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European Journal of Pharmacology 331 (1997) 285–293

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Properties of a new radioiodinated antagonist for human vasopressin V_2 and V_{1a} receptors

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Received 17 March 1997; accepted 21 May 1997

Abstract

A vasopressin receptor antagonist, [1-(β-mercapto-β,β-pentamethylenepropionic acid), 2-O-ethyl-D-tyrosine, 4-valine, 9-tyrosylamide] arginine vasopressin (d$(CH_2)_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP), has been prepared. This antagonist is a potent antiantidiuretic, antivasopressor and antioxytocic peptide with pA_2 values of 7.69–7.94 and affinities of 1.12–11.0 nM. When radioiodinated at the phenyl moiety of the tyrosylamide residue at position 9, this peptide was demonstrated to bind to vasopressin V_2 and V_{1a} receptors with a dissociation constant of 0.22–0.75 nM. This ligand is a good tool for further studies on human vasopressin V_2 receptor localization and characterization, when used in combination with a selective vasopressin V_{1a} ligand. © 1997 Elsevier Science B.V.

Keywords: Vasopressin V_2 receptor, human; Vasopressin receptor antagonist, iodinated; Autoradiography

1. Introduction

The characterization of kidney vasopressin V_2 receptors responsible for the antidiuretic action of arginine vasopressin has been made possible by the availability of tritium-labelled agonists or antagonists (Bockaert et al., 1973; Guillon et al., 1982; Stassen et al., 1987; Marchingo et al., 1988; Trinder et al., 1991). These ligands have a high affinity (0.4 to 7 nM) for the rat vasopressin V_2 receptor. However, some of them have a relatively lower affinity for the human vasopressin V_2 receptors (31 and 51 nM for [^{3}H][1-deamino,D-Arg^8] AVP (Chini et al., 1995) and [^{3}H]desGly–NH_2^9–d$(CH_2)_5$[D-Ile^2,Ile^4]AVP (Ala, unpublished), respectively. For many years several groups have tried to design antagonists able to recognize the human vasopressin V_2 receptor with a high efficiency (Allison et al., 1988). These antagonists would be useful tools in studies on the physiological and pathophysiological roles of these receptors. Moreover the availability of a radioiodinated antagonist would help to decipher the structure–function relationships of these receptors. It would also facilitate the search of a putative extra-renal vasopressin V_2 receptor. Until recently however, the design of radioiodinatable ligands with high affinity and specificity for the rat and human vasopressin V_2 receptors has been unsuccessful. We now present a promising radioligand for the vasopressin V_2 receptors. To design this radioligand we had to choose among numerous published and unpublished V_2 antagonists (see Manning et al., 1995), a radioiodinatable molecule having a high affinity for the human vasopressin V_2 receptor. Only one molecule, [1-(β-mercapto-β,β-pentamethylenepropionic acid), 2-O-ethyl-D-tyrosine, 4-valine, 9-tyrosylamide] arginine vasopressin (d$(CH_2)_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP), fulfills the criteria. We now describe the synthesis, radioiodination and estimation of the activities of this radioiodinated vaso-

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PII S0014-2999(97)01021-2

pressin receptor antagonist. It has a high affinity for the rat and human vasopressin V_2 receptors. It has also a good affinity for the rat and human V_{1a} receptors.

2. Materials and methods

2.1. Synthesis of d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP

Duplicate synthesis of d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP by methods A and B were carried out. Methods A and B required the synthesis of the same common acyloctapeptide intermediate β-(benzyl)-β,β,cyclopentamethylene propionyl-O-ethyl-D-Tyr–Phe–Val–Asn–Cys(Bzl)–Pro–Arg(tosyl)–Tyr(Bzl)–NH$_2$ (1A and 1B). d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP has the structure shown in Fig. 1.

A preliminary report on this vasopressin V_2/V_{1a} radioligand has been presented elsewhere (Manning et al., 1995).

