Engineering of a VPAC2 Receptor Peptide Agonist To Impart Dipeptidyl Peptidase IV Stability and Enhance in Vivo Glucose Disposal

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VPAC2P-PEG is a VPAC2 receptor agonist peptide that acts as a glucose-dependent insulin secretagogue. Proteolysis by DPPIV may contribute to the in vivo clearance of VPAC2P-PEG. Here, the N-terminus of VPAC2P-PEG is modified by N-terminal acetylation to impart DPPIV resistance. The acetylated peptide, Ac-VPAC2P-PEG, is a selective and potent VPAC2 agonist, resistant to DPPIV proteolysis, and exhibits substantially improved half-life and glucose disposal in rodents. Ac-VPAC2P-PEG has therapeutic potential for diabetes management.

Introduction

Glucose-dependent insulin secretagogues provide an avenue for diabetes management without the risk of hypoglycemia. Such currently marketed therapies or compounds in late-stage development target the incretin GLP-1[a] with two distinct approaches.[1−3] The first strategy employs GLP-1 peptide mimetics that activate the GLP-1 receptor, whereas the second uses DPPIV inhibitors that stabilize endogenous GLP-1.[2,3] Nausea and vomiting, however, can accompany GLP-1 receptor stimulation.[1] An alternative approach to induce glucose-dependent insulin secretion is through VPAC2 receptor activation.[4] VPAC2 is related to the VPAC1 and PAC1 receptors,[5] and peptides such as PACAP27 show nonselective activation of all three receptors.[5] However, stimulation of VPAC1 may lead to unwanted effects such as glucose production.[4] It is desirable, therefore, to develop selective VPAC2 receptor agonists for use as insulin secretagogues.[4]

Peptide **1** (BAY 55-9837)[4] is a selective VPAC2 receptor agonist peptide that stimulates glucose-dependent insulin secretion in isolated human pancreatic islets, increases insulin synthesis in purified rat islets, and causes a dose-dependent increase in plasma insulin levels in fasted rats.[4] Peptide **1**, however, exhibits rapid clearance and requires continuous intravenous or subcutaneous infusion for efficacy in rodents.[4] The short in vivo lifetime of **1** is typical of unmodified peptides.[6] Peptide **1**, therefore, was modified in terms of amino-acid substitutions that improve stability in formulation and by PEGylation to improve in vivo lifetime.[7] The resulting peptide VPAC2P-PEG exhibits greatly improved in vivo efficacy and duration of action in rodents relative to **1**.[7,8]

VPAC2P-PEG may be susceptible to in vivo DPPIV proteolysis, because the N-terminus of the peptide is based on the in vitro DPPIV-substrate VIP.[9,10] The N-terminal amino group of a DPPIV-bound peptide forms hydrogen bonds with DPPIV, as seen in the crystal structures of DPPIV complexes[11,12] Disruption of these intermolecular interactions via acetylation of the N-terminal α-amino group is envisaged to destabilize complex formation and impart protease resistance.[13] We show here that N-terminal acetylation of VPAC2P-PEG produces a DPPIV-resistant peptide VPAC2 agonist that exhibits significantly enhanced in vitro DPPIV stability and in vivo efficacy.

Results and Discussion

The amino-acid sequence of Ac-VPAC2P is Ac-HSDAVFT-DQYTRLRKQVAAKKYLQSIKQKRYC, where Ac denotes the N-terminal α-amino acetyl group. Ac-VPAC2P-PEG is the same peptide modified at the C-terminal Cys with 43 kDa branched PEG. Electrospray mass spectrometry indicates that Ac-VPAC2P has the expected mass, with a mass within 1 Da of the expected value of 3916.5 Da. Both Ac-VPAC2P and Ac-VPAC2P-PEG are >97% pure, as assessed by C_{18} and cation-exchange HPLC.

