Design of Potent and Selective Agonists for the Human Vasopressin V_{1b} Receptor Based on Modifications of [Deamino-Cys^1]arginine Vasopressin at Position 4

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The glutamine^4 residue in [deamino-Cys^1]arginine vasopressin (dAVP) was replaced by a broad series of aliphatic, aromatic, polar, and charged amino acids to give the following peptides: d[Gly^4]AVP (**1**), d[Ala^4]AVP (**2**), d[Abu^4]AVP (**3**), d[Nva^4]AVP (**4**), d[Nle^4]AVP (**5**), d[Leu^4]AVP (**6**), d[Ile^4]AVP (**7**), d[Thi^4]AVP (**8**), d[Phe^4]AVP (**9**), d[Tyr^4]AVP (**10**), d[Trp^4]AVP (**11**), d[Asn^4]-AVP (**12**), d[Ser^4]AVP (**13**), d[Thr^4]AVP (**14**), d[Dap^4]AVP (**15**), d[Dab^4]AVP (**16**), d[Orn^4]AVP (**17**), d[Lys^4]AVP (**18**), d[Arg^4]AVP (**19**), d[Har^4]AVP (**20**), and d[Glu^4]AVP (**21**). All peptides were synthesized by solid-phase methods using BOC chemistry for all but one peptide (**8**), which required the use of Fmoc chemistry. The binding and functional properties of these position 4 substituted analogues of dAVP (d[X^4]AVP) and the previously reported d[Cha^4]AVP (Derick et al. *Endocrinology* **2002**, *143*, 4655−4664) were evaluated on human arginine vasopressin (AVP) V_{1a}, V_{1b}, and V_2 receptors and on the human oxytocin (OT) receptor expressed in living Chinese hamster ovary (CHO) cells. Binding studies revealed that broad modifications of the fourth residue of dAVP do not significantly alter affinity for the human V_{1b} receptor. Only aromatic (Phe, Tyr, Trp) or negatively charged (Glu) residues reduce V_{1b} affinity. By contrast, the human V_{1a} and more particularly the human V_2 and the OT receptors are more sensitive to many of these modifications. Thus, the replacement of the Gln^4 residue of dAVP by aliphatic (Leu, Cha) or positively charged (Orn, Lys, Arg, Har) amino acids led to analogues exhibiting drastic reductions of their affinity for the human V_{1a}, V_2, and OT receptors. Consequently, in addition to the previously reported d[Cha^4]AVP, peptides **6** and **17**−**20** display excellent selectivities for the human V_{1b} receptor. The key structural requirement responsible for optimal V_{1b} selectivity appears to be the length and branching of the aliphatic side chain of the fourth residue of dAVP. Functional studies performed on CHO cells expressing the different human AVP/OT receptors confirm the V_{1b} selectivity of peptides **6**, **17**, **18**, **20**, and d[Cha^4]AVP. However, d[Arg^4]AVP (**19**), which triggers an excellent coupling between the human V_2 receptor and adenylyl cyclase, was found to exhibit both V_{1b} and V_2 agonism in functional tests. More interestingly, these functional experiments revealed that, depending on the AVP/OT receptor, a given d[X^4]AVP analogue may behave as a full agonist or as a partial agonist. This strongly suggests that the fourth residue of dAVP plays an important role in the coupling between the hormone-receptor complex, the heterotrimeric G protein, and the effectors. In conclusion, the synthesis of these d[X^4]AVP analogues led to the discovery of new V_{1b} agonists with high affinity and greatly enhanced selectivities. Thus, in addition to d[Cha^4]AVP, d[Leu^4]-AVP (**6**), d[Orn^4]AVP (**17**), d[Lys^4]AVP (**18**), and d[Har^4]AVP (**20**) are useful new tools for studying the structure and the function of the human V_{1b} receptor.

Introduction

Arginine vasopressin (AVP), a cyclic nonapeptide neurohormone, first characterized and synthesized in the du Vigneaud laboratory,[1] exerts multiple physiological effects in different target organs by acting on four known G-protein-coupled receptors (GPCRs) (for reviews, see refs 2 and 3). Besides its well-known antidiuretic effects, mediated by its actions on V_2 receptors in the kidney, its vasoconstrictor blood pressure increasing effects mediated by activation of vascular V_{1a} receptors, and its uterine contracting effects mediated by its actions on oxytocin (OT) receptors,[4] AVP also controls CRH mediated ACTH release by its actions on V_{1b} receptors in the anterior pituitary.[4−6] Very recently the V_{1b} receptor has also been shown to regulate anxiety and depression in the rat.[7] At the peripheral level, this receptor isoform is also involved in many secretory processes in the adrenals and in the pancreas.[8,9] The V_{1b}, V_{1a}, V_2, and OT receptors from a variety of species, including human, have been cloned.[10−17] Localization, characterization, and structure function studies on the V_{1a}, V_2, and OT receptors[10−21] as well as extensive physiological studies on the roles of OT and AVP in different species including humans[22−27] have been greatly facilitated by the availability of selective peptide and

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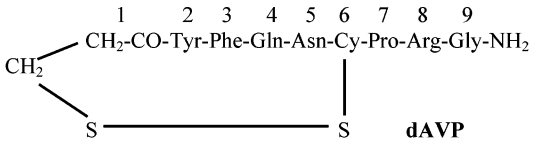
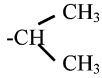
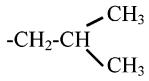
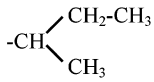
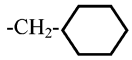
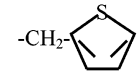
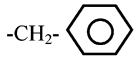
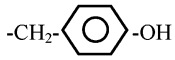
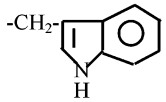
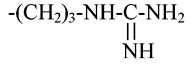
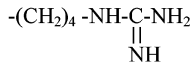
Table 1. 4-Substituted Analogues of Deamino[1]-arginine Vasopressin (dAVP) Synthesized and Evaluated for This Study



dAVP

No	Peptide	Side-chain structure at position 4
	Aliphatic AA	
1	d[Gly^4]AVP	-H
2	d[Ala^4]AVP	$-CH_3$
3	d[Abu^4]AVP	$-CH_2-CH_3$
4	d[Nva^4]AVP	$-(CH_2)_2-CH_3$
5	d[Nle^4]AVP	$-(CH_2)_3-CH_3$
*	d[Val^4]AVP	
6	d[Leu^4]AVP	
7	d[Ile^4]AVP	
*	d[Cha^4]AVP	
8	d[Thi^4]AVP	
	Aromatic AA	
9	d[Phe^4]AVP	
10	d[Tyr^4]AVP	
11	d[Trp^4]AVP	

No	Peptide	Side-chain Structure of position 4
	Polar AA	
	dAVP	$-CH_2-CH_2-CO-NH_2$
12	d[Asn^4]AVP	$-CH_2-CO-NH_2$
13	d[Ser^4]AVP	$-CH_2-OH$
14	d[Thr^4]AVP	
	Charged AA	
15	d[Dap^4]AVP	$-CH_2-NH_2$
16	d[Dab^4]AVP	$-(CH_2)_2-NH_2$
17	d[Orn^4]AVP	$-(CH_2)_3-NH_2$
18	d[Lys^4]AVP	$-(CH_2)_4-NH_2$
19	d[Arg^4]AVP	
20	d[Har^4]AVP	
21	d[Glu^4]AVP	$-CH_2-CH_2-COOH$

non-peptide agonists, antagonists, and radioligands for the V_{1a}, V_2, and OT receptors.[4,22,23,28−33] It should be noted also that besides their value as research tools, some V_{1a}, V_2, and OT receptor agonists and antagonists have found therapeutic use;[34−37] others are currently in clinical trials.[30,32,33] By constrast, structure−function studies on the V_{1b} receptor, physiological studies on the role of AVP in mediating V_{1b} receptor responses, and possible therapies based on these studies have been severely hampered by the lack of selective ligands for the V_{1b} receptor.[4,23,33] The design of selective ligands for the V_{1a}, V_2, and OT receptors was greatly aided by the availability of well characterized standard bioassays based on the vasopressor, antidiuretic, and oxytocic actions of AVP and OT in the rat.[29,38] The lack of a standard in vivo pituitary bioassay for the V_{1b} receptor has greatly hindered the design of selective ligands for the V_{1b} receptor.

The availability of cloned V_{1b} receptors from different species, including human,[16,17] has led to two recent breakthroughs in the decades-long search for selective V_{1b} receptor ligands: a selective non-peptide V_{1b} antagonist reported by the Sanofi-Synthelabo laboratory[7,33,39,40] and a selective peptide V_{1b} agonist reported by our laboratories.[40,41] The first selective agonist for the human V_{1b} receptor is [1-deamino-4-cyclohexyl-

Table 2. Binding Properties of d[X^4]AVP Analogues for Human Vasopressin and Oxytocin Receptors[a]