2.1.1. Method A: 8 + 1 coupling in solution

The partially protected precursor β-(benzylthio)-β,β,cyclopentamethylene propionyl-O-ethyl-D-Tyr–Phe–Val–Asn–Cys(Bzl)–Pro–Arg(tosyl)–OH (II) (synthesized by the solid phase method as previously described (Manning et al., 1987) was coupled with Tyr(Bzl)–NH$_2$ using a modification of previously described methodology (Elands et al., 1988) as follows. II (345 mg, 0.25 mmol) and *N*-hydroxybenzotriazole (42 mg, 0.275 mmol) and HCl · H–Tyr(Bzl)–NH$_2$ (153 mg, 0.5 mmol) were dissolved in warm (approx. 50°C) dimethylformamide (4 ml). Upon cooling to approx. 0°C, BOP (benzotriazol-1-yl-oxy-tris (dimethylamino) phosphonium hexafluorophosphate) (122 mg, 0.275 mmol) and DIEA (*N*,*N*-diisopropylethylamine) (0.19 mg, 1.1 mmol) were added and the resulting mixture stirred at RT overnight. Addition of 5% KHCO$_3$ (approx. 50 ml) and H$_2$O (approx. 300 ml) effected precipitation of the crude product. This was collected, dried and reprecipitated from dimethylformamide with EtOH/Et$_2$O. Upon drying in vacuo over P$_2$O$_5$, this gave the protected acyloctapeptide (1A), as a white powder (342 mg, 82%); m.p. 215–220°C. $[\alpha]_D^{24} = -21.1°$ ($c = 1$, dimethylformamide); thin-layer chromatography R_f(1-butanol/acetic acid/water (4:1:1)) = 0.85; R_f(1-butanol/acetic acid/water (4:1:5, upper phase)) = 0.88; R_f(chloroform/methanol 7:3) = 0.96; R_f(chloroform/methanol 9:1) = 0.30. An aliquot of IA (154 mg), dissolved in sodium dried liquid ammonia, was deblocked with sodium, reoxidized with potassium ferricyanide by a modified reverse procedure (Rivier et al., 1978), de-ionized and purified by a 2-step procedure on Sephadex G-15 as previously described (Manning, 1968; Manning et al., 1982, 1987; Elands et al., 1988) to give the desired product, d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP (peptide A) as a white fluffy powder: 21 mg, yield 18.5%, $[\alpha]_D^{24} = -89.5°$ ($c = 0.1$, 50% acetic acid); thin layer chromatography R_f(1-butanol:acetic acid:water (4:1:1)) = 0.39; R_f(1-butanol:acetic acid:water (2:1:1)) = 0.68; R_f(1-butanol/acetic acid/water (4:1:5, upper phase)) = 0.34; high-pressure liquid chromatography: t_R(min) = 48.61; electrospray mass spectrometry: 1242.5 $[M + H]^{1+}$.

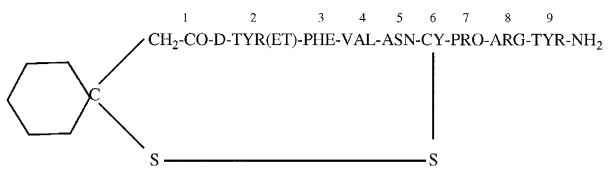

Fig. 1. Structure of the vasopressin receptor antagonist d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP.

2.1.2. Method B: Total synthesis on the resin

Boc–Tyr(Bzl)–resin (0.86 mmol) was converted to the protected acyloctapeptidyl–resin, β-(benzylthio)-β,β,cyclopentamethylenepropionyl-O-ethyl-D-Tyr–Phe–Val–Asn–Cys(Bzl)–Pro–Arg(tosyl)–Tyr(Bzl)–resin, (2.3 g; 0.86 mmol) by the manual method of solid-phase-synthesis, as previously described (Merrifield, 1964; Manning, 1968; Manning et al., 1982; Stewart and Young, 1984; Klein et al., 1995). Ammonolysis (Bodanszky and Sheehan, 1964; Manning, 1968), followed by extraction with hot dimethylformamide and precipitation with hot H$_2$O (approx. 500 ml), collection and drying over P$_2$O$_5$, gave the crude product. Reprecipitation from dimethylformamide/MeOH/Et$_2$O (approx. 15 ml/15 ml/200 ml), collection and drying in vacuo over P$_2$O$_5$ gave the purified protected acyloctapeptide (1B) as a white powder: 773 mg, yield 54.4%, m.p. 212–216°C; $[\alpha]_D^{25} = -19.6°$ ($c = 1$, dimethylformamide); thin-layer chromatography R_f(1-butanol/acetic acid/water (4:1:5, upper phase)) = 0.87; R_f(1-butanol/acetic acid/water (4:1:1)) = 0.81; R_f(BAWP:15:3:3:10) = 0.81. An aliquot of (1B) (150 mg) was deblocked, reoxidized, de-ionized and purified as described above for (1A) to give the desired product: d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP (peptide B) as a white fluffy powder 34 mg, yield 30.6%, $[\alpha]_D^{25} = -90°$ ($c = 0.1$, 50% acetic acid); thin-layer chromatography R_f(a)(1-butanol/acetic acid/water (4:1:5, upper phase)) = 0.38; R_f(b)(1-butanol/acetic acid/water (4:1:1)) = 0.44; R_f(c)(BAWP:15:3:3:10) = 0.6; high-pressure liquid chromatography: t_R(min) = 48.68; electrospray mass spectrometry: 1242.5 $[M + H]^{1+}$.

2.2. Bioassays

Peptides were assayed for antagonistic activities in the in vivo rat vasopressor assay, antidiuretic assay and in vitro rat oxytocic assay, by Schild's pA_2 method (Schild, 1947). The pA_2 is the negative logarithm of the molar

concentration of the antagonist that reduces the response to 2 × units of the agonist to equal the response to 1 × unit of the agonist in absence of antagonist. In practice, this effective concentration is estimated by finding concentrations above and below the effective concentration and interpolating on a logarithmic scale. In the rat in vivo assays, the effective dose is divided by an arbitrarily assumed volume of distribution of 67 ml/kg to yield approximately the molar concentration of the effective dose at the receptor. Synthetic arginine vasopressin and oxytocin which had been standardized in vasopressor and oxytocic units against the USP Posterior Pituitary Reference Standard were used as working standards in all bioassays. Vasopressor assays were performed on urethane-anesthetized and phenoxybenzamine-treated rats as described by Dekanski (1952). Antidiuretic assays were on water-loaded rats under ethanol anesthesia as described by Sawyer (1958). Oxytocic assays were performed on isolated uteri from diethylstilbestrol-primed rats in a Mg^{2+}-free van Dyke-Hasting's solution (Munsick, 1960). Each mean presented in Table 1 reflects results from at least four independent assay groups.

