Insights into the Structural Basis of Endogenous Agonist Activation of Family B G Protein-Coupled Receptors

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Agonist drugs targeting the glucagon-like peptide-1 (GLP1) receptor represent important additions to the clinical management of patients with diabetes mellitus. In the current report, we have explored whether the recently described concept of a receptor-active endogenous agonist sequence within the amino terminus of the secretin receptor may also be applicable to the GLP1 receptor. If so, this could provide a lead for the development of additional small molecule agonists targeting this and other important family members. Indeed, the region of the GLP1 receptor analogous to that containing the active WDN within the secretin receptor was found to possess full agonist activity at the GLP1 receptor. The minimal fragment within this region that had full agonist activity was NRTFD. Despite having no primary sequence identity with the WDN, it was also active at the secretin receptor, where it had similar potency and efficacy to WDN, suggesting common structural features. Molecular modeling demonstrated that an intradomain salt bridge between the side chains of arginine and aspartate could yield similarities in structure with cyclic WDN. This directly supports the relevance of the endogenous agonist concept to the GLP1 receptor and provides new insights into the rational development and refinement of new types of drugs activating this important receptor.

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MANY IMPORTANT NEUROENDOCRINE receptors belong to family B guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs). These include receptors for secretin, PTH, calcitonin, glucagon, glucagon-like peptides, vasoactive intestinal polypeptide (VIP), and gastric inhibitory polypeptide (1). Recently, we proposed a novel molecular mechanism for the activation of the secretin receptor, whereby natural agonist ligand binding to the receptor amino-terminal domain induced a conformational change in that domain, which exposed an endogenous agonist sequence that was totally distinct from the hormonal agonist (2). In that receptor, the minimally active sequence represented WDN, located between the second and third conserved cysteine residues. We also demonstrated similar activity for analogous regions of the VIP type 1 receptor (VPAC1) and calcitonin receptors, other closely related family B GPCRs that share the likely pharmacophore within this segment. This represents the tryptophan-aspartate sequence, without the asparagine that is glycosylated in the secretin receptor (2). Here, we have explored this potential mechanism of activation in the glucagon-like peptide-1 (GLP1) receptor, another family B receptor that is also missing the analogous tryptophan, only retaining the aspartate that is highly conserved in the family.

GLP1 is an important gluco-incretin hormone secreted from intestinal L cells in response to nutrient ingestion (3, 4). This peptide stimulates glucose-dependent insulin secretion, increases β-cell proliferation and sensitivity to glucose, inhibits glucagon secretion and gastric emptying, and reduces food intake (5). These effects make agonists quite promising for the treatment of patients with type 2 diabetes mellitus (6). Whereas peptide analogs of GLP1 requiring injection are already in clinical use, the possibility of the development of orally active small molecules with similar actions would be very important.

A unique structural feature that is characteristic of this group of GPCRs is a long extracellular amino-terminal domain that includes six conserved cysteines forming critical disulfide bonds (7–11). The importance of this region has been demonstrated for multiple members of this receptor family (12–17). Consistent with this are mutagenesis and chimeric receptor anal-
yses of the GLP1 receptor (18–21), although the first extracellular loop of that receptor may also contribute important residues (22). The isolated amino terminus of the GLP1 receptor has even been shown to bind GLP1 with high affinity (7, 23, 24).

Like natural ligands for other family B GPCRs, GLP1 is a moderately long peptide having a diffuse pharmacophoric region. Nuclear magnetic resonance (NMR) analysis of GLP1 reveals that residues 7–30 adopt a helical structure (25–28). It has been assumed that this carboxyl-terminal region interacts with the amino-terminal domain of this receptor, whereas the peptide amino terminus interacts with the receptor core (18, 29, 30). This theme has been proposed as a tethering mechanism for activation of some family members (31–33). The alternative mechanism for activation proposed for the secretin receptor involves a ligand-induced change in conformation of the receptor amino terminus exposing an intrinsic epitope (WDN) that interacts with the receptor core to act as an agonist (2).

Insights into structure of the amino-terminal region of family B GPCRs were advanced with solution of NMR structures of isolated amino-terminal regions of receptors for corticotrophin releasing factor (8, 34) and pituitary adenylate cyclase-activating polypeptide (PAC1) (11). Homology structures of analogous regions of the VPAC1 and secretin receptors have been proposed (35–37). Of note, the NMR structures have not included insight into the distal amino terminus, a region covalently labeled by many secretin photo-probes (35, 36). Although there is good agreement on general structure of the amino-terminal regions of these receptors, there are widely divergent proposals for orientation of these regions relative to the transmembrane helical bundle (8, 11, 34–37). Currently, sufficient experimental data do not exist to confirm these distinct models, reflecting potential problems in their generation or divergence in mechanisms of action.

