Design and Synthesis of the First Selective Agonists for the Rat Vasopressin V_{1b} Receptor: Based on Modifications of Deamino-[Cys^1]arginine Vasopressin at Positions 4 and 8†

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The neurohypophyseal peptides arginine vasopressin (AVP) and oxytocin (OT) mediate a wide variety of peripheral and central physiological and behavioral effects by acting on four different G-protein coupled receptors, termed V_{1a} (vascular), V_{1b} (pituitary), V_2 (renal), and OT (uterine). We recently reported that d[Cha^4]AVP (A), d[Leu^4]AVP (B), d[Orn^4]AVP (C), and d[Arg^4]AVP (D) have high affinity and are selective agonists for the human V_{1b} receptor. However, peptides A−D were subsequently shown to be potent antidiuretic agonists in the rat and are, thus, not selective V_{1b} agonists in the rat. Peptides A−D served as leads for the studies reported here. They were modified at position 8 by Lys, ornithine (Orn), diaminobutyric acid (Dab), and diaminopropionic acid (Dap) to give d[Cha^4,Lys^8]VP (**1**), d[Cha^4,Orn^8]VP (**2**), d[Cha^4,Dab^8]VP (**3**), d[Cha^4,Dap^8]VP (**4**), d[Leu^4,Lys^8]VP (**5**), d[Leu^4,Orn^8]VP (**6**), d[Leu^4,Dab^8]VP (**7**), d[Leu^4,Dap^8]VP (**8**), d[Orn^4,Lys^8]VP (**9**), d[Orn^4,Orn^8]VP (**10**), d[Arg^4,Lys^8]VP (**11**), d[Arg^4,Orn^8]VP (**12**), and d[Arg^4,Dab^8]VP (**13**). All peptides were synthesized by the Merrifield solid-phase method. Their binding and functional properties were evaluated in rat AVP V_{1a}, V_{1b}, and V_2 receptors and on the rat OT receptor expressed either in native tissues or in stably transfected cells. They were also examined in rat vasopressor, antidiuretic, and in in vitro (no Mg^{++}) oxytocic assays. Functional studies performed on chinese hamster ovary cells expressing the different AVP/OT receptors confirm that d[Cha^4,Lys^8]VP (**1**), d[Cha^4,Dab^8]VP (**3**), d[Leu^4,Lys^8]VP (**5**), and d[Leu^4,Dap^8]VP (**8**) are the first selective agonists for the rat V_{1b} receptor. These selective V_{1b} agonists are promising new tools for studies of the role of the V_{1b} receptor in the rat.

Introduction

Arginine vasopressin (AVP[a]) exerts three well-known peripheral physiological effects: antidiuresis, vasoconstriction, and the release of adrenocorticotropin hormone (ACTH; for reviews, see refs 1−4). In addition to these well-established functions, AVP is also involved peripherally in the regulation of insulin and glucagon release by the pancreas,[5−8] steroid, and catecholamine secretion by the adrenals,[9] glycogenolysis in the liver,[2] and the release of atrial natriuretic factor from the heart,[10] and paradoxically, it also elicits a vasodilatory response.[11,12] Centrally, AVP has multiple effects, including the regulation of memory, synaptic transmission, body temperature, pain, anxiety, and depression in rats and in humans (for reviews, see refs 13 and 14). The central effects of vasopressin and those of its closely related neurohypophyseal peptide, oxytocin (OT), have received intense investigative scrutiny in recent years.[15−38] The diverse peripheral and central effects of AVP and OT are mediated by four different 7-transmembrane G-protein-coupled receptors termed: V_2, V_{1a}, V_{1b} (V_3), and OT receptors.[1−4,39] V_2 receptors, present in the kidney, mediate the antidiuretic effects of AVP via the adenylate cyclase pathway.[1−4] V_{1a} receptors, present in many tissues, mediate the vascular effects of AVP by causing vasoconstriction of the vascular smooth muscle cells.[1−4] The OT receptor mediates the uterine contracting and milk let-down effects of OT.[1−4] In the central nervous system, V_{1a} receptors mediate the effects of AVP on anxiety.[14,24,38] V_{1b} receptors, present in the pituitary, pancreas, adrenals, and in the CNS, mediate the release of ACTH from the anterior pituitary,[1,2] steroids from the adrenal medulla,[9] and insulin and glucagon from the pancreas.[5−8] Recently, the V_{1b} receptor has also been shown to mediate anxiety and depression in rats[13,14,24,33,38,40] and also in humans.[13,22,34] By contrast, OT has been shown to relieve anxiety in rats,[41−43] mice,[28,44] monkeys,[45] and in humans.[46] The V_{1a}, V_{1b}, and OT receptors act via the phosphoinositide pathway.[1−4] The V_2, V_{1a}, V_{1b}, and OT receptors from a variety of species, including rat and human,

† Dedicated to the memory of Professor Bruce Merrifield.

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[a] Abbreviations: Symbols and abbreviations are in accordance with the recommended 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. AcOH, acetic acid; ACTH, adrenocorticotropin hormone, human; AVP, arginine^8 vasopressin; dAVP, deamino-[Cys^1]arginine vasopressin; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; cAMP, cyclic adenosine monophosphate; Cha, 1-amino-cyclopentane-1-carboxylic acid (cyclohexylalanine); CHO cells, chinese hamster ovary cells; Dab, L-2,4-diaminobutyric acid; Dap, L-2,3-diaminopropionic acid; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMEM, Dubbelco's modified Eagles medium; E, maximal efficiency; Et_3N, triethylamine; Et_2O, ethyl ether; HBS, Hank's buffered saline; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; hV_x-R, human VP receptors; 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; Mob, *p*-methoxybenzyl; Mpr, 3-mercaptopropionyl; ONp, *p*-nitrophenylester; OT, oxytocin; rV_x-R, rat VP receptors; SI, selectivity index; TLC, thin-layer chromatography; Tos, tosyl; VP, vasopressin; VT, vasotocin; V_{1a}, vascular; V_{1b}, pituitary; V_2, renal; Z, benzyloxycarbonyl.

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Table 1. Pharmacological Activities in Rat Bioassays of Lys[8], Orn[8], Dab[8], and Dap[8] Analogues of dAVP, d[X[4]]AVP Analogues **A**−**D** (where X = Cha, Leu, Orn, and Arg) and Related Analogues

no.	peptide	antidiuretic activity (units/mg)	vasopressor activity (units/mg)	oxytocic activity (in vitro) no Mg^{++} (units/mg)
	AVP[a]	323 ± 16	369 ± 6	13.9 ± 0.5
	dAVP[b]	1745 ± 385	346 ± 13	63 ± 4
	d[Lys[8]]VP[c]	550 ± 1.7[k] (301 ± 11)	145 ± 7[k] (126 ± 2)	12 ± 0.5
	d[Orn[8]]VP[d]	486 ± 17[k] (202 ± 23)	355 ± 11[k] (355 ± 30)	15 ± 2
	d[Dab[8]]VP[e]	463 ± 24[k] (1373 ± 128)	385 ± 12[k] (450 ± 30)	25 ± 7
	d[Dap[8]]VP[f]	165 ± 12[k] (1079)	8.7 ± 0.7[k] (14.0)	0.9
	dDAVP[g]	1200 ± 126	0.39 ± 0.02	1.5
	d[Val[4]]AVP[h]	1150 ± 110	51 ± 2	26
	dVDAVP[h]	1230 ± 170	<0.01	∼8
	d[D-3-Pal[2]]AVP[i]	∼1	antagonist $pA_2 = 6.22$[n]	ND[o]
A	d[Cha[4]]AVP[j]	133.6 ± 5.61	0.067 ± 0.005	ND[o]
1	d[Cha[4],Lys[8]]VP[k]	0.82 ± 0.01	0.043 ± 0.008	$pA_2 = 6.48 \pm 0.03$[p] $M = 3.41^{-7} \pm 0.2$
2	d[Cha[4],Orn[8]]VP[k]	1.0 ± 0.2	0.22 ± 0.02	$pA_2 = 6.55 \pm 0.05$[p] $M = 3.04^{-7} \pm 0.36$
3	d[Cha[4],Dab[8]]VP[k]	1.0 ± 0.1	0.18 ± 0.02	$pA_2 = 6.35 \pm 0.3$ $M = 4.67^{-7} \pm 0.34$
4	d[Cha[4],Dap[8]]VP[k]	0.3 ± 0.1	∼0.002	$pA_2 = 6.32 \pm 0.03$[p] $M = 4.87^{-7} \pm 0.3$
B	d[Leu[4]]AVP[l]	378 ± 23.67	3.11 ± 0.11	0.67 ± 0.12
5	d[Leu[4],Lys[8]]VP[k,m]	10.5 ± 0.9[k] 5−6[m]	0.9 ± 0.1[k] 0.55[m]	0.054 ± 0.002[k]
6	d[Leu[4],Orn[8]]VP[k]	5.7 ± 0.9	3.1 ± 0.1	mixed pA_2 ∼< 4.89 agonistic activity, ∼0.04
7	d[Leu[4],Dab[8]]VP[k]	5.7 ± 0.5	3.5 ± 0.2	0.27 ± 0.03
8	d[Leu[4],Dap[8]]VP[k]	0.7 ± 0.1	0.05 ± 0.01	0.046
C	d[Orn[4]]AVP[l]	260.32 ± 22.4	1.54 ± 0.13	6.49 ± 0.63
9	d[Orn[4],Lys[8]]VP[k]	7.8 ± 0.4	0.23 ± 0.02	3.1 ± 0.1
10	d[Orn[4],Orn[8]]VP[k]	7.9 ± 0.5	1.9 ± 0.1	2.8 ± 0.1
D	d[Arg[4]]AVP[l]	748.32 ± 12.96	159.93 ± 6.5	0.027 ± 0.003
11	d[Arg[4],Lys[8]]VP[k]	784 ± 54	83 ± 4	0.15 ± 0.02
12	d[Arg[4],Orn[8]]VP[k]	823 ± 133	173 ± 3	0.06 ± 0.01
13	d[Arg[4],Dab[8]]VP[k]	431 ± 58	190 ± 5	0.14 ± 0.02

