Biochem. J. (1996) 317, 577–582 (Printed in Great Britain)

Molecular recognition of peptide and non-peptide ligands by the extracellular domains of neurohypophysial hormone receptors

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This study was designed to ascertain whether the extracellular loops of vasopressin/oxytocin receptors bind ligands and, if so, to locate the molecular determinants of this ligand–receptor interaction. Ligand-binding studies were employed using a rat liver V_{1a} vasopressin receptor preparation and both peptide and non-peptide receptor ligands. Synthetic peptides corresponding to defined regions of the extracellular surface of the neurohypophysial hormone receptors recognized radioligands. These receptor mimetics inhibited the binding of radioligands to the V_{1a} receptor with apparent affinities (pK_i) ranging from 3.1 to 6.75. The same mimetics had no effect on the binding of angiotensin II to the rat AT_1 receptor, indicating specificity for V_{1a} receptor ligands. A mimetic peptide (DITYRFRGPDWL) of the first extracellular loop (ECII) of the V_{1a} vasopressin receptor also inhibited vasopressin-stimulated, but not angiotensin II-stimulated, glycogen phosphorylase in isolated rat hepatocytes. In contrast, scrambled ECII mimetics displayed greatly reduced affinity for vasopressin. In addition, the role of peptide side-chain versus main-chain atoms in the binding of ligands by vasopressin receptors was addressed using retro–inverso peptide mimetics. Our findings indicate a precise orientation of the extracellular receptor surface (particularly the ECII domain) which facilitates the initial 'capture' of both peptide and non-peptide ligands. Moreover, the data indicate that the main-chain atoms of both a major binding-site determinant in the first extracellular loop of the receptor and the neurohypophysial hormones contribute significantly to the ligand–receptor interaction. These findings also suggest that soluble receptor-binding domains have therapeutic potential.

INTRODUCTION

The structurally similar mammalian neurohypophysial peptide hormones [Arg[8]]vasopressin (AVP) and oxytocin (OT) have distinct physiological roles which are mediated by discrete receptors. AVP increases blood pressure, antidiuresis and glycogenolysis whereas OT stimulates lactation and uterine contraction. In common with other hormones and neurotransmitters, subtypes of vasopressin receptor (VPR) have been distinguished by pharmacological criteria [1,2]. More recently, the V_{1a}, V_{1b} and V_2 subtypes of VPR together with the oxytocin receptor (OTR) have been cloned [3–6]. Identification of the mechanism by which AVP and OT selectively bind to their respective G-protein-coupled receptors (GPRs) will augment the development of pharmaceuticals to combat a range of disease states including those of the cardiovascular system in which AVP and/or OT have a pathophysiological role [7].

It is now well established that the binding of biogenic amines to GPRs involves interaction of ligand with the hydrophobic transmembrane domains of the respective receptor [8]. However, size considerations and molecular modelling indicate that peptide ligands are unlikely to be accommodated solely within the transmembrane domains of GPRs in a manner analogous to the binding of biogenic amines [9]. Analysis of the primary amino acid sequence of cloned AVP and OT receptor proteins identifies two highly conserved domains in the putative first (ECII) and second extracellular loops (ECIII) [10,11]. We reasoned that this conservation of sequence reflected a common function. To test the hypothesis that these domains play a role in ligand binding, we have synthesized series of peptide mimetics corresponding to the four extracellular domains of the rat V_{1a} VPR ($rV_{1a}R$). These were then employed in radioligand-binding studies to determine whether molecular recognition occurs between the extracellular loops of neurohypophysial receptors and peptide/non-peptide ligands.

Mimetic peptides synthesized entirely from D-amino acids in the reverse order of the usual L-homologue (retro–inverso peptides) can retain biological activity [12]. The orientation of the amino acid side chains of retro–inverso peptides is identical with that of the naturally occurring peptide, whereas carbonyl and amine groups forming backbone amide bonds are reversed [12,13]. Retro–inverso peptides are therefore useful probes for determining the relative contribution of side-chain and main-chain interactions in a molecular recognition process. Thus we chose to adopt this strategy to elucidate further the molecular recognition events which mediate binding of neurohypophysial hormone analogues to the V_{1a} VPR.