2.3. $Na^{125}I$ iodination

The peptides A and B were radioiodinated at position 9 on the 9-tyrosinamide by means of the oxidant 1,3,4,6-tetrachloro-3α, 6α-diphenyl–glycouryl (iodo-gen, Pierce) in the presence of 1 mCi $Na^{125}I$ (IMS30, Amersham) as described by Elands et al. (1988). Immediate separation of the reaction products was obtained by injection on a high pressure liquid chromatography column (Waters C_{18} μBondapak). The reaction products were separated with a linear gradient of 0–60% solvent B (trifluoroacetic acid 0.1% in acetonitrile) in solvent A (trifluoroacetic acid 0.1%). The specific radioactivity of the monoiodinated antagonist (purified by 2 subsequent high-pressure liquid chromatography runs) was 2200 Ci/mmol.

2.4. Membrane preparation

Human vasopressin V_{1a} and V_{1b} receptor cDNAs, and human oxytocin receptor cDNA were kindly provided by Drs. M. Thibonnier, H. Kawashima and T. Kimura, respectively. Human vasopressin V_2 receptor was amplified from human genomic DNA as previously described (Chini et al., 1995). Rat vasopressin V_{1a} and V_2 receptor cDNAs were kindly provided by Drs. A. Morel and J.M. Elalouf, respectively. These receptors (Kimura et al., 1992; Morel et al., 1992; Birnbaumer et al., 1992; Lolait et al., 1992; Thibonnier et al., 1994; Sugimoto et al., 1994) were expressed into COS 7-, CHO- or L-cells and the cells were harvested, washed two times in phosphate-buffered saline without Ca^{2+} and Mg^{2+}, polytron-homogenized in lysis buffer (15 mM Tris–HCl pH 7.4, 2 mM $MgCl_2$, 0.3 mM EDTA) and centrifuged at 100 × g for 5 min at 4°C as described (Chini et al., 1995). Supernatants were recovered and centrifuged at 44 000 × g for 20 min. Pellets were washed in a buffer A (50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$) and centrifuged at 44 000 × g for 20 min. Membranes were resuspended in a small volume of buffer A and protein contents were determined. Aliquots of membranes were used immediately or stored in liquid nitrogen.

2.5. Binding assays

Binding assays were performed at 30°C using [^{125}I]HO-phenylacetyl-O-methyl-D-tyrosine–Phe–Gln–

Table 1
Pharmacological properties of radioiodinatable vasopressin V_2 and V_{1a} antagonist d(CH_2)$_5$[O-ethyl-D-Tyr(Et)^2,Val^4,Tyr–NH_2^9]AVP

No.	Peptide	Antiantidiuretic (Anti-V_2) effective dose		Antivasopressor (Anti-V_{1a}) effective dose		Antioxytocic (in vitro)
		(ED) [a] (nmol/kg)	pA_2 [b,c]	(ED) [a] (nmol/kg)	pA_2 [b,c]	pA_2 (no Mg^{2+})
I	d(CH_2)$_5$[O-ethyl-D-Tyr^2,Val^4]AVP [d]	1.10 ± 0.18	7.81 ± 0.07	0.45 ± 0.11	8.22 ± 0.12	8.34 ± 0.10
A	Tyr–NH_2^9 analog of I [e,f]	0.95 ± 0.12	7.86 ± 0.06	0.79 ± 0.08	7.94 ± 0.04	ND
B	Tyr–NH_2^9 analog of I [e,g]	1.31 ± 0.14	7.71 ± 0.05	0.89 ± 0.07	7.88 ± 0.04	7.69 ± 0.06
		Inhibition constants (K_i in nM)				
		hV_2-COS 7	hV_{1a}-COS 7	hV_{1b}-COS 7	hOT-L	
A	Tyr–NH_2^9 analog of I [e,f]	1.12 ± 0.10	0.33 ± 0.06	> 1000	11.0 ± 0.4	

Membranes from cells expressing the human vasopressin and oxytocin receptors were incubated with 2 nM [^{3}H]vasopressin, 50 pM [^{125}I]HO-LVA, 2 nM [^{3}H]vasopressin and 100 pM [^{125}I]OTA, respectively, and varying concentrations of peptide IA. Values are expressed in nM ± S.D. and are the mean of three independent determinations in triplicate.

[a] The effective dose is defined as the dose (in nmol/kg) that reduced the response seen with 2 × units of agonist to equal the response seen with x units of agonist administered in the absence of the antagonist.