[a] Original synthesis reported in ref 57. Data from ref 87. [b] Original synthesis reported in ref 72. Repeated synthesis and data from ref 86. [c] Original synthesis and data reported in ref 73. [d] Original synthesis and data reported in ref 74. [e] Original synthesis and data reported in ref 75. [f] Original synthesis and data reported in ref 76. [g] Original synthesis reported in ref 95a. Repeated synthesis and data from ref 86. [h] Original synthesis reported in ref 96a. Data from ref 86. [i] Original synthesis and data reported in ref 97. [j] Original synthesis and data reported in ref 65. [k] This publication. [l] Original synthesis reported in ref 67. Data reported in ref 68. [m] Original synthesis and data reported in ref 81. [n] In vivo pA_2 values are estimates since the [*M*] for the in vivo pA_2 is estimated by dividing ED (effective dose) by the estimated volume of distribution of 67 mL/kg. ED is defined as the dose (nmol/kg intravenously) of the antagonist that reduces the response to 2*x* units of agonist to the response with *x* units of agonist administered in the absence of the antagonist. [o] ND = not determined. [p] In vitro pA_2 values represent the negative logarithm to the base 10 of the average molar concentration [*M*] of the antagonist that reduces the response to 2*x* units of agonist to equal the response seen with *x* units of agonist administered in the absence of the antagonist.

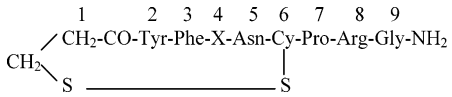
have been cloned during the past decade or so.[47−54] To date, the receptor that mediates the vasodilating effects of AVP has not been characterized.[55] Following the original syntheses of both OT[56] and AVP,[57] structure−activity studies, based on rat bioassays, which measure antidiuretic, vasopressor, and in vitro oxytocic activities,[58−61] uncovered structural modifications of these two peptides, which led to the design of agonists and antagonists that possess potent and selective antidiuretic, pressor, and oxytocic activities in the standard rat bioassays (for reviews, see refs 1 and 58−61). In fact, these findings led to the delineation of the V_2, V_{1a}, and OT receptors long before these receptors were cloned.[1,2] Although it was known that AVP could cause the release of ACTH (see ref 62a,b and references therein), it was not until the mid-eighties that the receptor that mediates this response was characterized pharmacologically and shown to be different than the V_{1a}, V_2, and OT receptors.[63,64] Because it was found to utilize the same second messenger system as the V_{1a} receptor, this AVP receptor was termed the V_{1b} receptor.[64] There is no routine bioassay for the ACTH releasing effect of AVP. Consequently, with the exception of those VP analogues examined in corticotropin-releasing factor in vitro and in vivo assays prior to the early eighties (see ref 62a,b and references therein), virtually all of the many hundreds of VP analogues reported to date[58−61] are lacking quantitative data on their ACTH-releasing activities. Thus, the design of selective agonists and antagonists for the V_{1b} receptor has, until recently, been a very daunting challenge. It was not until the V_{1b} receptor was cloned in the mid-nineties[48,52,53] that it became possible, through the use of V_{1b} receptor binding and functional assays, to design selective agonists and an antagonist for the AVP V_{1b} receptor.[65−68] We recently reported that deamino-[cyclohexylalanine[4]]-arginine vasopressin (d[Cha[4]]AVP, A) is the first selective agonist for the human V_{1b} receptor.[65] This peptide has since become a valuable pharmacological tool in a variety of studies.[25,31,69,70a−d] A selective nonpeptide V_{1b} antagonist, shown to be effective in humans, was also recently reported.[66] This nonpeptide $hV_{1b}R$ antagonist was suggested to have beneficial effects for treating depression in humans.[71] We subsequently reported[67] the synthesis of 21 position-4 analogues of deamino-[Cys[1]]-arginine vasopressin (dAVP).[72] Virtually all of these analogues exhibit very high affinities for the human V_{1b} receptor.[67] Furthermore, a number of these analogues, most notably, d[Leu[4]]AVP, d[Orn[4]]AVP and d[Arg[4]]AVP, with high affinities for the human V_{1b} receptor, have very low affinities for the human V_{1a}, V_2, and OT receptors.[67] In functional assays, these three peptides are also highly selective for the human V_{1b} receptor with respect to the human V_{1a}, V_2, and OT receptors.[67] Thus, they and d[Cha[4]]AVP[65] are the first high affinity selective

Table 2. Binding Properties of Lys[8],Orn[8], Dab[8], and Dap[8] Analogues of d[X[4]]AVP Analogues **A**−**D** (where X = Cha, Leu, Orn, and Arg) for Rat Vasopressin and Oxytocin Receptors[a]

		affinity, K_i (nM)				rV$_{1b}$-R SI		
no.	peptide	rV$_{1b}$-R	rV$_2$-R	rV$_{1a}$-R	rOT-R	V$_2$/V$_{1b}$	V$_{1a}$/V$_{1b}$	OT/V$_{1b}$
	AVP[b,c]	0.29 ± .05	0.45 ± 0.1	1.54 ± 0.97	1.17 ± 0.11	1.5	5.4	5.9
	dAVP[d] (d[Gln[4]]AVP)	0.20 ± 0.03[b]	0.8 ± 0.2[d]	10.8 ± 2.6[b]	0.97 ± 0.16[b]	3.8	54	4.8
	dDAVP[e]		0.3[f]	100[g]				
	d[Val[4]]AVP[h]	0.25 ± 0.06	0.3 ± 0.1	60 ± 15	826 ± 150	1.2	240	3304
	dVDAVP[h]		0.26[f]	316[g]				
	d[D-3-Pal[2]]AVP[j]	112 ± 30	69 ± 17	450 ± 22		0.6	4.0	
A	d[Cha[4]]AVP[k]	1.4 ± 0.1[b]	12.7 ± 2.8[k]	2300 ± 600[b]	1430 ± 330[k]	9	1641	1021
1	d[Cha[4], Lys[8]]VP[b]	1.9 ± 0.4	600 ± 100	9100 ± 700	590 ± 90	317	4837	312
2	d[Cha[4], Orn[8]]VP[b]	3.0 ± 0.6	450 ± 150	2900 ± 40	550 ± 20	148	967	182
3	d[Cha[4], Dab[8]]VP[b]	0.8 ± 0.2	450 ± 90	4400 ± 200	430 ± 60	566	5542	547
4	d[Cha[4], Dap[8]]VP[b]	10 ± 2	1050 ± 300	1800 ± 500	1200 ± 200	101	175	122
B	d[Leu[4]]AVP[l]	0.04 ± 0.01	3.1 ± 0.8	1252 ± 214	481 ± 49	77	31 300	12 025
5	d[Leu[4], Lys[8]]VP[b,m]	0.16 ± 0.1	100 ± 25	3800 ± 600	64 ± 10	631	23 662	400
6	d[Leu[4], Orn[8]]VP[b]	0.26 ± 0.02	425 ± 100	1400 ± 500	55 ± 6	1638	5523	213
7	d[Leu[4], Dab[8]]VP[b]	0.25 ± 0.02	210 ± 20	1000 ± 200	41 ± 10	828	4112	164
8	d[Leu[4], Dap[8]]VP[b]	0.38 ± 0.03	240 ± 50	3300 ± 500	134 ± 22	624	8603	352
C	d[Orn[4]]AVP[l]	0.45 ± 0.12	3.4 ± 0.3	900 ± 245	997 ± 196	7.6	2000	2215
9	d[Orn[4], Lys[8]]VP[b]	0.45 ± 0.04	51 ± 18	2800 ± 1000	22 ± 6	113	6227	49
10	d[Orn[4], Orn[8]]VP[b]	0.91 ± 0.10	76 ± 17	1300 ± 400	37 ± 9	83	1412	41
D	d[Arg[4]]AVP[l]	0.13 ± 0.12	0.20 ± 0.02	12.9 ± 0.4	3550 ± 790	1.5	99	27 323
11	d[Arg[4], Lys[8]]VP[b]	0.6 ± 0.2	3.8 ± 0.9	130 ± 7	125 ± 7	6.7	228	219
12	d[Arg[4], Orn[8]]VP[b]	0.6 ± 0.2	3.1 ± 1.5	67 ± 16	130 ± 30	5.1	112	218
13	d[Arg[4], Dab[8]]VP[b]	0.19 ± 0.04	1.8 ± 0.3	25 ± 10	195 ± 30	9.3	129	1026

[a] Bindings assays were performed on crude plasma membranes as described in the legend of Figure 1 and in the Experimental Section. K_i values are the mean ± SEM of at least three independent experiments, each performed in triplicate. For each analogue, the rV$_{1b}$-R SI was calculated as follows: SI = (K_i analogue for rV$_x$-R)/(K_i analogue for rV$_{1b}$-R), where rV$_x$-R is the rV$_{1a}$, rV$_2$, or rOT receptor. [b] This publication. [c] Original synthesis reported in ref 57. Repeat synthesis reported in ref 87. [d] Original synthesis reported in ref 72. Data from ref 65. [e] Original synthesis reported in ref 95a. [f] Data from ref 95b. [g] Data from ref 96b. [h] Original synthesis reported in ref 96a; data from ref 65, unless otherwise noted. [j] Original synthesis reported in ref 97. Data from ref 65. [k] Original synthesis reported in ref 65. Data from ref 65. [l] Original synthesis reported in ref 67. Data from ref 68. [m] Original synthesis reported in ref 81.

