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Synthesis and binding characteristics of two sulfhydryl-reactive probes for vasopressin receptors

Imre Pávó, Elzbieta Kojro and Falk Fahrenholz

Max Planck Institut für Biophysik, Kennedyallee 70, W-6000 Frankfurt am Main 70, Germany

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The present study describes the synthesis and receptor binding affinities of the sulfhydryl-reactive vasopressin analogs deamino[Dab(N^{δ}-*N*-maleoyl-β-alanin e)^4]AVP (**1a**) and deamino[Lys(N^{ε}-*N*-maleoyl-β-alanine)^8]VP (**2a**). The analogs were obtained by introducing the sulfhydryl-reactive maleoyl-β-alanyl group at the δ-amino group of Dab^4 in deamino[Dab^4]AVP (**1**) and at the ε-amino group of Lys^8 in deamino[Lys^8]VP (**2**), which were synthesized by the solid-phase method. Furthermore, the analog modified at Dab^4 was prepared as tritium labeled compound (**1b**) after catalytic iodine tritium exchange at Tyr^2 in deamino[Dab^4]AVP. The sulfhydryl-reactive vasopressin analogs retained high binding affinity for the V_2 vasopressin receptor in membranes derived from bovine kidney inner medulla. Apparent dissociation constants K_d of 45 nM (compound **1a**) and 15 nM (compound **2a**) were determined. Incubation of the ligand receptor complexes at pH 5.5 resulted in dissociation of the sulfhydryl-reactive vasopressin analogs from the V_2 receptor. No indications of a covalent reaction between analogs **1a**, **2a** and **1b** and sulfhydryl groups in or close to the hormone binding site of the V_2 receptor were found.

Vasopressin; Vasopressin V_2 receptor; Sulfhydryl reagent

1. INTRODUCTION

An effect of sulfhydryl group modification on radioligand binding has been demonstrated for a wide variety of membrane receptors (for review see [1]). One of the most widely used sulfhydryl group reagents is *N*-ethylmaleimide. The reaction is irreversible and under mild conditions at neutral pH specific for sulfhydryl groups. In several cases, e.g. the D-1 dopamine receptor [2] and opioid receptors [3,4], NEM-sensitive sulfhydryl groups are critical for receptor binding. In the case of the β2-adrenergic receptor the substitution of conserved extracellular cysteines with alanine, results in a reduced affinity of the receptor for its ligands [5].

The neurohypophyseal peptide hormone vasopressin stimulates water reabsorption in the mammalian kidney collecting tubule and in the amphibian urinary bladder (for review see [6]). An earlier study showed that in isolated toad bladder NEM inhibits the effect of vasopressin [7]. Recently Birnbaumer et al. reported the cloning of the human V_2 receptor [8]. The receptor has 10 cysteine residues. Further experiments are required to identify the determinants for ligand binding. We recently demonstrated that renal V_2 vasopressin receptors in isolated membranes or intact cells can be inactivated by pretreatment with several sulfhydryl reagents, and especially with NEM. Vasopressin analogs with agonistic or antagonistic properties protected against this inactivation, probably by inhibiting the reaction of NEM with sulfhydryl groups [9]. However, it is not clear whether the critical sulfhydryl groups are located in the ligand binding domain or whether the protective ligands can change the receptor conformation in such way that sulfhydryl groups, outside the binding site, become less accessible to NEM.

Sulfhydryl-reactive vasopressin analogs could be useful tools to answer this question. If a critical sulfhydryl group is in the binding site of the V_2 receptor, then sulfhydryl-reactive ligands may bind to it covalently. Subsequently, these vasopressin receptor–ligand complexes could not dissociate and the receptor molecules become unable to bind vasopressin. If the critical sulfhydryl groups are outside the V_2 receptor binding site, then the sulfhydryl-reactive vasopressin analogs would fail to block the binding site. After dissociation of the analogs, the intact receptor molecule could again react with its specific ligands.

In the present report we demonstrate the synthesis of two sulfhydryl-reactive vasopressin analogs. For this purpose, we introduced the maleoyl-β-alanyl residue in vasopressin analogs, either in the side chain of position 4 or 8. We also synthesized one of the analogs in radio-

Correspondence address: F. Fahrenholz, Max Planck Institut für Biophysik, Kennedyallee 70, W-6000 Frankfurt am Main 70, Germany.