[b] Estimated in vivo pA_2 values represent the negative logarithms of the 'effective dose' divided by the estimated volume of distribution (67 ml/kg).

[c] Means + SE.

[d] Data from Manning et al. (1982).

[e] This publication.

[f] 8 + 1 synthesis (Method A).

[g] Total synthesis on resin (Method B).

Asn–Arg–Pro–Arg–NH_2 ([^{125}I]HO-LVA) (Barberis et al., 1995) (50 pM, hV_{1a}), [^{125}I]d(CH_2)$_5$[Tyr(Me)^2,Thr^4,Tyr–NH_2^9]OVT ([^{125}I]OTA) (Elands et al., 1988) (100 pM, hOT) or [^{3}H]vasopressin (Chini et al., 1995) (2 nM, hV_{1b}, hV_2) as the radioligands and 1–10 μg of membrane proteins. Membranes were incubated in 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin and varying concentrations of peptide A in a total volume of 200 μl. Incubation lasted 60 min for [^{125}I]HO-LVA and [^{125}I]OTA or 30 min for [^{3}H]vasopressin. Non-specific binding was determined in the presence of a 250-fold excess of unlabelled peptides. Bound and free radioactivity were separated by filtration over Whatman GF/C filters, pre-soaked (for at least 2 h) either in 10 mg/ml bovine serum albumin ([^{3}H]vasopressin and [^{125}I]OTA) or 0.5% polyethyleneimine ([^{125}I]HO-LVA).

The dose-dependent specific [^{125}I]d(CH_2)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP binding at equilibrium to human vasopressin V_2 and V_{1a} receptor was performed with 2 μg protein/assay, using concentrations of iodinated ligand from 50 to 900 pM. Bound and free radioactivity were separated by filtration over Whatman GF/C filters, pre-soaked in 10 mg/ml bovine serum albumin. The ligand binding data were analyzed with non-linear model fitting programs (Kinetic, Ebda, Ligand, Lowry-Release 2; Biosoft).

2.6. Inositol phosphates assays

Measurements of accumulation of inositol phosphates were performed as described (Berridge et al., 1982). Briefly, the CHO cells expressing human vasopressin V_{1a} receptor were plated in supplemented Dulbecco's modified Eagle medium using 6-well plates, and myo-[2-^{3}H]inositol (Dupont NEN) was added to the medium for the last day of culture (final concentration: 2 μCi/ml). Before the experiment, the medium was replaced by medium free of inositol and fetal calf serum for 1 h. Cells were washed twice in phosphate-buffered saline. Then, the antagonist (10^{-10} to 10^{-6} M) and LiCl (10 mM) were added 15 min before adding 10^{-9} M vasopressin. The cells were incubated for another 15 min and the reaction was terminated by adding perchloric acid. After stopping the reactions, inositol phosphates were extracted, recovered on strong anion-exchange chromatography columns, and counted. All assays were performed in triplicate on at least three separate batches of cells.

2.7. cAMP assays

The CHO cells expressing human vasopressin V_2 receptor were plated in supplemented Dulbecco's modified Eagle medium using 6-well plates and [^{3}H]adenine (Isotopchim) was added to the medium (3 μCi/ml) for the last day of culture. Cells were washed twice in phosphate-buffered saline and then phosphate-buffered saline supplemented with glucose 5.5 mM, isobutyl–methylxanthine 1 mM, with or without the peptide A (at concentrations ranging from 10^{-10} to 10^{-6} M), was added 15 min before adding 10^{-9} M vasopressin. After a further 10 min incubation period, the reaction was terminated by replacing the incubation medium with 1 ml of 5% trichloroacetic acid. ATP and cAMP were added to the acid extracts at the concentration of 0.5 mM.

Relative intracellular cAMP levels were determined by measuring the formation of [^{3}H] cAMP from the prelabelled adenine nucleotide pool. Labelled cAMP was separated by sequential chromatography on Dowex and alumina columns as previously described (Salomon et al., 1974). Radioactivity present in the cAMP fractions was expressed as percent of the sum of radioactivity recovered in the cAMP fraction and radioactivity which was not retained by the Dowex column. The latter mainly corresponds to labelled ATP.

2.8. Autoradiography

Localization of [^{125}I]d(CH_2)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP binding sites in the rat kidney was performed according to previously published protocols (Barberis et al., 1995). Briefly, 14-μm-thick unfixed kidney sections from Sprague–Dawley adult male rats were collected on slides and incubated with 50 pM of [^{125}I]d(CH_2)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP either alone or in the presence of a competing peptide. Adjacent sections were incubated with the same amount of radioiodinated ligand in the presence of 20 or 100 nM of either [1-deamino,8-D-Arg]vasopressin or [2-Phe,8-Orn]vasotocin. Non-specific binding was determined by incubating adjacent sections with the same amount of [^{125}I]d(CH_2)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP in the presence of 2 μM non-radioactive vasopressin. After one hour incubation at room temperature sections were rinsed, dried and placed in an X-ray cassette in contact with βmax Hyperfilm (Amersham) for 3–5 days. Autoradiograms were obtained by developing films with Kodak D 19 during 5 min.