EXPERIMENTAL

Materials

[Phe-3,4,5-^{3}H][8-arginine]vasopressin ([^{3}H]AVP; 64.8 Ci/mmol), the labelled V_{1a}-selective antagonist [Phe-3,4,5-^{3}H][1-β-mercapto-β,β-cyclopentamethylenepropionic acid, 2-*O*-methyltyrosine]AVP {[^{3}H][d$(CH_2)_5$Tyr(Me)^2]AVP; 50.7 Ci/mmol} and [Tyr-3,5-^{3}H]angiotensin II ([^{3}H]AngII; 71.5 Ci/mmol) were from DuPont–NEN (Stevenage, Herts., U.K.). The non-peptide V_{1a}-

Abbreviations used: AngII, angiotensin II; AVP, [8-arginine]vasopressin; [d$(CH_2)_5$Tyr(Me)^2]AVP, [1-β-mercapto-β,β-cyclopentamethylenepropionic acid, 2-*O*-methyltyrosine]AVP; PhaaALVP, *linear* [1-phenylacetylamido, 2-*O*-methyl-D-tyrosine, 6-arginine, 8-arginine, 9-lysinamide]vasopressin; SR 49059, (2*S*)-1-[(2*R*,3*S*)-5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzenesulphonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl]pyrrolidine-2-carboxamide; OT, oxytocin; (*R*) indicates a retro–inverso peptide synthesized in reverse sequence from D-amino acids; GPR, G-protein-coupled receptor; VPR, vasopressin receptor; $rV_{1a}R$, rat V_{1a} vasopressin receptor; rV_2R, rat V_2 vasopressin receptor; hOTR, human oxytocin receptor; GP_a, glycogen phosphorylase$_a$; EC, extracellular domain.

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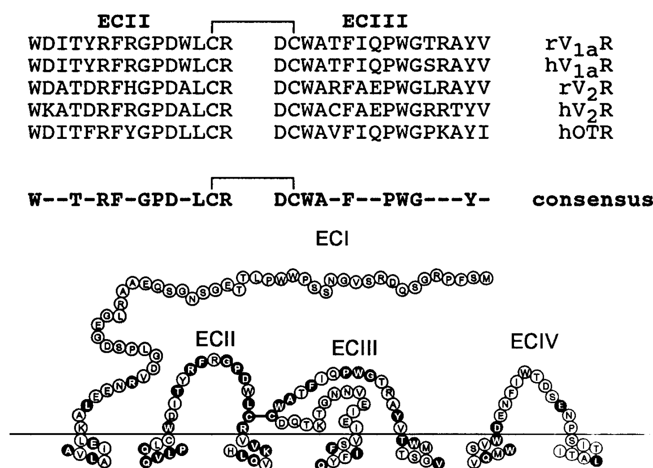

Figure 1 Homology of neurohypophysial hormone receptor extracellular domains

Sequence alignment (top) of neurohypophysial hormone receptors [3–6, 10] indicates that two extracellular domains, ECII and ECIII, are highly conserved. By analogy with other GPRs, it is likely that these extracellular loops are connected by a disulphide bond between conserved cysteine residues to form a spatially contiguous domain. The predicted structure of the extracellular surface of the $V_{1a}R$ (bottom) is comprised of four domains (ECI–ECIV) linking the seven transmembrane helices. Residues drawn as filled circles are conserved in the five mammalian neurohypophysial hormone receptors indicated. This figure highlights the unique homology within the ECII/ECIII contiguous domain of this subfamily of GPRs.

selective radioligand [prolinamide-3,4-^{3}H] (2*S*)-1-[(2*R*,3*S*)-5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzenesulphonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl]pyrrolidine-2-carboxamide ([^{3}H]SR 49059; 43.0 Ci/mmol) and α-D-[U-^{14}C]glucose 1-phosphate (313 mCi/mmol) were purchased from Amersham (Little Chalfont, Bucks., U.K.). Unlabelled [d(CH_2)$_5$Tyr(Me)^2] AVP was from Bachem (Torrance, CA, U.S.A.).

Peptide synthesis

Receptor mimetic peptides corresponding to the extracellular domains of neurohypophysial hormone receptors (Figure 1) and retro–inverso vasopressin analogues were synthesized on a 0.1–0.2 mmol scale using conventional solid-phase methodology by Alta Bioscience (Birmingham, U.K.). All receptor mimetic peptides were carboxyamidated. Retro–inverso-AVP [(*R*)AVP, see Figure 4] was cyclized by air oxidation in 0.1 M NH_4HCO_3. [19]. $V_{1a}R$ ECII/ECIII mimetic peptide (see Table 1), which has an intermolecular disulphide bond, was prepared by air oxidation of an equimolar mixture of the ECII and ECIII domain mimetics. The required peptide was resolved from ECII and ECIII homodimers by reverse-phase HPLC. All peptides were purified to homogeneity using semipreparative-scale reverse-phase HPLC. Peptide identity and purity were confirmed by a combination of analytical HPLC, amino acid analysis and mass spectroscopy [19]. Aqueous stock solutions of peptides (0.1–5 mM) were stored at −20 °C.