agonists for the human V_{1b} receptor. Due to species differences between the human and the rat V_2 receptors, these four peptides were subsequently shown not to be selective V_{1b} agonists in the rat.[68] While they were shown to exhibit high affinities for the rat V_{1b} receptor, they were also found to possess potent antidiuretic activities in the rat bioassay and to have high affinities for the rat V_2 receptor.[68] In searching for clues to the design of selective agonists for the rat V_{1b} receptor, we selected the four peptides that exhibited the highest selectivity for the human V_{1b} receptor, namely, d[Cha[4]]AVP (**A**), d[Leu[4]]AVP (**B**), d[Orn[4]]AVP (**C**), and d[Arg[4]]AVP (**D**).[67] Using these four peptides (**A**−**D**) as leads, we were confronted with the intriguing challenge of where and how they could be modified to give peptides that retain the V_{1b} receptor agonism and diminished V_{1a} and OT receptor agonism of peptides **A**−**D**, while eliminating the potent rat V_2 receptor agonism of peptides **A**−**D**.[67,68] Because previous studies had shown that a basic L-amino acid at position 8 is a requirement for the ACTH-releasing activity of AVP analogues in the rat,[62a,b] we focused on finding basic amino acid replacements for the Arg[8] residue in peptides **A**−**D**. We decided to replace the Arg[8] residue in peptides **A**−**D** with basic amino acids that have incremental differences in the sizes of their side chains. We thus selected the basic amino acids, Lys, ornithine (Orn), diaminobutyric acid (Dab), and diaminopropionic acid (Dap), which have, respectively, 4, 3, 2, and 1 methylene groups in their side chains. These four amino acids had previously been incorporated in dAVP[72] to give, respectively, d[Lys[8]]VP,[73] d[Orn[8]]VP,[74] d[Dab[8]]VP,[75] and d[Dap[8]]-VP.[76] These four position 8 analogues of dAVP were resynthesized for this study. In rat antidiuretic assays carried out on the resynthesized peptides, all four analogues exhibit substantial reductions in antidiuretic activity relative to that exhibited by dAVP[72] (Table 1). The greatest reduction is exhibited by d[Dap[8]]-VP, the analogue with the shortest side-chain at position 8. Peptides **A**−**D**[67,68] have the following general structure:



where X[4] = Cha (**A**); Leu (**B**); Orn (**C**), and Arg (**D**).

All four amino acids, Lys, Orn, Dab, and Dap, were incorporated at position 8 in peptides **A** and **B** to give peptides **1**−**8**. The Lys[8] and Orn[8] substituents were incorporated in peptide **C** to give peptides **9** and **10**. The Lys[8], Orn[8], and Dab[8] substituents were incorporated in peptide **D** to give peptides **11**−**13**. The 13 peptides designed according to this rationale are as follows:

	peptides **1**−**13**	side chain at position 8
1	d[Cha[4],Lys[8]]VP	−CH$_2$−CH$_2$−CH$_2$−CH$_2$−NH$_2$
2	d[Cha[4],Orn[8]]VP	−CH$_2$−CH$_2$−CH$_2$−NH$_2$
3	d[Cha[4],Dab[8]]VP	−CH$_2$−CH$_2$−NH$_2$
4	d[Cha[4],Dap[8]]VP	−CH$_2$−NH$_2$
5	d[Leu[4],Lys[8]]VP	−CH$_2$−CH$_2$−CH$_2$−CH$_2$−NH$_2$
6	d[Leu[4],Orn[8]]VP	−CH$_2$−CH$_2$−CH$_2$−NH$_2$
7	d[Leu[4],Dab[8]]VP	−CH$_2$−CH$_2$−NH$_2$
8	d[Leu[4],Dap[8]]VP	−CH$_2$−NH$_2$
9	d[Orn[4],Lys[8]]VP	−CH$_2$−CH$_2$−CH$_2$−CH$_2$−NH$_2$
10	d[Orn[4],Orn[8]]VP	−CH$_2$−CH$_2$−CH$_2$−NH$_2$
11	d[Arg[4],Lys[8]]VP	−CH$_2$−CH$_2$−CH$_2$−CH$_2$−NH$_2$
12	d[Arg[4],Orn[8]]VP	−CH$_2$−CH$_2$−CH$_2$−NH$_2$
13	d[Arg[4],Dab[8]]VP	−CH$_2$−CH$_2$−NH$_2$

We now report the synthesis and the binding affinities in rat V_{1b}, V_{1a}, V_2, and OT receptor assays of peptides **1**−**13** (Table 2). We also report their antidiuretic, vasopressor, and oxytocic activities in rat bioassays[77−80] (Table 1). It may be recalled that one of these peptides, d[Leu[4],Lys[8]]VP (**5**), had been previously reported in 1973.[81] This was at a time when the existence of the AVP V_{1b} receptor was neither hypothesized or known. So,

Table 3. Functional Properties of V_{1b} Specific Analogues d[Cha^4,Lys^8]VP (1), d[Cha^4,Dab^8]VP (3), d[Leu^4,Lys^8]VP (5) and d[Leu^4,Dap^8]VP (8) and Related Peptides (A) and (B) for Rat Vasopressin V_2 and V_{1b} Receptors

			V_2 receptor (rat kidney)			V_{1b} receptor (At-T20 cells transfected with rat V_{1b}-R)		
no.	peptides	antidiuretic activity (units/mg)	binding [^{3}H] AVP K_d (nM)	adenylate cyclase (AC) activity K_{act}(nM)	maximal AC activity (% of max AVP response)	binding [^{3}H] AVP K_d (nM)	phospholipase C activation K_{act} (nM)	maximal PLC activity (% of max AVP response)
	AVP[a]	323 ± 16[b]	0.45 ± 0.10	0.32 ± 0.08[c]	100	0.29 ± 0.05	2.3 ± 0.3	100
A	d[Cha^4]AVP[a]	133 ± 6[c]	12.7 ± 2.8	117 ± 15[c]	86	1.40 ± 0.09	0.6 ± 0.1	110 ± 3
1	d[Cha^4, Lys^8]VP[a]	0.82 ± 0.01	596 ± 123	ND[e]	ND[e]	1.88 ± 0.39	2.7 ± 0.4	73 ± 6
3	d[Cha^4, Dab^8]VP[a]	1.0 ± 0.1	447 ± 91	174 ± 25	75 ± 4	0.79 ± 0.17	1.4 ± 0.2	97 ± 4
B	d[Leu^4]AVP[a]	378 ± 24[d]	3.1 ± 0.8	ND[e]	ND[e]	0.04 ± 0.01	0.4 ± 0.1	128 ± 6
5	d[Leu^4, Lys^8]VP[a]	10.5 ± 0.9	101 ± 25	196 ± 71	76 ± 5	0.16 ± 0.09	1.5 ± 0.4	124 ± 4
8	d[Leu^4, Dap^8]VP[a]	0.7 ± 0.1	237 ± 47	300 ± 49	97 ± 3	0.38 ± 0.03	1.6 ± 0.1	108 ± 5

[a] This publication, unless otherwise noted. [b] Data from ref 87. [c] Data from ref 65. [d] Data from ref 68. [e] ND = not determined.

Table 4. Physicochemical Properties of Free Peptides **1**−**13**

			TLC,[b] R_f								
no.	peptide	yield[a] (%)	a	b	c	d	e	HPLC t_R (min)	formula	MW	MW found
1	d[Cha^4,Lys^8]VP	33.0	0.32	0.24	0.54	0.31		23.5	$C_{50}H_{71}O_{11}N_{11}S_2$	1066.3	1065.9
2	d[Cha^4,Orn^8]VP	48.0	0.33	0.10	0.43	0.25		20.1	$C_{49}H_{69}O_{11}N_{11}S_2$	1052.3	1051.0
3	d[Cha^4,Dab^8]VP	28.1	0.32		0.50	0.17	0.41	20.1	$C_{48}H_{67}O_{11}N_{11}S_2$	1038.3	1038.2
4	d[Cha^4,Dap^8]VP	17.8	0.36		0.42	0.28	0.51	20.2	$C_{47}H_{65}O_{11}N_{11}S_2$	1024.3	1024.2
5	d[Leu^4,Lys^8]VP	59.9	0.30	0.21	0.49	0.29		17.6	$C_{47}H_{67}O_{11}N_{11}S_2$	1026.3	1026.0
6	d[Leu^4,Orn^8]VP	44.6	0.31	0.21	0.50	0.28		17.8	$C_{46}H_{65}O_{11}N_{11}S_2$	1012.2	1014.5
7	d[Leu^4,Dab^8]VP	46.6	0.31		0.41	0.23	0.51	17.5	$C_{45}H_{63}O_{11}N_{11}S_2$	998.2	998.5
8	d[Leu^4,Dap^8]VP	46.3	0.32		0.39	0.24	0.45	17.1	$C_{44}H_{61}O_{11}N_{11}S_2$	984.2	984.3
9	d[Orn^4,Lys^8]VP	48.9	0.16		0.28	0.06	0.10	14.0	$C_{46}H_{66}O_{11}N_{12}S_2$	1027.3	1027.3
10	d[Orn^4,Orn^8] VP	37.5	0.12	0.10	0.27	0.13		14.0	$C_{45}H_{64}O_{11}N_{12}S_2$	1013.2	1013.6
11	d[Arg^4,Lys^8]VP	68.9	0.19		0.32	0.06		13.7	$C_{47}H_{68}O_{11}N_{14}S_2$	1069.3	1070.0
12	d[Arg^4,Orn^8] VP	49.5	0.18	0.05	0.29	0.12	0.18	13.9	$C_{46}H_{66}O_{11}N_{14}S_2$	1055.3	1056.1
13	d[Arg^4,Dab^8]VP	56.5	0.20		0.35	0.03	0.04	17.4	$C_{45}H_{64}O_{11}N_{14}S_2$	1041.3	1041.2

[a] Peptides **1**−**13** were obtained from the corresponding protected peptides **I**−**XIII**, Table 5. Yields are based on the protected peptide in the reduction−reoxidation step in each case and are uncorrected for AcOH 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 CH_3CN) over 30 min with a flow rate of 1.0 mL/min, as detailed in the Experimental Section, was applied.