We now find that the endogenous agonist hypothesis is indeed applicable to the GLP1 receptor, with peptides representing portions of the amino terminus possessing full agonist activity. This supports a general theme for family B GPCRs and provides novel leads for the development of small molecule agonists acting at potential drug targets within this important family.

RESULTS

Establishment of the GLP1 Receptor-Expressing Cell Line

A Chinese hamster ovary (CHO) cell line stably expressing the GLP1 receptor (CHO-GLP1R) was established and characterized. Figure 1 shows that these cells bound GLP1 with high affinity ([inhibition constant (K) = 1.1 ± 0.2 nM]), and GLP1 stimulated a biological response with high potency (EC_{50} = 45.8 ± 6.2 pM).
at their carboxyl terminus (Fig. 3). These were used to evaluate whether a covalent bond that establishes a loop structure would enhance biological activity, as it did for the endogenous secretin receptor peptide (2).

We started with a tripeptide (FDE) focusing on the most conserved Asp (Fig. 3). Unlike the WDN sequence from an analogous region of the secretin receptor, this tripeptide, both linear and cyclic, had barely detectable biological activity at the GLP1 receptor. Extending another residue (Tyr) in the carboxyl-terminal direction did not significantly improve the activity. However, the biological activity was significantly improved by extending it to include more residues at the amino-terminal end of this sequence (Fig. 3). Full agonist activity was achieved when three residues (NRT) were included at the amino terminus of the peptides. The carboxyl-terminal residues, glutamic acid and tyrosine, were not required for full agonist activity as long as NRTFD was present. The minimum sequence required for full agonist activity was the pentapeptide NRTFD [GLP1R (63–67)].

Interestingly, unlike the endogenous agonist WDN, which requires cyclization to elicit full efficacy at the secretin receptor, linear NRTFD was 12 times more potent than NRTFD cyclized through its ends. Figure 3 shows that further shortening of NRTFD sequence resulted in loss of biological activity.

**Structure-Activity Relationship Studies of the Endogenous GLP1 Receptor Peptide**

We prepared a series of synthetic peptides for structure-activity relationship studies focusing on the small-terminal residues, glutamic acid and tyrosine, were not required for full agonist activity as long as NRTFD was present. The minimum sequence required for full agonist activity was the pentapeptide NRTFD [GLP1R (63–67)].

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**Table 1. Identification of the Minimally Active Region**

| Peptides          | Sequences   | Intracellular cAMP response at 100 µM | Concentration to achieve 50% GLP1 E<sub>max</sub> (µM) |
|-------------------|-------------|--------------------------------------|-----------------------------------------------------|
| GLP1R(63-70), cyclic | XNRFDEYAD    | 101 ± 6                              | 9.1 ± 1.5                                           |
| GLP1R(63-68), cyclic | XFDE0       | 5 ± 2                                | > 100                                               |
| GLP1R(63-69), cyclic | XFDE0       | 4 ± 1                                | > 100                                               |
| GLP1R(63-66), cyclic | XTFDE0      | 37 ± 4                               | > 100                                               |
| GLP1R(64-69), cyclic | XRTFDE0     | 46 ± 5                               | > 100                                               |
| GLP1R(63-69), cyclic | XNRTFDE0    | 100 ± 6                              | 15.5 ± 3.0                                          |
| GLP1R(63-68), cyclic | XNRTFDE0    | 98 ± 4                               | 14.6 ± 3.6                                          |
| GLP1R(63-67), cyclic | XNRTFDE0    | 99 ± 3                               | 13.1 ± 2.3                                          |
| GLP1R(63-67), cyclic | XNRTFDE0    | 97 ± 5                               | 17.0 ± 3.2                                          |
| GLP1R(63-65), cyclic | NRT         | 102 ± 7                              | 1.8 ± 0.2                                           |
| GLP1R(64-66), cyclic | RTF         | 102 ± 6                              | 1.2 ± 0.2                                           |
| GLP1R(64-67), cyclic | RTFD        | 103 ± 6                              | 1.3 ± 0.2                                           |
| GLP1R(65-67), cyclic | XTFD        | 47 ± 6                               | > 100                                               |
| GLP1R(65-66), cyclic | XTFD        | 38 ± 4                               | > 100                                               |
| GLP1R(65-66), cyclic | XTFD        | 38 ± 4                               | > 100                                               |
est segment of the endogenous GLP1 receptor agonist ligand, NRTFD. Data in Fig. 4 show that replacement of Arg64 with a similarly charged lysine, Asp67, with a glutamic acid, and Phe66 with a structurally similar tryptophan or tyrosine were not tolerated, and each significantly reduced the biological activity of the peptide (Fig. 4A). However, complementary swapping of positions for Arg64 and Asp67 was tolerated (Fig. 4B). Replacement of Asn63 with a structurally similar glutamine and moving this Asn to the carboxyl terminus maintained full efficacy and potency (Fig. 4C). However, change of Asn63 to a structurally distinct leucine or isoleucine substantially decreased biological activity (Fig. 4C). Finally, NRTFD and the reversed sequence that contained all D-amino acids (D-DFTRN) had much lower activity (Fig. 4D).