3. Results

3.1. Biological activities and affinities of d(CH_2)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP for human vasopressin and oxytocin receptors

The pharmacological properties of both preparations A and B of d(CH_2)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP and those of the parent peptide d(CH_2)$_5$[O-ethyl-D-Tyr^2,Val^4]AVP (Manning et al., 1982) are given in Table 1. The products from methods A and B were shown to be chemically, physically and pharmacologically indistinguishable. However, for efficiency and ease of synthesis, method B, i.e., total synthesis on the resin, is the preferred

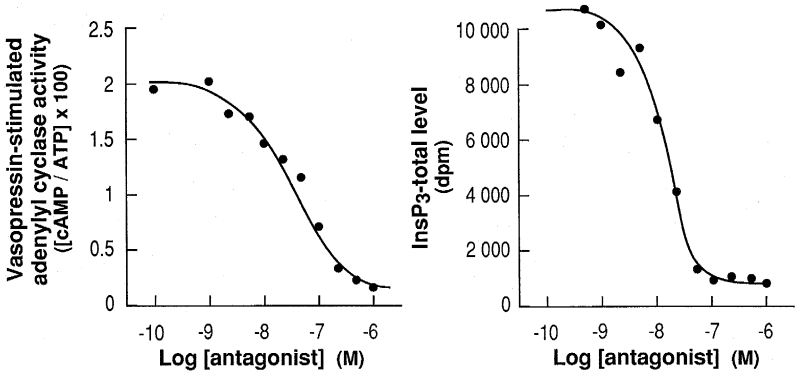

Fig. 2. Effects of $d(CH_2)_5$[o-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP on the cAMP accumulation induced by vasopressin in CHO cells expressing human vasopressin V_2 receptors (left) and on the inositol phosphate accumulation induced by vasopressin CHO cells expressing human vasopressin V_{1a} receptors (right). Human vasopressin V_2 and V_{1a} receptors were stimulated with 10^{-9} M vasopressin. The values in the graphs are from one experiment. They are representative of three independent determinations in triplicate.

method for the synthesis of the radioiodinatable precursor $d(CH_2)_5$[o-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP.

The parent peptide of A, $d(CH_2)_5$[o-ethyl-D-Tyr^2,Val^4]AVP is a potent antiantidiuretic, antivasopressor and antioxytocic peptide with pA_2 values of 7.81–8.32. Its derivative $d(CH_2)_5$[o-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP is also a potent antagonist with nearly identical pA_2 values (7.69–7.94) for the rat vasopressin V_{1a}, V_2 and oxytocin receptors.

The affinities of peptide A for human vasopressin and oxytocin receptors expressed in different cell lines were determined using [^{125}I]HO-LVA (V_{1a}), [^{3}H]vasopressin (V_{1b}, V_2) and [^{125}I] OTA (OT) as ligands. Peptide A exhibited a high affinity for both human vasopressin V_{1a} and V_2 receptors. The affinity for human oxytocin receptors was more than 10 times lower and that for human V_{1b} was much lower (Table 1).

3.2. Effects of $d(CH_2)_5$[o-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP (peptide A) on the cAMP accumulation induced by vasopressin-stimulated human vasopressin V_2 receptors

To determine the antagonistic properties of peptide A, we examined the activity of this compound on the vasopressin-induced adenylyl cyclase activity in CHO cells expressing the human vasopressin V_2 receptor. In these cells, vasopressin significantly increased the production of cAMP in a concentration-dependent manner. The maximal production obtained in the presence of 1 nM vasopressin corresponds to a 14-fold increase over basal concentration. The half maximal response (EC_{50}) to vasopressin was reached at a concentration of 0.1 nM. Peptide A significantly inhibited the cAMP accumulation induced by vasopressin (1 nM) in a concentration-dependant manner (Fig. 2, left-hand panel). The K_i value was 1.90 nM. Moreover the unlabelled iodinated peptide A was also able to antagonize the cAMP accumulation induced by vasopressin with a K_i value of 2.7 nM.

3.3. Effects of $d(CH_2)_5$[o-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP (peptide A) on the inositol phosphate accumulation induced by vasopressin-stimulated human V_{1a} receptors

Peptide A is also able to inhibit inositol phosphate accumulation induced by vasopressin acting on V_{1a} receptors. This is seen in the right-had panel of Fig. 2 where the vasopressin-induced accumulation of inositol phosphate in