Table 5. Physicochemical Properties of Protected Peptides **I**−**XIII**[a]

				TLC,[c] R_f				
no.	peptide[a]	yield[b] (%)	mp °C	a	b	c	d	e
I	Mpr(Meb)-Tyr(Bzl)-Phe-Cha-Asn-Cys(Bzl)-Pro-Lys(2Cl-Z)-Gly-NH_2	90.3	225−226	0.88	0.70	0.87	0.73	0.87
II	Mpr(Meb)-Tyr(Bzl)-Phe-Cha-Asn-Cys(Bzl)-Pro-Orn(Z)-Gly-NH_2	91.5	230−231	0.89	0.67	0.88	0.77	0.86
III	Mpr(Meb)-Tyr(Bzl)-Phe-Cha-Asn-Cys(Bzl)-Pro-Dab(Z)-Gly-NH_2	84.3	239−241	0.89		0.65	0.80	0.87
IV	Mpr(Meb)-Tyr(Bzl)-Phe-Cha-Asn-Cys(Bzl)-Pro-Dap(Z)-Gly-NH_2	84.4	244−246	0.78		0.65	0.75	0.87
V	Mpr(Meb)-Tyr(Bzl)-Phe-Leu-Asn-Cys(Bzl)-Pro-Lys(2Cl-Z)-Gly-NH_2	91.1	230−231	0.88	0.67	0.87	0.72	0.86
VI	Mpr(Meb)-Tyr(Bzl)-Phe-Leu-Asn-Cys(Mob)-Pro-Orn(Z)-Gly-NH_2	94.7	227−228	0.80	0.66	0.86	0.74	0.74
VII	Mpr(Meb)-Tyr(Bzl)-Phe-Leu-Asn-Cys(Bzl)-Pro-Dab(Z)-Gly-NH_2	73.0	235−237	0.83		0.96	0.76	0.81
VIII	Mpr(Meb)-Tyr(Bzl)-Phe-Leu-Asn-Cys(Bzl)-Pro-Dap(Z)-Gly-NH_2	77.8	247−249	0.82		0.87	0.72	0.76
IX	Mpr(Meb)-Tyr(Bzl)-Phe-Orn(Z)-Asn-Cys(Mob)-Pro-Lys(2Cl-Z)-Gly-NH_2	80.1	226−228	0.77		0.89	0.71	0.81
X	Mpr(Meb)-Tyr(Bzl)-Phe-Orn(Z)-Asn-Cys(Mob)-Pro-Orn(Z)-Gly-NH_2	92.9	222−224	0.81	0.66	0.86	0.74	0.86
XI	Mpr(Meb)-Tyr(Bzl)-Phe-Arg(Tos)-Asn-Cys(Bzl)-Pro-Lys(2Cl-Z)-Gly-NH_2	96.6	179−180	0.85	0.66	0.83	0.74	0.85
XII	Mpr(Meb)-Tyr(Bzl)-Phe-Arg(Tos)-Asn-Cys(Bzl)-Pro-Orn(Z)-Gly-NH_2	89.7	179−180	0.80	0.68	0.85	0.76	0.77
XIII	Mpr(Meb)-Tyr(Bzl)-Phe-Arg(Tos)-Asn-Cys(Bzl)-Pro-Dab(Z)-Gly-NH_2	80.0	183−185	0.76		0.83	0.72	0.78

[a] The protected peptides **I**−**XIII**, synthesized as described in the Experimental Section, are the immediate protected precursors for the peptides **1**−**13** 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.

the ACTH-releasing potency of this peptide was not investigated at that time. This peptide had been shown to exhibit negligible vasopressor activity and low antidiuretic activity in rat bioassays[81] (Table 1). It thus possessed the desired pharmacological properties needed for a selective V_{1b} agonist in the rat. The question now to be answered was, would this peptide, which possessed low bioactivities in the rat antidiuretic and vasopressor bioassays, retain the potent rat V_{1b} receptor agonism of its parent d[Leu^4]AVP?[68] We also report the functional properties for the rat V_{1b} and V_2 receptors of those peptides that exhibit high selectivity for the rat V_{1b} receptors with respect to the rat V_2, V_{1a}, and OT receptors in binding assays and that also exhibit very low activities in all three rat bioassays (Table 3).

Peptide Synthesis

The synthesis of the free peptides **1**−**13** (Table 4) was carried out utilizing the Merrifield solid-phase method[82−84] with the modifications previously described.[85−87] Starting from Boc-Gly resin, the protected precursors **I**−**XIII** (Table 5) were synthesized. HCl (1M)/acetic acid (AcOH) was used in all the deprotection steps. Neutralizations were carried out with 10% Et_3N/CH_2Cl_2 in all the neutralization steps. Coupling reactions were mediated mainly by DCC/HOBt[88] in CH_2Cl_2/DMF except for Boc-Asn, which was incorporated as its *p*-nitrophenyl ester[89,90] in DMF. Cleavage from the acylpeptide resin was by ammonolysis in methanol with DMF extraction[85,90] to give the

protected peptide amides **I**−**XIII** (Table 5). Na in liquid NH_3[56] was used to deblock each protected precursor, as previously described.[85−87] The resulting disulphydryl compounds were oxidatively cyclized with $K_3[Fe(CN)_6]$[91] using the modified reverse procedure.[92] The free peptides were desalted and purified by gel filtration on Sephadex G-15 and Sephadex LH-20, mainly in a two-step procedure[93] using 50% AcOH and 2 M AcOH as eluents, respectively, as previously described.[85−87] When necessary, an additional purification on Sephadex G-15 or Sephadex LH-20 with 0.2 M AcOH as eluent was carried out. The purity of the free peptides **1**−**13** (Table 4) was checked by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), and mass spectrometry. The four previously published position 8 analogues of dAVP, d[Lys^8]VP,[73] d[Orn^8]-VP,[74] d[Dab^8]VP,[75] and d[Dap^8]VP[76] (Table 2), were resynthesized by the solid-phase method as described for dAVP.[86] They were purified and analyzed as described for peptides **1**−**13**.

Bioassays. Peptides were assayed for agonistic activity in the rat antidiuretic assay, in the rat vasopressor assay, and in the in vitro rat oxytocic assay using the four-point assay design.[77−80] All experimental procedures were approved by the Institutional Committee for the Care and Use of Animals at Weill Medical College of Cornell University. Synthetic AVP and OT, which had been standardized in vasopressor and oxytocic units against the USP Posterior Pituitary Reference Standard, were used as working standards in all bioassays. Antidiuretic assays were on water-loaded rats under ethanol anesthesia, as described by Sawyer.[77] Vasopressor assays were performed on urethane-anesthetized and phenoxybenzamine-treated rats, as described by Dekanski.[78] Oxytocic assays were performed on isolated uteri from diethylstilbestrol-primed rats in a Mg^{2+}-free van Dyke−Hasting's solution.[79] When standard errors are presented in the tables, the means reflect results from at least four independent assay groups. A preliminary report on the bioassay activities and rat receptor affinities of some of these peptides has been reported.[94]

Receptor Binding and Functional Assays. These are described in the Experimental Section. They were carried out as previously described.[65,68,95b,96b,99,100]

Results

The rat V_{1b}, V_2, V_{1a}, and OT receptor affinities for the position 8 modified peptides **1**−**13**, together with those of their parent peptides d[Cha^4]AVP (**A**), d[Leu^4]AVP (**B**), d[Orn^4]AVP (**C**), and d[Arg^4]AVP (**D**)[67,68] and the related peptides, AVP,[57,87] dAVP,[72,86] dDAVP,[86,95a] dVDAVP,[96a] and d[D-3-Pal^2]AVP[97] are given in Table 2 and Figure 1. Their antidiuretic, vasopressor, and oxytocic (in vitro, no Mg^{++}) activities in rat bioassays together with those of the related analogues above and of d[Lys^8]-VP,[73] d[Orn^8]VP,[74] d[Dab^8]VP,[75] and d[Dap^8]VP[76] (resynthesized and original values) are given in Table 1. It should be noted that we obtained different values for the antidiuretic activities of these four resynthesized position 8 modified analogues compared to those from the original syntheses[73−76] (Table 1). In units/mg, with the original values in brackets, these values are as follows: d[Lys^8]VP, 550 (301);[73] d[Orn^8]VP, 486 (202);[74] d[Dab^8]VP, 463 (1373);[75] and d[Dap^8]VP, 165 (1079).[76] Thus, for the Lys^8 and Orn^8 analogues, our values are higher than those originally reported.[73,74] For the Dab^8 and Dap^8 analogues, the values are strikingly lower than the original values.[75,76] We have no explanation for these striking differences in antidiuretic activities. The vasopressor activities of the resynthesized position 8 analogues of dAVP are in general much closer to the values in the original publications[73−76] (Table 1).

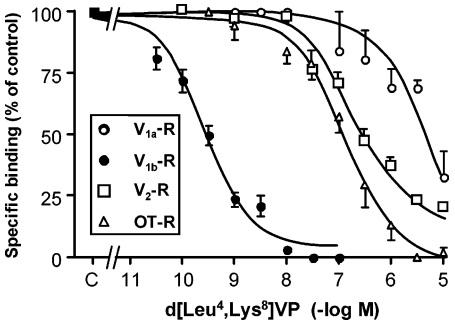


Figure 1. Binding properties of d[Leu^4,Lys^8]VP for rat AVP/OT receptors. The binding properties of d[Leu^4,Lys^8]VP were determined by binding competition experiments using [^{3}H]AVP as radioligand and membranes from At-T20 and CHO transfected cells (rV_{1b}-R and rOT-R, respectively) or from native tissues (rV_{1a}-R and rV_2-R), as described in the Experimental Section. Specific binding was plotted against the concentration of the unlabeled analogue used in the assay and expressed as percent of the specific binding measured in the presence of vehicle (**C**). Results are the mean ± SEM of at least three independent experiments, each performed in triplicate.

