Volume 229, number 2, 251–255 FEB 05651 March 1988

^{125}I-d(CH$_2$)$_5$[Tyr(Me)^2,Tyr(NH$_2$)^9]AVP: iodination and binding characteristics of a vasopressin receptor ligand

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Received 18 January 1988

A radioiodinated vasopressin antagonist, d(CH$_2$)$_5$[Tyr(Me)^2,Tyr(NH$_2$)^9]AVP has been prepared. Iodination was carried out at the phenyl moiety of the tyrosylamide residue at position 9, followed by HPLC purification. Non-radiolabelled monoiodinated antagonist was used as a reference for identification. ^{125}I-d(CH$_2$)$_5$[Tyr(Me)^2,Tyr(NH$_2$)^9]AVP binding appeared to take place with a dissociation constant of 0.28 ± 0.09 nM (K_d ± SD) to V_1 vasopressin receptors on rat liver membranes.

Vasopressin; Vasopressin receptor; Iodinated vasopressin antagonist; (Liver, Kidney, Uterus)

1. INTRODUCTION

Receptors for the vasopressins, arginine (AVP) and lysine (LVP) vasopressin have been studied extensively in liver, blood vessels and kidney. They have been well characterized using [^{3}H]AVP and/or [^{3}H]LVP (review [1]). Many studies on vasopressin receptors in tissues where they are less abundant have been hampered by the low specific radioactivity of tritiated vasopressins. Radioiodinated vasopressin can be easily prepared but appears unsuitable for receptor studies because of a marked loss in affinity due to iodination. We and others [2,3] have shown that iodination of several vasopressin analogues, especially analogues with antagonistic properties affect only slightly their affinity for vasopressin receptors. Based on these observations we report here, for the first time, the synthesis and radioiodination of a vasopressin antagonist ([1-(β-mercapto-β,β-cyclopentamethylene propionic acid), 2-(*O*-methyl)-tyrosine,9-tyrosylamide]arginine vasopressin: d(CH$_2$)$_5$[Tyr-(Me)^2, Tyr(NH$_2$)^9]AVP), with high affinity for vasopressin receptors and high specific radioactivity.

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2. MATERIALS AND METHODS

2.1. *Synthesis*

The protected precursor required for the synthesis of the AVP antagonist was β-(benzylthio)-β,β-cyclopentamethylene propionyl-Tyr(Me)-Phe-Gln-Asn-Cys(Bzl)Pro-Arg(Tos)-Tyr(Bzl)-NH$_2$ (I). It was synthesized by coupling Tyr(Bzl)-NH$_2$ to the previously reported partially protected acyl heptapeptide precursor of desGly(NH$_2$)^9-d(CH$_2$)$_5$[Tyr(Me)^2]AVP [4], as previously described for the related d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4, Tyr(NH$_2$)^9]OVT protected precursor [5]. The crude protected precursor I (455 mg) was purified by precipitation from dimethylformamide/methanol/ether, wt 275 mg (42.3% yield), m.p. 186–191°C (decomp.), $[\alpha]D^{20} = -32.7°$ (C_1, DMF). Thin layer chromatography in three different systems showed that this material was pure: R_f(A) = 0.67; R_f(B) = 0.92; R_f(C) = 0.60 [A = butanol/acetic acid/water, 4:1:5 (upper phase); B = butanol/acetic acid/pyridine/water, 15:3:3:10); C = chloroform/methanol, 7:3]. An aliquot of I was reduced with Na/li-