		affinity (K_i) (nM)				hV_{1b}-R selectivity index (SI = $K_i[V_X]/(K_I[V_{1b}])$)		
compd	peptide	hV_{1b}-R	hV_{1a}-R	hV_2-R	hOT-R	V_{1b}/V_{1a}	V_{1b}/V_2	V_{1b}/OT
	AVP[b]	0.68 ± 0.01[c]	1.1 ± 0.1[c]	1.2 ± 0.2[c]	1.7 ± 0.5	1.6	1.8	2.5
	dAVP[c,d]	0.37 ± 0.06[c]	3.8 ± 0.1[c]	5.0 ± 0.4[c]	5.0	10.2	13.5	13.5
	dDAVP[e,f]	5.8 ± 1.3	62.4 ± 17.6[f]	23.3 ± 2.6		11	4	
	d[D-3-Pal^2]AVP[c,g]	13.8 ± 0.6[c]	34.2 ± 0.2[c]	9600 ± 1800[c]		2	695	
				Aliphatic Amino Acids				
1	d[Gly^4]AVP	22.9 ± 2.1	276 ± 18	554 ± 71	556 ± 77	12	24	24
2	d[Ala^4]AVP	6.5 ± 0.8	24.8 ± 5.7	13.7 ± 0.9	48.7 ± 25.2	4	2	7
3	d[Abu^4]AVP[h]	1.2 ± 0.2	19.7 ± 1.3	2.1 ± 0.3	224 ± 38	16	2	187
4	d[Nva^4]AVP	0.78 ± 0.18	22.5 ± 5.2	24.1 ± 6.1	131 ± 32	29	31	169
5	d[Nle^4]AVP	0.43 ± 0.09	28.1 ± 5.6	117 ± 21	143 ± 6	65	273	331
	d[Val^4]AVP[c,i]	0.29 ± 0.09[c]	11.4 ± 1.6[c]	1.2 ± 0.1[c]	136	39	4	468
6	d[Leu^4]AVP	0.23 ± 0.03	44.1 ± 6.1	245 ± 22	211 ± 26	191	1065	917
7	d[Ile^4]AVP	0.42 ± 0.05	16.8 ± 3.0	9.9 ± 2.1	102 ± 27	40	24	243
	d[Cha^4]AVP[c,j]	1.2 ± 0.1[c]	151 ± 11[c]	750 ± 120[c]	240 ± 50	125	625	200
8	d[Thi^4]AVP	4.8 ± 1.5	63.3 ± 4.4	4416 ± 1440	369 ± 37	13	920	77
				Aromatic Amino Acids				
9	d[Phe^4]AVP	9.5 ± 2.4	80 ± 15	1067 ± 197	164 ± 65	8	112	17
10	d[Tyr^4]AVP	14.8 ± 0.9	36.9 ± 3.0	621 ± 24	126 ± 7	2	42	9
11	d[Trp^4]AVP	7.8 ± 2.4	75 ± 13	80 ± 17	223 ± 88	10	10	29
				Polar Amino Acids				
12	d[Asn^4]AVP	1.5 ± 0.4	1.5 ± 0.2	50 ± 9	25.6 ± 7.9	1	33	17
13	d[Ser^4]AVP	2.0 ± 0.3	13.0 ± 1.9	24.7 ± 3.9	206 ± 60	6	12	103
14	d[Thr^4]AVP[k]	1.0 ± 0.3	2.9 ± 0.9	1.5 ± 0.9	91 ± 10	3	2	91
				Charged Amino Acids				
15	d[Dap^4]AVP	3.4 ± 0.7	19.7 ± 4.4	36.8 ± 0.8	1033 ± 320	6	11	304
16	d[Dab^4]AVP	12.8 ± 3.3	36.3 ± 1.3	568 ± 51	482 ± 189	3	44	38
17	d[Orn^4]AVP	0.49 ± 0.03	88 ± 17	1125 ± 33	326 ± 13	179	2295	665
18	d[Lys^4]AVP	1.8 ± 0.2	463 ± 94	11170 ± 1280	1429 ± 233	257	6206	794
19	d[Arg^4]AVP[l]	0.37 ± 0.02	54.2 ± 6.6	131 ± 22	1083 ± 95	147	354	2927
20	d[Har^4]AVP	0.52 ± 0.06	48.2 ± 17	1386 ± 97	1364 ± 31	93	2665	2623
21	d[Glu^4]AVP	156 ± 5	436 ± 33	1090 ± 122	664 ± 149	3	7	4

[a] Binding assays were performed on plasma membranes from CHO cells stably transfected with the human VP/OT receptors (see Experimental Section). K_i values are the mean ± SEM of at least three independent experiments each performed in triplicate. For each analogue, the hV_{1b}-R selectivity index (SI) was calculated as follows: SI = (K_i analogue for V_X-R)/(K_i analogue for V_{1b}-R), where V_X-R is the hV_{1a}, hV_2, or hOT receptor. [b] Original synthesis reported in ref 1. Repeated here as in ref 56. [c] Data from ref 41. [d] Original synthesis reported in ref 42. [e] Original synthesis reported in ref 65. [f] Data from ref 66a. [g] Original synthesis reported in ref 64. [h] Original synthesis reported in ref 45. [i] Original synthesis reported in ref 43. [j] Original synthesis reported in ref 41. [k] Original synthesis reported in ref 44. [l] Original synthesis reported in ref 46.

alanine]arginine vasopressin (d[Cha^4]AVP).[41] This analogue of [1-deamino]arginine vasopressin (dAVP),[42] designed by replacing the Gln^4 residue in dAVP by Cha^4, exhibits a nanomolar affinity for the human V_{1b} receptor and greatly reduced affinities for the human V_{1a}, V_2, and OT receptors.[41] These properties are in striking contrast to those of AVP and dAVP, both of which exhibit high affinities for all four human V_{1b}, V_{1a}, V_2, and OT receptors[41] (Table 2). Clearly, the replacement of the Gln^4 residue in dAVP by the much more lipophilic amino acid cyclohexylalanine to give d[Cha^4]AVP led to an excellent retention of affinity for the V_{1b} receptor and dramatic reductions in affinities for the V_{1a}, V_2, and OT receptors, thus giving rise to a highly selective ligand for the human V_{1b} receptor[41] (Table 2). An examination of the affinities of d[Val^4]AVP, previously reported to be a highly potent antidiuretic agonist in the rat,[43] in human receptor assays[41] had also pointed to the importance of lipophilicity at position 4 in leading to an enhancement of human V_{1b}/V_{1a} selectivity[41] (Table 2). d[Val^4]AVP, while retaining high affinities for both the human V_{1b} and V_2 receptors, exhibits a slightly diminished affinity for the human V_{1a} receptor. In light of our findings on the human receptor affinities for both d[Cha^4]AVP and d[Val^4]AVP, we were eager to explore the effects on human V_{1b} receptor affinity and selectivity of other position 4 modifications in dAVP. The structure of dAVP is given in Table 1. In this study, the Gln^4 residue in dAVP has been replaced by a broad series of aliphatic, aromatic, polar, and charged amino acids (Table 1). The following amino acids were selected as Gln^4 replacements in dAVP; (aliphatic) Gly, Ala, Abu, norvaline (Nva), norleucine (Nle), Leu, Ile, thienylalanine (Thi); (aromatic) Phe, Tyr, Trp; (polar) Asn, Ser, Thr; (charged) diaminopropionic acid (Dap), diaminobutyric acid (Dab), Lys, Arg, homoarginine (Har), and Glu. We now report the synthesis, receptor affinities, and selectivities for the human AVP and OT V_{1b}, V_{1a}, V_2, and OT receptors of the 21 position 4 modified analogues of dAVP given in Table 1. Three of these peptides, d[Abu^4]AVP (**3**), d[Thr^4]AVP (**14**), and d[Arg^4]AVP (**19**) had been previously synthesized and shown to be potent antidiuretic agonists in rat bioassays.[44−46] Functional studies were also carried out on the six most selective of these 4-substituted analogues of dAVP (Table 3). Preliminary receptor binding affinities for 17 of these peptides have been reported.[47]

Peptide Synthesis

The synthesis of the free peptides **1**−**21** (Table 4) was carried out utilizing two solid-phase methods.[48−53] For the synthesis of peptides **1**−**7** and **9**−**21**, the Merrifield

Table 3. Functional Properties of V_{1b}-Specific d[X^4]AVP Analogues for Human Vasopressin and Oxytocin Receptors[a]

		InsPs assays						cAMP assays	
		hV_{1b}-R		hV_{1a}-R		hOT-R		hV_2-R	
compd	peptide	K_{act} (nM)	E (%)	K_{act} (nM)	E (%)	K_{act} (nM)	E (%)	K_{act} (nM)	E (%)
	AVP	2.0 ± 0.6[b]	100	0.7 ± 0.1	100	5−12	50	1.0 ± 0.2	100
	d[Cha^4]AVP	1.0 ± 0.2[b]	95 ± *2*	127 ± 54	22 ± *3*	n.m.	0	177 ± 28	73 ± 8
6	d[Leu^4]AVP	0.8 ± 0.1	95 ± *2*	100 ± 21	56 ± *3*	155 ± 58	17 ± *1*	70 ± 9	132 ± 2
17	d[Orn^4]AVP	1.6 ± 0.2	92 ± *4*	62 ± 15	69 ± *3*	184 ± 107	25 ± *2*	74 ± 25	104 ± 4
18	d[Lys^4]AVP	5.4 ± 1.0	92 ± *4*	294 ± 68	46 ± *3*	n.m.	0	2065 ± 117	70 ± 4
19	d[Arg^4]AVP	1.7 ± 0.4	84 ± *1*	25 ± 9	69 ± *3*	663 ± 47	6 ± *1*	6.9 ± 2.4	90 ± 7
20	d[Har^4]AVP	1.3 ± 0.1	93 ± *4*	28 ± 7	79 ± *2*	n.m.	0	419 ± 97	95 ± 9

[a] CHO cells expressing the different human AVP/OT receptors were incubated with or without increasing amounts of the analogues to be tested (see Experimental Section). The activity constant (K_{act}) of each d[X^4]AVP analogue was deduced from functional dose−response curves illustrated in Figure 2. The maximal efficiency (E) is the maximal response induced by a given d[X^4]AVP analogue expressed as percent of the maximal response induced by 1 μM AVP (V_{1a}-R, V_{1b}-R, V_2-R) or OT (hOT-R). Data are the mean ± SEM of three distinct experiments each performed in triplicate. n.m.: nonmeasurable. [b] Preliminary data given in ref 41.