Pharmacological characterization of peptides

As a strategy for studying molecular recognition between receptor mimetic peptides and radioligands, we used a ligand-binding protocol previously described in detail [19]. The following equilibrium would be established in the presence of a mimetic which recognizes the radioligand, where R is the receptor and L* is the free radioligand:

$$\text{mimetic–L*} \rightleftharpoons \text{mimetic} + \text{R} + \text{L*} \rightleftharpoons \text{R–L*}$$

Consequently, any interaction between a radioligand and a receptor mimetic peptide would reduce radioligand occupancy of the receptor (R–L*). All studies were performed using a partially purified preparation of rat liver membranes [20] containing 600 fmol $rV_{a1}R$/mg of total protein. The ranges of free radioligand concentration used were: [^{3}H]AVP, 0.42–0.62 nM; [^{3}H]-[d(CH_2)$_5$Tyr(Me)^2]AVP 0.27–0.57 nM; [^{3}H]SR 49059, 0.37–0.58 nM. Non-specific binding was defined using 4 μM unlabelled [d(CH_2)$_5$Tyr(Me)^2]AVP. After incubation for 90 min at 30 °C, to allow radioligand binding to reach equilibrium, membranes were pelleted by centrifugation. Free radioligand and mimetic–L* complexes were then removed by washing [19]. Membranes were solubilized, and bound radioactivity was determined by liquid-scintillation spectroscopy. To evaluate specificity, an identical protocol was used to determine whether peptide mimetics of neurohypophysial hormone receptors would reduce the specific binding of 0.47–0.82 nM [^{3}H]AngII to the rat liver AT_1 receptor. Apparent inhibition constants were determined by non-linear regression after the fitting of a simple Langmuir binding isotherm to experimental data using the Fig. P program (Biosoft, Milltown, NJ, U.S.A.).

The binding affinities of retro–inverso vasopressin analogues for the $rV_{1a}R$ were determined by competition binding experiments using a protocol identical with that described above. Parallel studies utilized a rat kidney medulla preparation [19] to determine the affinities of retro–inverso analogues for the rat V_2 VPR (rV_2R). Dissociation binding constants (K_d) were calculated from IC_{50} values using experimentally determined affinity constants for the binding of [^{3}H]AVP (K_d at $V_{1a}R = 0.68$ nM; K_d at $V_2R = 1.31$ nM [19]).

Stimulation and assay of glycogen phosphorylase$_a$ (GP_a) activity

Rat hepatocytes (> 95 % viable) were freshly prepared from the livers of Wistar rats by collagenase digestion and elutriation [21]. Cells (4.5×10^6/ml in physiological buffer) were stimulated with either 0.5 nM AVP or 10 nM AngII for 2 min at 37 °C as described previously [22], after which time an aliquot was rapidly frozen in liquid N_2. GP_a (EC 2.4.1.1) activity was subsequently assayed in these samples by measuring the incorporation of radioactive glucose from α-D-[U-^{14}C]glucose 1-phosphate into glycogen at 30 °C [22]. The effect of receptor mimetic peptides on this hormone-stimulated GP_a activity was studied by preincubating AVP or AngII with increasing concentrations of mimetic peptide for 30 min at 37 °C before exposure of the hepatocytes to hormone. This allowed mimetic–ligand complexes to form before the cells were challenged.

RESULTS

Localization of binding-site determinants using peptide mimetics

As all of the peptides that we synthesized corresponded to receptor extracellular loops, the inherent solubility of the majority of the mimetic peptides enabled them to be used at final concentrations of 0.1 mM or less in binding assays. This enabled us to determine accurately the apparent K_i for most peptides. Mimetic peptides corresponding to the entire extracellular surface of the $rV_{1a}R$ (Figure 1) as well as the ECII domain of the rV_2R and a human oxytocin receptor (hOTR) were tested for their ability to recognize ligands as revealed by reduced occupancy of the $rV_{1a}R$ (Figure 2). Our data show that some, but not

