Published in final edited form as:

Peptides. 2010 June ; 31(6): 1094–1098. doi:10.1016/j.peptides.2010.02.024.

Refinement of the pharmacophore of an agonist ligand of the secretin receptor using conformationally-constrained cyclic hexapeptides

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Abstract

There is a compelling need for the development of small molecule agonists acting at family B G protein-coupled receptors. A possible lead for the development of such drugs was reported when it was recognized that sequences endogenous to the amino terminus of the secretin receptor and certain other receptors in this family possess weak full agonist activity (Dong et al., *Mol Pharmacol* 2006). In the current report, we extended those observations by building the active dipeptide motif found in the secretin receptor (WD) into each position around a conformationally-constrained *D*-amino acid-containing cyclic hexapeptide, and determining the biological activity of each peptide at the secretin receptor. Indeed, only two positions for WD around this constrained ring resulted in biological activity at the receptor, providing further insights into the structural specificity of this phenomenon. Molecular modeling supported the presence of a unique WD backbone conformation shared only by these active peptides, and provided a more constrained template for future receptor-active agonist drug development.

Keywords

G protein-coupled receptor; secretin receptor; receptor activation; cyclic peptide; hexapeptide; molecular modeling

1. Introduction

The natural agonist ligands of family B guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) are all moderately long peptides, having diffuse pharmacophoric domains [24]. Such peptides are not bioavailable after oral administration, and can only be utilized as drugs when they are administered parenterally. Indeed, exenatide (exendin-4) is a peptide agonist of the glucagon-like peptide-1 receptor that is currently approved for parenteral administration for the therapy of type 2 diabetes mellitus [17]. A small molecule with similar biological activity that could be administered orally would have substantial advantages. Indeed, family B GPCRs contain several very attractive drug targets and could benefit substantially from leads for small molecule agonist development.

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There was much excitement recently when a small cyclic peptide analogue of the WDN region within the amino terminus of the family B secretin receptor was recognized as possessing full agonist activity at that receptor [7]. It was not clear that conformational change to expose this endogenous ligand represented a physiologic mechanism for activation of the secretin receptor, somewhat analogous to the protease-activated receptors in which an endogenous peptide is made accessible by proteolytic cleavage for binding to and activating those receptors [16,21], or whether its identification was a serendipitous observation of a lead for small molecule agonists of receptors in this family. Indeed, the analogous regions of other family B GPCRs, including VPAC1, calcitonin, and glucagon-like peptide-1 receptors, all possessed similar activities at their own receptors, with some of these also having activity at other family B GPCRs [4,7,8]. Recent observations suggest that this is not an endogenous physiologic mechanism, but rather a set of observations that holds promise for directing further drug development [6].

We have attempted to gain insights into the structural features critical for the biological activity of WDN at the secretin receptor. Our recent work demonstrated that the side chain of the asparagine residue was not critical for the activity of this molecule, since we could demonstrate similar degrees of stimulation with or without glycosylation [8]. In the natural receptor, this residue is, in fact, glycosylated [5]. In the current work, we focused on the WD portion of this lead compound. We pursued a strategy that has previously been successful in refining the pharmacophore of other small peptide ligands of other receptors [3,9,11,26], utilizing conformationally-constrained hexapeptides containing a single *D*-amino acid that vary the location of the active peptide sequence around the ring. Indeed, this experimental strategy was successful in identifying a subset of such peptides that possessed biological activity. Molecular modeling of these peptides suggested a structural basis for these observations. This confirms and extends earlier observations, and provides a more constrained template for future receptor-active agonist drug development.

2. Materials and Methods

2.1. Materials

Amino acids for peptide synthesis were purchased from Advanced ChemTech (Louisville, KY). Ham's F-12 medium, penicillin G/streptomycin, and soybean trypsin inhibitor were from Invitrogen (Carlsbad, CA). Fetal clone II culture medium supplement was from Hyclone laboratories (Logan, UT). Bovine serum albumin was from Serologicals Corp. (Norcross, GA). All other reagents were analytical grade.

2.2. Cells

The Chinese hamster ovary (CHO) cell line stably expressing the wild type rat secretin receptor (CHO-SecR) that we established previously [25] was used as a source of receptor for the current study. Cells were cultured at 37 °C in an environment containing 5 percent CO₂ on tissue culture plasticware in Ham's F-12 medium supplemented with 5% Fetal Clone II. Cells were passaged approximately twice a week.