Abbreviations: ACN, acetonitrile; AVP, [8-arginine]vasopressin; BOC, tert-butyloxycarbonyl; Dab, 2,3-diaminobutyric acid; DCC, dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; EPPS, *N*-(2-hydroxyethyl)-piperazine-*N*′-(3-propanesulfonic acid); NEM, *N*-ethylmaleimide; TFA, trifluoroacetic acid.

labeled form. Our results with these analogs suggest that the sulfhydryl groups important for ligand binding to the V_2 receptor are either located outside the binding site, or not accessible by sulfhydryl-reactive groups in position 4 or 8 of the vasopressin sequence.

2. MATERIALS AND METHODS

2.1. *Materials and HPLC analysis*

Merrifield resin was purchased from Bio-Rad Laboratories GmbH, München, Germany. BOC amino-protected amino acids were purchased from BACHEM Biochemica GmbH, Heidelberg, Germany. *N*-Maleoyl-β-alanine and *N*-hydroxysuccinimide were the products of Fluka Chemie AG, Buchs, Switzerland. [^{3}H]AVP (20 Ci/mmol) was purchased from Amersham, Buckinghamshire, England. All other chemicals used were of analytical grade. HPLC retention time determinations were performed with a Knauer system on a 250 × 4.6 mm Lichrosorb 10 RP 18 column with a 22.5 ml linear gradient of 18% to 72% ACN in 0.09% TFA and a flow rate of 1.5 ml/min.

2.2. *Synthesis*

The parent peptides deamino[Dab^4]AVP **(1)** and deamino[Lys^8]VP **(2)** were prepared as described previously [10]. The purity was checked with TLC, HPLC, UV spectroscopy and amino acid analysis. HPLC retention time was 6.07 min for **1** and 6.55 min for **2**. The tritium labeled peptide deamino[[3,5-^{3}H]Tyr^2,Dab^4]AVP was prepared from deamino[Dab^4]AVP **(1)** by catalytic dehalogenation of its diiodo-derivative, following a procedure described previously [11]. Specific activity 32.2 Ci/mmol. The synthesis **of** *N*-maleoyl-β-alanine-*N*-succinimidyl ester **(3)** was a modification of the procedure of Keller and Rudinger [12]. 170 mg (1 mmol) *N*-maleoyl-β-alanine was treated in 5 ml ethyl acetate with 126 mg (1.1 mmol) *N*-hydroxysuccinimide. After stirring on ice for 1 h and at room temperature for 5 h, the solution was filtered and evaporated to dryness. The crystalline product (240 mg), more than 95% pure, was analyzed by TLC and HPLC. R_t 5.50 min, yield: 90%.

Synthesis of deamino[Dab(*N*$^\delta$-*N*-maleoyl-β-alanine)^4]AVP **(1a)** and deamino[Lys(*N*$^\varepsilon$-*N*-maleoyl-β-alanine)^{8}VP **(2a)** (Fig. 1): 2 mg (approximately 2 μmol) peptide (**1** or **2**) was dissolved in 100 μl DMF. By the addition of 2 μl *N*-methylmorpholine, the pH was adjusted to 7.5–8.0. To this solution, 2.66 mg (10 μmol) **3** was added. After reaction for 2 h at room temperature and overnight on ice, the reaction mixture was diluted to 5 ml with 10% ACN in water and purified on a Lichrosorb 10 RP-18 column (250 × 22 mm) and subjected to isocratic elution with 28.8% ACN in 0.09% TFA at a flow rate of 5 ml/min. The products were analyzed by HPLC, FAB spectroscopy, UV spectroscopy, amino acid analysis, and found to be more then 95% pure. R_t: 7.23 min (**1a**) and 7.11 min (**2a**). Yields: 28% (**1a**) and 22% (**2a**). 1 mM water solutions of peptides **1a** and **2a** were incubated with 10 mM 2-mercaptoethanesulfonate for 30 min at 30 °C and the reaction mixtures were analyzed by HPLC.