Table 4. Physicochemical Properties of Free Peptides **1**−**21**

	peptide	yield (%)[a]	$[\alpha]_D^{25}$ (c 0.1, 1 N AcOH) (deg)	TLC, R_f [b] a	b	c	d	e	HPLC[c] t_r (min)	formula	MW calcd	MW found (ESMS)
1	d[Gly^4]AVP	56.9	−75.0	0.27	0.17	0.24		0.44	14.5*	$C_{43}H_{59}0_{11}N_{13}S_2$	998.2	998.5
2	d[Ala^4]AVP	66.1	−76.0	0.25	0.17	0.33		0.40	14.8*	$C_{44}H_{61}O_{11}N_{13}S_2$	1012.2	1012.5
3	d[Abu^4]AVP[d]	31.0	−101.0	0.23	0.16	0.30	0.36	0.51	31.0	$C_{45}H_{63}O_{11}N_{13}S_2$	1026.2	1026.4
4	d[Nva^4]AVP	38.4	−116.0	0.30	0.24	0.53		0.50	16.4*	$C_{46}H_{65}O_{11}N_{13}S_2$	1040.3	1040.5
5	d[Nle^4]AVP	45.0	−104.0	0.29	0.20	0.37	0.40	0.56	28.5	$C_{47}H_{67}O_{11}N_{13}S_2$	1054.3	1054.4
6	d[Leu^4]AVP	48.5	−97.0	0.42	0.38	0.62		0.69	28.2	$C_{47}H_{67}O_{11}N_{13}S_2$	1054.3	1054.4
7	d[Ile^4]AVP	23.8	−149.0	0.27	0.19	0.34	0.39	0.54	26.5	$C_{47}H_{67}O_{11}N_{13}S_2$	1054.3	1054.4
8	d[Thi^4]AVP[e]	9.2	−88.0	0.28	0.20	0.35	0.40	0.55	28.6	$C_{48}H_{63}O_{11}N_{13}S_3$	1094.3	1094.4
9	d[Phe^4]AVP	51.6	−98.0	0.37	0.20	0.33		0.50	29.7	$C_{50}H_{65}O_{11}N_{13}S_2$	1088.3	1088.5
10	d[Tyr^4]AVP	69.2	−88.0	0.31	0.20	0.33		0.44	16.0*	$C_{50}H_{65}O_{12}N_{13}S_2$	1104.3	1104.5
l1	d[Trp^4]AVP	61.0	−84.0	0.32	0.26	0.35		0.50	18.2*	$C_{52}H_{66}O_{11}N_{14}S_2$	1127.3	1127.5
12	d[Asn^4]AVP	37.2	−78.0	0.24	0.10	0.20	0.37	0.46	19.9*	$C_{45}H_{62}O_{12}N_{14}S_2$	1055.2	1055.5
13	d[Ser^4]AVP	29.8	−128.0	0.27	0.21	0.45		0.49	21.6	$C_{44}H_{61}O_{12}N_{13}S_2$	1028.2	1028.4
14	d[Thr^4]AVP[d]	65.8	−112.0	0.35	0.16	0.42		0.52	22.0	$C_{45}H_{63}O_{12}N_{13}S_2$	1042.2	1042.4
15	d[Dap^4]AVP	36.8	−74.0	0.21	0.03	0.10	0.35	0.31	13.6	$C_{44}H_{62}O_{11}N_{14}S_2$	1027.2	1027.5
16	d[Dab^4]AVP	44.0	−76.0	0.16		0.05	0.33	0.15	13.7*	$C_{45}H_{64}O_{11}N_{14}S_2$	1041.2	1041.5
17	d[Orn^4]AVP	35.1	−78.0	0.15		0.05	0.32	0.14	13.3*	$C_{46}H_{66}O_{11}N_{14}S_2$	1055.3	1055.5
18	d[Lys^4]AVP	33.4	−94.0	0.13		0.06	0.30	0.16	19.7*	$C_{47}H_{68}O_{11}N_{14}S_2$	1069.2	1069.5
19	d[Arg^4]AVP[d]	62.4	−80.0	0.23		0.10	0.36	0.05	13.7*	$C_{47}H_{68}O_{11}N_{16}S_2$	1097.3	1097.5
20	d[Har^4]AVP	67.8	−77.0	0.17		0.14	0.37	0.10	13.9	$C_{48}H_{70}O_{11}N_{16}S_2$	1111.3	1111.6
21	d[Glu^4]AVP	33.8	−88.0	0.22	0.13	0.21	0.35	0.50	20.9*	$C_{46}H_{63}O_{12}N_{13}S_2$	1072.2	1071.4

[a] Yields are based on the protected peptide in the reduction−reoxidation step in each case and are uncorrected for acetic acid and water content. [b] Solvent systems and conditions are given in the Experimental Section. [c] All peptides were at least 95% pure. For elution, a linear gradient from 90:10 to 30:70 (0.05% aqueous TFA/0.05% TFA in MeCN) over 60 min (when unmarked) or 30 min (marked with ∗) with a flow rate of 1.0 mL/min was applied. [d] Peptides **3** (d[Abu^4]AVP),[45] **14** (d[Thr^4]AVP),[44] and **19** (d[Arg^4]AVP)[46] were resynthesized for this investigation. [e] Synthesized using Fmoc strategy and Rink amide resin (see Experimental Section).

solid-phase method[48−50] using the Boc approach with the modifications previously described[54−56] was used. Peptide **8** was synthesized using the Rink amide resin[52] by the Fmoc approach.[51,53] Starting from Boc-Gly resin, the protected precursors **I**−**VII** and **IX**−**XXI** (Table 5) were synthesized. HCL/AcOH (1 M) was used in all the deprotection steps. Neutralizations were carried out with 10% Et_3N/CH_2Cl_2 in all the steps except the last three steps for peptides **XVI** and **XXI** (after the incorporation of Boc-Dab(Fmoc) and Boc-Glu(Fm), respectively) in which 10% DIPEA/CH_2Cl_2 was employed. All couplings were performed by DCC/HOBT,[57] except for Boc-Asn, which was incorporated as its *p*-nitrophenyl ester [58,59] in DMF. Cleavage from the acylpeptide resin was by ammonolysis in methanol with DMF extraction[54,59] to give the protected peptide amides **I**−**VII** and **IX**−**XXI** (Table 5). Na in NH_3[1,60] was used to deblock each protected precursor as previously described.[54−56] As noted above, the Rink amide resin[52] and the Fmoc strategy[51,53] was used for the synthesis of peptide **8** (Table 4). All deprotections were carried out with 20% piperidine/DMF.[51] Coupling reactions were performed primarily by DCC/HOBt in DMF [57] except for Fmoc-Asn, which was incorporated as its *p*-nitrophenyl ester[61,71] in DMF. The simultaneous cleavage of the peptide from the resin and its deprotection was mediated by Reagent K.[53] The resulting disulfydryl compounds from the Reagent K treatment and the Na/liquid NH_3 treatment were oxidatively cyclized with $K_3[Fe(CN)_6]$ using the modified reverse procedure.[62] The free peptides were desalted and purified by gel filtration on Sephadex G-15 and Sephadex LH-20 mainly in a two-step procedure[63] using 50% AcOH and 2 M AcOH as eluents, respectively, as previously described.[54−56] When necessary, an additional purification on Sephadex G-15 or/and Sephadex LH-20 with 0.2 M AcOH as eluent was carried out. The purity of the free peptides **1**−**21** (Table 4) was checked by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and electron spray mass spectrometry (ESMS).

Results

Affinities and Selectivities of d[X4]AVP Analogues for Vasopressin and Oxytocin Receptors

Table 5. Physicochemical Properties of Protected Peptides **I**−**VII** and **IX**−**XXI**

compd	peptide[a]	yield (%)[b]	mp (°C)	$[\alpha]_D^{25}$ (*c* 1.0, DMF) (deg)	TLC,[c] R_f a	b	c	d
I	Mpr(Meb)-Tyr(Bzl)-Phe-Gly-Asn-Cys(Mob)-Pro-Arg(Tos)-Gly-NH_2	92.0	175−177	−37.2	0.65	0.54	0.66	0.65
II	Mpr(Meb)-Tyr(Bzl)-Phe-Ala-Asn-Cys(Mob)-Pro-Arg(Tos)-Gly-NH_2	88.2	208−210	−32.4	0.79	0.60	0.72	0.69
III	Mpr(Bzl)-Tyr(Bzl)-Phe-Abu-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	89.3	225−226	−32.3	0.70	0.56	0.73	0.76
IV	Mpr(Bzl)-Tyr(Bzl)-Phe-Nva-Asn-Cys(Mob)-Pro-Arg(Tos)-Gly-NH_2	71.4	225−227	−34.9	0.68	0.56	0.71	0.75
V	Mpr(Bzl)-Tyr(Bzl)-Phe-Nle-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	93.2	223−224	−37.1	0.73	0.57	0.73	0.76
VI	Mpr(Bzl)-Tyr(Bzl)-Phe-Leu-Asn-Cys(Mob)-Pro-Arg(Tos)-Gly-NH_2	81.8	228−230	−36.9	0.70	0.63	0.71	0.71
VII	Mpr(Bzl)-Tyr(Bzl)-Phe-Ile-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	77.2	235−237	−35.5	0.72	0.58	0.72	0.75
IX	Mpr(Bzl)-Tyr(Bzl)-Phe-Phe-Asn-Cys(Mob)-Pro-Arg(Tos)-Gly-NH_2	87.2	218−220	−34.3	0.77	0.51	0.72	0.70
X	Mpr(Meb)-Tyr(Bzl)-Phe-Tyr(Bzl)-Asn-Cys(Mob)-Pro-Arg(Tos)-Gly-NH_2	87.9	238−240	−39.9	0.67	0.63	0.69	0.59
XI	Mpr(Meb)-Tyr(Bzl)-Phe-Trp-Asn-Cys(Mob)-Pro-Arg(Tos)-Gly-NH_2	91.8	205−207	−31.2	0.80	0.63	0.68	0.74
XII	Mpr(Meb)-Tyr(Bzl)-Phe-Asn-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	64.2	215−217	−31.4	0.67	0.52	0.72	0.72
XIII	Mpr(Bzl)-Tyr(Bzl)-Phe-Ser(Bzl)-Asn-Cys(Mob)-Pro-Arg(Tos)-Gly-NH_2	80.4	202−204	−26.1	0.86	0.75	0.73	0.72
XIV	Mpr(Bzl)-Tyr(Bzl)-Phe-Thr(Bzl)-Asn-Cys(Mob)-Pro-Arg(Tos)-Gly-NH_2	76.6	216−218	−19.4	0.73	0.59	0.72	0.77
XV	Mpr(Bzl)-Tyr(Bzl)-Phe-Dap(Z)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	78.2	210−212	−31.8	0.75	0.67	0.75	0.84
XVI	Mpr(Bzl)-Tyr(Bzl)-Phe-Dab-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	92.1	196−198	−30.8	0.51	0.43	0.56	0.62
XVII	Mpr(Bzl)-Tyr(Bzl)-Phe-Orn(2Cl-Z)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	86.3	213-215	−27.4	0.74	0.65	0.75	0.81
XVIII	Mpr(Meb)-Tyr(Bzl)-Phe-Lys(2Cl-Z)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	91.6	203−205	−28.4	0.76	0.59	0.75	0.78
XIX	Mpr(Meb)-Tyr(Bzl)-Phe-Arg(Tos)-Asn-Cys(Mob)-Pro-Arg(Tos)-Gly-NH_2	84.5	170−172	−48.6	0.59	0.54	0.62	0.63
XX	Mpr(Meb)-Tyr(Bzl)-Phe-Har(Tos)-Asn-Cys(Mob)-Pro-Arg(Tos)-Gly-NH_2	79.6	178−180	−25.2	0.69	0.53	0.54	0.66
XXI	Mpr(Meb)-Tyr(Bzl)-Phe-Glu-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2	98.4	193−194	−27.4	0.69	0.54	0.70	0.73

[a] The protected peptides **I**−**VII** and **IX**−**XXI** are the immediate protected precursors for the peptides **1**−**7** and **9**−**21** given in Table 4. [b] Yields are based on the amino acid content of the resin. [c] Solvent systems are described in the Experimental Section.