2.3. Peptides

We previously reported full agonist activity at the secretin receptor of a fragment of the amino terminus of that receptor [7]. The endogenous receptor sequence had no sequence identity or homology with the natural secretin agonist ligand. Attempts to identify the minimal fragment of the receptor sequence possessing agonist activity focused on WDN, with improvement in potency by cyclization through side chains of a diamino propionic acid moiety at the amino terminus and an additional aspartate at the carboxyl terminus. The asparagine residue in this cyclic WDN moiety was shown to not be a critical component of the pharmacophore of this

agonist when it was demonstrated that it could be glycosylated (a modification present at this residue within the receptor) without loss of activity [8]. Thus, the WD dipeptide sequence was considered as the pharmacophore.

To gain further insight into the conformation of this sequence in its active state, we synthesized a series of linear and cyclic hexapeptides incorporating the WD motif at each position, with a *D*-amino acid (lower case letters in the peptide sequences in Table 1) in the carboxyl terminus. The cyclic peptides were prepared by establishing a bond between the α -amino group and the α -carboxyl group at each end of the linear peptides. Synthesis was performed using manual solid-phase techniques with 2-chlorotrityl chloride resin and Fmoc-protected amino acids. After completion of the synthesis of the linear peptides, the amino-terminal Fmoc protection was removed using 20% piperidine in dimethylformamide, before removal from the resin with 20% trifluoroethanol in dichloromethane. When preparing cyclic peptides, bonds between the α -amino group and the α -carboxyl group were allowed to form by incubating approximately 5 mg/ml of peptide with benzotriazole-1-yloxy-tris (dimethyl amino)phosphonium hexafluorophosphate, 1-hydroxybenzotriazole and *N,N*'-diisopropylethylamine for 2 h, following the procedure described by Blackburn and Kates [2]. The side chain protecting groups, BOC for Trp and *t*-butyl ester for Asp, were subsequently removed with a trifluoroacetic acid mixture containing 6.25% (wt/vol) phenol, 2% (vol/vol) triisopropylsilane, 4% (vol/vol) thioanisole, 4% (vol/vol) distilled water, and 83% (vol/vol) trifluoroacetic acid.

All peptides were purified to homogeneity (A_{280} absorbance profile reflecting purity > 99%) by reversed-phase HPLC utilizing an octadecylsilane column (Vydac) [23], with the identities of the peptides established by mass spectrometry. Peptide purifications were achieved utilizing a background of 0.1% trifluoroacetic acid with acetonitrile, utilizing a flow rate of 1 ml/min while monitoring UV absorbance ($A_{280\text{ nm}}$). A gradient of acetonitrile concentration was utilized for peptide elution, starting with 10% acetonitrile and increasing to 60% acetonitrile at a rate of 1% per min. The peptides were eluted at the following times: 23.7 and 25.4 min for WDWDWd and c[WDWDWd]; 8.9 and 13.1 min for WDAAAa and c[WDAAAa]; 9.5 and 12.5 min for AWDAAAa and c[AWDAAAa]; 10.5 and 11.3 min for AAWDAAa and c[AAWDAAa]; 10.3 and 12.7 min for AAAWDa and c[AAAWDa]; 9.1 and 12.8 min for AAAAWd and c[AAAAWd]; and 12.2 and 19.6 min for DAAAAAd and c[DAAAAAd], respectively.

2.4. cAMP assays

Biological activities of the WD hexapeptides were assessed by measuring cAMP responses in secretin receptor-bearing CHO-SecR cells. Approximately 8,000 cells per well were grown in 96-well plates for 48 h. Cells were washed twice with PBS and stimulated for 30 min at 37 °C with increasing concentrations of the WD hexapeptides in Krebs-Ringers/HEPES medium containing 25 mM HEPES (pH 7.4), 104 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 0.01% soybean trypsin inhibitor, 0.2% bovine serum albumin, 0.1% bacitracin, and 1 mM 3-isobutyl-1-methylxanthine. Reactions were terminated by removing the medium and lysis in 6% ice-cold perchloric acid for 15 min with vigorous shaking. Lysates were adjusted to pH 6 with 30% KHCO₃, and the cAMP levels were assayed in a 384-well white Optiplat using a LANCE kit from PerkinElmer (Boston, MA).

2.5. Molecular modeling

Molecular mechanics simulations of the structurally-constrained *D*-amino acid-containing cyclic hexapeptides were performed using Internal Coordinate Mechanics (ICM program from Molsoft, LLC, LaJolla, CA) [1]. This method uses an efficient biased probability Monte Carlo global optimization algorithm to minimize the ECEPP/3 energy [18–20] in torsion angle coordinates. The linear peptide was first converted into a cyclic one by imposing distance restraints on the terminal residue atoms in order to enforce proper peptide bond geometry. The

simulations were run for 10^8 function calls with a temperature parameter for Metropolis Monte Carlo sampling of 500K. During the simulations, a non-redundant set of the lowest energy conformations, differing by at least 5° RMSD in the backbone ϕ and ψ angles, were collected for use in subsequent analysis. Structures were then analyzed based on peptide biological activity, seeking unique WD backbone conformations shared by the active peptides that were not observed in any of the inactive peptides in the series.