Synthesis of deamino[[3,5^{3}H]Tyr^2,Dab(*N*δ-*N*-maleoyl-β-alanine)^4]AVP **(1b)**: 300 μl ACN/water solution of 86 μmol deamino[[3,5-^{3}H]Tyr^2,Dab^4]AVP was dried under a nitrogen stream in a 1 ml Eppendorf cup. The residual material was dissolved in 50 μl DMF, and 10 μl DMF containing 250 μg (1 μmol) **3** and 9 μl *N*-methylmorpholine was added to the solution. The solution was vortexed for 14 h at room temperature prior to drying under a nitrogen stream. The residue was dissolved in 13.5% ACN containing 0.09% TFA and purified by HPLC on a Lichrosorb 10 RP 18 (250 × 4.6 mm) column with 45 ml linear gradient of 13.5% to 67.5% ACN in 0.09% TFA at a flow rate of 1.5 ml/min. 500 μl fractions were collected and aliquots were counted for radioactivity. Fractions 30–34, the peak of the radioactivity were combined. The retention time of the radioactive product on HPLC was the same as R_t of **1a**. The recovery of the radioactivity was 16%.

2.3. *Membrane preparation and receptor binding assays*

Bovine kidney inner medulla plasma membranes were prepared by a two-step centrifugation procedure [13]. The 10,000 ×*g* pellet resulting from this procedure had a specific binding capacity of approximately 1.3 pmol [^{3}H]AVP/mg protein.

Binding tests with plasma membranes were performed by incubating membranes (100 μg of protein) in 100 μl binding buffer (50 mM EPPS pH 8.3 and 5 mM $MgCl_2$) containing 10 nM [^{3}H]AVP in the presence (non-specific binding) or in the absence (total binding) of 1 μM AVP for 30 min at 30°C. To terminate the binding tests, the mixtures were diluted with 5 ml of ice-cold washing buffer (10 mM EPPS pH 8.3, 2 mM $MgCl_2$ and 0.1% BSA), immediately filtered under vacuum through Whatman GF/F filters, and rinsed twice with 5 ml ice-cold washing buffer. The filters were placed in counting vials with scintillation liquid and analyzed by liquid scintillation spectrometry. Protein determination was performed by means of a modified fluorescamine assay [14].

Increasing concentrations of [^{3}H]AVP or **1b** were used in the presence (non-specific binding) or in the absence (total binding) of a 100-fold excess of AVP to determine the binding affinity.

The receptor affinity was determined in competition binding experiments of the unlabeled vasopressin analogs. The binding assay suspensions contained different concentrations of unlabeled peptide and 10 μM AVP.

In test for irreversible receptor-ligand complex formation, membranes (100 μg protein in 100 μl binding buffer) were preincubated with the peptide ligands (AVP, **1**, **2**, **1a**, **2a**, or [^{3}H]AVP and **1b**) for 30 min at 30°C. After incubation with the ligand, the membranes were collected by centrifugation, and resuspended in 500 μl 250 mM ammoniumacetate buffer, pH 5.5, to allow non-covalently bound ligand to dissociate from the receptor. After 5 min at 30 °C, these membranes (with the exception of membranes pretreated with [^{3}H]AVP or **1b**) were collected by centrifugation, resuspended in binding buffer, and binding tests with [^{3}H]AVP were performed. In the case of membranes incubated with [^{3}H]AVP or **1b**, the pH 5.5 treatment was followed directly by filtration through the GF/F filter.

All assays were performed in duplicate. The experiment was carried out twice. Binding curves were fitted to a logistic function with a weighted iterative least-squares procedure based on the method of steepest descent [15]. The figures and tables relate to representative experiments.

3. RESULTS

The reactivity of the maleoyl group-containing peptides was examined with 2-mercaptoethanesulfonate [12]. Peptides **1a** and **2a** reacted quantitatively with the sulfhydryl-containing reagent under the same conditions (pH, time, temperature) as used in the membrane receptor binding tests. In aqueous solution at neutral pH and at room temperature, the peptides were stable for more then 24 hours. The reactions were monitored by HPLC (data not shown).

The affinities of the new sulfhydryl-reactive non-radioactive vasopressin analogs **(1a** and **2a)** and their parent peptides (**1** and **2**) were tested by competitive binding assay on bovine kidney membrane containing V_2 receptors (Fig. 2). The affinity of the radioactive maleoyl vasopressin derivative deamino[[3,5-^{3}H]Tyr^2,Dab(*N*δ-*N*-maleoyl-β-alanine)^4]AVP **(1b)** was determined directly in a dose-dependent binding assay, using 100-fold AVP to determine the non-specific binding (Fig. 3). The affinities of the labeled and unlabeled derivative of deamino[Dab^4]AVP were approximately the same. In