The functional properties in rat V_{1b} and V_2 receptor assays of the peptides, which are the most selective in the rat receptor binding assays, namely, d[Cha^4,Dab^8]VP (**3**), d[Leu^4,Lys^8]VP[81] (**5**), and d[Leu^4,Dap^8]VP (**8**) together with those of A, B, and d[Cha^4,Lys^8]VP (**1**), are given in Table 3 and Figure 2.

Examination of the rat V_{1b} receptor affinities of peptides **1**−**13** in Table 2 shows that, with the exception of peptide **4**, d[Cha^4,-Dab^8]VP, all of these peptides exhibit high affinities for the rat V_{1b} receptor. Ten peptides (**3**, **5**−**13**) exhibit subnanomolar affinities for the rat V_{1b} receptor. The Lys^8, Orn^8, Dab^8, and Dap^8 analogues of d[Cha^4]AVP and d[Leu^4]AVP exhibit low affinities for the rat V_2, V_{1a}, and OT receptors. By contrast, the position 8 analogues of C and D retain high affinities for the rat V_2 receptors and, thus, do not exhibit the desired V_{1b}/V_2 selectivity. Thus, in binding assays, only peptides **1**−**8** exhibit the desired high selectivity for the rat V_{1b} receptor with respect to the rat V_2, V_{1a}, and OT receptors. We selected four of these, **1**, **3**, **5**, and **8**, for examination in functional assays (see below).

Examination of the rat bioassay data for peptides **1**−**13**, presented in Table 1, shows that the four position 8 analogues of d[Cha^4]AVP (**A**; peptides **1**−**4**) all exhibit the desired reduction in antidiuretic activity compared to that exhibited by **A** (133.6 units/mg). The four peptides **1**−**4** exhibit antidiuretic activities in the range of 0.3 to 1.0 unit/mg. These four peptides also exhibit very weak vasopressor activities (0.002 to 0.22 unit/mg). Peptides **1**−**4** exhibit weak OT antagonism in in vitro (no Mg^{++}) assays. As noted earlier, peptide **5**, d[Leu^4,Lys^8]VP, had previously been reported to exhibit 5−6 units/mg of antidiuretic activity.[81] In this study, we report a value of 10.5 units/mg for the resynthesized peptide. These values show that replacing the Arg^8 residue by a Lys^8 residue in the recently reported parent peptide d[Leu^4]AVP,[67] which has an antidiuretic activity = 378 units/mg,[68] led to a drastic reduction in its antidiuretic activity. The vasopressor activity of d[Leu^4,Lys^8]VP was originally reported to be 0.55 unit/mg.[81] We report here vasopressor potency of 0.9 unit/mg for d[Leu^4,Lys^8]VP. We also report that d[Leu^4,Lys^8]VP exhibits very weak in vitro (no Mg^{++}) oxytocic activity (0.054 unit/mg). The three remaining analogues of d[Leu^4]AVP (**B**), peptides **6**−**8**, also exhibit reductions in antidiuretic potencies relative to **B**. With an antidiuretic potency of 0.75 unit/mg, the d[Leu^4,Dap^8]VP analogue (**8**) exhibits the greatest reduction. With vasopressor potencies of 3.1 units/mg and 3.5 units/mg, the Orn^8 and Dab^8 analogues of **B** do not show reductions in vasopressor potencies relative to that

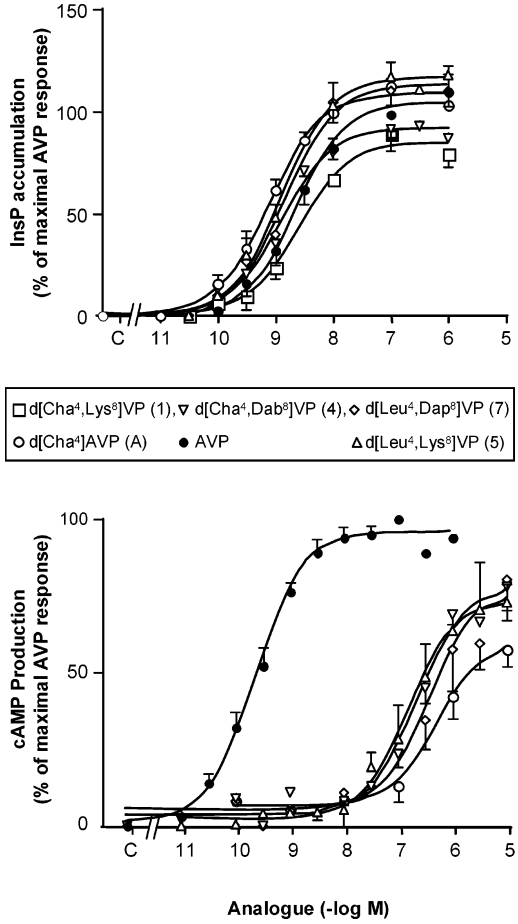


Figure 2. Agonist properties of some d[X^4,Y^8]VP analogues for the rat V_{1b} and V_2 receptors. Upper panel: [^{3}H] myo inositol prelabeled At-T20 cells stably transfected with the rV_{1b}-R were incubated 15 min at 37 °C in an HBS medium supplemented with 15 mM LiCl with or without (**C**) increasing amounts of AVP or d[X^4,Y^8]VP analogues. Total InsP, which accumulated, was determined in each experimental condition. Results, expressed as % of maximal InsP response obtained with 100 nM AVP, are the mean ± SEM of at least three independent experiments, each performed in triplicate. Lower panel: Rat kidney membranes naturally expressing the rV_2-R were incubated at 37 °C with or without (**C**) increasing amounts of AVP or d[X^4,Y^8]VP analogues in the presence of α[^{32}P]-ATP. [^{32}P]-cAMP produced was determined as described in the Experimental Section and expressed as % of maximal cAMP response obtained with 100 nM AVP. Results are the mean ± SEM of at least three distinct experiments, each performed in triplicate.

exhibited by **B** (3.11 units/mg). With a vasopressor potency of 0.05 unit/mg, the d[Leu^4,Dap^8]VP analogue (peptide **8**) exhibits a striking reduction in vasopressor potency relative to **B** (vasopressor activity = 3.11 units/mg). All three peptides **6**−**8** exhibit very weak oxytocic activities; 0.04, 0.27, and 0.046 unit/mg, respectively. With antidiuretic activities of 7.8 units/mg and 7.9 units/mg, peptides **9** and **10**, the Lys^8 and Orn^8 analogues of d[Orn^4]AVP (**C**), exhibit reductions in antidiuretic potencies relative to **C** (antidiuretic activity = 260 units/mg). These two analogues also exhibit ∼50% reductions in in vitro (no Mg^{++}) oxytocic activities relative to **C** (oxytocic activity = 6.49 units/mg). The Lys^8 analogue (**9**) exhibits diminished vasopressor potency (0.23 units/mg) relative to **C** (vasopressor activity = 1.54 units/mg), whereas the Orn^8 analog exhibits slightly higher vasopressor potency (1.9 units/mg). Replacement of the Arg^8 residue in d[Arg^4]AVP (**D**) by Lys and Orn to give peptides **11** and **12** led in each instance to enhancements of antidiuretic activity. Peptide **D** exhibits antidiuretic activity = 748 units/mg. Peptides **11** and **12** exhibit antidiuretic activity of 784 and 823 units/mg, respectively. Replacement of the Arg^8 residue in **D** by a Dab^8 residue to give peptide **13** reduced the antidiuretic activity. However, with an antidiuretic activity of 431 units/mg, peptide **13** is more potent than AVP (antidiuretic activity = 332 unit/mg). All three analogues of **D** (peptides **11**−**13**) exhibit substantial vasopressor potencies of 83, 173, and 190 units/mg, respectively, with one (peptide **11**) being lower and two (peptides **12** and **13**) being higher than **D** (vasopressor potency = 160 U/mg). Thus, in reviewing the rat receptor affinity data in Table 2 and the rat bioassay data in Table 1, it became clear that although the majority of these 13 peptides have high affinities for the rat V_{1b} receptor, only the two position 8 analogues of d[Cha^4]AVP (**A**), namely, d[Cha^4,Lys^8]VP (**1**) and d[Cha^4,Dab^8]VP (**3**), and two of the analogues of d[Leu^4]-AVP (**B**), namely, d[Leu^4,Lys^8]VP (**5**), and d[Leu^4,Dap^8]VP (**8**), exhibit the desired reductions in rat antidiuretic and vasopressor activities to merit further scrutiny in functional assays.

Functional Properties of d[Cha^4,Lys^8]VP (1), d[Cha^4,Dab^8]-VP (3), d[Leu^4,Lys^8]VP (5), and d[Leu^4,Dap^8]VP (8; Table 3, Figure 2). To further analyze the V_{1b} and V_2 agonist properties in the rat of the four analogues that exhibit the best affinities and selectivities for the rat V_{1b} receptor with respect to the rat V_2 receptor, namely, d[Cha^4,Lys^8]VP (**1**), d[Cha^4,-Dab^8]VP (**3**), d[Leu^4,Lys^8]VP (**5**), and d[Leu^4,Dap^8]VP (**8**), we tested their abilities to stimulate phospholipase C(PLC) (inositol phosphates accumulation) on At-T20 cells transfected with the V_{1b} receptor and adenylate cyclase (cyclic AMP accumulation) on rat kidney membranes expressing the V_2 receptor. In the PLC functional assays, there is a good correlation between their binding affinities for the rat V_{1b} receptor and their K_{act} (concentration of agonist leading to half-maximal activity) values (Table 3). Similarly, in the adenylate cyclase assay, peptides **3**, **5**, and **8** exhibit a good correlation between their K_{act} values and their binding affinities for the rat V_2 receptor. Thus, in contrast to their parent peptides, d[Cha^4]AVP (**A**) and d[Leu^4]AVP (**B**), peptides **1**, **3**, **5**, and **8** are excellent high affinity selective agonists for the rat V_{1b} receptor.