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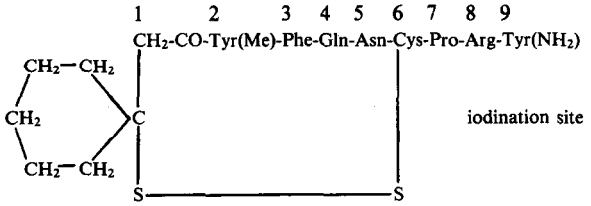
00145793/88/$3.50

quid NH_3, reoxidized with $K_4[Fe(CN)_6]$, de-ionized, purified on Sephadex G-15 and lyophilized, as described for the parent Gly-NH_2 analogue, $d(CH_2)_5[Tyr(Me)^2]AVP$ [6] to give the desired product $d(CH_2)_5[Tyr(Me)^2,Tyr(NH_2)^9]AVP$ (AVP antagonist) as a fluffy white powder, 26.4 mg (22% yield), $[\alpha]D^{23}$ = −60.8° ($C_{0.2}$, 1 N NaOH), R_f(A) = 0.30; R_f(B) = 0.57; R_f(D) = 0.35 [D = butanol/acetic acid/water, 4:1:1]. The structure of the peptide is the following:



2.2. *Chloramine-T iodination*

AVP antagonist was iodinated on the 9-tyrosylamide by means of the oxidant Chloramine-T (Merck, FRG). AVP antagonist (2 nmol for ^{125}I-Na-iodination or 10 nmol for NaI-iodination, in 10 μl methanol) was incubated in 30 μl of 1 M KH_2PO_4 buffer (pH 7.5) with 10 μg Chloramine-T and 1 mCi ^{125}I-Na (IMS-300, Amersham, Bucks, England) or 375 nm NaI (a quantity corresponding to 5 mCi ^{125}I-Na). Buffer pH was 7.5 to prevent oxidation of the disulphur bridge by Chloramine-T. After 30 s the reaction was stopped by immediate injection onto a Waters C_{18}-μBondapak reversed-phase column. HPLC was performed with a 35 min linear gradient of 40–75% trifluoroacetic acid (TFA) 0.067% in acetonitrile 60% (solvent B), in TFA 0.067% (solvent A). Flow rate was 1 ml/min. The ^{125}I-labelled peptide was further purified on a second Waters C_{18}-μBondapak reversed-phase column. The monoiodinated antagonist (I-AVP antagonist) was identified by the bathochrom shift of the UV-spectrum due to the monoiodination (λ_{max} = 304 nm).

2.3. *Bioassays*

Antagonistic activities were determined from bioassays on female Sherman rats weighing 185–225 g as described in [5].

2.4. *Peptides*

[^{3}H]AVP was obtained from New England Nuclear (Boston, MA). Specific radioactivity was 67.7–70.0 Ci/mmol. [^{3}H]-[4-threonine,7-glycine]oxytocin ([^{3}H])-[Thr^4,Gly^7]OT) was prepared as described in [7]. Specific radioactivity was 23.3 Ci/mmol. The labelled peptides were purified by HPLC and affinity-chromatography using a neurophysin-Sepharose column [8]. AVP was purchased from Bachem (Bubendorf, Switzerland).

2.5. *Membrane preparation*

Female Wistar rats (200 g body wt) were purchased from Iffa Credo (Lyon, France). Rat liver membranes were obtained according to the method described by Neville [9] up to step 11 and stored in liquid nitrogen until use. Rat kidney membranes were prepared as described by Butlen et al. [10]. Estrogenized rat uterus membranes were prepared as described by Elands et al. [7].