**Evaluation of Potential Interactions between Ligands Structurally Related to GLP1 and NRTFD**

We have proposed that the endogenous peptide agonist within the receptor amino terminus, the NRTFD sequence within the GLP1 receptor, acts at a distinct region of the receptor from the natural peptide agonist, GLP1. Here, we examine the potential interactions between ligands that are structurally related to GLP1 and NRTFD. Data are shown in Fig. 5. GLP1 and NRTFD had independent actions at the GLP1 receptor that were additive up to full efficacy, with neither agonist resulting in potentiation or inhibition of the action of the other. The maximal cAMP response observed was determined by the density of GLP1 receptors. NRTFD also had no effect on GLP1 binding to the GLP1 receptor (data not shown).

We also studied the GLP1-like peptide antagonist, exendin(9–39). This antagonized the action of GLP1 in a concentration-dependent manner but had no effect on the ability of NRTFD to stimulate a response (Fig. 5). We studied peptides related to NRTFD that have low levels of activity, NKTFD, NRTWD, and peptides with D-amino acids NRTFD and DFTRN. None of the low-activity NRTFD variants had any effect on GLP1 action (data not shown). These data support the concept that NRTFD binds to an allosteric site on the GLP1 receptor and does not interact with the orthosteric site where GLP1 acts.

**Biological Activity of the Endogenous GLP1 Receptor Peptide at Functionally Impaired Mutant GLP1 Receptor**

We also studied mutations within NRTFD of the GLP1 receptor (R64K, F66W, and D67E) expressed in COS cells to gain further insights into this mechanism. Mutation of Arg64 to lysine and Phe66 to tryptophan had little negative effect on receptor binding (data not shown) and biological activity (Fig. 6). Mutation of Asp67 to glutamic acid resulted in markedly impaired binding (data not shown) and biological activity (Fig. 6). Mutation of Asp67 to glutamic acid resulted in markedly impaired binding (data not shown) and biological responses to the natural agonist ligand (Fig. 6). Asp67 corresponds...
to a highly conserved residue in this receptor family that is mutated in the GH-releasing hormone receptor, resulting in the little mouse phenotype (38, 39). The analogous residues in the receptors for VIP and glucagon have also been shown to be critical (40, 41). Notably, the D67E receptor mutant responded with full efficacy to stimulation with the endogenous GLP1 receptor peptide, NRTFD, with similar potency to that of wild-type, R64K, and F66W receptors (Fig. 6).

**Cross-Reactivity of Family B GPCR Endogenous Agonists**

To examine whether the endogenous GLP1 receptor peptide is active at other family B GPCRs, we tested NRTFD at secretin, VPAC1, and calcitonin receptors. We also tested the endogenous receptor peptides from secretin, VPAC1, and calcitonin receptors at the GLP1 receptor. Data in Fig. 7 show that NRTFD was also active at secretin and VPAC1 receptors, whereas it was not active at the calcitonin receptor. Similarly, the endogenous receptor peptides from secretin and VPAC1 receptors were active at the GLP1 receptor, whereas the endogenous calcitonin receptor peptide was not active at the GLP1 receptor. Whereas NRTFD had similar potencies in stimulating biological responses at secretin and VPAC1 receptors to their respective endogenous ligands, cyclic WDN from the secretin receptor had much lower potency than NRTFD and the endogenous VPAC1 peptide at the GLP1 receptor.
Structural Similarity between the Secretin and GLP1 Receptor Endogenous Peptides

Molecular models of structurally constrained cyclic WDN and noncovalently cyclized NRTFD are shown in Fig. 8. Remarkably, there is substantial similarity observed, based on the salt bridge that modeling approaches suggest can form in linear NRTFD. This results in similar geometry and presentation of the phenylalanine in similar position to the tryptophan in the more tightly constrained cyclic WDN.