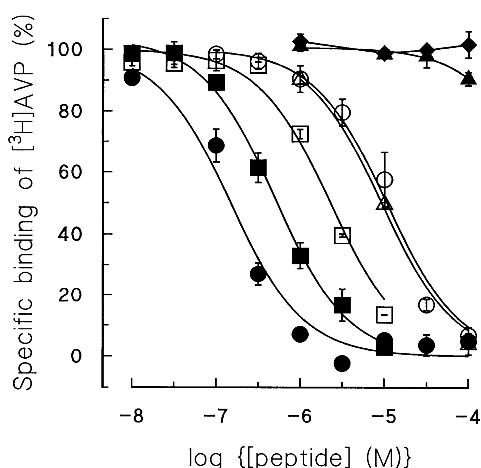

Figure 2 Pharmacological characterization of receptor mimetic peptides

Dose-dependent inhibition of specific [^{3}H]AVP binding (0.42–0.62 nM) to the rat liver V_{1a} VPR by receptor mimetic peptides. All results are means ± S.E.M. ($n = 3$), and the curves show the best fit of a simple Langmuir binding isotherm to the experimental data. Non-specific binding was less than 14% of total binding. ●, DITYRFRGPDWL, $rV_{1a}R^{102–113}$; ■, DITFRFYGPDLL, $hOTR^{100–111}$; ▲, DATDRFHGPDAL, $rV_2R^{100–111}$; ○, TYRFRGPD, $rV_{1a}R^{104–111}$; □, QDCWATFIQP, $rV_{1a}R^{193–203}$; △, DRSVGNSSPWWPLTTE, $rV_{1a}R^{9–24}$; ◆, (*R*)peptide LWDPGRFRYTID, $rV_{1a}R^{113–102}$.

all, mimetic peptides inhibited the specific binding of the agonist [^{3}H]AVP with apparent pK_i values ranging from 3.1 to 6.75 (Figure 2). The binding of V_{1a}-selective antagonists was also investigated. Both the peptide antagonist [^{3}H]-[d(CH_2)$_5$Tyr(Me)^2]AVP [23] and the non-peptide antagonist [^{3}H]SR 49059 [24] were recognized by mimetic peptides (Table 1). Comparison of the apparent pK_i values of mimetic peptides corresponding to the extracellular surface of the $rV_{1a}R$ (Table 1) indicates that the first extracellular loop (ECII, Figure 1) contains a major binding-site determinant with further contributions provided by the domains ECI and ECIII. The peptide DENFIWTDSEN, which mimics the relatively short ECIV domain of the $rV_{1a}R$, was without effect. The location of the domains observed to interact with ligands, as well as an indication of the relative contribution each makes to this interaction, is shown schematically in Figure 3. The pharmacological properties of the $rV_{1a}R$- and hOTR-derived ECII mimetic peptides were very similar, binding all three classes of ligand investigated with comparable pK_i values. In contrast, molecular recognition of the rV_2R ECII mimetic peptide DATDRFHGPDAL was restricted to the endogenous agonist [^{3}H]AVP (Figure 2, Table 1). Interestingly, this V_2R mimetic exhibited a lower apparent affinity for [^{3}H]AVP than the corresponding ECII mimetic of either the $rV_{1a}R$ or the hOTR. The ECII/ECIII dimeric mimetic peptide also bound the three classes of radioligand and did not discriminate between them (Table 1). The specificity of the interaction between the ECII mimetic peptide and ligands was demonstrated by using mimetic analogues. Scrambled analogues of the ECII mimetic DITYRFRGPDWL had greatly reduced affinity for all ligands studied (Table 1).

In contrast with the L-enantiomer DITYRFRGPDWL, the $rV_{1a}R$ ECII retro–inverso mimetic [(*R*)LWDPGRFRYTID] did not ‘recognize’ any vasopressin receptor ligand (Table 1). Parallel experiments revealed that none of the peptide mimetics

Table 1 Comparative inhibition constants for receptor mimetic peptides

Inhibition constants derived from experiments shown in Figure 2 are converted into apparent pK_i values for ease of comparison. Data shown are arithmetic means from three to five independent determinations of the apparent K_i value. A value of < 2 or < 3 indicates that no inhibition of radioligand binding occurred at a peptide concentration of 10^{-4} M or 10^{-5} M respectively. Peptides are grouped by domains as indicated in Figure 1. Numbers correspond to the position of residues in the appropriate receptor sequence [10,14–18]. The sequence of the peptide DITYRFRGPDWL, which was designed to mimic the ECII domain of the $rV_{1a}R$, is identical in the human V_{1a} vasopressin receptor.