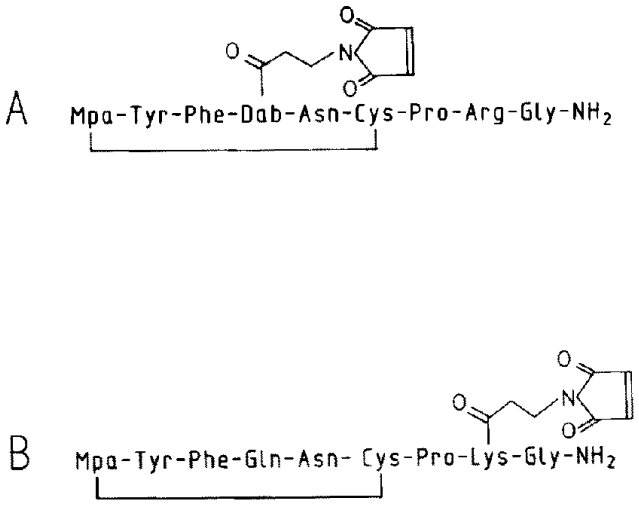

Fig. 1. Structures of deamino[Dab(N^{δ}-*N*-maleoyl-β-alanine)^4]AVP (**1a**, A) and desamino[Lys(N^{ε}-*N*-maleoyl-β-alanine)^{8}VP (**2a**, B)

both reactive analogs introduction of the maleoyl-β-alanyl group reduced the affinity only moderately (2- to 3-fold) as compared to the parent peptide (Table I).

The complex of the radioactive sulfhydryl-reactive peptide **1b** with the V_2 receptor dissociated during incubation at pH 5.5. After this treatment, the receptors were able to bind the specific ligand [^{3}H]AVP (results not shown). Analysis of membranes incubated with the tritium labelled reactive analog **1b** by SDS-gel electrophoresis did not reveal a covalent reaction of the analog with kidney membrane proteins (data not shown).

Pretreatment of the V_2 receptors with saturation concentrations of unlabeled sulfhydryl-reactive peptides **1a** and **2a** also resulted in non-covalent acid-labile complexes. After the pH 5.5 treatment of these complexes,

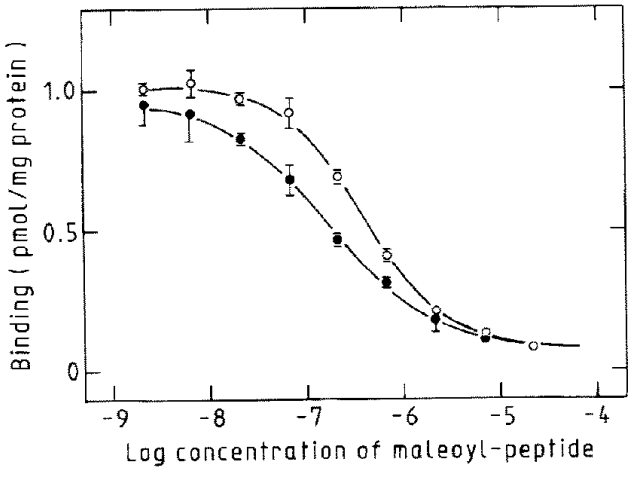

Fig. 2. Relative potencies of sulfhydryl-reactive vasopressin analogs deamino[Dab(N^{δ}-*N*-maleoyl-β-alanine)^4]AVP **1a** (■) and deamino[Lys(N^{ε}-*N*-maleoyl-β-alanine)^{8}VP **2a** (●) for inhibiting [^{3}H]AVP binding. A fixed concentration of [^{3}H]AVP (10 nM) was incubated with the V_2 receptor containing bovine kidney membrane in the presence of various concentrations of unlabeled peptides.