Discussion

Effects of Position 8 Modification in Peptides A−D on Rat V_{1b} Receptor Affinities and Selectivities. In searching for clues to the design of selective agonists for the rat V_{1b} receptor, we selected as leads four peptides, d[Cha^4]AVP (**A**), d[Leu^4]-AVP (**B**), d[Orn^4]AVP (**C**), and d[Arg^4]AVP (**D**), which are highly selective for the human V_{1b} receptor with respect to the human V_2, V_{1a}, and OT receptors.[67] Although these four peptides were subsequently found to exhibit high affinities for the rat V_{1b} receptor,[68] they were also found to exhibit high affinities for the rat V_2 receptor and to exhibit potent antidiuretic activities in rat bioassays.[68] Consequently, they are not selective for the V_{1b} versus the V_2 receptor in the rat. To use these four peptides as leads for the design of ligands that are selective for the rat V_{1b} receptor, we needed to retain their affinities for the V_{1b} receptor while diminishing their affinities for the rat V_2 receptor. In the study reported here, the Arg^8 residue in peptides **A**−**D** was replaced with one or more of the following basic amino acids: Lys, Orn, Dab, and Dap. The resulting peptides **1**−**13** (Table 2) exhibit varying degrees of high V_{1b} receptor affinity and selectivity. With the exception of peptides **11**−**13**, all peptides exhibit very low affinities for the rat V_{1a} receptor. Thus, peptides **1**−**10** are highly selective for the rat V_{1b} receptor with respect to the rat V_{1a} receptor. All 13 peptides exhibit diminished affinities for the rat OT receptor, especially in relationship to

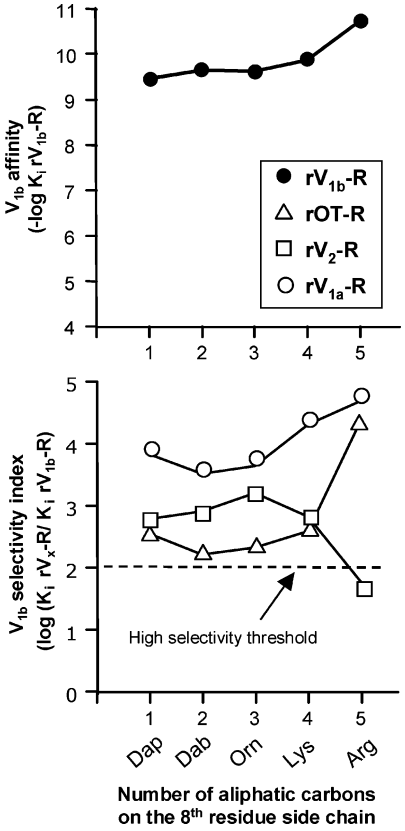


Figure 3. Influence of the number of aliphatic carbons in the side-chain at position-8 of d[Leu^4,Y^8]VP analogues on rat V_{1b} receptor properties. The affinity (upper panel) and the V_{1b} receptor SI relative to other VP/OT receptor isoforms (lower panel) for given d[Leu^4,Y^8]-VP analogues were plotted as a function of the number of aliphatic carbons present on the side chain of their eight residue. K_i values and V_{1b} SI were from Table 2.

dAVP (K_i = 0.97 nM). They thus exhibit varying degrees of selectivity for the rat V_{1b} receptor with respect to the rat OT receptor. Peptides **1**−**8**, the position 8 analogues of d[Cha^4]AVP (**A**) and d[Leu^4]AVP (**B**), all exhibit diminished affinities for the rat V_2 receptor and, in binding assays, are consequently highly selective for the rat V_{1b} receptor with respect to the rat V_2 receptor. These are the first such peptides to exhibit high affinity and selectivity for the rat V_{1b} receptor with respect to the rat V_{1a}, V_2, and OT receptors.

Effects on Rat V_{1b} Receptor Affinity of Position 8 Modifications in d[Cha^4]AVP (A; Peptides 1−4, Table 2). d[Cha^4]AVP (**A**) exhibits a K_i value for the rat V_{1b} receptor = 1.40 nM.[68] Replacement of the Arg^8 residue in d[Cha^4]AVP by Lys, Orn, Dab, and Dap to give peptides **1**−**4** led to varying degrees of retention of rat V_{1b} receptor affinity (Table 2). The Lys^8 and Dab^8 substituents are clearly superior to the Orn^8 and Dap^8 substituents in retaining or enhancing rat V_{1b} receptor affinity. d[Cha^4,Lys^8]VP (**1**) and d[Cha^4,Dab^8]VP (**3**) are the most promising peptides to emerge from this series of position 8 analogues of **A**. These findings show that when the position 4 residue in dAVP is occupied by Cha, the affinity for the rat V_{1b} receptor is highly dependent on the structure of the basic amino acid at position 8.

Effects on Rat V_{1b} Receptor Affinity of Position 8 Modifications in d[Leu^4]AVP (B; Peptides 5−8, Table 2; Figure 3). d[Leu^4]AVP (**B**) exhibits a very high K_i value for the rat V_{1b} receptor = 0.04 nM.[68] Replacement of the Arg^8 residue in B by Lys, Orn, Dab, and Dap led to excellent retention of rat V_{1b} receptor affinities by the resultant peptides **5**−**8**. All four peptides **5**−**8** exhibit rat V_{1b} receptor affinities equivalent to that of AVP (K_i = 0.29 nM). The high affinity of the Dap^8 analogue of **B**, peptide **8**, is in striking contrast to the relatively low affinity of the Dap^8 analogue of **A**, peptide **4**. These findings demonstrate that the effects of the Dap^8 modification in d[X^4]-AVP analogues on rat V_{1b} receptor affinity is very dependent on the structure of the amino acid at position 4 and on the number of aliphatic carbons of the residue at position 8 (Figure 3, upper panel). These findings show that when position 4 in dAVP is occupied by Leu, position 8 can be occupied with a wide variety of basic amino acids with retention of high affinity for the rat V_{1b} receptor. These findings are in contrast to those observed when position 4 in dAVP is occupied by Cha (see above).

Effects on Rat V_{1b} Receptor Affinity of Position 8 Modifications in d[Orn^4]AVP (C; Peptides 9 and 10). d[Orn^4]-AVP (**C**) exhibits subnamolar affinity for the rat V_{1b} receptor (K_i = 0.45 nM).[68] Replacement of the Arg^8 residue in **C** by Lys and by Orn to give peptides **9** and **10** resulted in retention of subnanomolar affinities for the rat V_{1b} receptor. The affinity of peptide **9** for the rat V_{1b} receptor is equivalent to that of **C**. The affinity of peptide **10** for the rat V_{1b} receptor is about half that of **C**. These finding show that Arg^8, Lys^8, and Orn^8 substitutions in combination with an Orn^4 substitution in dAVP are well tolerated by the rat V_{1b} receptor.

Effects on Rat V_{1b} Receptor Affinity of Position 8 Modifications in d[Arg^4]AVP (D; Peptides 11−13, Table 2). d[Arg^4]AVP (**D**) exhibits subnamolar affinity for the rat V_{1b} receptor (K_i = 0.13 nM).[68] Replacement of the Arg^8 residue in **D** by Lys, Orn, and Dab to give peptides **11**−**13** was very well tolerated. Peptides **11**−**13** all exhibit subnamolar affinities for the rat V_{1b} receptor. These findings show that when position 4 in dAVP is occupied by Arg, position 8 can be occupied by Arg, Lys, Orn, and Dab with retention of high affinity for the rat V_{1b} receptor.

These data prove conclusively that the four lead peptides **A**−**D** could be modified at position 8 to give a series of peptides that, with the exception of three peptides, the Lys^8, Orn^8, and Dap^8 analogues of A (peptides **1**, **2**, and **4**), exhibit subnanomolar affinity for the rat V_{1b} receptor.

Effects of Position 8 Modifications in Peptides A−D on Rat V_2 Receptor Affinities (Table 2; Figure 3). As is clear from the rat V_2 receptor affinity data in Table 2, the effects of the Lys^8, Orn^8, Dab^8, and Dap^8 replacements for Arg^8 in peptides **A**−**D** depends very much on the nature of the amino acid at position 4. When these substitutions were carried out in the Cha^4 and Leu^4 analogues (peptides **A** and **B**), the resulting peptides **1**−**8** exhibited significant reductions in rat V_2 receptor affinities, whatever the size of the position 8 residue (1−4 aliphatic carbons; Figure 3, lower panel). By contrast, when these substitutions were carried out in the Orn^4 and Arg^4 analogues (peptides **C** and **D**), the resulting peptides **9**−**13** exhibit only very modest reductions in rat V_2 receptor affinities relative to those exhibited by the parent peptides **C** and **D**.

Effects on Rat V_2 Receptor Affinities and Selectivities of Position 8 Modifications in d[Cha^4]AVP (A; Peptides 1−4, Table 2). d[Cha^4]AVP (**A**) exhibits a K_i value for the rat V_2 receptor = 12.7 nM.[65] Replacement of the Arg^8 residue in **A** by Lys, Orn, Dab, and Dap to give peptides **1**−**4** brought about striking reductions in affinities for the rat V_2 receptor. These reductions combined with the high affinities for the rat V_{1b} receptor lead to strikingly high rat V_{1b}/V_2 receptor selectivities exhibited by peptides **1**−**4** relative to that of **A**

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Effects on Rat V_2 Receptor Affinities and Selectivities of Position 8 Modifications in d[Leu^4]AVP (Peptides 4−8, Table 2). d[Leu^4]AVP (**B**) exhibits a K_i value for the rat V_2 receptor = 3.1 nM.[68] Peptides **5**−**8** exhibit reductions in rat V_2 receptor affinities relative to **B**. Thus, the replacement of the Arg^8 residue in **B** with Lys, Orn, Dab, and Dap resulted in the desired reductions in affinities for the rat V_2 receptor. These reductions combined with the high affinities for the rat V_{1b} receptor led to striking enhancements in rat V_{1b}/V_2 receptor selectivities of peptides **5**−**8** relative to **B**.

