC-21268. At least seven concentrations of each ligand were tested, and the points were chosen to be the linear portion of displacement curve. K_i values were generated using the iterative curve-fitting program LIGAND [13]. Each value represents either the mean or the mean ± S.E.M. of the data from at least 2–3 different experiments performed in duplicate.

Tris–HCl, 1 mM $MgCl_2$, pH 7.4) [12]. The mixture was then centrifuged at 1000×*g* at 4°C for 10 min to remove nuclei. The supernatant was centrifuged at 35 000×*g* for 20 min at 4°C and the pellet was resuspended in the binding buffer (100 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% (w/v) bovine serum albumin). The protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

2.5. Radioligand binding assay

Radioligand binding studies were performed as described previously [9]. Briefly, membrane aliquots (50 μg of protein) were incubated for 30 min at 30°C with [^{3}H]AVP, and either with or without a competing drug, in a final volume of 250 μl of the binding buffer. After dilution with ice-cold buffer, samples were immediately filtered through Whatman GF/C glass fiber filters with a Brandel cell harvester (Model-30, Gaithersburg, MD, USA). Each filter was collected and the radioactivity was measured. Competition experiments were carried out at about 8.2 nM [^{3}H]AVP which is approximately equal to the K_d of the ligand. At this concentration, non-specific binding, defined as that occurring in the presence of 10 μM AVP, represented less than 20% of total binding. Binding assays were performed in duplicate at all times. Data were analyzed by the iterative non-linear regression program, LIGAND [13].

TMDVI

	298	302	310
hV1a	VIV T	AYI V	CW APFFI I QMWS VWD
	303	307	315
r V1a	VIV S	AYI L	CW APFFI V QMWS VWD

TMDVII

	337
hV1a	L G SLNSCCN PWIYMFFSGH LLQ
	342
r V1a	L A SLNSCCN PWIYMFFSGH LLQ

Fig. 2. Alignment of TMD VI and VII domains of the wild-type human and rat V1a receptors. Four amino acids, Thr-298, Val-302, Ile-310 and Gly-337 in TMD VI–VII of the human V1a receptor (indicated by lines), are different from those of the rat V1a receptor. They were individually changed to the corresponding amino acids of the rat V1a receptor, Ser-303, Leu-307, Val-315 and Ala-342, respectively.

3. Results and discussion

3.1. Affinities of OPC-21268 for chimeric human/rat V1a receptors

As previously reported [9], the non-peptide V1a receptor-selective antagonist, OPC-21268, exhibited a markedly higher affinity for the wild-type rat V1a receptor (K_i = 380 nM) than for the wild-type human V1a receptor (K_i = 140 μM). To determine the domain(s) responsible for this species-dependent difference in the affinity of OPC-21268 for V1a receptors, we constructed several chimeric human/rat V1a receptors (CRs). Structures of various human/rat V1a CRs (CR1–CR9) are shown in Fig. 1. CR1–CR4 are human V1a receptors into which progressively larger regions of the rat V1a receptor, from the amino-terminal extracellular tail to TMD VII, were introduced. The affinity of OPC-21268 for the human V1a receptor was not affected by the replacement of the region spanning from the amino-terminal extracellular tail to ECL II with that of the rat V1a receptor (CR1, CR2). In addition, only a slight increase in the affinity of OPC-21268 was observed by replacing the region spanning from the amino-terminal extracellular tail to ICL III of the human V1a receptor with that of the rat V1a receptor (CR3). However, the replacement of the entire human V1a receptor, except for the putative intracellular carboxy-terminus, with the rat V1a receptor (CR4), resulted in a greatly increased affinity for OPC-21268 (a 350-fold increase in K_i compared with that for the wild-type human V1a receptor). The affinity of OPC-21268 for CR4 (240 nM) was close to that for the wild-type rat V1a receptor (380 nM) (Table 1).

Next, we examined the affinity of OPC-21268 for CR5–CR9. In these CRs, a part of the human V1a receptor, from the amino-terminal extracellular tail to TMD VII, was introduced into the rat V1a receptor (Fig. 1). The rat V1a-selective binding of OPC-21268 was relatively unaffected by the replacement of the region from the amino-terminal extracellular tail to ICL III of the rat V1a receptor with that of the human V1a receptor (CR5, CR6 and CR7). The affinity of OPC-21268 for the rat V1a receptor was slightly decreased by the replacement of the region from the amino-terminal extracellular tail to TMD VI of the rat V1a receptor with the homologous region from the human V1a receptor (CR8). However, CR9, a CR containing the putative intracellular carboxy-terminus of the rat V1a receptor with the rest of the receptor coming from the human V1a receptor, showed a greatly decreased affinity for OPC-21268 (a 316-fold decrease in K_i compared with that for the wild-type rat V1a receptor). The affinity of OPC-21268 for CR9 (120 μM) was close to that for the wild-type human V1a receptor (140 μM) (Table 2). Taken together, these results suggest that the domains responsible for the high affinity binding of OPC-21268 for the rat V1a receptor may reside in the region from TMD VI to VII (TMD VI–