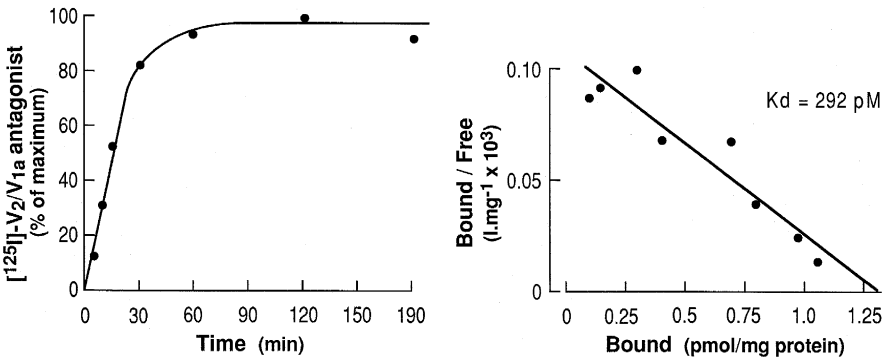

Fig. 3. Time-course of the association (left) and concentration-dependence (right) of [^{125}I]$d(CH_2)_5$[o-ethyl-D-Tyr^2,Val^4,Tyr–NH_2^9]AVP binding to human vasopressin V_2 receptors expressed in CHO cells. The values represented in the graphs are from one experiment. They are representative of 4 independent experiments in triplicate.

COS cells expressing the human V_{1a} receptor is inhibited by the vasopressin receptor antagonist. The maximal production obtained in the presence of 1 nM vasopressin corresponds to a 11.4-fold increase over basal concentration. The half maximal response (EC_{50}) to vasopressin was reached at a concentration of 0.34 nM. Peptide A significantly inhibited the inositol phosphates accumulation induced by vasopressin (1 nM) in a concentration-dependent manner. The K_i value was 15 nM.

3.4. Affinities of [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP for human and rat V_2 and V_{1a} receptors

Specific [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP binding to human vasopressin V_2 receptors expressed in CHO cells was a fairly slow process. At 30°C and for 180 pM ligand, a value close to the equilibrium dissociation constant, a maximal value was reached after 1 h and was stable thereafter up to 3 h (Fig. 3, left-hand panel). The estimated half-time was about 17 min. All further experiments were performed at 30°C and the duration of membrane incubation in the presence of [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP was 1 h.

Specific [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP binding was saturable. The results shown in Fig. 3B indicate that dose-dependent binding was observable in a concentration range from 50 to 900 pM. At 250 pM, a concentration close to the observed dissociation constant, the non-specific binding component of [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP binding represented 50.3% of total binding. Analysis of the data with 'Ligand' indicated a dissociation constant of 260 ± 35 pM. When the human vasopressin V_2 receptors were expressed in COS 7 cells their affinity for the [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP was a little lower (Table 2). [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP also binds to rat V_2 and human and rat V_{1a} receptors with a high affinity (220 to 370 pM, Table 2). Radioiodinated peptides A and B have the same affinities for vasopressin V_{1a} and V_2 receptors and were pharmacologically indistinguishable.

