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Exploration of the Endogenous Agonist Mechanism for Activation of Secretin and VPAC1 Receptors Using Synthetic Glycosylated Peptides

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Abstract

Current understanding of the molecular basis of activation of class II G protein-coupled receptors remains limited, despite recent solution of NMR and crystal structures of amino-terminal domains of several family members. One mechanism proposed for the activation of these receptors involves an agonist-stimulated change in conformation of the receptor amino terminus. This results in the exposure of a “hidden endogenous agonist” (WDN sequence in secretin and VPAC1 receptors) within the receptor amino terminus that interacts with the receptor core, thereby changing its conformation and exposing its G protein-binding region. The Asn in this WDN sequence is known to be glycosylated in both secretin and VPAC1 receptors, raising concern about whether this posttranslational modification might interfere with the proposed mechanism. Therefore, we prepared glycosylated forms of cyclic WDN and the longer cyclic peptide, LWDNM, and tested them for agonist activity at secretin and VPAC1 receptor-bearing cell lines. Both glycosylated peptides stimulated full cAMP responses in the cell lines. Clearly, glycosylation did not interfere with this mechanism and may actually facilitate the correct orientation of the pharmacophore of the endogenous agonist ligand. These data provide further evidence for this proposed mechanism for the activation of this family of receptors.

Keywords

G protein-coupled receptors; Secretin receptor; VPAC1 receptor; Receptor activation; Glycosylation

Introduction

The receptors for secretin and vasoactive intestinal polypeptide (VIP) are prototypic members of the class II family of G protein-coupled receptors (GPCRs). This family includes many important therapeutic targets, such as receptors for parathyroid hormone, calcitonin, corticotrophin-releasing factor (CRF), pituitary adenylate cyclase-activating polypeptide (PACAP), glucagon, gastric inhibitor polypeptide (GIP), and glucagon-like peptide 1 (GLP1) (Mayo et al. 2003; Ulrich et al. 1993b).

Understanding of the mechanism of ligand binding and receptor activation of the class II GPCRs should facilitate the rational design of drugs acting at these receptors. However, the molecular basis of these events for this family of receptors is not well understood. A

characteristic structural feature of the receptors in this family is a long and complex amino-terminal tail domain that has been shown to play a critical role in ligand binding and receptor activation. Recent studies of the NMR structure of the amino-terminal regions of the CRF (Grace et al. 2004, 2007) and PAC1 (Sun et al. 2007) receptors and the crystal structure of the amino terminus of the GIP receptor (Parthier et al. 2007) have substantially advanced our understanding of the structure of the amino-terminal domain of class II GPCRs. However, our understanding of the spatial relationship of this domain and the receptor core and the mechanism of receptor activation of receptors within this family of GPCRs remains quite limited.

Two mechanisms for the activation of class II GPCRs have been proposed. One involves the “ligand tethering” mechanism in which the carboxyl regions and midregions of the ligand peptide bind to the amino-terminal domain of the receptor, whereas the amino terminus of the ligand interacts with the receptor core to induce receptor activation (Bisello et al. 1998; Dong et al. 2004a, b). The other proposed mechanism involves an “endogenous, hidden agonist” (WDN sequence in the secretin receptor) that is exposed upon ligand-induced conformational change in the receptor amino-terminal domain that acts as an agonist ligand interacting with the region of the receptor core to activate the receptor (Dong et al. 2006). The “endogenous agonist” mechanism was based on studies on the secretin receptor and it was demonstrated to also be applicable to the calcitonin and VPAC1 receptors (Dong et al. 2006).

It is well established that the Asn⁴⁹ within the putative WDN endogenous agonist sequence of the secretin receptor (Dong et al. 2006) and the analogous Asn⁶⁹ within the VPAC1 receptor are glycosylated in these mature receptors. The glycosylation of these Asn residues within the secretin and VPAC1 receptors changes their electrophoretic migration by 9–10 kDa (Dong et al. 2007; Tan et al. 2006). This has raised the question whether glycosylation might affect the interaction of the WDN peptide with the core region of the secretin receptor (Dong et al. 2006; Laburthe et al. 2007). To directly test this concern, we have synthesized cyclic WDN with a glycosylated Asn⁴⁹ and tested it for its agonist activity in both secretin and VPAC1 receptor-bearing cell lines. In addition, we have also synthesized and tested a longer cyclic peptide, LWDNM, which incorporated a glycosylated Asn⁴⁹. In this work, we have shown that these glycosylated putative endogenous agonist candidate peptides maintained their abilities to stimulate cAMP responses in both the secretin and VPAC1 receptor-bearing cell lines. This further supports the proposed mechanism for the activation of the class II GPCRs by an endogenous agonist sequence.