Effects on Rat V_2 Receptor Affinities and Selectivities of Position 8 Modifications in d[Orn^4]AVP (C; Peptides 9 and 10) and in d[Arg^4]AVP (D; Peptides 11−13, Table 2). With K_i values of 3.4 nM and 0.2 nM, peptides **C** and **D** exhibit high and very high affinities, respectively, for the rat V_2 receptor. Replacement of the Arg^8 residue in d[Orn^4]AVP (**C**)[68] by Lys and Orn resulted in only modest reductions in rat V_2 receptor affinities relative to that exhibited by **C** (Table 2). Consequently, peptides **9** and **10** do not exhibit the same gains in rat V_{1b}/V_2 receptor selectivity as peptides **1**−**8**. The affinities of peptides **11**−**13** for the rat V_2 receptor, while reduced in comparison with the very high affinity of the parent peptide **D**, nonetheless, are in the same high range as peptides **B** and **C**. Thus, peptides **11**−**13** exhibit very low gains in rat V_{1b}/V_2 receptor selectivities (Table 2).

It is clear from the rat V_2 receptor affinities and rat V_{1b}/V_2 receptor selectivities of the 13 position 8 analogues of **A**−**D** in Table 2 that the neutral lipophilic substitutions, Cha and Leu, are superior to the basic amino acids, Orn and Arg, at position 4 in leading to peptides that exhibit reduced rat V_2 receptor affinities and high rat V_{1b}/V_2 receptor selectivities.

Effects of Position 8 Modifications of Peptides A−D on Antidiuretic Activities: Comparisons with Rat V_2 Receptor Affinities (Peptides 1−13, Tables 1 and 2; Figure 4). As noted earlier, all four parent peptides, d[Cha^4]AVP (A), d[Leu^4]AVP (**B**), d[Orn^4]AVP (**C**), and d[Arg^4]AVP (**D**),[68] although possessing excellent affinities for the rat V_{1b} receptor, are clearly not selective agonists for the rat V_{1b} receptor, because they also exhibit potent antidiuretic activities. Replacement of the Arg^8 residue in d[Cha^4]AVP (**A**) with Lys, Orn, Dab, and Dap resulted in each case in drastic reductions in antidiuretic potencies (Table 1). These findings all correlate very well with the low affinities for peptides **1**−**4** in the rat V_2 receptor binding assays (Table 2, Figure 4). Replacement of the Arg^8 residue in d[Leu^4]AVP (**B**) by Lys, Orn, Dab, and Dap also brought about marked reductions in antidiuretic activities. The original synthesis of d[Leu^4, Lys^8]VP (peptide **5**) reported antidiuretic activity of 5−6 units/mg.[81] In peptides **4**−**8**, with the exception of d[Leu^4, Dap^8]VP (peptide **8**), the low rat receptor affinities of peptides **5**−**7** do not correlate well with their rat antidiuretic activities. These findings are illustrated in Figure 4, upper panel, showing that receptor affinities may not always accurately reflect agonistic properties. In striking contrast to their effects in **A**, **B**, and **C**, replacement of the Arg^8 residue in d[Arg^4]AVP (**D**) by Lys and Orn to give peptides **11** and **12** effected unexpected increases in antidiuretic activities. With antidiuretic activities of 784 and 823 units/mg, peptides **11** and **12** are among the most potent antidiuretic agonists reported to date. These two peptides and indeed peptide **D** are clearly far more potent antidiuretic agonists than AVP (antidiuretic activity = 332 units/mg).[86] These findings on the contrasting effects on antidiuretic activities of the Lys^8, Orn^8, Dab^8, and Dap^8 substitutions in dAVP$^{73-76}$ and in d[Arg^4]AVP (**D**) with those obtained for those same substitutions in peptides **A**−**C**, (d[Cha^4]AVP, d[Leu^4]-AVP, and d[Orn^4]AVP), illustrate the need for caution in making generalizations on the effects of a series of substitutions based on making these changes in only one peptide.

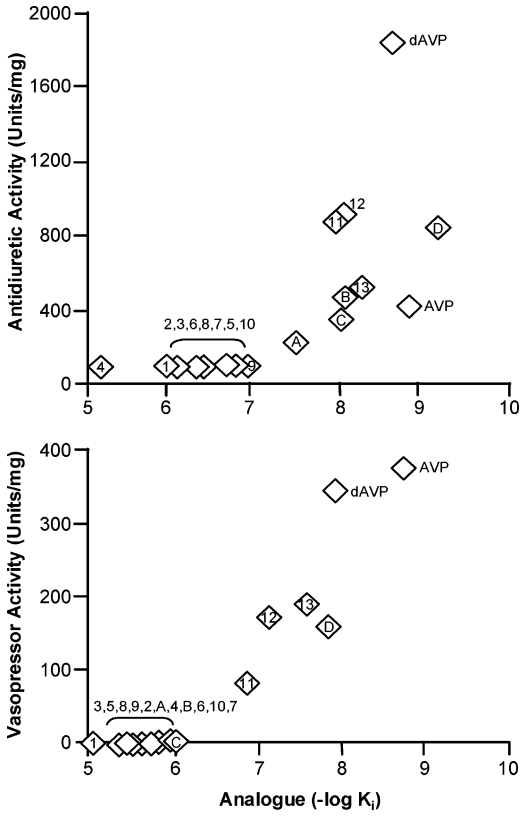

Figure 4. Correlation between antidiuretic and vasopressor activities of d[X^4,Y^8]VP analogues and their corresponding binding properties (K_i). Data from this figure are from Tables 1 and 2. The correspondence between peptides (**A**−**D** and **1**−**13**) is given in Table 2.

Effects of Position 8 Modifications of Peptides A and B on Vasopressor Activities (Peptides 1−8, Table 1, Figure 4). d[Cha^4]AVP (**A**) exhibits very weak vasopressor activity. Replacement of the Arg^8 residue in **A** by Lys, Orn, Dab, and Dap resulted in two peptides (**1** and **4**) with reductions of vasopressor potencies and two peptides (**2** and **3**) with very modest increases in vasopressor potencies. This pattern was also observed with d[Leu^4]AVP (**B**). The Lys^8 and Dap^8 analogues of **B** (peptides **5** and **8**) exhibit diminished vasopressor potencies. Whereas, the Orn^8 and Dab^8 analogues (peptides **7** and **8**) exhibit vasopressor potencies that are virtually equipotent with that of **B**. The greatest reductions in vasopressor potencies in peptides **A** and **B** were clearly brought about by the Dap^8 modification, which has a single carbon in its side chain. The Lys^8 residue with four carbons also brought about a marked reduction in vasopressor potency in peptide **B**. By contrast, the Orn^8 and Dab^8 residues, with three and four carbons in their side chains, led to slight enhancements in vasopressor potency in **A** and good retention of vasopressor potency in **B**. These patterns of reduced and retained or enhanced vasopressor activities brought about by the Lys, Orn, Dab, and Dap substituents at position 8 in d[Cha^4]AVP (**A**) and in d[Leu^4]AVP (**B**), closely mirror the effects on vasopressor activities of these four substituents at position 8 in dAVP$^{73-76}$ (Table 1). Thus, there is no correlation between the number of carbons in the side chain at position 8 and the vasopressor potency of the resultant analogue. As noted above for the rat V_2 receptor, there is not always a good correlation between binding data for the rat V_{1a} receptor and for rat vasopressor activities (see Figure 4, lower panel).

Conclusion

We report here the first analogues of AVP, which are selective agonists for the rat V_{1b} receptor with respect to the rat V_{1a}, V_2, and OT receptors. These peptide analogues were designed by using as leads the four peptides, d[Cha[4]]AVP (**A**), d[Leu[4]]AVP (**B**), d[Orn[4]]AVP (**C**), and d[Arg[4]]AVP (**D**), which were the first high affinity selective agonists for the human V_{1b} receptor.[67] Peptides **A**−**D** exhibit properties very similar to those of the potent selective antidiuretic agonist dDAVP,[86,95(a)] widely used clinically for the treatment of diabetes insipidus.[1] Recently, dDAVP was shown to be a full V_{1b} agonist in humans and a partial V_{1b} agonist in rats.[98] Replacement of the Arg[8] residue in peptides **A**−**D** by Lys, Orn, Dab, and Dap resulted in peptides **1**−**13** (Table 1). With the exception of peptides **1**, **2**, and **4**, all of these peptides exhibit subnanomolar affinities for the rat V_{1b} receptor (Table 2). Examination of these 13 peptides in rat V_{1a}, V_2, and OT receptor binding assays and in rat antidiuretic, vasopressor, and oxytocic (in vitro; no Mg^{++}) bioassays uncovered four peptides: d[Cha[4],Lys[8]]VP (**1**), d[Cha[4],Dab[8]]VP (**3**), d[Leu[4],Lys[8]]VP (**5**), and d[Leu[4],Dap[8]]VP (**8**), which exhibit very high affinities for the rat V_{1b} receptor and very low affinities for the rat V_{1a}, V_2, and OT receptors. These four peptides exhibit high selectivities for the rat V_{1b} receptor with respect to the rat V_2, V_{1a}, and OT receptors. In functional assays, these four peptides are potent agonists for the rat V_{1b} receptor and weak agonists for the rat antidiuretic, vasopressor, and OT receptors. One of these peptides, d[Leu[4],Lys[8]]VP, was first reported in 1973.[81] Long before the discovery of the AVP V_{1b} receptor,[63,64] it was shown to be a weak antidiuretic and vasopressor agonist in rat bioassays.[81] We now report that it is also a weak oxytocic agonist and that it is a potent and a highly selective agonist for the rat V_{1b} receptor. The selective agonists for the rat V_{1b} receptor reported here are potentially useful new tools for studies on the role of the V_{1b} receptor in modulating anxiety in the rat. They also offer promising new leads to the design of peptide antagonists for the rat V_{1b} receptor and of radiolabeled and fluorescent ligands for the rat V_{1b} receptor.