(Table 2). We report here the pharmacological properties of 21 4-substituted analogues of dAVP (affinities and selectivities) for the human V_{1b} (hV_{1b}-R), V_{1a} (hV_{1a}-R), V_2 (hV_2-R), and OT (hOT-R) receptors. The affinity and selectivity data for the d[X^4]AVP analogues of dAVP are summarized in Table 2. Previously reported data for AVP, dAVP, d[Val^4]AVP, and d[Cha^4]AVP[41] are also presented in these tables. The human V_{1b}, V_{1a}, and V_2 receptor affinity data for d[D-3-pyridylalanine]^{2}arginine vasopressin (d[D-3-Pal^2]AVP)[41,64] and for [1-deamino-8-D-arginine]vasopressin (dDAVP)[65,66] are also presented in Table 2.

Examination of the receptor affinities for all 21 new d[X^4]AVP analogues showed that, with the exception of the Glu^4 analogue (**21**), the hV_{1b}-receptor tolerated a broad latitude of structural change at position 4 in dAVP while still retaining the ability to bind to these peptide ligands. Thus, six of the analogues that have a variety of aliphatic amino acids at position 4 have affinities for the hV_{1b}-R ranging from 0.23 to 1.2 nM. Four of the analogues that have basic amino acids at position 4 also exhibited hV_{1b}-R binding affinities in the nanomolar range. Similar results were obtained when the Gln^4 residue of dAVP is replaced by polar residues such as Thr, Asn, and Ser. By contrast, the three analogues with the aromatic amino acids Phe, Tyr, and Trp at position 4 bind to the hV_{1b}-receptor with reduced affinities (K_i values ranging from 7.8 to 14.8 nM). All of the position 4 analogues of dAVP exhibit significantly diminished affinities for the hV_{1a}, hV_2, and hOT receptors relative to those of AVP and dAVP. A number of these peptides combine a high affinity for the hV_{1b}-receptor with greatly reduced affinities for the human hV_{1a}, hV_2, and hOT receptors. Consequently, in addition to the previously reported d[Cha^4]AVP,[41] five of the peptides reported here, d[Leu^4]AVP (**6**), d[Orn^4]AVP (**17**), d[Lys^4]AVP (**18**), d[Arg^4]AVP (**19**), and d[Har^4]AVP (**20**) exhibit striking gains in selectivity for the hV_{1b}-receptor (Figure 1 and Table 2).

Functional Properties of Position 4 Substituted Analogues of dAVP (Table 3). To further analyze the agonist character of the hV_{1b}-R, analogues that exhibit the best affinities and selectivities, namely, d[Cha^4]AVP, d[Leu^4]AVP, d[Orn^4]AVP, d[Lys^4]AVP, d[Arg^4]AVP, and d[Har^4]AVP (Table 2), we tested their abilities to stimulate phospholipase C (PLC) (inositol phosphates accumulation) and adenylyl cyclase (cyclic AMP accumulation) on CHO cells expressing the hV_{1a}, hV_{1b}, hOT, and hV_2 receptors (see Experimental Section). On cells expressing the hV_{1b}-R, all the peptides dose-dependently stimulated InsPs accumulation with a nanomolar activity constant (K_{act}) (see Table 3). Moreover, like AVP, they maximally stimulated InsPs accumulation to the same maximal extent (maximal efficiency, *E*, ranging from 84% to 95% of the maximal response induced by AVP). Such results indicate that all the compounds tested behave as potent agonists for the hV_{1b}-R isoform. These six analogues also stimulated InsPs accumulation on CHO cells expressing the hV_{1a}-R (Figure 2). However, they exhibit much weaker agonism for the hV_{1a}-R than for the hV_{1b}-R. Three of them, d[Leu^4]AVP, d[Cha^4]AVP, and d[Lys^4]AVP, behave as partial agonists (Figure 2). d[Orn^4]AVP, d[Arg^4]AVP, and d[Har^4]AVP displayed the best coupling properties between the hV_{1a}-R and PLC (maximal efficiency, *E*, ranging from 69% to 79%, Table 3). By comparison, d[Cha^4]AVP and d[Leu^4]AVP (**6**) were less efficient (maximal efficiency of 22% and 56%, respectively, Table 3). Concerning the hOT-R, only d[Leu^4]AVP and d[Orn^4]-AVP weakly but significantly stimulated InsPS accumulation (Table 3). The other compounds had no significant effect. Finally, the six compounds tested also dose-dependently stimulated cAMP accumulation on CHO cells expressing the hV_2-R (Figure 2). Surprisingly, the K_{act} values obtained were lower compared to their binding K_i values determined on CHO plasma membranes expressing hV_2-R (compare data from Tables 2 and 3 for d[Leu^4]AVP, d[Orn^4]AVP, d[Lys^4]AVP, d[Arg^4]AVP, and d[Har^4]AVP). The most striking gains in the coupling properties were observed for analogues with a basic amino acid at position 4. Thus, d[Orn^4]AVP and d[Arg^4]AVP have K_i/K_{act} ratios of 15.2 and 18.9,

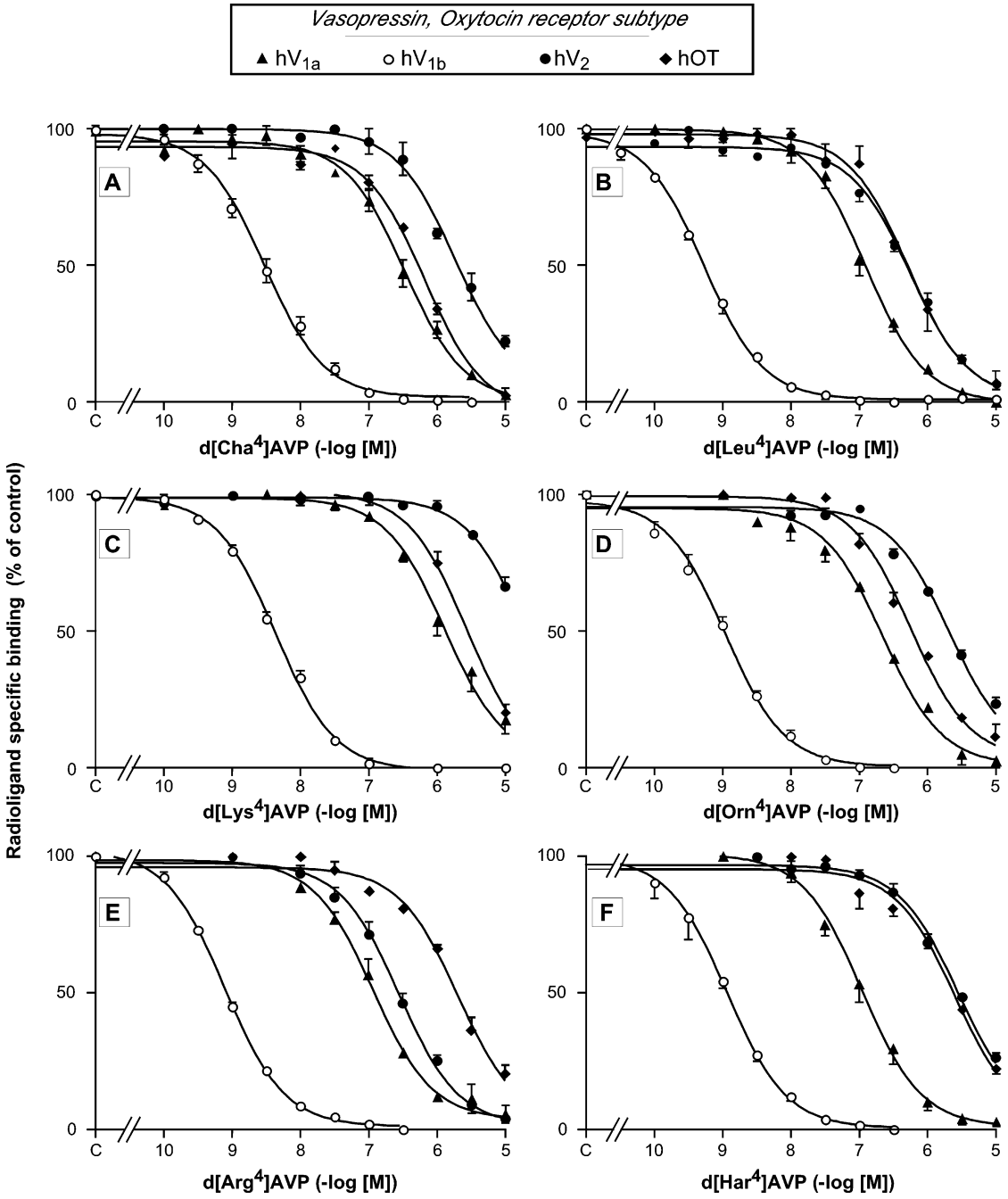


Figure 1. Binding properties of some d[X^4]AVP analogues for human AVP/OT receptor isoforms. The binding properties of the d[X^4]AVP analogues were determined by competition experiments. Plasma membranes from CHO cells expressing the different human AVP/OT receptors subtypes were incubated with 0.5−1.2 nM [^{3}H]AVP (hV$_{1a}$, hV$_{1b}$, and hV$_2$ receptors) or 150pM [^{125}I]-OTA (hOT receptors) in the presence or absence (control = C) of increasing concentrations of the unlabeled d[X^4]AVP analogue as described in the Experimental Section. Nonspecific and total binding were determined with or without 1 μM unlabeled AVP (hV$_{1a}$, hV$_{1b}$, and hV$_2$ receptor) or OT (hOT receptors). Results, expressed as percent of the maximal control specific binding, are the mean ± SEM of at least three independent experiments each performed in triplicate.

respectively. d[Cha^4]AVP, d[Leu^4]AVP, d[Lys^4]AVP, and d[Har^4]AVP also displayed a good coupling but with lower K_i/K_{act} ratios ranging from 3.3 to 5.4. All six analogues are full hV$_2$-R agonists. Each of their maximal efficiencies relative to AVP is greater than 70% and, surprisingly, in the case of d[Leu^4]AVP, reaches 132% (Figure 2 and Table 3).