Materials and Methods

Materials

Rat VIP was purchased from Bachem (Torrance, CA, USA). Rat secretin was prepared in our laboratory, as described previously (Ulrich et al. 1993a, b). Ham's F-12 medium, penicillin G/streptomycin, lipofectamine transfection reagent, and soybean trypsin inhibitor were from Invitrogen (Carlsbad, CA, USA). Fetal clone II culture medium supplement was from Hyclone Laboratories (Logan, UT, USA). Bacitracin and 3-isobutyl-1-methylxanthine were from Sigma-Aldrich. Bovine serum albumin was from Serologicals (Norcross, GA, USA). Amino acids for peptide synthesis were purchased from Advanced ChemTech (Louisville, KY, USA). All other reagents were of analytical grade.

Preparation of glycosylated asparagine for incorporation during peptide synthesis

We developed a method for the synthesis of *N*- α -Fmoc-*N*- β -(3,4,6-tri-*O*-acetyl-1-2-[acetylamino]-deoxy-2- β -glucopyranosyl)-L-asparagine (Fmoc-Asn(Ac₃AcNH- β -Glc)-OH) to

be incorporated into peptides to reflect the proximal glycosylation unit utilized in N-linked glycosylation. 2-Acetamido-2-deoxy- β -D-glucopyranose was refluxed with acetyl chloride at room temperature overnight and extracted with iced chloroform to yield 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- α -D-glucopyranosyl chloride (Horton and Wolfrom 1962). This product was then converted to 2-acetamido 3,4,6-tri-*O*-acetyl-2-deoxy- α -D-glucopyranosyl azide by reacting the product with sodium azide and ammonium formate for 3 h at 80–85°C before being poured into ice-cold water and extracted with chloroform. The 2-acetamido 3,4,6-tri-*O*-acetyl-2-deoxy- α -D-glucopyranosyl azide was then converted to 2-acetamido 3,4,6-tri-*O*-acetyl-2-deoxy- α -D-glucopyranosyl amine by hydrogenolysis using platinum oxide (Yamamoto et al. 1965). *N*- α -Fmoc-L-aspartic acid α -t-butyl ester (Fmoc-Asp-OtBu) was activated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and *N,N'*-diisopropylethylamine and added to 2-acetamido 3,4,6-tri-*O*-acetyl-2-deoxy- α -D-glucopyranosyl amine to yield Fmoc-Asn-(Ac₃AcNH- β -Glc)-OtBu. The OtBu was removed with 50% TFA/CH₂Cl₂ and the resultant product, Fmoc-Asn-(Ac₃AcNH- β -Glc)-OH, was directly incorporated into the peptide during solid-phase peptide synthesis, as described below. The expected molecular mass was verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Peptide synthesis

Endogenous agonist peptides for secretin (SecR(48–50), WDN) and VPAC1 (VPAC1(51–71)) receptors (Fig. 1) that were prepared previously (Dong et al. 2006) were used in this study. The glycosylated cyclic WDN (SecR(48–50)) and LWDNM (SecR(47–51)) listed in Fig. 1 were synthesized using the procedures we previously described for the noncyclic WDN peptide (Dong et al. 2006), except that Fmoc-Asn-(Ac₃AcNH- β -Glc)-OH was used to replace Fmoc-Asn(trt)-OH during synthesis. All peptides were purified to homogeneity by reversed-phase HPLC using techniques that have been described previously (Powers et al. 1988). The expected molecular masses of the peptides were verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Cell lines

A Chinese hamster ovary (CHO) cell line stably expressing the wild-type rat secretin receptor (CHO-SecR) (Ulrich et al. 1993a) and a baby hamster kidney (BHK) cell line stably expressing the rat VPAC1 receptor (BHK-VPAC1) (Dong et al. 2006) were used as the sources of receptors for the cAMP study. Cells were cultured at 37°C in a 5% CO₂ environment on Falcon tissue culture plastic ware in Ham's F-12 (for CHO-SecR) or culture medium consisting of equal parts Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (for BHK-VPAC1) supplemented with 5% fetal clone II. Cells were passaged approximately twice a week.