DISCUSSION

Understanding the molecular basis of activation of a receptor can provide a rational basis for development and refinement of receptor-active agonist drugs. A possible lead for small molecule agonists for family B GPCRs that normally are activated by moderately large peptide ligands came from the recent observation that a small portion of the amino terminus of the secretin receptor possesses full agonist activity upon binding to the core transmembrane domain of the same receptor (2). This intramolecular interaction was proposed as a key component of the molecular basis of activation of that receptor after binding of the natural ligand to the receptor amino terminus. That work also provided an indication that endogenous agonist activity was also present in the analogous region of calcitonin and VPAC1 receptors. Here, we have explored whether this concept is also relevant to the GLP1 receptor that is recognized as a very attractive target for agonist drugs useful in the therapy of diabetes mellitus.

The concept of an endogenous agonist and the relevance of the WDN sequence within the secretin receptor to other family members have been questioned based on two lines of reasoning. This first concern is focused on the aspartate residue within this sequence, because it was shown to be involved in an intradomain salt bridge in the first NMR structures of calcitonin and VPAC1 receptors. Here, we have explored whether this concept is also relevant to the GLP1 receptor that is recognized as a very attractive target for agonist drugs useful in the therapy of diabetes mellitus.

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The aspartate in this domain of family B GPCRs can be quite important in this receptor family. Attention was first drawn to it when the mutation in the little mouse was reported to involve this residue in the GHRH receptor (38, 39). Subsequent mutations of the analogous aspartate residue in the VPAC1 and glucagon receptors were also reported to disrupt function (40, 41). The current report shows that this is also true of the GLP1 receptor. Of particular interest, such a mutation in the secretin receptor was found to have no detrimental effect on its secretin binding or secretin-stimulated biological activity (2). It is noteworthy that the NMR structure of the amino terminus of the corticotrophin releasing factor receptor demonstrated the involvement of this aspartate in a salt bridge with the basic residue within the amino terminus, Arg101, but that this proposed bond was on the surface of the structure (8). Solvent-exposed salt bridges are known to be easily disrupted. It is also unclear that such a bond would exist within the intact receptor. Indeed, a very recent report of a crystal structure of the amino terminus of another family member, the receptor for gastric-inhibitory polypeptide, had no analogous salt bridge present (42).

In the initial three-dimensional structure proposed for the secretin receptor, there appears to be adequate room for glycosylation of this asparagine to extend between the highly structured disulfide-bonded amino-terminal domain and the core transmembrane domain of that receptor (35). Indeed, preliminary data have shown that glycoforms of WDN do retain full agonist activity at that receptor (our unpublished work in progress).

When the tripeptide present within the amino terminus of the GLP1 receptor (FDE centered on Asp67) that is analogous to secretin receptor WDN was tested for agonist activity, none was found. However, the phenomenon of endogenous agonist activity was supported by a slightly longer fragment of this same region of the GLP1 receptor. In a series of studies looking for the minimal fragment with full agonist activity, a five-residue peptide was identified that resides just to the amino-terminal side of the FDE tripeptide. It is particularly interesting that this sequence, NRTFD, has no apparent primary sequence identity with WDN. Further, forming a cyclic peptide covalently closed at the ends of this active segment (cyclic NRTFD) reduced its activity, unlike WDN where such cyclization stabilized and enhanced its activity.

It is intriguing that preliminary molecular modeling of linear NRTFD showed that it is capable of forming an intradomain salt bridge between side chains of arginine and aspartate that results in a structure similar to cyclic WDN in the area of the aromatic residue key to the pharmacophore. Of interest, such an intrapeptide bond could not be formed when the NRTFD model was chemically cyclized through its ends. Consistent...
with these preliminary models, linear NRTFD was found to be active not only at the GLP1 receptor, but also at secretin and VPAC1 receptors. This, too, suggests the possibility of common structures with a shared pharmacophore. It is notable that, like WDN observed previously, NRTFD had no activity at the calcitonin receptor. It will be important to examine that activity in the presence of different levels of expression of receptor activity-modifying proteins, given their ability to change the structural specificity of that receptor. Refinement of the endogenous agonist structures will be extremely important in understanding their active conformations. Such structural data could provide important insights for rational development and refinement of small molecule agonists that share this mechanism of action.

