The Effects of N-Terminal Part Modification of Arginine Vasopressin Analogues with 2-Aminoindane-2-carboxylic Acid: A Highly Potent V_2 Agonist

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In this study we present the synthesis and some pharmacological properties of nine new analogues of arginine vasopressin modified in the N-terminal part of the molecule with 2-aminoindane-2-carboxylic acid (Aic). The peptides were tested for their in vitro uterotonic and in vivo pressor and antidiuretic activities. One of the new peptides, [Mpa^1,Aic^2,Val^4,D-Arg^8]VP, exhibited an antidiuretic activity similar to that of [Mpa^1,D-Arg^8]VP, thus being one of the most potent antidiuretic vasopressin analogues reported to date.

Introduction

Arginine vasopressin (AVP[a]), a neuropeptide, exerts its action through at least three types of G-protein-coupled receptors with seven transmembrane spanning domains. V_1 vascular receptors (also known as V_{1a}), which mediate vasoconstriction, are located in vascular smooth muscle and are also found in kidney, myometrium, bladder, hepatocytes, platelets, and spleen.[1] V_{1b} receptors (also known as V_3) mediate the ACTH-releasing effects of AVP from the anterior pituitary gland.[2] These receptors are responsible for the action of vasopressin in the central nervous system, where vasopressin modulates the memory, blood pressure, body temperature, and release of pituitary hormones.[3] Renal V_2 receptors activate water reabsorption in the renal collecting duct. In this way, V_2 receptors mediate the antidiuretic response to AVP.[4]

Many of the vasopressin agonists and antagonists have been designed and synthesized in the course of extensive investigations of the structure−activity relationships.[5−8] A lot of modifications were N-terminal ones.[7,9] All these efforts resulted in probably the best understanding of such relationships among peptide hormones. However, the design of analogues, which are very active and truly selective for AVP receptors, still remains an area of great interest.

The most straightforward approach for subtle peptide modifications is the introduction of changes into the side chains of amino acids in predetermined positions. By incorporation of nonproteinogenic amino acid residues, it is possible to introduce bulky groups or sterically restricted elements with the aim to restrict the conformational flexibility of a certain part of the peptide chain. Conformational restrictions may increase receptor binding selectivity, metabolic stability, or intrinsic activity and lead to highly potent agonists or antagonists. In our laboratory, we have shown that such an approach could result in analogues with very interesting pharmacological properties.[10−15]

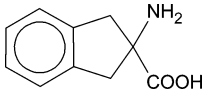

Figure 1. Structure of 2-aminoindane-2-carboxylic acid (Aic).

Recently, we replaced the residues at position 2 or 3 of AVP and some of its agonistic and antagonistic analogues with 1-aminocyclohexane-1-carboxylic acid (Acc) or 1-aminocyclopentane-1-carboxylic acid (Apc).[13−15] Acc and Apc were chosen to reduce the flexibility of peptides by implanting a sterically constrained residue, thus forcing the peptide backbone and side chains to adopt specific orientations. Both modifications are an example of the $C^{\alpha}\leftrightarrow C^{\alpha}$ cyclization, whereby a dialkylated glycine residue is converted into a cyclic side chain. The Acc and Apc substitutions in position 2 selectively altered biological activity. Some of the analogues were highly potent V_2 agonists with different pressor and uterotonic potencies.[13−15] The [Apc^2,D-Arg^8]VP analogue was of particular interest because it turned out to be both potent and a highly selective antidiuretic agonist.[15]

These findings prompted us to continue our studies by the introduction of new sterically restricted residues into this part of the molecule with the aim to find out how the modification performed would reflect in the values of biological potency of the analogues. We synthesized and determined some pharmacological properties of nine new analogues of AVP substituted at positions 2 or 3 with 2-aminoindane-2-carboxylic acid (Aic, see Figure 1). In addition to the reduction of flexibility, this new amino acid should enhance the resistance of the resulting peptides to enzymes. We prepared the following analogues: [Aic^2]AVP (**1**), [Mpa^1,Aic^2]AVP (**2**), [Aic^2,D-Arg^8]VP (**3**), [Mpa^1,Aic^2,D-Arg^8]VP (**4**), [Aic^2,Val^4]AVP (**5**), [Mpa^1,Aic^2,-Val^4,D-Arg^8]VP (**6**), [Aic^3]AVP (**7**), [Mpa^1,Aic^3]AVP (**8**), and [Aic^3,D-Arg^8]VP (**9**).