3. Results

We previously showed that the cyclic WDN peptide from the amino terminus of the secretin receptor functioned as an endogenous agonist ligand at this receptor [7] and that the glycosylation of the Asn was not critical for this activity [8]. This helped to focus attention on the WD sequence within this region as the site of activity. In this work, we focused on this motif and made a hexapeptide, WDWDWd, in both linear and cyclic forms. While the linear form of the peptide had no biological activity, the cyclic form exhibited full agonist activity at the CHO-SecR cells with potency not significantly different from the cyclic WDN peptide (Figure 1 and Table 1). This suggests that the architecture of this constrained cyclic structure is adequate to present this peptide to the secretin receptor in its active form.

A series of cyclic hexapeptides containing only a single WD and the remaining residues as alanines was prepared to determine the critical positions of the WD within the structure. Figure 2 and Table 1 show that only cyclic peptides with WD incorporated in positions 2 and 3 (c[AWDAAa]) or 3 and 4 (c[AAWDAAa]) had full agonist activity at the secretin receptor, although both had lower potency than their parental peptide, c[WDWDWd]. Of note, the linear forms of these two peptides also had agonist activity at the secretin receptor, but they were less potent and efficacious than their cyclic counterparts. Incorporation of WD in other positions resulted in almost complete loss of agonist activity at the secretin receptor.

Molecular mechanics simulations of all cyclic peptides were also performed in order to discover any backbone conformations of the WD residues that might be shared by the active peptides but not by the inactive peptides in this series. WD backbone angles that were present in the active peptides and not present in the inactive peptides were identified using a 40° cutoff. Only residues 3 and 4 in c[WDWDWd], at the corresponding positions of WD in c[AAWDAAa], were considered. Interestingly, only a single tight cluster of backbone angles that satisfied these criteria was found, even though the unbound peptides are fairly flexible and so assume a wider range of low energy conformations. The (ϕ, ψ) angles for tryptophan were approximately $(-146^\circ, 170^\circ)$, while the corresponding angles for aspartate were $(-59^\circ, -42^\circ)$. Furthermore, low energy conformations from a simulation of the active cyclic WDN peptide also shared similar backbone angles for the tryptophan and aspartate residues. Figure 3 shows representative structures for the three active cyclic peptides from this study as well as the cyclic WDN peptide, with the WD residues that define the pharmacophore superimposed on each other. These results suggest a possible backbone conformation that is shared only by the active peptides that is also energetically favorable. This may be consistent with the active bound conformation of these peptides.

4. Discussion

Cyclic peptides possess several advantages as receptor ligands over their linear counterparts. They can have higher affinity and specificity than flexible linear peptides, due to the reduced conformational freedom and lower entropic penalties of the cyclic peptides, assuming that their conformations match the receptor-bound conformations of the linear peptides. Also, their lack of free amino- and carboxyl-termini renders them resistant to proteolytic degradation by exopeptidases. In addition, short cyclic peptides have been found to permeate the cell

membrane better than their linear counterparts and thus can also have improved bioavailability after oral administration [22].

NMR studies have shown that including a *D*-amino acid in short cyclic pentapeptides or hexapeptides can stabilize a characteristic backbone conformation by inducing a bII' turn motif with the *D*-amino acid in the *i*+1 position [10,12,14]. Thus, this class of cyclic peptides is thought to provide a defined backbone scaffold for presenting different short peptide binding motifs. Often, so-called "spatial screening" is used to search for high affinity ligands by positioning the motif of interest at different positions relative to the *D*-amino acid [13]. A number of such small cyclic peptides ligands have been developed to bind to diverse target proteins. These include RGD (Arg-Gly-Asp)-containing integrin ligands [11,12,15], analogues of the Leu-Asp-Thr sequence of mucosal addressin cell adhesion molecule-1 (alpha(4)beta(7) integrin antagonists) [3], somatostatin receptor agonists [26], human thymopoietin III analogs [14], and CXCR4-chemokine antagonists [9]. Motivated by these previous successes for this class of cyclic peptides, we have synthesized and tested cyclic hexapeptides with a single *D*-amino acid that contain the secretin receptor endogenous agonist WD motif.

