Defining the ligand-binding site for vasopressin receptors: a peptide mimetic approach

J. Howl*, R. A. Parslow and M. Wheatley
School of Biochemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

Introduction
The diverse functions of the neurohypophysial peptide hormones [arginine⁵]vasopressin (AVP) and oxytocin (OT) are mediated by a family of receptor proteins expressed in both the central nervous system and peripheral tissues [1,2]. cDNA clones encoding five vasopressin receptors (VPRs) and two oxytocin receptors (OTRs) have now been isolated from rodent, porcine and human sources [3–9]. These proteins are members of the larger family of G-protein-coupled receptors (GPCRs), which have seven putative transmembrane domains. Sequence comparison of the neurohypophysial peptide hormone receptors reveals two highly conserved domains in the putative first (ECII) and second (ECIII) extracellular (EC) loops which we [9] and others [10–12] have hypothesized to play a role in ligand binding. To test this hypothesis, we synthesized peptide mimics of the extracellular domains of neurohypophysial peptide hormone receptors. The rationale employed in this investigation was to determine whether molecular recognition occurs between isolated receptor domains and peptide/non-peptide ligands. Using this approach, we have identified binding-site determinants in the extracellular domains of the rat V₁s, VPR (rV₁s,R) which selectively 'recognize' neurohypophysial hormones. We propose that these domains are closely associated in the native receptor protein to form an extracellular ligand-binding site.

Experimental
Peptide mimics of extracellular receptor domains (Table 1) were prepared by Alta Bioscience, University of Birmingham, using conventional solid-phase methodology. Carboxy-amidated peptides were purified by semi-preparative-scale h.p.l.c. and lyophilized [13]. The purity and identity of each peptide was confirmed by a combination of analytical h.p.l.c., amino acid analysis and m.s. [13]. Peptides were dissolved at a concentration of 0.5–5 mM and stored at −20°C.

Effect of receptor mimics on ligand binding
As a strategy to study molecular recognition, we employed a ligand-binding protocol in which the interaction of a radiolabelled ligand with a receptor mimic would reduce radioligand occupancy of the rV₁s,R. To aid comparison of binding data, all assays were performed as previously described [13–15] using a single preparation of rat liver membranes with a V₁s,R capacity of 600 fmol/mg of membrane protein. We chose to use the V₁s,R preparation as both peptide and non-peptide high-affinity radio-ligands for this receptor are commercially available. This meant that we could undertake a comprehensive dissection of ligand-recognition determinants. The radioligands used were the natural agonist [Phe-3,4,5-[³H]AVP (64.8 Ci/mmol), [Phe-3,4,5-[³H]](d(CH)₅Tyr(Me))²AVP (1-β-mercaptopro-β-cyclpentamethylenepropionic acid, 2-O-methyltyrosine[AVP], a V₁s,R-selective peptide antagonist [16], 50.7 Ci/mmol) and the non-peptide V₁s,R antagonist [prolinamide-3,4-[³H]]SR 49059 (2(S)-1-[(2R,3S)-5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulphonyl)-3-hydroxy-2,3-dihydro-1H-indole-2-carbonyl]-pyrrolidine-2-carboxamide, 43.0 Ci/mmol [17]). To evaluate specificity, an identical protocol was used to determine whether synthetic peptide mimics of neurohypophysial peptide receptors would reduce the specific binding of [³H]angiotensin II ([³H]AngII, 71.5 Ci/mmol) to the rat liver AT₁ 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).

Effect of receptor mimics on hormone-stimulated glycojen phosphorylase activity
Glycogen phosphorylase (GP₁) activity was assayed in preparations of rat hepatocytes by measuring...
the incorporation of radioactive glucose from [α-32P]U-1-14Cglucose 1-phosphate into glycogen [18]. In order to determine whether receptor mimic peptides could inhibit AVP- and AngII-stimulated GP activity, we compared two rV₁R ECII mimics. To allow formation of peptide-ligand complexes, peptides were pre-incubated with 0.5 nM AVP or 10 nM AngII at 37°C in physiological buffer, then stored on ice until required. Rat hepatocytes (4.5 × 10⁶ cells/ml) were incubated at 37°C for 20 min before the addition of agonists or agonist/peptide mixtures. After a further 2 min, incubations were terminated by rapid freezing in liquid N₂. Subsequently, GP activity was assayed in homogenized cells as previously described [18].