Table 2
Binding affinities of AVP and OPC-21268 for the wild-type human, the wild-type rat and the chimeric human/rat V1a receptors

	OPC-21268 K_i (nM)	AVP K_i (nM)	OPC/AVP
Rwt	380 ± 7	1.4 ± 0.6	270
CR5	440 ± 9	3.1 ± 0.3	140
CR6	230 ± 45	0.75 ± 0.15	300
CR7	2 900 ± 360	2.1 ± 0.6	1 400
CR8	2 500 ± 400	0.81 ± 0.07	3 100
CR9	120 000 ± 11 000	1.7 ± 0.4	71 000
Hwt	140 000	1.4 ± 0.2	100 000

Cell membranes from COS-7 cells, transfected with the pME 18S expression vector containing the wild-type human, the wild-type rat and the chimeric human/rat V1a receptors (CRs 5–9), were incubated with ligand, [^{3}H]AVP, in the absence or presence of increasing concentration of AVP and OPC-21268. At least seven concentrations of each ligand were tested, and the points were chosen to be the linear portion of displacement curve. K_i values were generated using the iterative curve-fitting program LIGAND [13]. Each value represents either the mean or the mean ± S.E.M. of the data from at least 2–3 different experiments performed in duplicate.

Table 3
Binding affinities of AVP and OPC-21268 for the wild-type human, the wild-type rat V1a receptors and amino acid-substituted human V1a receptor mutants

	OPC-21268 K_i (nM)	AVP K_i (nM)	OPC/AVP
Hwt	140 000	1.4 ± 0.4	100 000
I310V	110 000	1.1 ± 0.2	100 000
V302L	94 000	0.26 ± 0.04	360 000
T298S	81 000	0.47 ± 0.10	170 000
G337A	3 000	0.48 ± 0.12	6 300
Rwt	380 ± 7	1.6 ± 0.3	240

Cell membranes from COS-7 cells, transfected with the pME 18S expression vector containing the wild-type human, the wild-type rat and amino acid-substituted human V1a receptors, were incubated with ligand, [^{3}H]AVP, in the absence or presence of increasing concentrations of AVP and OPC-21268. At least seven concentrations of each ligand were tested, and the points were chosen to be the linear portion of displacement curve. K_i values were generated using the iterative curve-fitting program LIGAND [13]. Each value represents either the mean or the mean ± S.E.M. of the data from at least 2–3 different experiments performed in duplicate.

VII). In all CRs and wild-type V1a receptors, AVP possessed similar binding affinities ($K_i = 0.26 \sim 1.6$ nM).

3.2. Analysis of amino acid-substituted mutant receptors

To identify the amino acid(s) responsible for the markedly higher affinity binding of OPC-21268 for the rat V1a receptor, four amino acids in TMD VI–VII of the wild-type human V1a receptor, that are different from those of the wild-type rat V1a receptor, were individually, as well as in combination, changed to corresponding amino acids of the rat V1a receptor. The positions of the mutated amino acids are indicated in Fig. 2. The K_d values of [^{3}H]AVP for the amino acid-substituted mutant V1a receptors were not significantly different from that for the wild-type human V1a receptor (data not shown). As shown in Table 3, the mutation of Ile-310 to Val (I310V), Val-302 to Leu (V302L) and Thr-298 to Ser (T298S) resulted in no significant differences in the binding affinity of OPC-21268, compared with that for the wild-type human V1a receptor. However, the change of Gly-337 to Ala (G337A) increased the affinity for OPC-21268 by 46-fold. In addition, we examined double mutants (V302L/G337A, I310V/G337A, T298S/G337A) and a triple mutant (T298S/V302L/G337A). The affinities of OPC-21268 for V302L/G337A (6300 ± 460 nM, $n = 3$) and I310V/G337A (4500 ± 120 nM, $n = 3$) mutants were not significantly different from that for the G337A mutant, while T298S/G337A (13 000 ± 1900 nM, $n = 3$) and T298S/V302L/G337A (38 000 ± 2400 nM, $n = 3$) mutants showed decreased affinities compared with that for the G337A mutant. These results suggest that the mutation of G337A is important in determining the binding affinity of OPC-21268 for the V1a receptor. In all mutants, AVP possessed a similar binding affinity (Table 3). Taken together, the results obtained from these amino acid-substituted mutant receptors indicated that Ala-342 in TMD VII of the rat V1a receptor is the major amino acid conferring high affinity binding of OPC-21268 to the vasopressin V1a receptor. This study provides valuable information on the structure–activity relationship of the vasopressin V1a receptor and its non-peptide, low-molecule ligand.

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