Ac-VPAC2P-PEG is a full agonist of the human VPAC2 receptor with an apparent EC_{50} of 13 ± 1 nM (Table 1), as assessed by cAMP accumulation in CHO cells expressing human VPAC2 (Figure 1). The VPAC2 receptor IC_{50} value for Ac-VPAC2P-PEG is 160 ± 20 nM, as assessed by the competitive displacement of the nonselective VPAC2 peptide agonist ^{125}I-labeled PACAP27. This IC_{50} value is very similar to that observed for the unacetylated analog VPAC2P-PEG of 200 ± 26 nM.[7]

PEGylation causes a decrease in VPAC2 receptor binding and activation. The unPEGylated peptide Ac-VPAC2P exhibits an approximately 20-fold more potent EC_{50} and a 6-fold more potent IC_{50} than Ac-VPAC2P-PEG (Table 1). The decrease in activity seen upon PEGylation is typical of many peptides and proteins,[14] although cases exist where site-specific PEGylation results in a relatively small effect on in vitro activity.[13,15−17]

The VPAC family of receptors comprises PAC1, VPAC1, and VPAC2.[5] The nonselective peptide agonist PACAP27 is a potent activator of all three receptors.[5] In the cAMP accumulation assay, PACAP27 stimulates all three receptors with high potency. The EC_{50} values for PAC1, VPAC1, and VPAC2 are 0.13 ± 5, 0.6 ± 0.1, and 0.087 ± 0.025 nM, respectively (Table 1). In contrast, Ac-VPAC2P-PEG activates only the VPAC2 receptor (EC_{50} = 13 ± 1 nM) and exhibits EC_{50} values >10

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[a] Abbreviations: AUC, area under the curve; DPPIV, dipeptidyl peptidase IV; GLP-1, glucagon-like peptide 1; HPLC, high-performance liquid chromatography; IPGTT, intraperitoneal glucose tolerance test; MALDI, martix assisted laser desorption ionization; PAC1, PACAP type 1 receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide; VPAC1, VIP/PACAP type 1 receptor; VPAC2, VIP/PACAP type 2 receptor.

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Table 1. Receptor Activation (EC_{50}) and Binding (IC_{50}) Properties of Ac-VPAC2P, Ac-VPAC2P-PEG, and PACAP27[a]

	Ac-VPAC2P-PEG		Ac-VPAC2P		PACAP27	
	EC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)	IC_{50} (nM)
VPAC2	13 ± 1	160 ± 20	0.6 ± 0.3	27 ± 3	0.087 ± 0.025	13 ± 5
PAC1	>10 000	>10 000	n.d.[b]	n.d.[b]	0.13 ± 0.05	3 ± 1
VPAC1	>10 000	>10 000	n.d.[b]	n.d.[b]	0.6 ± 0.1	12 ± 1

[a] See Figure 1 and Supporting Information. [b] n.d. = not determined.

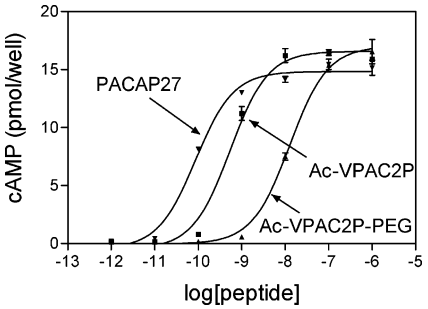

Figure 1. VPAC2 receptor activation by Ac-VPAC2P and Ac-VPAC2P-PEG as measured by cAMP accumulation in CHO cells expressing human VPAC2. Ac-VPAC2P and Ac-VPAC2P-PEG stimulate the VPAC2 receptor with apparent EC_{50} values of 0.6 ± 0.3 and 13 ± 1 nM, respectively. The nonselective VPAC2 receptor agonist peptide PACAP27 exhibits an apparent EC_{50} of 87 ± 25 pM.

μM for the PAC1 and VPAC1 receptors (Table 1). Similar results are seen in ^{125}I-PACAP competitive-binding assays. Ac-VPAC2P-PEG does not displace ^{125}I-PACAP27 from the human PAC1 or VPAC1 receptors (IC_{50} > 10 μM; Table 1). In the same competitive-binding assay, PACAP27 exhibits IC_{50} values of 3 ± 1 and 12 ± 1 nM for the human PAC1 and VPAC1 receptors, respectively (Table 1). Thus, in common with the parental peptides **1** and VPAC2-PEG,[4,7] Ac-VPAC2P-PEG is a specific VPAC2 receptor agonist.

To evaluate the contribution of N-terminal α-amino acetylation to DPPIV stability, mass spectrometry was used to identify the DPPIV products of the peptides VPAC2P and Ac-VPAC2P that lack PEG. The unPEGylated peptides were used to facilitate direct detection of DPPIV products with mass spectrometry. Incubation with DPPIV of the unacetylated and unPEGylated peptide VPAC2P results in products corresponding to the loss of the first two or four amino acids after 24 h (Figure 2A), as expected for a DPPIV substrate. In contrast, significant formation of proteolysis products is not observed for the acetylated peptide Ac-VPAC2P (Figure 2B).