Discussion

We previously showed that substitution of the Gln^4 residue of dAVP by a cyclohexylalanine residue leads to a peptide, d[Cha^4]AVP, with high affinity for the hV$_{1b}$-receptor and good selectivity with respect to the other AVP/OT receptor isoforms.[41] In this study, we have examined the effects of a variety of other amino acid substitutions at position 4 in dAVP on hV$_{1b}$-R affinity, selectivity, and ability to stimulate InsPs and cAMP accumulation.

Influence of the Chemical Nature of the Amino Acid at Position 4 in dAVP on Human AVP/OT Receptor Affinities (Tables 1 and 2). AVP exhibits a high affinity for all the human V$_{1b}$, V$_{1a}$, V$_2$, and OT

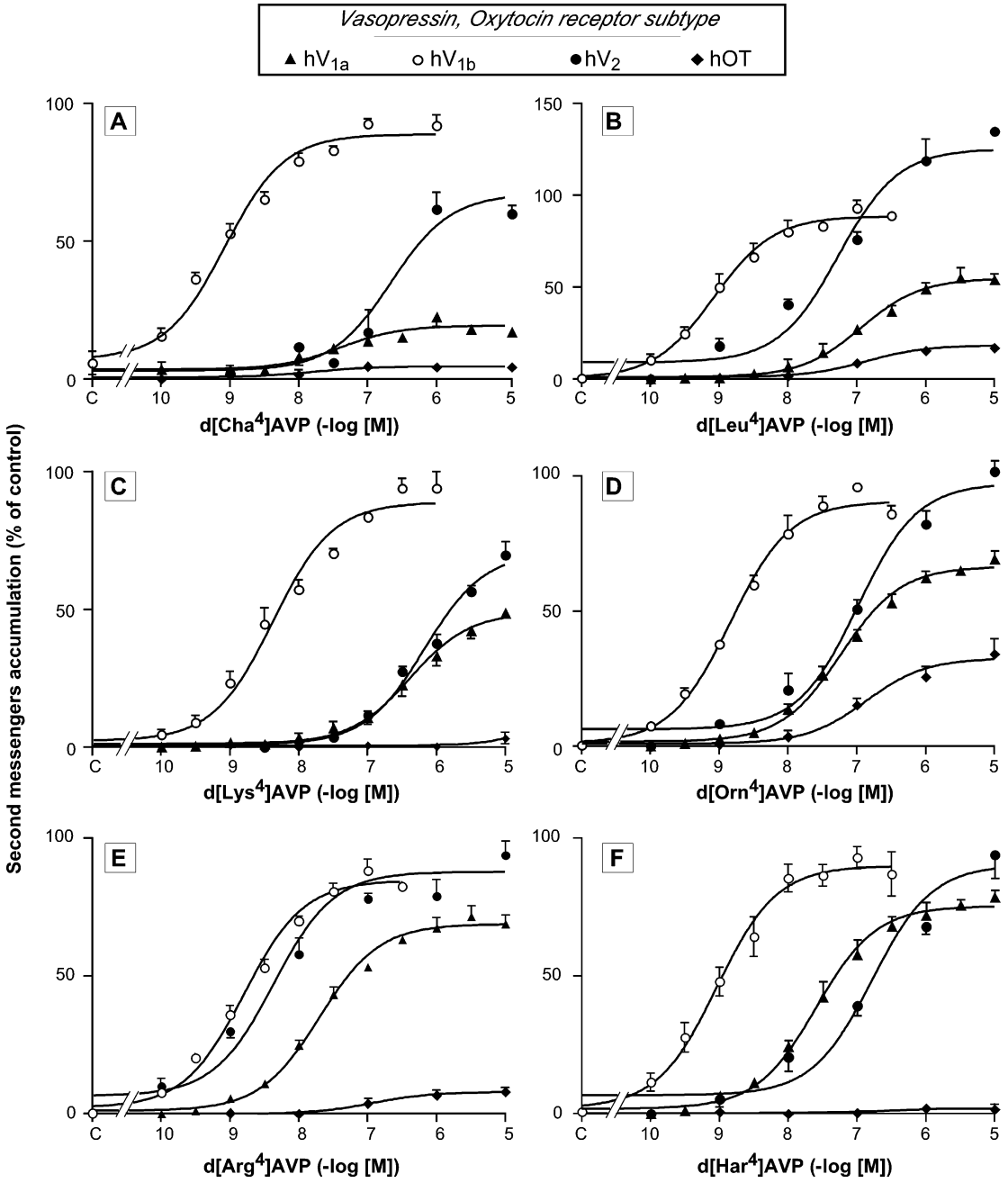


Figure 2. Agonist properties of some d[X^4]AVP analogues for human AVP/OT receptor isoforms. CHO cells that express the different human AVP/OT receptor isoforms were incubated at 37 °C in an HBS medium with or without (C) increasing concentrations of d[X^4]AVP analogues. Second messenger accumulation (inositol phosphates from CHO cells expressing the hV_{1a}, hV_{1b}, and hOT receptors or cyclic AMP from CHO cells expressing the hV_2 receptor) was then determined as described in the Experimental Section. Results expressed as percent of the maximal second messenger accumulation induced by 1 μM AVP (hV_{1a}, hV_{1b}, and hV_2 receptors) or OT (hOT receptors) are the mean ± SEM of at least three independent experiments each performed in triplicate.

receptors (Table 2). Deamination of the Cys^1 residue of the natural hormone leads to dAVP, a peptide exhibiting an enhanced affinity for the hV_{1b}-receptor and slightly diminished affinities for the hV_{1a}, hV_2, and hOT receptors relative to those exhibited by AVP[41] (Table 2). For most of the 21 d[X^4]AVP analogues reported here, replacement of the Gln^4 residue of dAVP by a broad series of aliphatic, aromatic, polar, and charged amino acids led to analogues that, with a few exceptions, exhibit excellent retention of hV_{1b}-R affinities. Thus, with the exception of the Gly^4, Ala^4, Glu^4, and the three aromatic position 4 analogues, all the remaining compounds tested display very low nanomolar affinities for the hV_{1b}-R. These findings indicate that the hV_{1b}-receptor is highly tolerant of structural changes at position 4 in dAVP, especially with aliphatic and positively charged amino acids. Only the presence of a negatively charged residue at position 4 (d[Glu^4]AVP) results in a drastic loss of hV_{1b}-R affinity. By contrast, all 21 d[X^4]AVP analogues exhibit significant losses of their affinities for the hOT-R relative to dAVP (see Table 2). Likewise, with the exception of the Asn^4 and Thr^4 peptides, all of the remaining 19 d[X^4]AVP analogues exhibited losses of hV_{1a}-R affinity relative to dAVP. For the hV_2-R, a global decrease of affinity was also observed for 17 of the 21 peptides tested. This loss of affinity is

much higher for peptides **8**, **9**, **17**, **18**, **20**, and **21**. By contrast, the d[Abu^4]AVP (**3**), d[Thr^4]AVP (**14**), and d[Val^4]AVP analogues exhibit good retentions of hV_2-R affinity with respect to AVP and dAVP.

These findings point to intriguing differences between the hV_{1b} and the other AVP receptor isoforms in their abilities to discriminate between different amino acids at position 4 in dAVP. It would appear from these affinity data that for interactions with the hV_{1b}-receptor, the position 4 residue in dAVP is minimally involved. By contrast, for interactions with the hV_{1a}, hV_2, and hOT receptors, the chemical nature of the position 4 residue is of critical importance. As was suggested by Mouillac and co-workers, the Gln^4 residue of AVP appears to participate in H bond interactions with the Gln and Thr residues of the hV_{1a}-R.[18] This would explain the high hV_{1a}-R affinities of the d[Asn^4]AVP and d[Thr^4]AVP analogues. Replacement of the Gln^4 residue by an aliphatic or a charged residue, however, would not allow the hV_{1a}-receptor to establish adequate interactions with the fourth amino acid of such d[X^4]AVP analogues. This is probably also the case for the hV_2 and hOT receptor subtypes.

Influence of the Chemical Nature of the Amino Acid at Position 4 in dAVP on Human V1b Receptor Selectivity (Tables 1 and 2). V_{1b}/V_{1a} Selectivity. Replacement of the Gln^4 residue of dAVP by some aliphatic amino acids, namely, Nle^4, Val^4, Leu^4, Ile^4, and Cha^4 greatly increases the V_{1b}/V_{1a} selectivity (Table 2). Similar increases are also observed when the Gln^4 residue is replaced by positively charged residues such as Orn,^{4} Lys^4, Arg^4, and Har^4 (Table 2). These gains in V_{1b}/V_{1a} selectivity are due to a retention of high V_{1b} receptor affinities combined with diminished V_{1a} receptor affinities. By contrast, polar (Asn^4, Ser^4, Thr^4) or aromatic (Phe^4, Tyr^4, Trp^4) residue substitutions at position 4 do not significantly increase V_{1b}/V_{1a} selectivity. These observations suggest that in contrast to the hV_{1b}-receptor, the hV_{1a}-receptor is intolerant of basic amino acids and some aliphatic amino acid substitutions at position 4 in dAVP.

V_{1b}/V_2 Selectivity. Most of the position 4 substituted analogues of dAVP exhibit gains in V_{1b}/V_2 receptor selectivity relative to dAVP (Table 2). Among these, peptides **5**, **6**, **8**, **9**, **17**−**20**, and d[Cha^4]AVP exhibit striking enhancements in V_{1b}/V_2 receptor selectivity, ranging from 112 for d[Phe^4]AVP (**9**) to 6082 for d[Lys^4]-AVP (**18**). These gains in V_{1b}/V_2 selectivity are due to retention of high V_{1b} receptor affinity combined with drastic losses in V_2 receptor affinities. The V_{1b}/V_2 selectivity relative to that of dAVP is not enhanced and is in fact reduced in only four of these new d[X^4]AVP analogues, namely, d[Ala^4]AVP, d[Abu^4]AVP, d[Thr^4]-AVP, and d[Glu^4]AVP. These data show that the nature of the amino acid at position 4 in dAVP plays a critical role in V_{1b}/V_2 selectivity. This appears to be correlated with the size and the degree of branching of the side chain at position 4. Side chains with three or less aliphatic carbons with or without an ω-NH_2 group are not selective. Side chains with more than three aliphatic carbons (e.g., Leu^4) or extended with an ω-NH_2− (e.g., Orn,^{4} Lys^4) are highly selective. As observed for the hV_{1a}-receptor, the hV_2-receptor is intolerant of basic amino acid and some aliphatic amino acid substitutions at position 4 in dAVP.