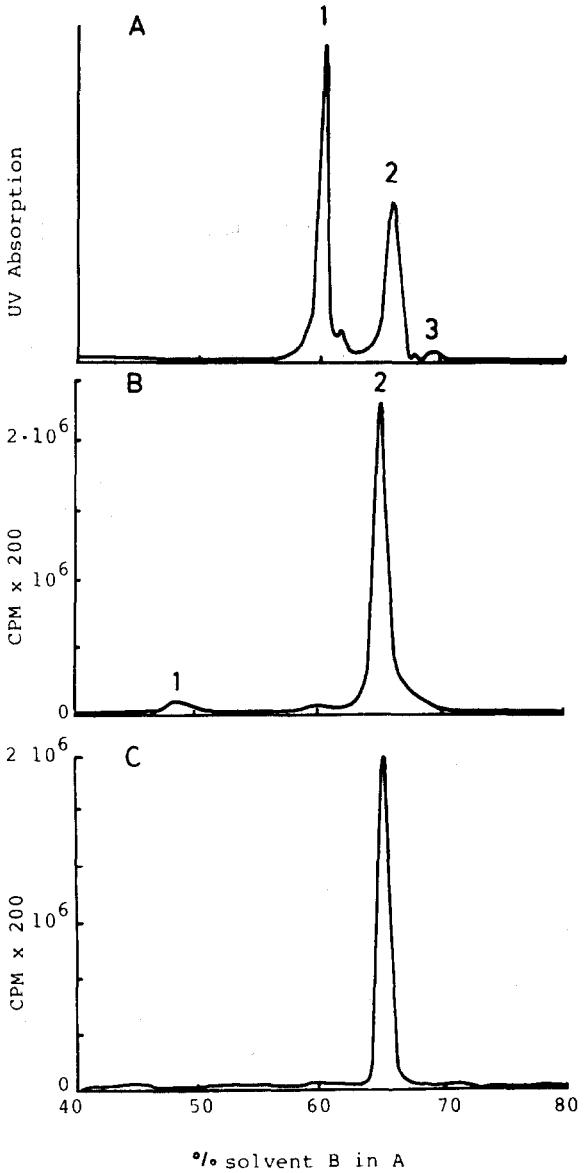


Fig.1. Elution profiles of an NaI- and an ^{125}I-Na-iodination of the AVP antagonist. (a) NaI-iodination. Separation was performed with a linear 40–75% gradient of solvent B (0.067% TFA, in 60% acetonitrile) in solvent A (0.067% TFA). abs is the absorption measured at 254 nm. Peak 1 is the non-iodinated, peak 2 the monoiodinated, and peak 3 the diiodinated AVP antagonist. (b) ^{125}I-Na-iodination. Separation was performed with a linear 40–75% gradient of solvent B (0.067% trifluoroacetic acid in 60% acetonitrile) in solvent A (0.067% TFA). Quantities are expressed in cpm. Peak 1 is non-covalently bound ^{125}I and peak 2 the ^{125}I-AVP antagonist. (c) Purification of ^{125}I-labelled AVP antagonist after a BSA incubation of 15 min. HPLC was performed as in 1b. Quantities are expressed in cpm.

2.6. *Binding assays*

Membranes (10–15 mg for liver, 10–65 mg for kidney and 15 mg for uterus membranes) were incubated in 50 mM Tris-HCl (pH 7.4), 1, 5 or 10 mM $MgCl_2$ (for, respectively, kidney, liver and uterus), 1 mg/ml BSA and varying concentrations of labelled and unlabelled peptides. Incubation at 30°C lasted 30 min for the tritiated peptides and 40 min for the ^{125}I-labelled AVP antagonist. The final assay volumes were respectively 200 and 80 μl. Non-specific binding was determined in the presence of a 250-fold excess of I-AVP antagonist or a 1000-fold excess of AVP. Bound and free radioactivity were separated by filtration over Gelman (GA-3) filters for the tritiated peptides and over Whatman GF/C filters, presoaked in 10 mg/ml BSA (for at least 2 h) for the radioiodinated peptides, as described [11]. Binding constants for the non-labelled AVP antagonist and the I-AVP antagonist were determined in competition experiments in the presence of 0.4–1.7 nM [^{3}H]AVP for liver membranes, 1.0 nM [^{3}H]AVP for kidney medulla membranes and 1.9–3.1 nM [^{3}H]-[Thr^{4}Gly^7]OT for uterus membranes. Results of dose-dependent binding and competition experiments were analyzed using a non-linear weighted regression analysis carried out with ligand [12]. K_d values are expressed in nM ± SD.