	Apparent pK_i		
Peptide	AVP	[d(CH_2)$_5$Tyr(Me)^2]AVP	SR 49059
ECI			
DRSVGNSSPWWPLTTE ($rV_{1a}R^{9–24}$)	4.94	5.13	5.13
EGSNGSQEAARLGEGD ($rV_{1a}R^{24–39}$)	< 2	< 2	< 2
DSPLGDVRNEELAK ($rV_{1a}R^{39–52}$)	< 2	< 2	< 2
ECII			
DITYRFRGPDWL ($rV_{1a}R^{102–113}$)	6.75	6.57	6.73
TYRFRGPD ($rV_{1a}R^{104–111}$)	4.71	4.56	4.70
DITFRFYGPDLL ($hOTR^{100–111}$)	6.25	6.43	6.76
DATDRFHGPDAL ($rV_2R^{100–111}$)	3.1	< 2	< 2
Scrambled peptides			
DDFGILPRRTWY ($rV_{1a}R^{102–113}$)	3.99	3.76	3.94
WRTDFLRIDPYG ($rV_{1a}R^{102–113}$)	< 2	< 2	3.52
(*R*)peptide			
LWDPGRFRYTID ($rV_{1a}R^{113–102}$)	< 2	< 2	< 2
ECIII			
EVNNGTKTQDCWAT ($rV_{1a}R^{185–198}$)	4.55	4.61	3.89
EVNNGTKTQD ($rV_{1a}R^{185–194}$)	< 2	< 2	< 3
QDCWATFIQP)$rV_{1a}R^{193–202}$)	5.70	5.54	5.46
WATFIQPWGT ($rV_{1a}R^{196–205}$)	4.65	4.49	4.60
ECIV			
DENFIWTDSEN ($rV_{1a}R^{313–323}$)	< 3	< 3	< 3
ECII/ECIII			
DITYRFRGPDWLCR ($rV_{1a}R^{102–115/194–205}$) DCWATFIQPWGT	5.56	5.49	5.73

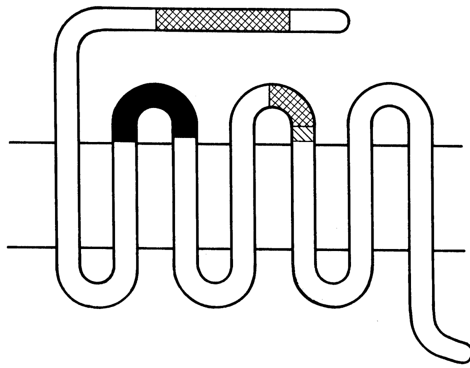

Figure 3 Schematic representation of the location of the subdomains within the extracellular surface of the $V_{1a}R$ which bind peptide and non-peptide ligands

Regions within the extracellular domains which bind ligands are indicated. The degree of shading is proportional to the relative affinity of that domain for ligands, as revealed by radioligand-binding studies with peptide mimetics. ■, Highest affinity binding; ⊠, intermediate affinity binding; ▧, lowest affinity binding. Peptide mimetics corresponding to the unshaded areas did not bind any of the ligands used in the binding studies.

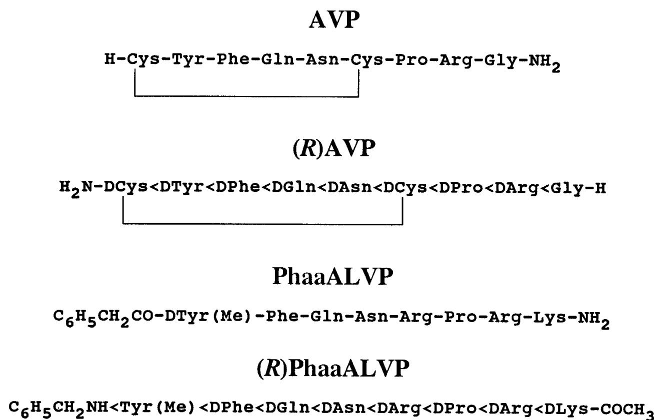

Figure 4 Molecular structure of (*R*)analogues of vasopressin

The structures of (*R*)peptides are compared with their corresponding L-enantiomers. Arrowheads indicate the positions of reversed amide bonds. To preserve the side-chain orientation of the linear peptide (*R*)PhaaALVP, D-Tyr(Me) at position 2 of PhaaALVP is replaced by Tyr(Me) in (*R*)PhaaALVP. Both AVP and PhaaALVP are C-terminal amidated peptides (indicated as $-NH_2$). The N- and C-terminals of AVP are effectively reversed in the synthesis of (*R*)AVP. Cys[1] of AVP is replaced by phenylacetamide in PhaaALVP. This structure is mimicked in the synthesis of (*R*)PhaaALVP by chemically replacing the carboxylic hydroxy group of Tyr(Me) with benzylamine. The α-nitrogen of D-Lys in (*R*)PhaaALVP is acetylated.

used in this study bound AngII, as they did not inhibit the specific binding of [^{3}H]AngII to the rat liver AT_1 receptor.