V_{1b}/OT Selectivity. With the exception of the Ala^4, Tyr^4, and Glu^4 peptides, all of the other position 4 substituted analogues of dAVP exhibit an enhancement of the V_{1b}/OT selectivity relative to dAVP. This enhancement is modest for polar and aromatic analogues. By contrast, compounds that have an aliphatic residue (d[Leu^4]AVP, d[Val^4]AVP) or a basic residue (d[Orn^4]-AVP, d[Lys^4]AVP, d[Arg^4]AVP, d[Har^4]AVP) in position 4 displayed much higher V_{1b}/OT selectivities. The gains in V_{1b}/OT selectivity are due to retention of high V_{1b} affinities combined with drastic reductions in OT-R affinities. These results suggest that the hOT receptor is more generally intolerant of position 4 modifications of dAVP than the V_{1a} and V_2 receptor isoforms.

Altogether, these data show that the nature of the amino acid at position 4 in dAVP plays a critical role in endowing the resultant ligand with high selectivity for the hV_{1b}-receptor. An examination of the data presented in this study allowed us to determine the structural features at position 4 in dAVP that result in d[X^4]AVP analogues that exhibit good V_{1b} affinity and selectivity. As illustrated in Figure 3A, the hV_{1b}-R affinity increases with the size of the aliphatic moiety of the fourth residue and reaches nanomolar values when at least two aliphatic carbons are present. The enhancement of V_{1b} selectivity with respect to the three other AVP/OT receptors is also dependent on this parameter (Figure 3B). At least three to four aliphatic carbons (d[Nva^4]-AVP and d[Nle^4]AVP, respectively) are necessary to get compounds with a high V_{1b}/V_{1a} and V_{1b}/V_2 selectivity. By contrast, the V_{1b}/OT selectivity is more sensitive to this parameter, since d[Abu^4]AVP (two aliphatic carbons) still exhibits a good selectivity.

Analogues possessing basic residues at position 4 (d[Orn^4]AVP, d[Lys^4]AVP, d[Arg^4]AVP, d[Har^4]AVP) are also very selective for the hV_{1b}-R subtype and display nanomolar affinities for this receptor isoform. Yet, they all possess an aliphatic moiety of at least three carbons. To determine whether their enhanced V_{1b} selectivity and good affinity are due to the presence of a basic residue or to an aliphatic moiety at position 4, we compared the binding properties of d[Nle^4]AVP (**5**) and d[Orn^4]AVP (**17**). As illustrated in Table 1, the side chain of norleucine (Nle) and ornithine (Orn) have approximately the same steric bulk but the ϵ methyl group of Nle is replaced by an ω amino group in Orn. The two analogues have similar affinities for the hV_{1b}-receptor. Interestingly, the V_{1b}/V_{1a}, V_{1b}/V_2, and V_{1b}/OT selectivities displayed by d[Orn^4]AVP are slightly greater than those exhibited by d[Nle^4]AVP (see Table 2). Such observations indicate that the aliphatic moiety of the fourth residue is mainly responsible for the high hV_{1b}-receptor affinity and selectivity. The presence of an ω-NH_2 group reinforces this V_{1b}-R selectivity. Thus, steric factors combined with lipophilicity rather than the presence of a positive charge at position 4 would appear to be the critical structural features required for high hV_{1b}-R affinity and selectivity. This assumption is verified by comparing the pharmacological properties of d[Dap^4]AVP and d[Orn^4]AVP, both of which possess on their respective position 4 side chains an ω amino group but a variable number of aliphatic carbons (see

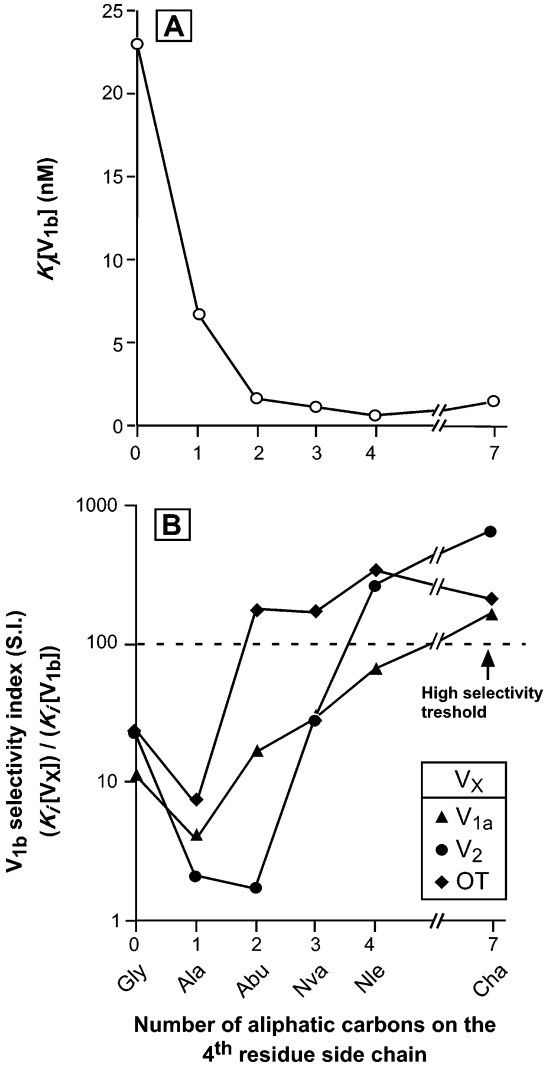


Figure 3. Influence of the number of aliphatic carbons present at the fourth residue side chain of d[X^4]AVP analogues on human V_{1b} receptor properties. The affinity (A) and the V_{1b} selectivity index relative to the other AVP/OT receptor subtypes (B) of a given d[X^4]AVP analogue were plotted as a function of its number of aliphatic carbons present on the side chain of its fourth residue. K_i values and V_{1b} selectivity index were from Table 2. Analogues were numbered as described in Tables 1 and 2. Symbols not numbered refer to d[Cha^4]AVP.

Tables 1 and 2). The d[Orn^4)AVP analogue exhibits significantly greater hV_{1b}-receptor affinity and selectivity than d[Dap^4]AVP. Thus, enhanced hV_{1b}-R selectivity clearly requires an aliphatic side chain of at least three carbons on the fourth residue of dAVP.

It may be instructive to compare the hV_{1b}-receptor, hV_{1a}-receptor, and hV_2-receptor affinity and selectivity data for the most selective hV_{1b}-receptor agonists reported here (i.e., **6**, **17**, **18**, and **20**) with data for two previously reported V_{1b} agonists d[D-3-Pal^2]AVP[41,64] and dDAVP[65,66] (Table 2). dDAVP,[65,66] also known as desmopressin, is widely used as a selective V_2 agonist for the treatment of diabetes insipidus and nocturia.[34,35] We have shown that d[D-3-Pal^2]AVP exhibits affinities of 13.8, 34.2, and 9600 nM, respectively, for the hV_{1b}-receptor, hV_{1a}-receptor, and hV_2-receptor.[41] Although d[D-3-Pal^2]AVP exhibits good selectivity for the hV_{1b}-receptor with respect to the hV_2-receptor, its hV_{1b}-receptor affinity is much weaker than the most selective

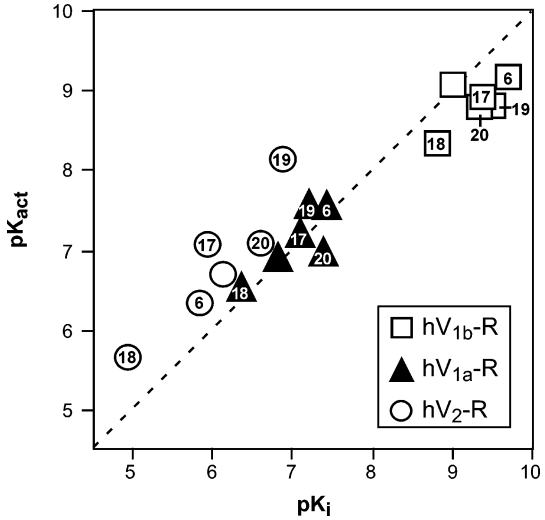


Figure 4. Coupling function of d[X^4]AVP analogues for the human AVP receptor isoforms. For a given AVP/OT receptor, the K_i of the different d[X^4]AVP analogues were plotted against their respective K_{act} values. Data were from Tables 2 and 3, respectively. Analogues were numbered as described in Table 2. Symbols not numbered correspond to d[Cha^4]AVP.

d[X^4]AVP analogues reported here. i.e., **6**, **17**, **18**, and **20** (Table 2). Furthermore, it is essentially nonselective for the hV_{1b}-receptor with respect to the hV_{1a}-receptor (Table 2). The human receptor affinity data for dDAVP [66a] (Table 2) show that although it has a relatively high affinity for the hV_{1b}-receptor (5.8 nM) compared to its affinities for the hV_{1a}-receptor (62.4 nM) and the hV_2-receptor (23.3 nM), its hV_{1b}-receptor affinity and selectivities are much weaker than the most selective d[X^4]AVP analogues reported here, i.e., **6**, **17**, **18**, and **20**. These comparisons show that the d[Leu^4]AVP, d[Orn^4]AVP, d[Lys^4]AVP, and d[Har^4]AVP analogues reported here exhibit much higher affinities and selectivities for the hV_{1b}-receptor than d[D-3-Pal^2]AVP and dDAVP.