Results

The nine new analogues of AVP (**1**−**9**) were synthesized by Fmoc strategy, purified, and characterized. Their physicochem-

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[a] Abbreviations: The symbols of the amino acids and peptides are in accordance with the 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature and "A Revised Guide to Abbreviations in Peptide Science" published in *J. Pept. Sci.* **2003**, *9*, 1−8. Other abbreviations are the following: Acc, 1-aminocyclohexane-1-carboxylic acid; Aic, 2-aminoindane-2-carboxylic acid; Apc, 1-aminocyclopentane-1-carboxylic acid; AVP, arginine vasopressin; dDAVP, desmopressin [Mpa^1,D-Arg^8]VP; DIEA, diisopropylethylamine; DMF, dimethylformamide; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; Mpa, 3-mercaptopropionic acid; NMP, 1-methyl-2-pyrrolidone; PhOH, phenol; TBTU, 2-1*H*-(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

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Table 1. Physicochemical Properties of Peptides **1**−**9**

analogue		formula	HPLC T_R[a,b]	MW	$[M + H]^+$
[Aic^2]AVP	1	$C_{47}H_{65}N_{15}O_{11}S_2$	7.8 (11.0)	1080.2	1080.3
[Mpa^1,Aic^2]AVP	2	$C_{47}H_{64}N_{14}O_{11}S_2$	11.7 (13.9)	1065.2	1065.5
[Aic^2,D-Arg^8]VP	3	$C_{47}H_{65}N_{15}O_{11}S_2$	10.2 (11.2)	1080.2	1080.3
[Mpa^1,Aic^2,D-Arg^8]VP	4	$C_{47}H_{64}N_{14}O_{11}S_2$	11.8 (14.2)	1065.2	1065.2
[Aic^2,Val^4]AVP	5	$C_{47}H_{66}N_{14}O_{10}S_2$	11.2 (13.3)	1051.2	1051.3
[Mpa^1,Aic^2,Val^4,D-Arg^8]VP	6	$C_{47}H_{65}N_{13}O_{10}S_2$	13.9 (16.9)	1036.2	1036.1
[Aic^3]AVP	7	$C_{47}H_{65}N_{15}O_{12}S_2$	10.6 (12.7)	1096.2	1096.1
[Mpa^1,Aic^3]AVP	8	$C_{47}H_{64}N_{14}O_{12}S_2$	12.5 (21.2)	1081.2	1081.2
[Aic^3,D-Arg^8]VP	9	$C_{47}H_{65}N_{15}O_{12}S_2$	10.5 (12.1)	1096.2	1096.5

[a] Linear gradient 20−80% [B] for 20 min. [b] For values in parentheses, linear gradient 20−80% [B] for 30 min for analogues **1**−**6** and linear gradient 10−60% [B] for 30 min for analogues **7**−**9**.

Table 2. Pharmacological Properties of New Analogues of AVP (IU/mg or pA_2)