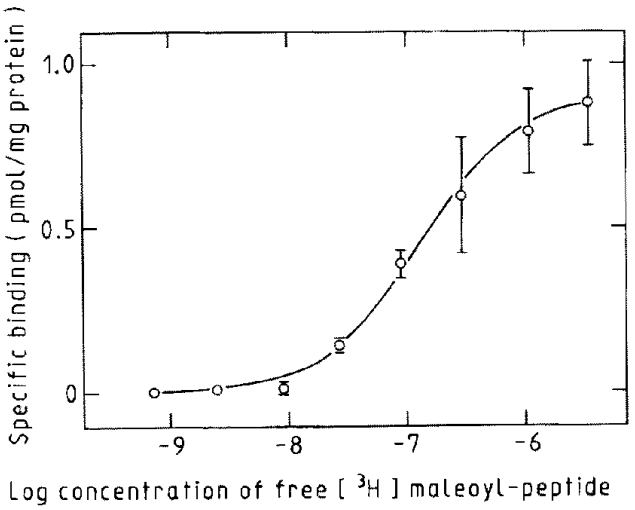

Fig. 3. Dose-dependent binding of deamino[[3,5-^{3}H]Tyr^2,Dab(*Nδ*-*N*-maleoyl-β-alanine)^4]AVP (**1b**) to the bovine renal V_2 receptor. The results show the concentration of specific binding to the membranes in several concentrations of the peptide.

similar to the complexes between the parent peptides **1** and **2**, AVP and the receptor, were able to bind [^{3}H]AVP (Table II).

4. DISCUSSION

Our recent results suggest that sulfhydryl groups play an important role in the binding of ligands to the renal V_2 receptor. It was possible to inactivate V_2 receptor molecules with NEM, a specific sulfhydryl reagent. However, it is not clear whit which domain of the V_2 receptor molecule NEM reacts, i.e. where the critical sulfhydryl groups are located.

Photorcactive analogs of the parent peptides **1** and **2**, modified after UV radiation, bound covalently to renal V_2 receptors [10]. There are various chemical possibilities for binding photoreactive groups to the receptor molecules, but they are not clearly defined.

The new peptides presented in this work could bind

Table I

Affinities (K_d values) of vasopressin analogs and their sulfhydryl-derivatives to the V_2 receptors in bovine renal medullary membranes (K_d values were determined for binding and competition curves described in section 2)

Peptide	K_d (nM)	K_d/K_d-AVP
AVP	1.5 ± 0.3	1
d[Dab^4]AVP (**1**)	25.0 ± 4.0	16.7
d[Lys^8]VP (**2**)	4.8 ± 0.8	3.2
d[Dab(N^{δ}-*N*-maleoyl-β-alanine)^4]AVP (**1a**)	44.5 ± 7.8	29.6
d[[3,5-^{3}H]Tyr^2,Dab(N^{δ}-*N*-maleoyl-β-alanine)^4]AVP (**1b**)	34.5 ± 4.7	22.6
d[Lys(N^{ε}-*N*-maleoyl-β-alanine)^{8}VP (**2a**)	14.9 ± 2.6	9.9

d = deamino-

Table II

Specific [^{3}H]AVP binding to bovine kidney V_2 receptors after preincubation the membrane with vasopressin analogs. Control: binding capacity remaining after preincubation with AVP. The differences are statistically not significant. Experiments were performed as written in section 2.

Ligand	Conc. of ligand (nM)	Receptor binding remaining after precincubation with ligand (% of control)
AVP	10	100 ± 9
d[Dab^4]AVP (**1**)	100	92 ± 8
d[Lys^8]VP (**2**	30	94 ±11
d[Dab(N^δ-*N*-maleoyl-β-alanine)^4]AVP (**1a**)	100	84 ±14
d[Lys(N^ε-*N*-maleoyl-β-alanine)^{8}VP (**2a**)	50	82 ±13

d = deamino-

covalently the V_2 receptor if a free sulfhydryl group is located in the binding site of the receptor molecule and if the reaction of the sulfhydryl with the maleoyl group is not sterically inhibited. The sulfhydryl-reactive peptides might be useful tools to determine wether the critical sulfhydryl group is inside or outside the binding site of the receptor molecule.

The maleoyl-peptides used in this study have their sulfhydryl-reactive moieties in different positions. Peptides **1a** and **1b** carry the maleoyl-β-alanyl group in position 4, inside the 20-membered ring structure of the vasopressin molecule containing the 6 N-terminal amino acids. Peptide **2a** is a peptide containing the maleoyl-β-alanyl group in position 8, in the flexible C-terminal tripeptide of the vasopressin molecule. None of them reacted covalently with the receptor molecule. The receptor-ligand complexes of the V_2 receptor and peptides **1a**, **1b** and **2a** dissociated in acidic buffer, and the V_2 receptor, after this treatment, proved to be capable to bind its ligand. Our findings suggest that the critical sulfhydryl groups, important for ligand binding, are either outside the binding site of the receptor molecule or inaccessible for the maleoyl residue in position 4 or 8 of the vasopressin molecule.

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