Binding affinity of retro–inverso vasopressin analogues

The structures of the retro–inverso vasopressin analogues used in this study are shown in Figure 4. The affinities of these peptides for $V_{1a}R$ and rV_2R were determined by competition binding experiments (Figure 5). The cyclic ligand, (*R*)AVP, exhibited extremely low affinity for both receptor subtypes ($K_d > 10^{-4}$ M). The linear retro–inverso peptide [(*R*)PhaaALVP; Figure 4], a homologue of the high-affinity V_{1a}-selective antagonist [PhaaD-Tyr(Me)^{2}Arg^{6}Lys(NH_2)]AVP [25], selectively bound to the $rV_{1a}R$. The affinity (K_d) of (*R*)PhaaALVP was 780 ± 30 nM ($n =$

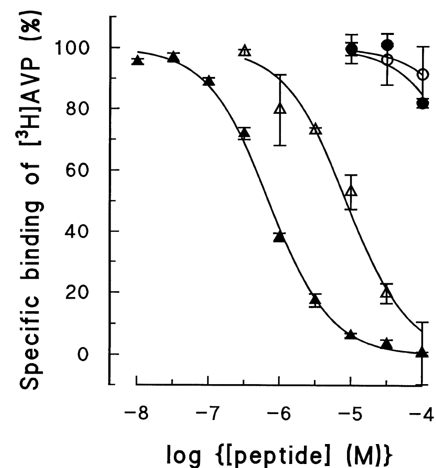

Figure 5 Determination of dissociation constants of vasopressin (*R*)analogues

Membrane preparations of rat liver (closed symbols, V_{1a} receptor) and rat kidney medulla (open symbols, V_2 receptor) were incubated in the presence of 0.32–0.89 nM [^{3}H]AVP and various concentrations of unlabelled peptides: ○, ●, (*R*)AVP; △, ▲, (*R*)PhaaALVP.

3) and 3.4 ± 1.0 μM ($n = 4$) for binding to the $rV_{1a}R$ and rV_2R respectively (mean ± S.E.M.). (*R*)PhaaALVP was a full antagonist at V_{1a} receptors. At 50 μM, (*R*)PhaaALVP prevented AVP-stimulated inositol 1,4,5-trisphosphate accumulation in [^{3}H]inositol-labelled WRK-1 cells (results not shown).

Inhibition of a cellular response to AVP by a receptor mimetic peptide

In addition to studying the effect of mimetic peptides on ligand recognition by VPRs, we also investigated the effect of mimetics on a functional response to AVP. The stimulation of glycogenolysis by various hormones, including AVP, in rat hepatocytes is well characterized ([22] and references therein). The 12-mer mimetic peptide DITYRFRGPDWL, which corresponds to the ECII domain of the $rV_{1a}R$ and possesses a major ligand-binding epitope (Figure 2), effectively prevented AVP-stimulated GP_a activity in rat hepatocytes with an IC_{50} value of 7.9 μM (Figure 6). The truncated ECII mimetic TYRFRGPD, which bound AVP with an apparent pK_i value two orders of magnitude higher than the 12-mer (Table 1), was correspondingly less effective at inhibiting the cellular response to AVP (Figure 6). Neither of these $rV_{1a}R$ ECII domain mimetics inhibited stimulation of GP_a by AngII (Figure 6).

DISCUSSION

The primary event in receptor activation by agonists is the formation of receptor–ligand complexes. A prerequisite to a full understanding of this phenomenon and to the logical design of ligand-based therapeutic agents is the identification of those domains within the receptor protein that constitute the ligand-binding site. To this end, we have synthesized a series of overlapping peptides which correspond to the extracellular loops of the $rV_{1a}R$ (Figure 1). Collectively, these mimic the molecular surface presented to ligands by the receptor at the early stages of ligand binding. In this paper we show that some, but not all, of the $rV_{1a}R$ mimetic peptides interact specifically with both peptide and non-peptide VPR ligands (Figure 2, Table 1). In contrast, none of these peptides recognized AngII.

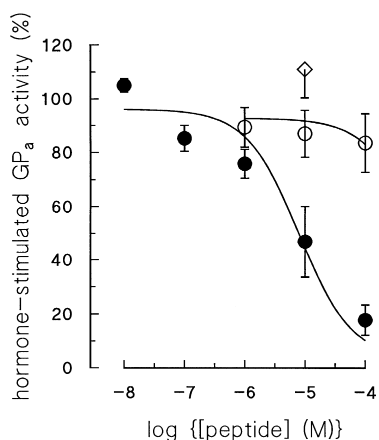

Figure 6 Dose-dependent selective inhibition of AVP-stimulated GP_a activity in rat hepatocytes

The effect of $rV_{1a}R$ ECII domain mimetic peptides on hormone-stimulated GP_a activity was investigated. Mimetic peptides were present at the concentrations indicated, and GP_a activity was stimulated and assayed as described in the Experimental section. AVP (0.5 nM) and AngII (10 nM) increased GP_a activity by $250 \pm 40\%$ and $270 \pm 60\%$ respectively over a basal value of 0.54 ± 0.19 μmol/min per 10^6 cells (mean ± S.E.M.; $n = 3$). ●, DITYRFRGPDWL + AVP; ○, TYRFRGPD + AVP; ◇, DITYRFRGPDWL + AngII.