Experimental Section

Materials. All reagents used were analytical grade. Most standard chemicals were purchased from Sigma (St. Louis, MO), Roche Molecular Biochemicals (Mannheim, Germany), or Merck & Co., Inc. (Darmstadt, Germany), unless otherwise indicated. AVP came from Bachem (Bubendorf, Switzerland). [^{3}H]AVP (60−80 Ci/mmol) was from Perkin-Elmer Life Sciences (Coutaboeuf, France). DMEM (Dubbelco's modified Eagles medium)-F12 was from Sigma (St. Louis, MO), geneticine and Lipofectine Plus Reagent were from Gibco, L-glutamine was purchased from In vitrogen (Cergy Pontoise, France), Nu-serum was from Becton Dickinson Biosciences (Ozyme, Saint Quentin en Yvelines, France), and Fetalclone III was from Hyclone (Perbio, Brévières France). Dowex AG1-X8 formate form 200−400 mesh was purchased from Bio-Rad Laboratories, Inc. (Munchen, Germany). The Merrifield resin was purchased from Calbiochem−Novabiochem Corp. (San Diego, CA). The Boc-Asn-ONp was provided by Bachem Bioscience, Inc. (King of Prussia, PA). The Boc-Phe, Boc-Leu, and Boc-Pro derivatives were provided by Bachem (Torrance, CA). All other amino acid derivatives were purchased from Chem-Impex International, Inc. (Wood Dale, IL). 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 (4:1:2); (d) 1-butanol/AcOH/H_2O/pyridine (15:3:3:10); and (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.[84] 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 CH_3CN, linear gradient over 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. Mass spectra were done by the Tuft's Core Facility, Physiology Department (Boston, MA) on a MALDI-TOF Voyager mass spectrometer (Perspective Biosystems/Applied Biosystems) using dihydrobenzoic acid as the matrix.

Solid-Phase Synthesis Procedures. Peptides **1**−**13** (Table 4) were synthesized using the Merrifield method[82−84] with the modifications previously described.[85−87] Starting with 0.25 nM of Boc-Gly resin, eight cycles of deprotection, neutralization, and coupling were carried out by the DCC/HOBt[88] or the active ester[89−90] procedure. The side chain protection of the Boc-amino acids incorporated was as follows: Mpr(Meb), Tyr(Bzl), Orn(Z), Arg(Tos), Cys(Mob) or Cys(Bzl), Lyz(2-Cl-Z), Dab(Z), and Dap-(Z). Ammonolysis in MeOH[85,90] was used to cleave the protected peptides from the resin. The protected precursors obtained by ammonolysis were extracted with hot DMF and isolated by precipitation with hot water, as previously described.[85−87] They were purified by reprecipitations with DMF/MeOH/Et_2O until adjudged pure by TLC[85] to give the required protected peptides **I**−**XIII** (Table 5). All protected precursors **I**−**XIII** were deblocked using sodium in liquid ammonia,[56] as previously described.[85−87] The resulting disulphydryl compounds were oxidatively cyclized with $K_3[Fe(CN)_6]$[91] using the modified reverse procedure.[92] The free peptides were purified by a two-step gel filtration procedure[93] 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**−**13** are given in Table 4.

Mpr(Meb)-Tyr(Bzl)-Phe-Leu-Asn-Cys(Bzl)-Pro-Dap(Z)-Gly-NH_2 (VIII, Table 5). Boc-Gly resin (0.5 g, 0.35 mmol) was subjected to eight cycles of deprotection, neutralization, and coupling with Boc-Dap(Z), Boc-Pro, Boc-Cys(Bzl), Boc-Asn-ONp, Boc-Leu, Boc-Phe, Boc-Tyr(Bzl), and Mpr(Meb), respectively. The resulting protected peptidyl resin was cleaved by ammonolysis, as previously described.[85−87] The protected peptide was extracted with hot DMF (30 mL), and the product was precipitated by the addition of hot water (ca. 300 mL). After cooling, the product was collected, dried in vacuo over P_2O_5, and reprecipitated from methanol (30 mL) and ether (ca. 200 mL). Collection and drying in vacuo over P_2O_5 gave the required nonapeptide amide 490 mg, yield 77.8% (**VIII**, Table 5). The same procedure was used for the synthesis and purification of all other protected nonapeptide amides **I**−**VII**, **IX**−**XIII** (Table 5). The physicochemical properties of the protected peptides **I**−**XIII** are given in Table 5.

d[Leu[4],Dap[8]] Vasopressin (8, Table 4). The Na/liquid NH_3 procedure[56] was used for the deprotection of all protected nonapeptide amides **I**−**XIII**, as described here for peptide **VIII**. A solution of protected nonapeptide amide (**VIII**, 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 about 30 s.[85−87] NH_4Cl was added to discharge the color. Reoxidation of the deblocked disulphydryl peptide **8** was performed with $K_3[Fe(CN_6)]$[91] by the modified reverse procedure[92] as follows. The resulting disulphydryl peptide residue was dissolved in 25 mL of 50% AcOH, and the solution was diluted with 50 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 **8** 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). The combined filtrate and washings were lyophilized. The resulting powder was desalted on a Sephadex G-15 column (110 × 2.7 cm), eluting with aqueous AcOH (50%), with a flow rate of 5 mL/h[93].

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 × 1.5 cm), eluting with aqueous AcOH (2 M 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 **8** (48.7 mg, 46.3%; Table 4). The deprotection, reoxidation, and purification of peptides **1**−**7** and **9**−**13** (Table 4) were carried out in essentially the same manner.

Cell Transfection. Mouse corticotroph At-T20 cells (ATCC, CRL-1795) were transfected using the cDNA coding for the rat V1b-R.[53] This cDNA called rV3 was amplified by polymerase chain reaction, inserted into the HindIII-Xbal sites of the expression vector pcDNA3, and verified by restriction enzyme digestion and DNA sequencing. Transfection was achieved using the Lipofectamine Plus reagent according to the instruction of the manufacturer. Cells stably expressing pcDNA-rV3 were selected using the geneticine added in the cell culture medium. The clones able to bind [^{3}H]AVP were purified by the limited dilution techniques and screened again by the [^{3}H]AVP assay.

Cell Culture. Chinese hamster ovary (CHO) cells stably expressing the rat isoforms of the vasopressin V_{1a}, V_{1b}, V_2, and OT receptors were cultured in DMEM supplemented with 10% of fetal calf serum, 2 mM L-glutamine, 500 units/mL penicillin and streptomycin, and 1× nonessential amino acids in an atmosphere of 95% air and 5% CO_2 at 37 °C, as previously described (65).

Mouse corticotroph At-T20 cells stably expressing the rat V_{1b} receptor isoform were cultured in the same conditions as the CHO cells but in a DMEM/F12 medium supplemented with 7.5% Fetalclone II and 7.5% Nu-serum, 0.5 mM L-glutamine and 200 μg/mL geneticine.

Binding Experiments. CHO and At-T20 cells, stably transfected with vasopressin or OT receptors, were used to prepare crude plasma membrane, as previously described (65). Rat kidney (inner medulla) and liver membrane preparations naturally expressing V_2 and V_{1a} receptor isoforms were obtained as previously described.[99] Membrane binding assays were performed as previously described[99] using [^{3}H]AVP as radioligand. Membranes (5−50 μg protein) were incubated 60 min at 30 °C (membranes from transfected cells) or at 37 °C (membranes from native tissues) in a medium containing 50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mg/mL bovine serum albumin (BSA), and 0.01 mg/mL leupeptine. The binding properties of the unlabeled analogues were determined by competition experiments. Briefly, 0.5 to 3 nM of [^{3}H]AVP was added in the incubation medium with (nonspecific binding) or without (total binding) 1 μM of unlabeled AVP and increasing amounts of the unlabeled analogues to be tested or with vehicle (control). Radioactivity found associated to plasma membranes was determined by filtration through GF/C filters. Specific binding was calculated as the difference between total and nonspecific binding and expressed as percent of control specific binding.

Functional Assays. Total inositol phosphate (InsP) accumulation was determined as previously described.[9] Briefly, At-T20 cells expressing the rat vasopressin V_{1b} receptor were cultured in 24-well plates at 100 000 cells/well. Cells were grown for 48 h in DMEM/F12 medium supplemented with 7.5% Fetalclone II, 7.5% Nu-serum, and 0.5 mM L-glutamine and with 1 μCi/mL *myo*-[2-^{3}H]-inositol. Cells were then washed twice with a Hank's buffered saline medium (HBS), equilibrated at 37° in HBS for 30 min and incubated for 15 min in HBS supplemented with 15 mM LiCl, 1 mg/mL glucose, 1 mg/mL BSA, and 2.1 g/L $NaHCO_3$. Cells were then stimulated for 15 min with increasing concentrations of the analogues to be tested. The reaction was stopped with 5% (v/v) perchloric acid. InsP accumulated were extracted and purified on Dowex AG1-X8 anion exchange chromatography column and counted.

Adenylate cyclase activity was assessed on plasma membranes from rat kidney and was determined by measuring the conversion of α[^{32}P]-ATP to [^{32}P]-cAMP, as previously described.[95b,102]

Data analysis were analyzed by GraphPad Software, Inc., Prism (GraphPad Software, Inc., San Diego, CA), as previously described.[65] The inhibitory dissociation constants (K_i) for unlabeled AVP analogues were calculated from binding competition experiments according to the Cheng and Prusoff equation: $K_i = IC_{50}/(1 + [L]/K_d)$,[101] where IC_{50} is the concentration of unlabeled analogue leading to half-maximal inhibition of specific binding, [L] is the concentration of the radioligand present in the assay, and K_d is its affinity for the VP receptor studied. Concentrations of VP analogue leading to half-maximal stimulation of second messenger accumulations (K_{act}) were calculated from functional studies using GraphPad Software, Inc., Prism. Results are expressed as the mean ± SEM of the number of distinct experiments indicated.

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