		activity			
				antidiuretic[b]	
analogue		oxytocic uterus in vitro test no Mg^{2+}	pressor	IU/mg $t_{1/2}$ 60 ($t_{1/2}$ 200)	% of dDAVP $t_{1/2}$ 60 ($t_{1/2}$ 200)
AVP[a]		17	412	465	10 (0.2)
[Mpa^1,D-Arg^8]VP (dDAVP)[a]		1.5−5.1	∼0.39	800−50 000[f]	100 (100)
[Acc^2]AVP[c]		pA_2 ∼ 5.6	56.6	750−900 (∼9300)	∼15 (4)
[Apc^2]AVP[d]		pA_2 = 6.0 and 0.2 IU/mg	13.4 ± 3.8	∼1800 (1800)	50 (1.4)
[Aic^2]AVP	**1**	pA_2 = 7.27 ± 0.22	9.4 ± 2.8	∼450 (45 000)	∼10 (10)
[Mpa^1,Acc^2]AVP[c]		pA_2 = 6.1 and 0.7 IU/mg	17.2 ± 0.8	∼4500 (50 000)	∼100 (20)
[Mpa^1,Apc^2]AVP[d]		pA_2 = 7.1	15.5 ± 3.5	∼1000 (9000)	∼25 (5)
[Mpa^1,Aic^2]AVP	**2**	pA_2 = 7.50 ± 0.16	5.3 ± 2.5	∼450 (45 000)	∼10 (10)
[Acc^2,D-Arg^8]VP[c]		pA_2 ∼ 5.7	pA_2 ∼ 5.8	750−900 (∼9300)	∼15 (4)
[Apc^2,D-Arg^8]VP[d]		0	0	∼1000 (9000)	∼25 (5)
[Aic^2,D-Arg^8]VP	**3**	pA_2 = 6.86 ± 0.17	0	∼45 (4500)	∼1 (1)
[Mpa^1,Acc^2,D-Arg^8]VP[e]		pA_2 = 6.5 and 0.3 IU/mg	pA_2 = 5.70	∼4500 (50 000)	∼100 (20)
[Mpa^1,Apc^2,D-Arg^8]VP[d]		pA_2 = 6.5	0	∼1000 (9000)	∼25 (5)
[Mpa^1,Aic^2,D-Arg^8]VP	**4**	pA_2 = 7.31 ± 0.25	pA_2 = 5.60	∼450 (45 000)	∼10 (10)
[Acc^2,Val^4]AVP[e]		pA_2 = 6.9	0.9 ± 0.2	∼2300 (23 000)	∼50 (10)
[Apc^2,Val^4]AVP[d]		pA_2 = 6.6	0.20 ± 0.02	∼1000 (9000)	∼25 (5)
[Aic^2,Val^4]AVP	**5**	pA_2 = 7.93 ± 0.17	0	∼450 (45 000)	∼10 (10)
[Mpa^1,Acc^2,Val^4,D-Arg^8]VP		pA_2 = 7.81	pA_2 = 6.14	∼4500 (50 000)	∼50−100 (10)
[Mpa^1,Aic^2,Val^4,D-Arg^8]VP	**6**	pA_2 = 8.06 ± 0.11	pA_2 = 6.25	∼4500 (450 000)	∼50−100 (100)

[a] Values are taken from ref 5. [b] The activities obtained by comparing doses of the analogues and AVP or dDAVP resulting in an antidiuresis time of $t_{1/2}$ = 60 min in arbitrary units or % of activity, respectively; in parentheses, the activities obtained by comparing doses of the analogues and AVP or dDAVP resulting in an antidiuresis time of $t_{1/2}$ = 200 min. [c] Values taken from ref 13. [d] Values taken from ref 15. [e] Values taken from ref 14. [f] The antidiuretic activity from antidiuretic test on anaesthetized rats.

ical properties are presented in Table 1. The values of the molecular ions were as expected and the purity was higher than 97%.

The activities of the new analogues were determined in the in vitro rat uterotonic test in the absence of magnesium ions, the rat pressor test, and in the antidiuretic assay using conscious rats, as described in Experimental Section. The results of the pharmacological evaluation of peptides **1**−**6**, together with relevant data for AVP and some related peptides, are presented in Table 2. The analogues **7**−**9** were devoid of any activity and thus are not included in the table.