The previous study focused on the secretin receptor included photoaffinity labeling of that receptor using analogs of cyclic WDN that were radioiodinated and incorporated photolabile benzoyl-phenyl-lalanine at either side of the active region as sites of attachment (2). Both of these probes covalently labeled the third extracellular loop above transmembrane segment 6. Of great interest, a model was recently proposed for the secretin receptor that accommodates a broad variety of constraints, including nine sites of intrinsic photoaffinity labeling through positions spread throughout the secretin pharmacophore, three intradomain disulfide bonds within the receptor amino terminus, and 16 distance constraints coming from fluorescence resonance energy transfer between positions spread throughout secretin and positions in its extracellular loops (36). Indeed, this model placed the WDN sequence adjacent to the helical bundle domain. It would be easy to envision interaction of the endogenous agonist with the third loop of the receptor that was photoaffinity labeled or with the second loop that is spatially approximated with it.

It is notable that these receptor peptides have relatively low potencies and affinities, as well as being nonselective. This is not a problem for action of the endogenous peptides within the receptors, because they are tethered into place and can only sample a small spatial volume, giving them higher functional affinities and specificities. Clearly the affinities and specificities of these peptides will have to be improved if small molecule ligands based on these structures are to become useful drugs. Unfortunately, there is not yet an adequately detailed model of the helical bundle domain of any family B GPCR to provide insight into the specific site of docking these endogenous agonist peptides. Such structural insights will be critical for rational refinement of these leads for development of receptor-active agonist drugs. They will also be critical for insights into how such agents might be made more selective and specific for a particular receptor.

MATERIALS AND METHODS

Material

Human GLP1(7–36)-amide (GLP1), exendin(9–39), human calcitonin, and rat VIP were purchased from Bachem (Torrance, CA). Rat secretin was synthesized in our laboratory (43). Fetal Clone II culture medium supplement was from Hyclone Laboratories (Logan, UT). BSA was from Serologically Corp. (Norcross, GA). The solid-phase oxidant, N-chlorobenzensulfonamide (Iodo-beads), was from Pierce Chemical Co. (Rockford, IL). All other reagents were analytical grade.

Peptide Synthesis

Candidate endogenous agonist peptides representing portions of the amino terminus of secretin, VPAC1, and calcitonin receptors were prepared previously (2). GLP1 receptor peptides were prepared from the region of this receptor analogous to secretin receptor WDN. These include both linear and cyclic peptides, with the latter prepared by cross-linking the side chains of diaminopropionic acid and aspartic acid moieties. Synthetic strategies involved manual solid-phase peptide synthesis using Pal resin (Advanced ChemTech, Louisville, KY) and Fmoc-protected amino acids (44). When preparing cyclic peptides, side-chain protection using Nα-Fmoc-Nβ-4-methyltrityldiaminopropionic acid and Nα-Fmoc-L-aspartic acid β-2 phenylisopropyl ester was used, and the methyltryt and 2-phenylisopropyl protection groups were selectively removed while the peptide was still fully protected and attached to the resin using 1.8% trifluoroacetic acid in CH2Cl2 (45). Bonds between the side chains of diaminopropionic acid and aspartic acid were formed by coupling with benzotriazole-1-xyloxy-tris(dimethyl aminophosphonium hexafluorophosphate, 1-hydroxybenzotriazole and N,N′-disopropylethylamine for 2 h. The amine-terminal Fmoc protection was removed using 20% piperidine in dimethylformamide. The peptides were removed from the resin using trifluoroacetic acid containing 6.25% (wt/vol) phenol, 2% (vol/vol) trisopropylsilane, 4% (vol/vol) thioanisole, 4% (vol/vol) distilled water, and 83% (vol/vol) trifluoroacetic acid. All peptides were purified to homogeneity by reversed-phase HPLC (44). Expected molecular masses were verified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

Radioiodination of GLP1

GLP1(7–36)-amide was radioiodinated oxidatively on Tyr in position 19, using Na125I and a 15-sec exposure to N-chlorobenzene-sulfonamide (Iodo-beads). Products were purified to homogeneity by reversed-phase HPLC, yielding a specific radioactivity of 2000 Ci/mmol (43).