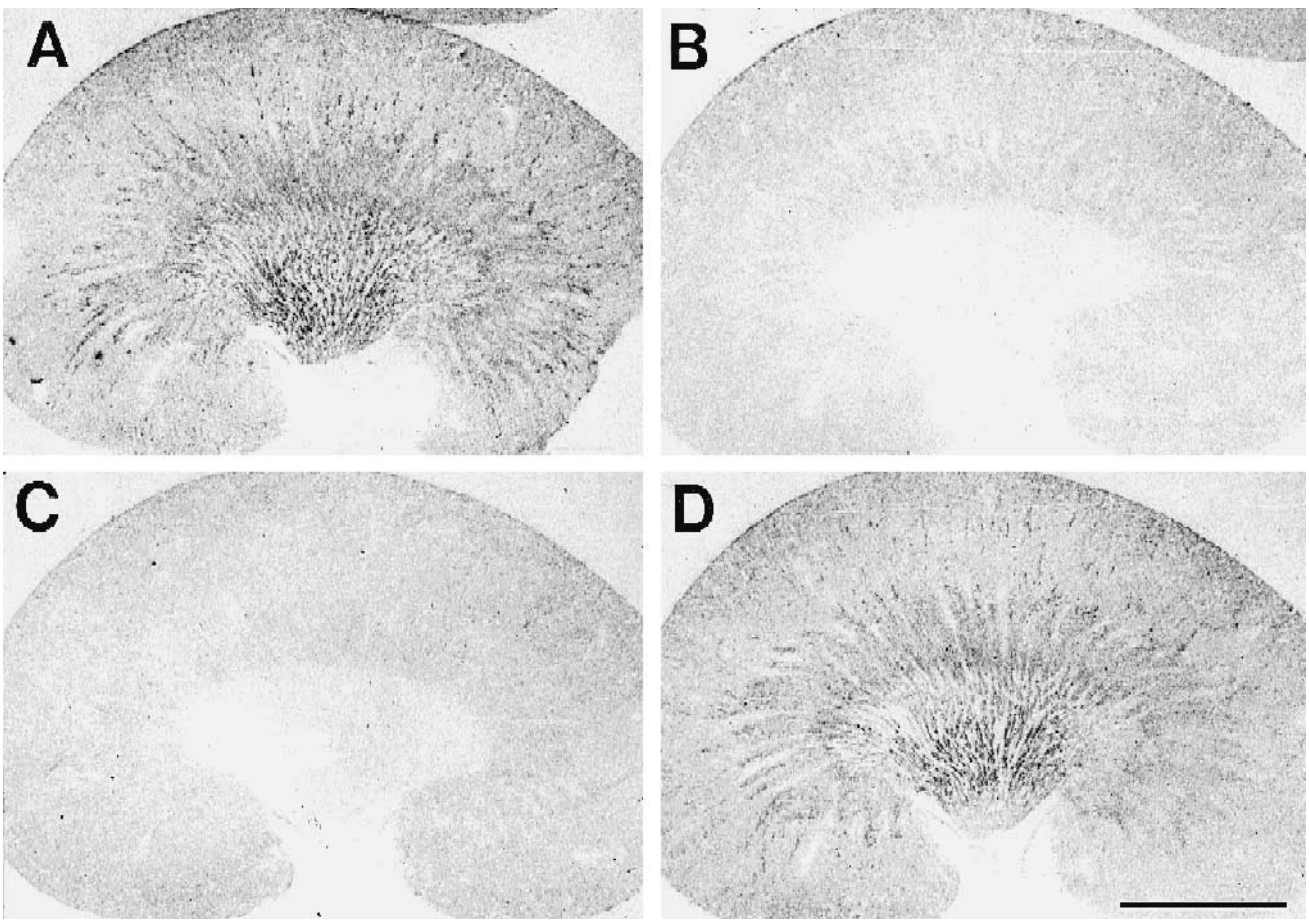

Fig. 4. Labelling of vasopressin receptors in rat kidney. Autoradiograms (A) to (D) were obtained from four adjacent sections incubated with [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP (50 pM) either alone (A) or in the presence of: an excess of nonradioactive vasopressin (2 μM) (B), a vasopressin V_2 agonist, [1-deamino, 8-D-Arg] vasopressin (100 nM) (C), or a vasopressin V_{1a} agonist, [2-Phe, 8-Orn]vasotocin (100 nM) (D). Calibration bar: 500 μm.

Table 2
Affinity constants of [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP for human and rat vasopressin V_2 and V_{1a} receptors expressed in stable or transient cell lines

Receptors	Cell line	K_d (pM)
hV_2	CHO	263 ± 35 (3)
hV_2	COS 7	750 ± 80 (3)
rV_2	COS 7	373 ± 21 (3)
hV_{1a}	COS 7	223 ± 66 (3)
rV_{1a}	COS 7	359 ± 170 (4)

3.5. Renal localization of [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP binding sites

Fig. 4 shows autoradiograms obtained from rat kidney sections incubated with [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP in the presence or absence of competing non-radioactive peptide. A dense labelling was observable in the medullopapillary portion of the kidney, showing a tubular pattern (Fig. 4A). A specific binding (although less intense) was also detected in the cortical portion of the kidney, which very likely corresponds to [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP binding to cortical collecting ducts. Binding in the inner medulla was more easily displaced by non-radioactive vasopressin than in the outer medulla and cortex (Fig. 4B). [1-deamino, 8-D-Arg]vasopressin, a vasopressin V_2 receptor agonist with a high affinity for human vasopressin V_2 receptor, was also able to completely displace the labelling when used at a concentration of 100 nM (Fig. 4C). On the contrary the vasopressin V_{1a} receptor agonist, [2-Phe,8-Orn]vasotocin, only slightly displaced the labeling present on the outer part of the medulla (Fig. 4D).

4. Discussion

The design of radioiodinatable ligands with high affinity and specificity for the vasopressin V_2 receptors has been elusive for a long time. Several antagonists have been developed that are selective for the vasopressin V_2 receptors in the rat (see Manning and Sawyer, 1993). However these antagonists do not recognize the human vasopressin V_2 receptors with a high affinity. Thus one of the more selective ligands for the rat vasopressin V_2 receptors (d(CH$_2$)$_5$[D-Ile^2,Ile^4] AVP, Manning et al., 1984) has a low affinity for the human vasopressin V_2 receptors (Manning et al., 1995). Here we have developed a radioiodinatable ligand (Fig. 1) which has a high affinity for the human vasopressin V_2 receptor. It has also a high affinity for the human V_{1a} receptor. Two methods have been used to synthesize this compound. Method A which necessitates more chemical manipulations than method B, was first used to prepare a series of different compounds with modifications at the C-terminal position (unpublished). Peptide A, a member of this series, was found to be a valuable compound to study human vasopressin V_2 receptors. Therefore method B, easier and more efficient, was developed to produce peptide B with a high yield (30.6% versus 18.5% for method A).

Potent antagonists of vasopressin V_2 and V_{1a} receptors have been developed in the past that bind specifically and with high affinity to vasopressin V_{1a} receptors of vascular smooth muscle cells and liver and to renal vasopressin V_2 receptors. The analog desGly-d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4]AVP (SK&F 101926) was reported to be a potent antagonist of vasopressin-stimulated adenylyl cyclase in rat, dog, squirrel monkey and human kidney, with a K_i value of 3.6 nM in human (Allison et al., 1988). Peptide A, which is the Tyr–NH$_2^9$ analog of SK&F 101926 is also a potent inhibitor of vasopressin-stimulated adenylyl cyclase in CHO cells expressing human vasopressin V_2 receptors, with a K_i value of 2.7 nM. The addition of a tyrosine residue at the carboxy end of the molecule does not change the potency of the vasopressin V_2 receptor antagonist, but it allows the iodination of the molecule.