Biological activity assay

The biological activities of the natural peptide ligands and synthetic peptides to stimulate receptor-bearing cell lines (CHO-SecR and BHK-VPAC1) to signal were assessed by measuring cAMP responses in these cells. Approximately 8,000 cells were placed in each well of 96-well plates. Two days after plating, cells were washed with PBS and stimulated for 30 min at 37°C with increasing concentrations of peptides (ranging from 10⁻¹² to 10⁻⁶ M for natural peptide ligands and from 10⁻⁸ to 10⁻⁴ M for synthetic endogenous receptor agonist peptides) in Krebs-Ringers/HEPES (KRH) 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. The peptide solution was then removed and 6% ice-cold perchloric acid was added to lyse the cells. After adjusting the pH to 6 with 30% KHCO₃,

the lysates were introduced directly into an assay of cAMP levels using a LANCETM cAMP-384 kit from PerkinElmer (Boston, MA, USA). The assay was performed as per manufacturer's instructions.

Results

Synthesis and characterization of the glycosylated endogenous secretin receptor peptides

The glycosylated Asn residue (Fmoc-Asn-(Ac₃AcNH-β-Glc)-OH) to be incorporated into the secretin receptor peptides was synthesized and had its chemical identity verified by mass spectrometry. This residue was incorporated into the cyclic WDN and LWDNM peptides (Fig. 1) using manual solid-phase synthesis. The identities of the peptides were verified by mass spectrometry.

Biological activities of the glycosylated endogenous secretin receptor peptides in the CHO-SecR cell line

Both the glycosylated cyclic WDN and LWDNM peptides were tested for their abilities to stimulate cAMP responses in the CHO-SecR cell line. Figure 2 shows that both peptides were fully efficacious agonists at the secretin receptor. The glycosylated cyclic LWDNM peptide had similar potency to that of nonglycosylated cyclic WDN and VPAC1(51–71) peptides, whereas the glycosylated cyclic WDN peptide had lower potency acting at this receptor (Fig. 2, Table 1).

Biological activities of the glycosylated endogenous secretin receptor peptides in the BHK-VPAC1 cell line

Both the glycosylated cyclic WDN and LWDNM peptides were also tested for their abilities to stimulate cAMP responses in the BHK-VPAC1 cell line. As shown in Fig. 3, they stimulated cAMP responses in these cells in a concentration-dependent manner. Both had similar potencies that were not significantly different from those of nonglycosylated WDN and VPAC1(51–71) peptides (Fig. 3, Table 1).

Discussion

The endogenous agonist mechanism of activation of class II GPCRs was recently proposed based on observations with peptides found within the amino terminus of the secretin receptor acting as full agonists through their interaction with the core of the receptor (Dong et al. 2006). This mechanism was further refined with the photoaffinity labeling of a region of the secretin receptor in the third extracellular loop above the top of the predicted fourth transmembrane segment (Dong et al. 2006). Similar biological activity observations have been made with endogenous amino-terminal peptides found in other members of this family of receptors, the calcitonin receptor and the VPAC1 receptor (Dong et al. 2006). However, all of these observations were made with linear and/or cyclic peptides that did not include a glycosylated asparagine residue, known to be present within this region of these receptors (Dong et al. 2006; Tan et al. 2006). This resulted in the question being raised of whether glycosylation would interfere with this phenomenon, making it unlikely to represent a natural mechanism of activation of these receptors (Laburthe et al. 2007).

In the current report, we prepared glycoforms of the previously studied peptides. Indeed, they were found to represent fully efficacious agonists, which elicited maximal cAMP responses like those observed with the best endogenous peptides previously studied. It is remarkable that some of these peptides were also similarly potent to the nonglycosylated forms of these peptides. Whereas the glycoforms used in these studies only mimicked the proximal carbohydrate unit attached to the asparagine in N-linked glycoproteins, this was

clearly enough to rule out the face of the peptides as a domain of interaction with the receptor core. It is most likely that the natural glycosylation of these residues helps to orient the endogenous agonist epitope for its optimal activity. Indeed, in the molecular model of the secretin receptor that we recently reported (Dong et al. 2007; Harikumar et al. 2007), there is adequate room for the heavy glycosylation of this asparagine residue to extend through the space between the amino terminus and receptor body domains.