Peptides modified at position 2 with Aic exhibited weak (**2** and **3**), moderate (**1** and **4**), or strong (**5** and **6**) antiuterotonic activities. Analogues **1** and **2** showed weak pressor agonism, whereas compounds **4** and **6** were weak pressor antagonists. Peptides **3** and **5** were inactive. In regard to the antidiuretic activity, new peptides were either potent (**1**−**5**) or highly potent (**6**) agonists with prolonged action. It is difficult to compare antidiuretic activity data of different analogues described in the past, as the published values were obtained using different procedures. In the test using conscious rats, the slopes of the dose−response curves for some compounds (e.g., [Mpa^1]AVP or [Mpa^1,D-Arg^8]VP) are much steeper than that of vasopressin. This means that the same increase in the applied dose of either of the two compounds produces a much greater increase in activity than in the case of vasopressin. Compounds with increased slope of the dose−response curves are considered to have protracted action (dDAVP). The slope of the dose−response curves of analogues **1**−**6** is also steeper than that of AVP, as was the case with Acc and Apc analogues described in ref 15. Thus, to compare the effects of the modifications with the parent compounds, we compared such doses of AVP or dDAVP and new analogues that gave the same antidiuretic response, that is, the doses that caused the time in which the rats excreted half of the water load ($t_{1/2}$) to be 60 and 200 min. For AVP, the activity was calculated to be 465 IU/mg and for dDAVP was assumed to be 100%. None of the new compounds exhibited diuretic or, in other words, anti-antidiuretic activity.

Discussion

As a continuation of our efforts to explore the role of Tyr^2 and Phe^3 of AVP molecule on pharmacological activities, we are now describing the qualities of a series of peptides substituted at positions 2 or 3 with compact amino acid Aic (see Figure 1). We assumed that it would reduce flexibility of peptides and force their backbones and side chains to adopt

specific orientations that should result in the change of pharmacological properties. As compared to the previously used amino acids Acc or Apc, the current modification does not change the character of the fragment of the molecule from aromatic to aliphatic. This amino acid has a bulky planar side chain that together with its rigid structure should substantially reduce conformational freedom of the N-terminal part of the peptides.

The results presented in Table 2 show that the antidiuretic potency of analogues **1**, **2**, **4**, and **5** is similar to that of AVP if calculated on the basis of the threshold doses, however, the analogues have steeper dose−response curves, thus having prolonged activity. Their activity at the level of 200 min is about 100 times higher than that of the AVP, that is, a hundred time less of the compounds is necessary to evoke the same effect, that is, $t_{1/2} = 200$ min. Analogue **3** displays only 10% of the activity of AVP, but it also acts longer. The most potent peptide **6** has almost nine times and 1000 times higher antidiuretic activity than AVP if comparing doses resulting in antidiuresis times of $t_{1/2} = 60$ min and $t_{1/2} = 200$ min, respectively. Moreover, when we look at the antidiuretic potency of the new analogues and that of dDAVP, analogue [Mpa^1,Aic^2,Val^4,D-Arg^8]VP (**6**) has comparable activity and a comparably steep dose−response curve. The previously synthesized Apc^2- or Acc^2-substituted analogues, considered to be models of the new compounds **1**−**5**, exhibited higher antidiuretic potencies than their Aic^2 counterparts, but four of the five Aic^2 peptides are longer acting than their Apc counterparts.

The data presented above show that the combination of Aic^2 modification together with either deamination of position 1 or Val^4 substitution did not result in an increased antidiuretic activity (peptides **2** and **5**). Moreover, Aic^2 substitution combined with the change of configuration of Arg^8 gave analogue **3** with substantially lower antidiuretic activity. These are interesting findings showing that Aic^2 modification by itself is not enhancing the interaction with the receptors, however, it possibly contributes to the prolongation of the effect by making the peptide more stable. A strong support for this hypothesis comes from the comparison of the activity of [Mpa^1,Aic^2,Val^4,D-Arg^8]VP and its Acc^2 counterpart,[13] see Table 2; the Aic^2 modification resulted in substantial prolongation of action. Finally, the moderate antidiuretic activity of **4**, the peptide that contains three modifications, for example, Mpa^1, Aic^2, and D-Arg^8, agree with the previous findings that change of the configuration of Arg in position 8 is important mainly for the increase of the selectivity (decrease of pressor potency).