Influence of the Chemical Nature of the Amino Acid at Position 4 in dAVP on the Functional Properties of the Human Vasopressin/Oxytocin Receptors (Tables 1 and 3). The functional experiments performed on CHO cells stably expressing the hV_{1b}-R revealed that the agonist properties of the six d[X^4]AVP analogues tested are similar to those of the natural hormone AVP (K_{act} and maximal efficiency). As shown in Table 3, the coupling properties between hV_{1b}-R and the phospholipase C (PLC) are not modified when the Gln^4 residue of dAVP is substituted by an aliphatic or a positively charged amino acid. Interestingly, a good correlation between the K_i and K_{act} values of the analogues tested (coupling function) is observed for the hV_{1b}-receptor (Figure 4). This suggests a linear coupling between hV_{1b}-receptor occupancy and PLC stimulation. By contrast, the d[X^4]AVP analogues displayed reduced efficiencies on the hV_{1a}-R subtype (Table 3). Three of them (d[Leu^4]AVP, d[Cha^4]AVP, and d[Lys^4]AVP) are only partial agonists (see Figure 2 and Table 3). Despite this loss in efficiency, a good correlation between K_i and K_{act} values of the analogues tested for the hV_{1a}-R is observed (Figure 4). Concerning the hV_2-R, all the analogues tested behave as full agonists. Yet, at variance with the hV_{1b} and hV_{1a} receptors, the correlation between the K_i and K_{act} values is weaker

(Figure 4). This is particularly the case for the d[Orn^4]-AVP, d[Lys^4]AVP, and d[Arg^4]AVP analogues. This suggests an excellent functional coupling between the agonist/hV_2-R complex and adenylyl cyclase. Such a phenomenon has already been observed for the rat V_2 receptor with d[Thi^3,Val^4,D-Arg^8]AVP, a structural analogue of AVP modified at positions 1, 3, 4, and 8.[29,67] This excellent coupling function will probably compromise the use of d[Arg^4]AVP as a selective hV_{1b}-R agonist in functional studies. Finally, among the six analogues tested, four did not exhibit significant agonist properties for the hOT-R. Previous studies demonstrated that AVP is a partial agonist of the OT receptor with an efficiency close to 50% compared to oxytocin.[68] Our results suggest that in addition to positions 3 and 8, the two structurally dissimilar positions in AVP and OT, position 4 is also of critical importance for the coupling between the hOT-R and PLC.

Taking into account both the binding properties of the 21 d[X^4]AVP analogues and the functional properties of the 6 most selective of these reported here, four of these, d[Leu^4]AVP (**6**), d[Orn^4]AVP (**17**), d[Lys^4]AVP (**18**), and d[Har^4]AVP (**20**) together with d[Cha^4]AVP[41] appear to be the most selective hV_{1b}-R compounds reported to date.

Conclusion

The design, synthesis, and pharmacological characterization of 21 position 4 substituted analogues of dAVP reported here led us to identify four new high-affinity specific agonists of the human V_{1b} receptor. In addition to the previously reported d[Cha^4]AVP,[41] the most promising pharmacological tools characterized in this study are d[Leu^4]AVP (**6**), d[Orn^4]AVP (**17**), d[Lys^4]-AVP (**18**), and d[Har^4]AVP (**20**). These exhibit much higher hV_{1b}-receptor affinities and selectivities than any previously reported hV_{1b}-receptor agonists.[41,64,66] In addition to the recently reported non-peptide V_{1b} antagonist SSR149415,[39] these new V_{1b}-receptor specific agonists will allow a better characterization of hV_{1b}-R functions, especially in extrapituitary tissues. The radiolabeling of these agonist compounds will also provide valuable tools for the localization of the V_{1b}-R subtype within tissues that express this receptor isoform. These new specific ligands will also facilitate structure−function studies to better characterize the molecular determinants of the human V_{1b} receptor responsible for its binding, activation, and signal transduction properties.

Since profound pharmacological species differences have been previously described between rat and human AVP/OT receptors,[4,41,66,67,69] the determination of the binding and functional properties of these new compounds on rat tissues is now highly warranted. Finally, the findings on the specific V_{1b} agonists reported here also provide valuable clues for the design of antagonists with high affinity and selectivity for the human V_{1b} receptor.

Experimental Section

Materials. All reagents used were analytical grade. The Merrifield resin and the Rink amide resin were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). The Mpr(Bzl) and Boc-Glu(Fm) were purchased from Bachem (Torrance, CA). The Mpr(Meb) was purchased from Chem-Impex International, Inc. (Wood Dale, IL). The Boc-Dab(Fmoc) was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). All other amino acid derivatives were purchased from Bachem (Torrance, CA) or Chem-Impex International, Inc. Peptides d[Abu^4]AVP[45] (**3**, Table 1), d[Thr^4]AVP[44] (**14**, Table 1), and d[Arg^4]AVP [46] (**19**, Table 1) were resynthesized as described below for d[Leu^4]AVP (**6**). Thin-layer chromatography (TLC) was run on precoated silica gel plates (60F-254, E. Merck) with the following solvent systems: (a) 1-butanol/AcOH/H_2O (4:1:5, upper phase); (b) 1-butanol/AcOH/H_2O (4:1:1); (c) 1-butanol/AcOH/H_2O/pyridine (15:3:3:10); (d) 1-butanol/AcOH/H_2O (4:1:2); (e) 1-butanol/AcOH/H_2O (2:1:1). Loads of 10−15 μg were applied, and chromatograms were developed at a minimal length of 10 cm. The chlorine gas procedure for the KI-starch reagent was used for detection.[50] Optical rotations were measured with a Rudolph Autopol III polarimeter. Analytical HPLC was performed on a Waters 810 instrument under the following conditions: 90:10 to 30:70 0.05% aqueous TFA/0.05% TFA in MeCN, linear gradient over 30 or 60 min at 1.0 mL/min (λ = 210 nm) on a Microsorb C_{18} column (Rainin Instrument Co., Inc.). All peptides were at least 95% pure. Electron spray mass spectrometry (ESMS) experiments were done by the University of Michigan Protein and Carbohydrate Structure Facility on a Vestec 201 single quadropole mass spectrometer using AcOH/H_2O/MeCN (4:46:50) as a solvent or by the University of Oklahoma Health Science Center Molecular Biology Resource Facility on a PE Sciex Q-STAR quadropole TOF mass spectrometer using 50:50 CH_3CN/H_2O with 0.5% AcOH as a solvent. ES mass spectra of the free peptides were in agreement with the composition of each peptide.

Solid-Phase Synthesis Procedures. For peptides **1**−**7** and **9**−**21** (Table 4) the Merrifield solid-phase method[48−50] with the modifications previously described[54−56] was used. For the synthesis of protected peptidyl resins, starting with the Boc-Gly resin, eight cycles of deprotection, neutralization, and coupling were carried out by the DCC/HOBt[57] or the active ester[58,59] procedure. With the exceptions noted below for peptides **XVI** and **XXI** (Table 5), side chain protection of the Boc-amino acids incorporated were as follows: Arg (Tos), Cys (Mob), Cys (Bzl), Tyr (Bzl), Ser (Bzl), Thr (Bzl), Dap (Z), Orn (2-CL-Z), Lys (2-Cl-Z), Har (Tos), Mpr (Meb), and Mpr (Bzl). Ammonolysis in MeOH[54,59] was used to split the protected peptides from the resin. For peptides **XVI** and **XXI** (Table 5), the Dab^4 and the Glu^4 residues were incorporated using Boc-Dab(Fmoc) and Boc-Glu(Fm),[70,71] respectively, by the DCC/HOBt[57] procedure. For the solid-phase synthesis of peptides **XVI** and **XXI**, the final three deprotections were performed with HCl(1 M)/AcOH as described above, but the subsequent neutralizations were carried out with 10% DIPEA/CH_2Cl_2.[72] The ammonolytic cleavage of peptides **XVI** and **XXI** was accompanied by removal of their respective side chain Fmoc and Fm protecting groups. The protected precursors obtained by ammonolysis were extracted with hot DMF and isolated by precipitation with hot water as previously described.[54−56] They were purified by reprecipitations with DMF/MeOH/Et_2O until adjudged pure by TLC[54] to give the required protected peptides **I**−**VII** and **IX**−**XXI** (Table 5). The protected precursors **I**−**VII** and **IX**−**XXI** were deblocked using sodium in liquid ammonia[1,60] as previously described.[54−56]

For peptide **8** (Table 4), 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxymethylpolystyrene resin (Rink amide resin)[52] and the Fmoc strategy[51,53] were used. Protection of the side chain functional groups of the amino acids was as follows: Bu^t for Tyr; Trt for Cys and Mpr; Pmc for Arg. The deprotections were carried out with 20% piperidine/DMF,[51,52] and the coupling reactions were mediated primarily by DCC/HOBt[57] except for the Fmoc-Asn, which was incorporated as its *p*-nitrophenyl ester[61,71] in DMF. For the synthesis of protected peptidyl resin, nine cycles of deprotection and coupling were performed. Cleavage of the peptide from the resin and simultaneous removal of the protecting groups were carried out by Reagent K (16.5 mL of TFA, 1 g of phenol, 1 mL of H_2O, 1 mL of thioanisole, and 0.5 mL of 1,2-ethanedithiol).[53]

The resulting disulfydryl compounds from both the Na/liquid NH_3 treatment and the Reagent K treatment were oxidatively cyclized with $K_3[Fe(CN)_6]$ using the modified reverse procedure.[62] The free peptides were purified by a two-step gel filtration procedure[63] on Sephadex G-15 (eluent 50% AcOH) and Sephadex LH-20 (eluent 2 M AcOH). For some peptides, an additional purification by gel filtration on Sephadex G-15 and/or LH-20 was used. The physicochemical data for the free peptides **1**−**21** are given in Table 4.

Mpr(Bzl)-Tyr(Bzl)-Phe-Leu-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (VI, Table 5). Boc-Gly resin (0.5 g, 0.35 mmol) was subjected to eight cycles of deprotection, neutralization, and coupling with Boc-Arg(Tos), Boc-Pro, Boc-Cys(Mob), Boc-Asn-ONp, Boc-Leu, Boc-Phe, Boc-Tyr(Bzl), and Mpr(Bzl), respectively. The resulting protected peptidyl resin was cleaved by ammonolysis as previously described.[54−56] The protected peptide was extracted with hot DMF (30 mL), and the product was precipitated by addition of hot water (ca. 300 mL). After cooling, the product was collected, dried in vacuo over P_2O_5, and reprecipitated from DMF (30 mL) and ether (ca. 200 mL). Collection and drying in vacuo over P_2O_5 gave the required nonapeptide amide (**VI**, Table 5). The same procedure was used for the synthesis and purification of the protected nonapeptide amides **I**−**V**, **VII**, and **IX**−**XXI** (Table 5). The ammonolytic cleavage of peptides **XVI** and **XXI** was accompanied by removal of their side chain Fmoc and Fm protecting groups, respectively. The physicochemical properties of the protected peptides **I**−**VII** and **IX**−**XXI** are given in Table 5.