Another interesting feature of the putative endogenous agonist WDN sequence within the secretin receptor amino terminus is the aspartate residue in the middle of this region. This residue has been shown to be functionally very important for some receptors in class II GPCRs (Carruthers et al. 1994; Couvineau et al. 1995; Godfrey et al. 1993; Lin et al. 1993). The best example of this is the mouse mutant of this aspartate in the GHRH receptor of the *little mouse* (Godfrey et al. 1993; Lin et al. 1993). In the secretin receptor, mutation of this aspartate did not interfere with receptor binding or biological activity (Dong et al. 2006). Nevertheless, in the first structures of the amino terminus of a member of this receptor family, the CRF2 β receptor (Grace et al. 2004; Grace et al. 2007), the analogous aspartate was reported to be involved in an intradomain salt bridge with a basic residue, possibly making it inaccessible for the proposed mechanism of action. It was reassuring that the higher resolution and more extensive crystal structure of the amino terminus of the GIP receptor that was solved recently had no such salt bridge present (Parthier et al. 2007). Thus, this region continues to represent a possibility for being involved in the putative endogenous agonist mechanism proposed.

The current report establishes that the endogenous agonist mechanism proposed for the secretin receptor that has been shown to also be relevant to several other receptors in this family continues to represent a viable mechanism. The theoretical concern that glycosylation of this peptide might interfere with its action can now be ruled out. Further refinement of the relevant structure of the endogenous agonist and the specific region of the receptor core that it interacts with will be important in the future.

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Secretin receptor peptides	Sequences
WDN, cyclic	[.....] XWDND
WDN(AcGlc), cyclic	[.....] XWDN*D
LWDN(AcGlc)M, cyclic	[.....] XLWDN*MD
VPAC1 peptide	#
VPAC1(51-71), linear	LEEAQLENETTGS [#] SKMW [#] DNLT

Figure 1.

Synthetic peptides used in cAMP assays. Shown are the sequences of the synthetic peptides used in this study. These include the sequences of the glycosylated endogenous cyclic WDN (WDN (AcGlc), cyclic) and LWDNM (LWDN(AcGlc)M, cyclic) peptides, along with the nonglycosylated secretin (WDN, cyclic) and VPAC1 (VPAC1(51–71)) receptor endogenous agonist peptides that were previously used (Dong et al. 2006). *X* denotes diaminopropionic acid; the *asterisk* indicates Ac₃AcNH-β-Glc; the *number sign* indicates that internal Cys was changed to Ser

Figure 2.

Biological activity of glycosylated endogenous secretin receptor peptides in CHO-SecR cells. Shown are curves of cAMP responses in CHO-SecR cells stimulated by the glycosylated cyclic WDN and LWDNM peptides. As controls, curves for CHO-SecR cells stimulated by the nonglycosylated cyclic WDN and linear VPAC1 (51–71) peptides are 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

Figure 3.

Biological activity of glycosylated endogenous secretin receptor peptides in BHK-VPAC1 cells. Shown are curves of cAMP responses in BHK-VPAC1 cells stimulated by the glycosylated cyclic WDN and LWDNM peptides. As controls, curves for BHK-VPAC1 cells stimulated by the nonglycosylated cyclic WDN and linear VPAC1(51–71) peptides are 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 VIP

Table 1

Biological activity of synthetic endogenous secretin receptor peptides

Cell line	Peptides	EC ₅₀	E _{max} (pmol/10 ⁶ cells)
CHO-SecR	Secretin	75.8±11.1 pM	202±31
	Cyclic WDN	6.2±2.0 μM	200±22
	VPAC1(51–71)	10.2±1.9 μM	201±28
	Glycosylated cyclic LWDNM	13.4±2.1 μM	203±25
	Glycosylated cyclic WDN	71.6±8.9 μM	202±27
BHK-VPAC1	VIP	82.0±10 pM	207±24
	Cyclic WDN	25.2±3.2 μM	169±21
	VPAC1(51–71)	45.1±4.7 μM	193±30
	Glycosylated cyclic WDN	32.0±2.9 μM	157±20
	Glycosylated cyclic LWDNM	18.4±2.3 μM	188±29

Shown are the EC₅₀ and E_{max} values for peptides to stimulate cAMP responses in CHO-SecR and BHK-VPAC1 cells. Basal cAMP levels for CHO-SecR and BHK-VPAC1 cells were 2.4±0.5 and 2.9±0.7 pmol/10⁶ cells, respectively.