As far as the pressor test is concerned, it is clear that in comparison to Tyr^2 the Aic^2 analogues, as their Apc^2 counterparts, exhibit either strongly diminished (**1**, **2**) activities or become converted into weak antagonists (**4**, **6**), while peptides **3** and **5** are inactive.

The change of configuration of Arg at position 8 in analogue **1** (analogue **3**, [Aic^2,D-Arg^8]VP) resulted in a 10-fold decreased antidiuretic activity and undetectable pressor activity. [Aic^2,D-Arg^8]VP is thus medium potent and a selective antagonist of oxytocin, while [Apc^2,D-Arg^8]VP is a highly selective V_2 agonist. Significant differences in pharmacological profiles of the two above-mentioned compounds are due to the absence or presence of a bulky aromatic ring at position 2 of [Apc^2,D-Arg^8]-VP and [Aic^2,D-Arg^8]VP, respectively.

In regard to the uterotonic test, the new compounds **1**−**6** have high antioxytocin potency, higher than that of Apc^2- or Acc^2-substituted analogues. This is a very interesting finding, as the analogues have neither a bulky substituent in position 1 nor a D-enantiomer in position 2. Restriction of the movability of the peptide bond between positions 1 and 2 or positions 2 and 3 might be responsible for this effect, however, this may be confirmed only by further studies using physicochemical methods.

As far as the Aic^3 analogues are concerned, this modification alone, or in combination with other substitutions, resulted in complete loss of all the activities studied.

Conclusion

The substitution of Tyr in position 2 with Aic resulted in similar character of changes of the pharmacological properties as substitutions with Apc and Acc. The enlargement of the cyclic side chain moiety, restoring its aromatic character, however, placing the aromatic ring closer to the backbone in comparison to Tyr, led either to retention or to decrease of antidiuretic activity, but all of the analogues had prolonged action. This may be due to the nonproteinogenic character of the amino acid. One of the new peptides, namely, [Mpa^1,Aic^2,Val^4,D-Arg^8]VP, has antidiuretic activity similar to that of dDAVP, thus being one of the most potent antidiuretic peptides reported to date. These analogues exhibit an antioxytocin activity that is higher compared to that of the Apc and Acc analogues.

In our opinion, these peptides deserve further investigations for changes in the three-dimensional shape of their molecules using NMR, CD, and theoretical molecular modeling methods and, optionally, crystallography. These methods may contribute to the explanation of the relations between the backbone structure and the functional group orientation and biological activity.

Experimental Section

Peptide Synthesis. All peptides were obtained by solid-phase synthesis using the *Symphony* synthesizer (Protein Technologies, Inc.) and Fmoc-chemistry on polystyrene resin (Fmoc-Gly TentaGel S RAM, capacity 0.22 mmol/g), according to standard procedures.[16] All amino acid derivatives were purchased from NovaBiochem, except for Fmoc-Aic, which was provided by Neosystem. Mpa(Trt) was obtained as described for Cys(Trt)[17] using 3-mercaptopropionic acid instead of L-cysteine hydrochloride. Mixtures of protected amino acid/TBTU/HOBt/DIEA (1:1:1:2) in DMF or protected amino acid/HATU/HOAt/DIEA (1:1:1:2) in DMF or in a mixture of DMF/NMP (1:1 v/v) containing 1% Triton were used for coupling. The Fmoc deprotection was accomplished using a 20% solution of piperidine in DMF. A solution of TFA/H_2O/TIS/PhOH (92.5:2.5:2.5:2.5) was used for the cleavage of peptides from the TentaGel resin (3 h). Solutions of the cleaved peptides were filtered and evaporated in vacuo to a volume of about 1 mL. Then the peptides were precipitated with diethyl ether to afford crude products. The resulting dithiols were oxidatively cyclized with 0.1 M I_2 in methanol using the standard procedure.