3. RESULTS AND DISCUSSION

3.1. *Chloramine-T iodination*

Chloramine-T iodination appeared to be a convenient and reproducible method. In order to avoid reducing agents, which can be potentially harmful to AVP analogues, to terminate the oxidation reaction, the reaction mixture was directly injected onto a HPLC column. Under the experimental conditions used, the disulphur bridge of the AVP antagonist molecule was not oxidized by Chloramine-T. An elution profile of an iodination with NaI (fig.1a) showed three peaks. Peak 1 (61% solvent B in A) was identified as the nonreacted starting material because of its co-migration with pure, unlabelled AVP antagonist, and the absence of a bathochrom shift of the UV spectrum (λ_{max} = 295 nm) due to the presence of a monoiodinated tyrosine. Peak 2 (65% B in A) was identified as the monoiodinated AVP antagonist on the basis of a bathochrom shift (λ_{max} = 304 nm). Peak 3 (68% B in A) appeared to be diiodinated AVP antagonist (λ_{max} = 310 nm). Only the tyrosyl residue at position 9 was iodinated. No iodination took place at the methyltyrosine in position 2 [5]. The unlabelled monoiodinated AVP antagonist was used to identify the ^{125}I-AVP antagonist on HPLC and to establish inhibition constants for AVP and OT receptors (table 1). HPLC separation of the reaction products of the ^{125}I-Na iodination showed two peaks of radioactivity (fig.1b). The first, identified as non-incorporated ^{125}I, eluted almost directly in one peak (47–50% B in A). Elution of the second peak, the ^{125}I-AVP antagonist, occurred at 65–66% B in A. Because the stoichiometry of the reaction was in disfavour of diiodination only very small amounts of diiodinated antagonist were produced. The compound was purified in a second HPLC step. The ^{125}I-antagonist coeluted with the 'cold' NaI-Chloramine-T monoiodinated AVP antagonist, on which basis the ^{125}I-AVP antagonist was identified.

3.2. *Biological activities and affinity determinations*

The vasopressin antagonist, d$(CH_2)_5$[Tyr-$(Me)^2$,Tyr$(NH_2)^9$]AVP appeared as a highly potent anti-vasopressor and anti-oxytocic peptide with

Table 1

Biological activities of AVP antagonist and I-AVP antagonist

Peptide	Anti-vasopressor pA_2	Anti-anti-diuretic pA_2	Anti-diuretic U/mg	Anti-oxytocic pA_2	
				0 mM Mg^{2+}	0.5 mM Mg^{2+}
AVP ant	8.47 ± 0.03	6.0	0.002	8.37 ± 0.03	8.22 ± 0.09
I-AVP ant	8.17 ± 0.06[a]	<6.3	0.007	7.50 ± 0.06	7.91 ± 0.06

[a] The iodinated analogue appeared to have a significantly longer duration of antagonistic activity in the vasopressor assay

Antagonistic properties represent pA_2 values ± SE (of at least 4 determinations). pA_2 is the negative log of A_2, which is the concentration of antagonist reducing the response to 2 × units of agonist to equal the response to 1 × unit administered before the antagonist

low anti-antidiuretic potency and very small antidiuretic activity (table 1). Iodination of the 9-tyrosylamide residue slightly reduced the antivasopressor and anti-oxytocic potencies and slightly enhanced the anti-antidiuretic potency and residual antidiuretic activity. The vasopressin antagonist and its iodinated counterpart inhibited [^{3}H]AVP binding to a V_1 (liver) and a V_2 (kidney medulla) VP receptor and [^{3}H]-[Thr^{4}Gly^7]OT binding to a OT (uterus) receptor. In line with in vivo data, high affinities for the V_1 and OT receptors were found (table 2). Unexpected was the observation that d(CH$_2$)$_5$[Tyr-(Me)^2,Tyr(NH$_2$)^9] also bind renal V_2 receptors with high affinity. At present no explanation can be given for this discrepancy between in vivo and in vitro data. Monoiodination resulted in increased affinities for V_1, V_2 and OT receptors, a phenomenon which has also been observed for other 9-tyrosylamide containing AVP antagonists [3]. Hill plot slopes did not differ significantly from unity, indicating an interaction with single populations of receptor sites in all cases. This was confirmed by regression analysis of the untransformed data, conducted with Ligand.