It is significant that mimetic peptides corresponding to the ECII domain of $V_{1a}R$ and hOTR displayed similar binding characteristics (Table 1), as this mirrors the pharmacology of the native receptors. For example, many peptide ligands (agonists and antagonists), including AVP and $[d(CH_2)_5Tyr(Me)^2]$AVP, which bind with high affinity to V_{1a} VPRs also exhibit high-affinity binding to OTRs [26]. In addition, the rV_2R ECII mimetic peptide DATDRFHGPDAL did not recognize either of the V_{1a}-selective ligands $[d(CH_2)_5Tyr(Me)^2]$AVP or SR 49059. This concurs with the low affinity of both of these ligands for native V_2Rs [23,24]. Our data (Figure 2, Table 1) indicate that the ECII domain (first extracellular loop) is a major binding-site determinant and furthermore that it contributes to the characteristic pharmacological profile of the receptor subtype. The dependence of ligand recognition by the ECII mimetic on the precise sequence of the peptide was demonstrated by the much reduced affinity of scrambled ECII mimetic peptides (Table 1). It is also suggested that part of the N-terminal domain, together with all of the first and part of the second extracellular loops, are spatially orientated so as to provide a ligand-recognition site (Figure 3). It is possible that the N-terminal subdomain forms a 'lid' over the top of the ligand-occupied receptor as has been suggested for the formyl peptide receptor [27]. Such interaction with receptor extracellular domains has now been demonstrated for a variety of peptide ligands, from small formyl peptides to large glycoproteins [28–35].

The putative disulphide bond between the conserved cysteine residues in ECII and ECIII would tether these two loops together. This would effectively maintain the juxtaposition of loops necessary for optimal binding. Indeed, the mutation C112R in the V_2R, which precludes formation of this putative disulphide bond, generates a non-functional VPR and gives rise to the X-linked disease nephrogenic diabetes insipidus [36]. The disulphide-linked heterodimeric peptide corresponding to ECII/ECIII exhibited an affinity for ligands which was intermediate between that of the ECII and ECIII mimetics (Table 1). The observation that the ECII/ECIII binding domain did not bind ligands more avidly than the ECII mimetic alone probably reflects a limitation of the mimetic peptide strategy. However, it is not surprising that the extracellular surface presented by the membrane-bound receptor protein is not exactly reproduced by the same peptides being present free in solution.

The importance of extracellular loops in ligand binding by VPRs is supported by sequence conservation. The human $V_{1a}R$ has 72% overall identity with the rat $V_{1a}R$ but the ECII domain is absolutely identical [37]. Vasotocin and isotocin are homologues of AVP found in fish and amphibians. The ECII and ECIII domains of various vasotocin receptors are conserved and homologous to mammalian VPRs [38]. More direct evidence is provided by the report that a photoaffinity ligand labelled the ECII domain of a bovine V_2R [39]. Furthermore a single amino acid change (R113W) in the ECII region of the human V_2R resulted in a 20-fold reduction in affinity for AVP [14]. As cited above, defective V_2Rs give rise to the disease nephrogenic diabetes insipidus. It is noteworthy that the only mis-sense mutations in extracellular domains of V_2Rs which result in this disease, reported to date, occur in the ECII, the ECIII and the middle section of the N-terminal domains [15].

The mimetic peptide studies presented in this paper also establish that the extracellular domains are not the sole providers of binding-site epitopes for the neurohypophysial hormone receptors. Indeed, the highest affinity observed for ligand–mimetic interaction was two to three orders of magnitude lower than that displayed by wild-type receptors. This suggests that other ligand–receptor interactions, probably within the hydrophobic transmembrane domains, must provide the additional binding energy in native receptors. This would be compatible with the amphipathic character of AVP [16], and moreover pharmacological considerations support this conclusion. SR 49059 is selective for V_{1a} VPRs relative to OTRs (K_i values of 1.6 nM and 130–1080 nM at $V_{1a}R$ and OTR respectively [24]). However, SR 49059 did not discriminate between mimetics corresponding to $V_{1a}R$ and OTR ECII domains (Table 1).