The crude products were desalted on a Sephadex G-15 column and eluted with aqueous acetic acid (30%) at a flow rate of 3 mL/h. After freeze-drying, the fractions comprising the major peak were purified by RP-HPLC (Waters chromatograph equipped with UV detector ($\lambda = 226$ nm), Vydac C_{18} column 15 μm, 7.8 × 300 mm in a gradient running from 20 to 50% [B] in [A] for 90 min for analogues **1**−**5**; from 5 to 35% for 90 min for analogues **7** and **9**; and from 10 to 35% for 80 min for analogues **6** and **8**, at a flow rate of 2.5 mL/min; [A] 0.1% aqueous trifluoroacetic acid (TFA), [B] acetonitrile/0.1% aqueous TFA (80:20, v/v)). The purity and identity of each peptide was determined by analytical HPLC (Vydac C_{18} column 5 μm, 4.6 × 250 mm, linear gradient from 20 to 80% [B] in [A] for analogues **1**−**6** and from 10 to 60% for analogues **7**−**9**, for 30 min at a flow rate of 1 mL/min) and FAB mass spectroscopy (MALDI TOF MS, molecular ion).

Biological Evaluation. Wistar rats were used in all experiments. Handling of the experimental animals was done under supervision of the Ethics Committee of the Academy of Sciences, the law of the Czech Republic No. 246/1992.

The uterotonic test was carried out in vitro on the strips of rat uterus in the absence of magnesium ions.[18,19] Rats in induced estrus by the injection of estrogen 48 h before the experiments were used. After decapitation, the uterine horns were excised, longitudinally cut, placed into a bathing chamber and hooked up to recorder of contractions. The height of the single isometric contraction of a uterine strip was measured. In principle, cumulative dosing was applied in the experiments, that is, doses of standard (in the presence or absence of analogues) or of the analogue were added successively to the uterus in the organ bath in doubling concentrations and at 1 min intervals without the fluid being changed until the maximal response was obtained. The dose−response curves were constructed. Synthetic OT was used as a standard.

The vasopressor test was performed using phenoxybenzamine-treated male rats[20] in urethane anesthesia. AVP was used as a standard. Changes of the arterial blood pressure were registered. Dose−response (single administration into vena femoralis) curves were constructed in the presence and absence of an antagonist. The antagonist was administered 1 min prior to the administration of a standard.

For agonists, the activity was expressed in IU/mg, for antagonists as pA_2. The pA_2 values represent the negative logarithm to the base 10 of the average molar concentration of an antagonist that reduces the response to two *x* units of the agonist to the response to *x* units of the agonist. The volume of distribution in the in vivo experiments is arbitrarily taken as 67 mL/Kg. The values reported are averages of three to five separate experiments. The responses to standard doses of OT or AVP were stable for several hours, without problems with tachyphylaxis. For details, see ref 21.

Tests to assess the antidiuretic or diuretic properties were conducted on conscious male rats in two variations of the modified Burn test.[22,23] In the standard manner with hydrated rats, the animals having fasted for 16 h were weighed and then given tap water through a stomach catheter. The water load was 4% of the body weight. Immediately after the water load, the tested substances (or physiological saline as control) were administered subcutaneously at doses of 0.001−100 nmol/kg. The rats were then placed in individual metabolic cages, and their urine was collected over a 5 h period. The time $t_{1/2}$ in which the rats excreted half the water load was determined and then plotted against the dose. As the dose−response curves were not parallel, such doses were chosen for comparison of the compounds potency yielding $t_{1/2}$ equal to 200 min and the so-called threshold doses yielding $t_{1/2}$ equal to 60 min (equal to the value of $t_{1/2}$ obtained with the physiological solution). On each day of the experiment, 21 rats divided into five groups of four or five animals were administered different doses of different compounds; each dose being tested in two or three independent experiments (different days, different rats). To test for diuretic effects with nonhydrated rats, no water load was given to the fasting animals. As standards, synthetic AVP and dDAVP were used. For details, see ref 23. The results were thus expressed in IU/mg in comparison to AVP (the value 465 IU/mg was taken for AVP for both $t_{1/2} = 60$ min and $t_{1/2} = 200$ min) and in percent of activity when compared to dDAVP (100% for both $t_{1/2} = 60$ min and $t_{1/2} = 200$ min).

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Supporting Information Available: HPLC data for peptides **1**−**9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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