Receptor-Expressing Cell Lines

Cell lines expressing the rat secretin receptor (CHO-SecR) (43), human calcitonin isomeric II receptor [human embryonic kidney (HEK)293-calcitonin receptor (CTR)], and rat VPAC1 receptor [baby hamster kidney (BHK)-VPAC1 (2)] were used as sources of receptors for the current study. Cells were cultured at 37°C in a 5% CO2 environment on Falcon tissue culture plasticware in Ham’s F-12 (for CHO-SecR) or DMEM (for HEK293-CTR) or culture medium consisting of equal parts DMEM and Ham’s F-12 nutrient mixture (for BHK-VPAC1) supplemented with 5% Fetal Clone II. Cells were passaged approximately twice a week.

A receptor-bearing CHO cell line stably expressing the human GLP1 receptor (CHO-GLP1R) was established for this
study. For this, non-receptor-bearing CHO-K1 cells were transfected with the human GLP1 receptor construct in the pcDNA3.1/Zeo(+) expression vector using Lipofectamine, and zeocin-resistant cells were selected using 0.5 mg/ml zeocin. Clonal populations of surviving cells were then selected by a series of limiting dilutions. Cell lines expressing an appropriate receptor density were cultured and used as source of receptor.

Three additional GLP1 receptor mutants were generated that included Arg64 to a lysine (R64K) or Phe66 to a tryptophan (F66W), or Asp67 to a glutamic acid residue (D67E), each representing mutation of residues within the Asn63–Asp67 (NRTFD) sequence of the GLP1 receptor. They were prepared using an oligonucleotide-directed approach with the QuickChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA), with their sequences verified by direct DNA sequencing. These mutants were expressed transiently in COS-1 cells (American Type Culture Collection, Manassas, VA) after transfection using a modification of the diethylamino-ethanol-dextran method (46). Cells were maintained under the same conditions as the HEK293-CTR cells described above.

**Binding Assay**

GLP1 binding to CHO-GLP1R cells was performed in a standard competition-binding assay, using conditions previously established (47). Approximately 200,000 CHO-GLP1R cells were incubated at room temperature for 1 h with a constant amount of 125I-labeled GLP1 (5–10 pM) in the presence of varied concentrations of unlabelled GLP1 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, and 0.2% BSA. Separation of free from bound radioligand was performed by washing the cells twice with ice-cold KRH medium. Cells were lysed with 0.5 M NaOH, and membrane-bound radioactivity was quantified. Nonspecific binding was assessed in the presence of 1 μM unlabeled GLP1 and represented less than 20% of total radioligand binding. Data were graphed using Prism software (GraphPad Software, San Diego, CA) and were analyzed using the LIGAND nonlinear least squares curve fitting program (48).

**Biological Activity Assay**

Biological activities of the ligands to stimulate receptor-bearing cells were assessed by measuring cAMP responses. Approximately 8000 cells per well were grown in 96-well plates for 48–72 h. Cells were washed twice with PBS and stimulated for 30 min at 37 °C with increasing concentrations of individual ligands (natural peptide ligand or candidate endogenous receptor agonist peptides) or ligands in pairs [GLP1 and NRTFD, or GLP1 and exendin9–39], or NRTFD and exendin9–39] in KRH medium containing 0.1% bacitracin, and 1 mM 3-isobutyl-1-methylxanthine. Reactions were terminated by removing the medium and lysis in ice-cold 6% 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 Optiplate using a LANCE kit from PerkinElmer (Boston, MA).

**Molecular Modeling**

All molecular modeling was done on a Linux workstation with Pentium IV Duo Core 3.0 GHz processors. Sampling of peptide conformations was performed by using the Monte Carlo biased-probability simulation in Internal Coordinate Mechanics (ICM) (49, 50). The initial structure of the linear peptide, NRTFD, with carboxyl-terminal amide protection was first created using the peptide construction module of ICM. The number of cycles for sampling was calculated as 50,000 times the number of unfixed variables. For each cycle, the number of minimization steps was 170 plus three times the number of unfixed variables. The simulation was carried out at 300K with energy calculated from the sum of the Van der Waals, hydrogen bonding, electrostatic, torsion energy, surface, and entropy terms. The simulation was repeated multiple times with random starting geometries, and the resulting lowest-energy conformations were found to converge. The chemical structure of cyclic WDN (diaminopropionic acid-WDN-D) (2) was created by using Molecular Editor of ICM. The Monte Carlo protocol for cyclic WDN was similar to that for linear NRTFD, except that the internal dihedral angles defining the coordinates of backbone atoms of the cyclic peptide were unfixed, and bond length and angle terms that keep the cyclic structure of the peptide were included in the energy calculation. The simulation converged and the lowest-energy conformation was retained for analysis.

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