AVP and $[d(CH_2)_5Tyr(Me)^2]$AVP bound to the same mimetic peptides. This was not unexpected as agonist and peptide antagonist binding is competitive. It is noteworthy, however, that this binding was also paralleled by the non-peptide antagonist SR 49059. Mutation studies have indicated that peptide and non-peptide antagonist binding epitopes are different for neurokinin-1 receptors [17,18], AT_1 receptors [40] and cholecystokinin B/gastrin receptors [41]. We propose that the binding of all three classes of ligand to extracellular receptor domains of VPR, revealed by peptide mimetics, represents the initial 'capture' of ligand by the receptor. The final 'docked' position of the ligand involves additional epitopes which contribute to the observed binding affinity and pharmacological selectivity of a given ligand. This 'capture and docking' model is compatible with peptide and non-peptide antagonists adopting different 'docked' positions and may be applicable to many peptide hormone receptors. Indeed, this multistep binding model is supported by studies on the luteinizing hormone receptor which has a large N-terminus of 341 residues. This first extracellular domain in isolation is soluble and was found to bind the hormone [42]. In addition, a truncated luteinizing hormone receptor with only a ten-residue N-terminus also bound luteinizing hormone, albeit with reduced affinity, and furthermore stimulated adenylate cyclase [43].

Although the ECII mimetic peptide corresponds to only 3% of the $rV_{1a}R$ [10], it bound ligands with quite high affinity (apparent $pK_i = 6.75$, Table 1). We predicted that soluble peptides containing significant binding-site epitopes would compete with VPRs for AVP, thereby concomitantly reducing receptor

occupancy and AVP-induced effects. This was indeed what we observed (Figure 6). Furthermore the inhibition of AVP-induced hepatic glycogenolysis by mimetic peptides paralleled their ligand-binding characteristics (Figure 6 and Table 1). Consequently, with respect to the $rV_{1a}R$ ECII domain mimetics, the 12-mer peptide (DITYRFRGPDWL) inhibited both [^{3}H]AVP binding to $rV_{1a}R$ and AVP-stimulated GP_a activity at lower concentrations than the 8-mer peptide (TYRFRGPD). The effect of the 12-mer mimetic was specific for AVP as it did not affect either [^{3}H]AngII binding to the AT_1 receptor or AngII stimulation of GP_a (Figure 6). This inhibition of a cellular response to AVP by a mimetic peptide establishes that soluble binding-site domains of peptide GPRs have potential as novel therapeutic agents. The binding of a peptide ligand to its receptor could involve interactions between both side-chain and main-chain epitopes. Retro–inverso mimetic peptides provide an attractive means of determining the relative contribution of both sets of atoms to this molecular recognition. As previously described [12,13], the net result of combining all D-enantiomers with reverse synthesis is to exchange the positions of the carbonyl and amine groups of the peptide bonds while preserving the orientation of the side-chain groups of each α carbon. Our data show that the retro–inverso mimetic of the $rV_{1a}R$ ECII domain did not bind any of the ligands used in this study (Figure 2, Table 1). This indicates that the binding-site epitope in ECII is not solely provided by the amino acid side chains but is also dependent on a correctly orientated main chain. The implication of this is that extracellular-loop main-chain atoms contribute to ligand recognition by the receptor. Likewise, (*R*)AVP did not bind to VPRs (Figure 5). It is noteworthy that the sequence -F-GPD-L- is conserved in all neurohypophysial hormone receptors cloned to date, which is perhaps indicative of a conserved function for this subdomain. We have evidence (J. Howl and M. Wheatley, unpublished work) from NMR studies, for a β-turn in the $rV_{1a}R$ ECII domain created by the GPD motif. The prolyl ring introduces local conformational constraints as it links the peptide nitrogen to the α carbon. It is possible that incorporating D-Pro into the retro–inverso mimetic of this region generates different local constraints compared with the L-Pro [13]. Thus the inactivity of the retro–inverso mimetic of the ECII domain may reflect, in part, a detrimental effect of utilizing D-Pro. This does not explain the lack of binding of (*R*)AVP as L-Pro at position 7 is not an absolute requirement for binding [26]. Indeed, the linear ligand (*R*)PhaaALVP has D-Pro corresponding to position 7 and still bound to $V_{1a}Rs$. It is possible that the disulphide bond present in (*R*)AVP, but absent from (*R*)PhaaALVP, introduced constraints which perturbed the pharmacophore.

In conclusion, this study has established that the extracellular domains of neurohypophysial hormone receptors bind both peptide and non-peptide ligands. We propose that this constitutes an initial 'capture' of ligand before the final 'docking' which involves additional epitopes [44,45]. Furthermore we have provided the first experimental data supporting the theoretical proposal [46] that soluble binding-site domains have therapeutic potential.

We thank Dr. J. C. Wilton for providing viable hepatocytes by elutriation, and Mrs R. A. Parslow for excellent technical assistance, and the BHF for financial support.

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Received 27 February 1995; accepted 25 March 1996