Peptide A (d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP) binds to the human vasopressin V_2 receptor with an affinity of 1.1 nM. This high affinity is also found for the parent peptide of A (d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4]AVP) which has a glycine in position 9, and binds to human vasopressin V_2 receptors with an affinity of 0.9 nM (unpublished). Therefore the addition of the residue tyrosine in position 9 of the molecule results in a radioiodinatable ligand having enhanced affinity for the human vasopressin V_2 receptors.

Iodination of some antagonists on the tyrosyl residue in position 2 has been shown to preserve a fairly high affinity for binding to vasopressin V_2 or V_{1a} receptors (Moore et al., 1984, Kelly et al., 1989). However, for antagonists of the antidiuretic response iodination resulted in either an increase or a decrease in affinity for rat kidney membranes (Jard et al., 1987). Radioiodination of antagonists on the tyrosyl amide residue replacing the glycyl amide residue in position 9 have already resulted in good ligands to study vasopressin and oxytocin receptors. This was the case of the d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Tyr–NH$_2^9$]OVT and Phaa-D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Arg–Tyr–NH$_2$, two radioiodinated ligands with high specificity and selectivity for the oxytocin and vasopressin V_{1a} receptors, respectively (Elands et al., 1988, Schmidt et al., 1991). Our ligand radioiodinated on the tyrosyl residue in position 9 has a high affinity for both vasopressin V_{1a} and V_2 receptors. However, when the human vasopressin V_2 receptor was expressed in CHO cells its affinity for the ligand was found to be higher than when expressed in COS 7 cells. The reason for this small difference is not known. It could well be due to differences in posttransductional modifications in the two different cell lines. The role of glycosylation in expression and properties of human vasopressin V_2 receptor has been recently evaluated (Innamorati et al.,

1996). It was found that the unglycosylated receptor was able to bind AVP with the same affinity as the glycosylated receptor. It is still possible that the complexity of the sugar moiety linked to asparagine is different in the two cell lines and affects the affinity for an antagonist.

The [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP is a useful radioligand to localize vasopressin V_2 receptors. Localization of binding sites of the ligand was investigated in the adult rat kidney by autoradiography. These experiments demonstrate that [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP is a suitable probe for investigating the distribution of V_2 receptors in complex tissue such as the kidney. Using the ligand at a low concentration and the selective vasopressin V_{1a} receptor agonist, [2-Phe,8-Orn]vasotocin or the selective vasopressin V_2 receptor agonist [1-deamino, 8-D-Arg]vasopressin, we could see a specific and intense labeling in the medullopapillary and cortical portions of the kidney, comparable to the labeling obtained with tritiated vasopressin (Tribollet et al., 1988). The labelling of the vasopressin V_2 receptor was completely displaced by [1-deamino, 8-D-Arg]vasopressin. No specific labeling which could be due to oxytocin receptors present in the cortex can be seen, confirming the low affinity of the ligand for the oxytocin receptor. The non-specific binding seen in the presence of a high concentration of AVP was higher in the cortex and outer medulla than in the inner medulla. This is most probably due to a difference in the structure of the tissue itself.

The new vasopressin V_2 receptor radioligand, [^{125}I]d(CH$_2$)$_5$[O-ethyl-D-Tyr^2,Val^4,Tyr–NH$_2^9$]AVP also clearly recognizes the vasopressin V_{1a} receptors. However, its high affinity for human vasopressin V_2 receptors and its high specific radioactivity make this ligand a good tool for further studies on human vasopressin V_2 receptor localization and characterization. It can be used to search for putative extrarenal vasopressin V_2 receptors in human, provided that the V_{1a} receptors are masked with a specific V_{1a} ligand such as the peptide antagonist HO-phenylacetyl-O-methyl-D-tyrosine–Phe–Gln–Asn–Arg–Pro–Arg–NH$_2$ (Barberis et al., 1995) or the non-peptide antagonist SR 49059 (Serradeil-Le Gal et al., 1993). Furthermore it provides useful clues to the design of high affinity highly specific radioiodinated ligands for the human vasopressin V_2 receptors.

Acknowledgements

This research was supported in part by grants from the Institut National de la Santé et de la Recherche Médicale, by grants DK-01940 (WHS) and GM-25280 (MM) and by grant 3100-028624.94 from the Swiss National Science Foundation. We thank Dr. M. Thibonnier for the human vasopressin V_{1a} receptor cDNA, Dr. T. Kimura for the human oxytocin receptor cDNA, Dr. H. Kawashima for the human vasopressin V_{1b} receptor cDNA, Dr. A. Morel for the rat vasopressin V_{1a} receptor cDNA and Dr. J.M. Elalouf for the rat vasopressin V_2 receptor cDNA. We also thank Mireille Passama for help in the illustrations. A.S.K. is a visiting investigator from the University of Gdańsk (Gdańsk, Poland).

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