Results
The inherent solubility of the majority of peptides synthesized in this study allowed us to routinely use concentrations of ≤0.1 mM in binding assays. Using this methodology, we were able to accurately determine the apparent Kᵦ for most of the peptides used. As shown in Figure 1, receptor mimic peptides inhibited radioligand binding in a dose-dependent manner. Comparative data (Table 1) indicate that peptide mimics of the ECII domain of the rV₁R and human OTR (hOTR) were the most effective inhibitors of the binding of all three radioligands. Thus, the 12-mers DITYRFRGPDWL (rV₁R) and DITFRFYGPDDL (hOTR) displayed apparent pKᵦ values in the range 6.25–6.75. Comparison of the two rV₁R ECII mimics clearly demonstrated that a reduction in size from the 12-mer (DITYRFRGPDWL) to the 8-mer (TYRFRGPD) reduced apparent affinity by two orders of magnitude. In contrast, the peptide mimic of the rat V₁VPR (rV₁R) ECII domain (DATDRFHGDAL) interacted weakly with only AVP and did not inhibit the binding of either [³²P][d(CH₂)₂Tyr(Me)²]AVP or [³H]SR 49059 (Table 1 and Figure 1).

Using a series of overlapping peptides to mimic the complete extracellular surface of the rV₁R, we were able to identify two further sites within the ECI and ECIII domains which also inhibited radioligand binding (Figure 1 and Table 1). The peptide DENFIWIATSE, which corresponds to the relatively short ECIV domain of the rV₁R, was without effect (Table 1). As a control, we tested all peptide mimics for their ability to reduce [³H]AngII binding. At concentrations of 10–100 μM none of the peptides used in this study reduced ligand occupancy of the rat liver AT₁ receptor.

As indicated in Figure 1(d), the ECII mimics DITYRFRGPDWL and TYRFRGPD both inhibited AVP-stimulated GP activity with IC₅₀ values of 7.9 and 400 μM respectively. Neither of these peptides inhibited AngII-stimulated GP activity.

Discussion
Our data demonstrate that the use of peptide mimetics is a successful method for determining the functional domains of GPCRs. The specificity of this approach is confirmed by our findings that not all of the peptides used in this study inhibited the binding of V₁R ligands and that none of them inhibited [³H]AngII binding to the rat AT₁ receptor. A synthetic peptide approach has previously been used to investigate the extracellular ligand-binding domain of GPCRs which bind relatively large glycoprotein hormones [19–21]. Our findings indicate that such an approach is also valid for the investigation of the ligand-binding domain of GPCRs which interact with smaller peptide hormones.

Our data reveal that conserved amino acids within the first extracellular loop and a section of the second loop (ECII and ECIII domains, Figure 2) comprise a major determinant of the extracellular ligand-binding site of the rV₁R. As similar domains are a feature of GPCRs of lower vertebrates which bind the hormone vasotocin [11], it is tempting to speculate that these domains have an ancient evolutionary origin as vasotocin is believed to be the ancestral precursor of the mammalian hormone AVP [22]. In order for the ECII and ECIII domains of the native V₁R to participate in ligand binding we propose that these extracellular loops are linked by a disulphide bridge between Cys-124 and Cys-205 (Figure 2) to form a spatially contiguous domain. In support of this contention, an intact disulphide bridge linking extracellular domains is a critical requirement for the binding of AngII [23] and natriuretic peptides [24] to their respective receptors.

Comparative data indicate a close similarity between the apparent Kᵦ values of rV₁R- and hOTR-derived ECII mimics (Table 1). This finding is significant because the majority of high-affinity peptide ligands for the V₁R also bind to OTRs [25]. In contrast, SR 49059 is a selective non-peptide antagonist which binds with high affinity to rat liver V₁R VPRs (Kᵦ 1.6 nM) but exhibits much reduced affinity (Kᵦ 130–1080 nM) at human OTRs [17]. Thus, additional factors must be involved in the binding of non-peptide ligands compared with peptide ligands and for determining the affinity of...
G-Protein-Linked Receptors