d[Leu^4]arginine Vasopressin (6, Table 4). The Na/liquid NH_3 procedure was used for the deprotection of all protected nonapeptide amides **I**−**V**, **VII**, and **IX**−**XXI** as described here for protected peptide **VI**. A solution of protected nonapeptide amide (**VI**, Table 5) (120 mg) in sodium-dried ammonia (ca. 400 mL) was treated at the boiling point and with stirring with sodium from a stick of metal contained in a small-bore glass tube until a light-blue color persisted in the solution for ca. 30 s.[1,60,54−56] NH_4Cl was added to discharge the color. Reoxidation of the deblocked disulfydryl peptide **6** was performed by the modified reverse procedure[62] as follows. The resulting disulfydryl peptide residue was dissolved in 25 mL of 50% AcOH, and the solution was diluted with 60 mL of H_2O. The peptide solution was added dropwise with stirring over a period of 15−30 min to a 600 mL aqueous solution that contained 20 mL of a 0.01 M solution of potassium ferricyanide. Meanwhile, the pH was adjusted to approximately 7.0 with concentrated ammonium hydroxide. Following oxidation, the free peptide **6** was isolated and purified as follows. After acidification with AcOH to pH 4.5 and stirring for 20 min with an anion-exchange resin (Bio-Rad, AG 3 × 4, Cl^- form, 5 g damp weight), the suspension was slowly filtered and washed with 0.2 M AcOH (3 × 30 mL) and the combined filtrate and washings were lyophilized. The resulting powder was desalted on a Sephadex G-15 column (110 cm × 2.7 cm), eluting with aqueous acetic acid (50%) with a flow rate of 5 mL/h.[63] The eluate was fractionated and monitored for absorbance at 254 nm. The fractions making up the major peak were checked by TLC, pooled, and lyophilized. The residue was further subjected to two consecutive gel filtrations on Sephadex LH-20 (100 cm × 1.5 cm), eluting with aqueous acetic acid (2 and 0.2 M), respectively, with a flow rate of 4 mL/min. The peptide was eluted in a single peak (absorbance at 254 nm). Lyophilization of the pertinent fractions gave the desired vasopressin analogue d[Leu^4]AVP (**6**, Table 4). The deblocking, reoxidation, and purification of peptides **1**−**5**, **7**, and **9**−**21** (Table 4) were carried out in essentially the same manner.

d[Thi4]arginine Vasopressin (8, Table 4). 4-(2′,4′-Dimethoxyphenyl-Fmoc-aminomethyl)phenoxymethylpolystyrene resin (Rink amide resin) (0.43 g, 0.22 mmol/g) was subjected to nine cycles of deprotection and coupling with Fmoc-Gly, Fmoc-Arg(Pmc), Fmoc-Pro, Fmoc-Cys(Trt), Fmoc-Asn-ONp, Fmoc-Thi, Fmoc-Phe, Fmoc-Tyr(Bu^t), and Mpr(Trt). The resulting protected peptidyl resin (0.98 g) was cleaved and simultaneously deprotected by treatment with Reagent K[53] as follows. The peptidyl resin was stirred with 10 mL of Reagent K for 4 h at room temperature, filtered, and washed twice with 5 mL of TFA. The combined filtrate and washings were precipitated with ether, and the collected product was washed with ether and dried in vacuo over P_2O_5. The resulting disulfydryl peptide (ca. 0.150 g) was reoxidized by the reverse procedure[62] as described above for peptide **6** to give the free peptide **8** (Table 4).

Cell Culture. Chinese hamster ovary (CHO) cells stably expressing the human isoforms of the vasopressin V_{1a}, V_{1b}, V_2, and OT[73] receptors were plated in 150 mm Petri dishes or 24-well plates depending upon the experiment to be conducted. Cells were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 500 units/mL penicillin and streptomycin, and 1X nonessential amino acids in an atmosphere of 95% air and 5% CO_2 at 37°C. Cells were cultured for 48−72 h and were then used for the different experimental assays.

Membrane Preparations and Binding Experiments. CHO cells, stably or transiently transfected with vasopressin or oxytocin receptors, were cultured for 48−72 h in 150 mm Petri dishes, harvested and then washed two times in phosphate-buffered saline without $CaC1_2$ and $MgC1_2$, Polytron homogenized in lysis buffer (15mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 0.3 mM EDTA), and centrifuged at 100*g* for 5 min at 4 °C. Supernatants were recovered and centrifuged at 44000*g* for 20 min at 4 °C. Pellets were washed in buffer A (50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$) and centrifuged at 44000*g* for 20 min at 4 °C. Membranes were suspended in a small volume of buffer A, and protein content was determined according to the Bradford method using bovine serum albumin as standard. Aliquots of membranes were used immediately or stored at −80 °C.

Membrane binding assays were performed as previously described[74] using [^{3}H]AVP or [^{125}I]-d(CH_2)$_5$[Tyr(Me)^2,Thr^4,Tyr-NH_2^{9}]OVT ([^{125}I]-OTA)[75] as radioligands for the vasopressin or oxytocin receptors, respectively. OTA was synthesized and radioiodinated to give [^{125}I]-OTA in our laboratories as previously described.[75] Membranes (1−20 μg of protein) were incubated 60 min at 30 °C in a medium containing 50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mg·mL^{-1} bovine serum albumin (BSA), 0.01 mg·mL^{-1} leupeptine, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The binding properties of the unlabeled analogues were determined by competition experiments. Briefly, 0.5−3 nM [^{3}H]AVP or 150 pM [^{125}I]-OTA was added in the incubation medium for the V_{1a}, V_{1b}, V_2, or OT receptors with (nonspecific binding) or without (total binding) 1 μM of unlabeled AVP or OT and increasing amounts of the unlabeled analogues to be tested. K_i values were calculated from the dose-displacement curves fitted with the Cheng and Prussof equation.[76] Affinities of [^{3}H]AVP and [^{125}I]-OTA were determined in saturation experiments using concentrations ranging from 0.1 to 10 nM and from 10 to 1500 pM, respectively. In each condition, total and nonspecific binding were determined. Radioactivity found associated with plasma membranes was determined by filtration through GF/C filters. Specific binding was calculated and expressed as percent of the maximal binding capacity determined without unlabeled analogue.

InsPs Assays. Inositol phosphates (InsPs) accumulation was determined as previously described.[77] Briefly, CHO cells expressing the human vasopressin/oxytocin receptors were cultured in 24-well plates at 20 000 cells/well. Cells were grown for 24 h in DMEM supplemented with 10% fetal calf serum. Cells were further incubated for another 24 h period in a serum and inositol-free medium supplemented with 1 μCi·mL^{-1} *myo*-[2-^{3}H]-inositol. Cells were then washed twice with a Hank's buffered saline medium (HBSM), equilibrated at 37 °C in HBSM for 30 min, and incubated for 15 min in HBSM supplemented with 10 mM LiCl, 1 mg/mL glucose, 1 mg/mL BSA, and 2.1 g/L $NaHCO_3$. Cells were then further stimulated for 15 min with increasing concentrations of the analogues to be tested. Reaction was stopped with 5% (v/v) perchloric acid. Total inositol phosphates (InsPs) accumulated were extracted

and purified on Dowex AG1-X8 anion exchange chromatography column and counted.

cAMP Assays. CHO cells were grown in the same conditions as those described for InsPs accumulation measurements with some minor differences. For the last day of culture, [^{3}H]-adenine (Amersham International, Buckinghamshire, U.K.) was added to the culture medium at a final concentration of 2 μCi/mL. Cells were washed twice in HBSM. To evaluate the basal or stimulated adenylyl cyclase activity, cells were further incubated in a Hank's buffered saline medium supplemented with 1 μM RO1724 (Calbiochem-Novabiochem Corp., San Diego, CA), 1 mg/mL glucose, 1 mg/mL BSA, and 2.1 g/L $NaHCO_3$, with or without increasing concentrations of the analogue to be tested. After a 15 min incubation period, the reaction was stopped by replacing the incubation medium by 1 mL of trichloroacetic acid (5%, v/v). ATP and cAMP were added to the acid extracts at a concentration of 0.5 mM. Relative intracellular cAMP levels were determined by measuring the formation of [^{3}H]-cAMP from the prelabeled adenine nucleotide pool. Labeled cAMP was separated by sequential chromatography on Dowex and alumina columns by the method of Salomon.[78] Radioactivity present in the cAMP fractions was expressed as percent of the sum of radioactivity recovered in the cAMP fraction and radioactivity that was not retained by the Dowex column which mainly corresponds to labeled ATP.

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Appendix

Abbreviations. Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem*. **1989**, *180*, A9−A11) and IUPHAR (*Trends Pharmacol. Sci.* **2001**). All amino acids are in the L-configuration unless otherwise noted. Other abbreviations are the following: Abu, L-α-aminobutyric acid; AcOH, acetic acid; ACTH, adrenocorticotropin hormone, human; AVP, arginine vasopressin; dAVP, [deamino-Cys^1]arginine vasopressin; d[X^4]AVP, position 4 substituted analogue of dAVP; dDAVP, [deamino-Cys^1,8-D-arginine]vasopressin; d[D-3-Pal^2]AVP, [deamino-Cys^1,2-D-3-pyridylalanine]arginine vasopressin; Boc, *tert*-butyloxycarbonyl; Bu^t, *tert*-butyl; Bzl, benzyl; cAMP, cyclic adenosine monophosphate; Cha, L-cyclohexylalanine; CHO cells, Chinese hamster ovary cells; CRH, corticotropin releasing hormone; 2Cl-Z, 2-chlorobenzyloxycarbonyl; Dab, L-2,4-diaminobutyric acid; Dap, L-2,3-diaminopropionic acid; DCC, dicyclohexylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; DMEM, Dulbecco's modified Eagle's medium; *E*, maximal efficiency; EDTA, ethylenenediamine tetraacetic acid; ESMS, electron spray mass spectrometry; Et_3N, triethylamine; Et_2O, ethyl ether; Fm, 9-fluorenylmethyl; Fmoc, 9-fluorenylmethoxycarbonyl; Har, L-homoarginine; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; InsPs, total inositol phosphates; K_{act}, concentration of agonist leading to half-maximal activity; K_d, concentration of peptide leading to half-maximal specific binding deduced from saturation experiments; K_i, concentration of peptide leading to half-maximal specific binding deduced from competition experiments; Meb, *p*-methylbenzyl; MeCN, acetonitrile; Mob, *p*-methoxybenzyl; Mpr, 3-mercaptopropionyl; Nle, L-norleucine; Nva, L-norvaline; ONp, *p*-nitrophenylester; OT, oxytocin; PMSF, phenylmethylsulfonyl fluoride; PLC, phospholipase C; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; SI, selectivity index; TFA, trifluoroacetic acid; Thi, L-thienylalanine; TLC, thin-layer chromatography; Tos, tosyl; Trt, trityl; VP, vasopressin; VT, vasotocin; V_{1a}, vascular; V_{1b}, pituitary; V_2, renal; Z, benzyloxycarbonyl.

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