Indeed, the architecture of the cyclic hexapeptide containing a single *D*-amino acid was compatible with maintaining full agonist activity of the WD. This peptide motif was only active in two of six positions around this conformationally-constrained structure. Of particular interest, molecular mechanics analysis of these peptides established that there was only a single cluster of conformations of the backbone of the WD sequence that was shared by all the active cyclic hexapeptides and the active cyclic WDN peptide described previously, but were absent from each of the inactive peptides utilized in the current series of studies. This structure is energetically favorable and may well be consistent with the active bound conformation of these peptides.

This set of observations again confirms and extends the original observation of the biological activity of cyclic WDN at the secretin receptor. This further establishes the structural specificity of the pharmacophore and should help to constrain the template that can be utilized in future receptor-active agonist drug development. It will be particularly important to attempt to further improve the affinity and potency of mimics and variants of this structure.

Acknowledgments

This work was supported by grants from the National Institutes of Health (DK46577) and from the Fitterman Foundation. The authors thank Ms. Mary Lou Augustine for her technical assistance.

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Fig. 1.

Biological activity of synthetic hexapeptides containing repeated WD sequences. Shown are concentration-dependent cAMP responses in CHO-SecR cells stimulated by both linear and cyclic WDWDWd peptides. As controls, data for stimulation of these cells with cyclic WDN [7] and secretin peptides are also shown. Values are expressed as means $\pm$ SEM of data from three independent experiments performed in duplicate with data normalized relative to the maximal response to secretin. The potencies of c[WDN] and c[WDWDWd] to stimulate cAMP responses at the secretin receptor were not significantly different ($P > 0.05$).

Fig. 2.

Biological activity of synthetic hexapeptides containing single WD sequences. Shown are concentration-dependent cAMP responses in CHO-SecR cells stimulated by both linear and cyclic hexapeptides. Values are expressed as means $\pm$ SEM of data from three independent experiments performed in duplicate with data normalized relative to the maximal response to secretin. Two-tailed t-tests were performed to determine if the cAMP responses observed were different from basal levels. * Denotes concentrations that were significantly above basal levels ($P < 0.05$). Table 1 includes EC_{50} data and demonstrates that the cyclic forms of AWDAaAa and AAWDAaAa were significantly different (higher potency) from the linear forms of those peptides ($P < 0.05$).

Fig. 3. Superposition of WD backbone conformations present only in active cyclic peptides. Representative low energy structures from molecular mechanics simulations are shown for the active cyclic peptides in the current study, c[WDWDWd] (blue), c[AWDAAa] (red), and c[AAWDAAa] (green), as well as active cyclic WDN (yellow) that was initially described [7]. For clarity, the tryptophan (W) and aspartate (D) side chains are only illustrated for one of the peptides. The positions of the α -carbons of the *D*-amino acids in the cyclic structures are noted by lower case identifiers (a, a, and d). The WD backbone atoms in all structures have been aligned to show the similar backbone structure for these residues that comprise the presumed pharmacophore. None of the inactive cyclic peptides had similar WD backbone conformations.

Table 1

Biological activity of synthetic linear and cyclic hexapeptides.

Peptides	EC ₅₀	E _{max} (pmol/10 ⁶ cells)
Secretin	76 ± 11 pM	198 ± 28
c[WDN]	5.0 ± 1.2 μM	199 ± 24
c[WDWDWd]	11.7 ± 3.0 μM	198 ± 33
WDWDWd	N.A.	10 ± 1.4
c[WDAAAa]	N.A.	28 ± 6.2
WDAAAa	N.A.	3.5 ± 0.9
c[AWDAAa]*	21 ± 4.2 μM	198 ± 44
AWDAAa	57 ± 6.2 μM	177 ± 41
c[AAWDAAa]*	23 ± 5.3 μM	199 ± 47
AAWDAAa	78 ± 10 μM	131 ± 30
c[AAAWDa]	N.A.	3.6 ± 0.4
AAAWDa	N.A.	4.0 ± 0.9
c[AAAAWd]	N.A.	2.5 ± 0.4
AAAAWd	N.A.	2.9 ± 0.5
c[DAAAAw]	N.A.	2.5 ± 0.5
DAAAAw	N.A.	3.4 ± 0.7

Shown are the EC₅₀ and E_{max} values for noted peptides to stimulate cAMP responses in CHO-SecR cells. Basal cAMP levels for the CHO-SecR cells was 2.6 ± 0.7 pmol/10⁶ cells. Upper case letters in peptide sequences denote L-amino acids, while lower case letters denote D-amino acids; c, identifies cyclic peptides.

* Denotes cyclic peptides that had EC₅₀ values that were significantly different from their respective linear forms, P < 0.05.