Figure I
Characterization of receptor mimics

(a–c) Dose-dependent inhibition of radioligand binding by receptor mimics. The ranges of free radioligand concentration used in these experiments were: (a) $[^3H]AVP$, 0.42-0.62 nM; (b) $[^3H][d(CH_2)_5Tyr(Me)]AVP$, 0.27-0.57 nM; (c) $[^3H]SR 49059$, 0.37-0.58 nM. Non-specific binding, defined by 4 uM unlabelled $[d(CH_2)_5Tyr(Me)]AVP$, was <14% of the total binding for $[^3H]AVP$, <21% for $[d(CH_2)_5Tyr(Me)]AVP$ and 50-61% for $[^3H]SR 49059$. Data represent mean ± S.E.M. (n = 3). All curves are representative of 3-5 independent determinations of the apparent $K_i$. Symbols: DITYRFRGPDWL $\gamma V_1R_{ECII}$; DITFRFYGPDLL $\gamma V_1R$; DATDRFHGPDAL $\gamma V_1R$; TYRFRGPD $\gamma V_1R^{EcII}$; QDCWATFIQP $\gamma V_1R^{EcII}$; DRSVGNSSPWPLTTE $\gamma V_1R^{AcI}$. A further description of the peptides is shown in the legend to Table I. (d) Inhibition of AVP-stimulated GP$_i$ activity by receptor mimics. In these experiments, AVP (0.5 nM) and AngII (10 nM) increased GP$_i$ activity to mean levels of 3-fold greater than basal. Both DITYRFRGPDWL and TYRFRGP showed a dose-dependent inhibition of AVP-stimulated GP$_i$ activity but had no effect upon AngII stimulation. Data represent mean ± S.E.M. (n = 3-6). Symbols: DITYRFRGPDWL + AVP; TYRFRGP + AVP; DITYRFRGPDWL + AngII.

SR 49059 for native receptors. The $\gamma V_1R$-derived ECII mimic interacts only weakly with AVP and does not recognize $[d(CH_2)_5Tyr(Me)]AVP$ or SR 49059 (Table I). It is noteworthy that both $[d(CH_2)_5Tyr(Me)]AVP$ and SR 49059 bind only weakly to the $\gamma V_1R$ subtype [17,18]. Thus, the properties of the ECII mimics appear to parallel some features of the pharmacology of native VPRs but do not exclusively determine ligand selectivity. As indicated in Figure 2, the amino acid sequence of the N-terminal ECI domain of the neurohypophysial peptide hormone receptors is not highly conserved in this family of receptor proteins. However, as revealed by the properties of the mimetic peptide DRSVGNSSPWPLTTE, this domain does contribute to the ligand-binding site of the $V_{1a}$ VPR. Part of this amino acid sequence, GNSSPWWPL, is preserved in the human $V_{1a}$.
Comparative inhibition constants for receptor mimetic peptides

Inhibition constants derived from experiments shown in Figure 1 are converted into apparent pK, values for ease of comparison. Data shown are arithmetic means from 3–5 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^{-8} M or 10^{-9} M respectively. Peptides are grouped by domains as indicated in Figure 2. Numbers correspond to the position of residues in the appropriate receptor sequence [3–5,8,9]. The sequence of the peptide DITYRFRGPDWL, which was designed to mimic the ECII domain of the rV1s [3,9], is conserved in the hV1sR [8]. Similarly, the hOTR peptide DITFRFYGPDDL [4] is conserved in a porcine OTR [7].

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Radioligand</th>
<th>([^{3}H])AVP</th>
<th>([^{3}H][d(CH_2)_5Tyr(Me)])AVP</th>
<th>([^{3}H]SR 49059)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRSVGNSSPWPTTE</td>
<td>rV1sR^{9-24}</td>
<td>4.94</td>
<td>5.13</td>
<td>5.13</td>
</tr>
<tr>
<td>EGSNSQEAARLGEDSR</td>
<td>rV1sR^{44-49}</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>&gt;2</td>
</tr>
<tr>
<td>DSPLGDVNEELAK</td>
<td>rV1sR^{19-42}</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>ECII</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DITYRFRGPDWL</td>
<td>rV1sR^{102-113}</td>
<td>6.75</td>
<td>6.57</td>
<td>6.73</td>
</tr>
<tr>
<td>TYRFRGPD</td>
<td>rV1sR^{104-111}</td>
<td>4.71</td>
<td>4.56</td>
<td>4.70</td>
</tr>
<tr>
<td>DITFRFYGPDDL</td>
<td>hOTR{06-111}</td>
<td>6.25</td>
<td>6.43</td>
<td>6.76</td>
</tr>
<tr>
<td>DATDRFGPDAL</td>
<td>rV2R^{100-111}</td>
<td>3.1</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>ECIII</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EVNNGTKTQDCWAT</td>
<td>rV1sR^{185-198}</td>
<td>4.55</td>
<td>4.61</td>
<td>3.89</td>
</tr>
<tr>
<td>EVNNGTKTQD</td>
<td>rV1sR^{185-194}</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>QDCWATFIQP</td>
<td>rV1sR^{197-203}</td>
<td>5.70</td>
<td>5.54</td>
<td>5.46</td>
</tr>
<tr>
<td>WATFIQPGWGT</td>
<td>rV1sR^{196-205}</td>
<td>4.65</td>
<td>4.49</td>
<td>4.60</td>
</tr>
<tr>
<td>ECIV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENFIWTDSEN</td>
<td>rV1sR^{113-323}</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

VPR (hV1sR), a receptor that exhibits similar but not identical ligand-binding selectivity to the rV1sR [18]. By analogy with conclusions drawn from studies with formyl peptide receptors [26,27], we suggest that the N-terminus ECI domain may provide a 'lid' over the major binding-site determinant of the ECII/ECIII contiguous domain.