3.3. *^{125}I-AVP antagonist binding*

A saturable specific ^{125}I-AVP antagonist binding to liver membrane preparations was easily revealed (fig.2) due to small non-specific binding. Analysis of the data with ligand indicated binding to one class of receptor sites with a dissociation constant of 0.28 ± 0.09 nM which was in good agreement with the value found in competition experiments (K_i = 0.27 ± 0.01, table 2). Binding capacity was 550 ± 85 fmol/mg protein, a value which is in the range of those previously determined with [^{3}H]vasopressin [10]. Radioiodinated d(CH$_2$)$_5$[Tyr-(Me)^2,Tyr(NH$_2$)^9] thus appears as a good labelled ligand for V_1 vasopressin receptors. It is worth noting that its affinity for V_1 receptors (K_d = 0.28 nM) is higher than that of [^{3}H]AVP (K_d = 2.0 nM, 1). This property together with a high specific radioactivity, 2000 Ci/mmol as compared to 50–70 Ci/mmol for [^{3}H]AVP will contribute to considerably reduce the detection limit for V_1 vasopressin receptors. However, the poor selectivity of this new radiolabelled ligand (high affinity for OT receptors and to a lesser extent for V_2 vasopressin receptors) will impose control experiments with more selective unlabelled ligands.

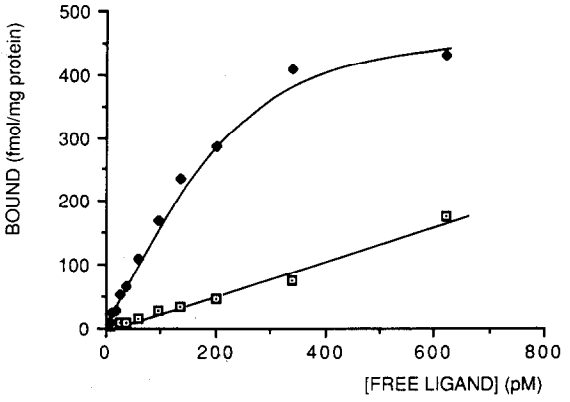


Fig.2. Dose-dependent binding of ^{125}I-labelled d(CH$_2$)$_5$[Tyr-(Me)^2,Tyr(NH$_2$)^9]AVP to liver membranes. The figure shows the results of a typical experiment. The amount of membrane protein was 9.8 μg. Filled symbols, specific binding; open symbols, non-specific binding.

Table 2

Affinities of AVP antagonist and I-AVP antagonist

Peptide	Inhibition constants (nM)		
	Liver (V_1)	Kidney (V_2)	Uterus (OT)
AVP ant	0.61 ± 0.15	4.0 ± 0.2	4.8 ± 2.5
I-AVP ant	0.27 ± 0.01	1.0 ± 0.1	0.13 ± 0.07

Inhibition constants of the AVP antagonist and I-AVP antagonist were determined in competition experiments. Liver, kidney medulla and uterus membranes were incubated with 0.4–1.7 nM [^{3}H]AVP for vasopressin receptors, 1.9–3.1 nM [^{3}H]-[Thr^4,Gly^7]OT for oxytocin receptors and varying concentrations of non- and monoiodinated AVP antagonist. Values are the mean of two independent determinations in triplicate ± SD. They were calculated using the non-linear weighted least square regression analysis of the untransformed data, conducted with ligand

Acknowledgements: This work was supported by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Fondation pour la Recherche Médicale and by a fellowship awarded to J.E. from the Institut National de la Santé et de la Recherche Médicale.

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