One other important conclusion to be drawn from this study on the rV1sR derives from our observation that the binding-site mimics inhibit, with similar potency, the binding of all three classes of radioligand. These results strongly imply that some of the binding-site determinants for vasopressin agonists and antagonists (peptide and non-peptide) are identical or that they overlap to a significant degree. This finding contrasts with studies on other GPCRs [28–31] which have inferred that the binding-site determinants for peptides and non-peptide ligands are different.

As predicted from our observation that soluble receptor binding-site mimics inhibit ligand binding to the rV1sR, our data indicate that the formation of a specific ligand–peptide complex selectively reduces agonist activity. These findings substantiate the hypothesis [32] that the soluble ligand-binding domains of receptors have therapeutic potential as a new class of drug.

There is now substantial evidence to support the contention that molecular recognition of the extracellular domains of GPCRs is a widespread mechanism by which peptide ligands selectively bind to GPCRs. Indeed, such a mechanism appears to mediate the binding of a variety of different agonists and antagonists which range in size from the relatively small tripeptide formyl peptides to glycoproteins with molecular masses of >30 000 [19–21,24,26–31, 33–38]. We conclude that GPCRs which selectively bind the neurohypophysial peptide hormones can be added to the growing list of cloned GPCRs for which extracellular domains contribute part of the ligand-binding site.
**Figure 2**

**Model of the rat V_{1a} VPR**

The predicted domain structure of the extracellular surface is illustrated. ECI-ECIV represent extracellular domains 1-4. Residues which are conserved in all mammalian VPRs [3,5-8] and OTRs [4,5] are shown with solid circles. Superimposed upon this structure (asterisks) are the span of peptides which contain binding-site determinants for VPR ligands.

This work was supported by the British Heart Foundation, Wellcome Trust and the Royal Society. We would like to thank Dr. J. C. Wilton for the provision of viable hepatocytes purified by elutriation.

Allosteric regulation of G-protein-linked receptors

N. J. M. Birdsell*†, F. Cohen*, S. Lazarenko† and H. Matsui†

*Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K., and †MRC Collaborative Centre, Mill Hill, London NW7 1AD, U.K.

Introduction

Allosteric effects are observed when there are cross-interactions between two binding processes which can occur simultaneously or sequentially; the binding of one ligand affects the binding of the second ligand. This is illustrated simply in Scheme 1 in which R can be a monomeric or multimeric protein and D and L can be small molecules or proteins. In general, allosteric interactions are manifest if it is possible to form the ternary complex, DRL and

\[ K_1 \neq K_2 \] (both affinity constants). If \( K_1 > K_2 \) (\( K_1 > K_3 \)), there is negative co-operativity between the binding of L and D to R. Conversely, if \( K_1 < K_2 \) (\( K_1 < K_3 \)) positive co-operativity is manifest. The degree of co-operativity, \( K_2/K_1 \) (which must be the same as \( K_4/K_3 \)), is dependent on both D and L. A special case occurs when \( K_1 = K_2 \) and there is

neutral co-operativity. In this instance, the equilibrium binding of D is unaffected by L although the binding kinetics of D may change. Compounds which exhibit neutral co-operativity are useful as antagonists of the co-operative effects of other ligands which bind to the same site but have \( K_2/K_1 \) values not equal to 1.

Consider the case of R being a receptor and L, the endogenous neurotransmitter or hormone. In conventional pharmacological terms, agonists mimic the actions of L by binding to the same site and activating the receptor. Antagonists compete with both L and other agonists, again by acting at the same site, but cannot activate the receptor. The temporal pattern of activation of receptors by L and agonists may differ. In vitro neurotransmitter receptors respond to the stimulated and often pulsatil release of L. This means that the receptors are not continuously and maximally stimulated. Exogenous agonists, in contrast, produce a sustained stimulation which can result in

Abbreviations used: NMS, N-methylscopolamine; TM, transmembrane.

†To whom correspondence should be addressed.

Received 11 August 1994