To evaluate the stability of PEGylated peptides, Edman sequencing was used to detect the DPPIV products of the PEGylated peptides.[13] If proteolysis occurs, the appearance of peptides beginning at DPPIV cleavage sites that are two amino-acid residues from the N-terminus will be observed. VPAC2P, which is neither acetylated nor PEGylated, is approximately 88% cleaved after incubation with DPPIV for 48 h at both Asp 3 and Val 5 (Figure 3). VPAC2P-PEG, which is PEGylated but not acetylated, is approximately 25% cleaved at Asp 3 (peptides corresponding to cleavage at Val 5 are not observed). For Ac-VPAC2P-PEG, proteolysis would be detected by the appearance of products with a free N-terminal α-amino group that can be detected with Edman sequencing (the α-amino acetyl group of Ac-VPAC2P-PEG blocks the Edman reaction). Peptides with free N-termini, such as those observed for VPAC2P-PEG, are not detected for Ac-VPAC2P-PEG after 48 h in the presence of DPPIV, suggesting that significant DPPIV proteolysis of Ac-VPAC2P-PEG is not occurring.

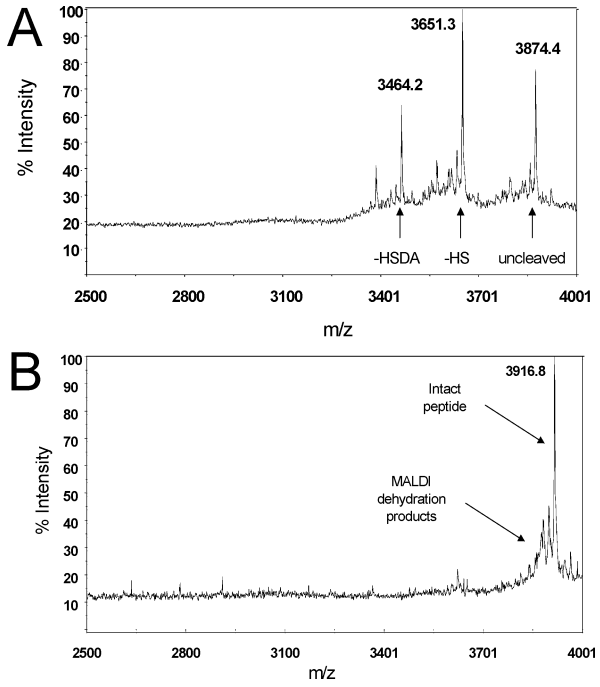

Figure 2. MALDI mass spectrometry analysis of DPPIV proteolysis of VPAC2P and Ac-VPAC2P. (A) Proteolysis products corresponding to cleavage of the first two (−HS) and four amino acids (−HSDA) of VPAC2P are observed after 24 h. (B) Significant levels of proteolysis products are not observed for Ac-VPAC2P after 24 h.

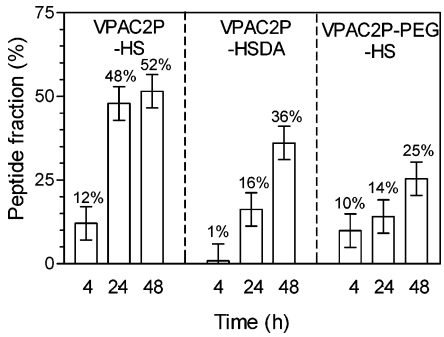

Figure 3. Edman analysis of proteolysis by DPPIV. Proteolysis products corresponding to cleavage of the first two (−HS) and four amino acids (−HSDA) of VPAC2P and of the first two amino acids (−HS) of VPAC2P-PEG are observed. Peptides with free N-termini such as those observed for VPAC2P-PEG are not detected for Ac-VPAC2P-PEG after 48 h in the presence of DPPIV, suggesting that significant DPPIV proteolysis of Ac-VPAC2P-PEG is not occurring.

Collectively, the MS and Edman sequencing results show that Ac-VPAC-PEG is resistant to in vitro DPPIV proteolysis, in contrast to the unacetylated peptide. The unacetylated peptide is somewhat protected from proteolysis by PEGylation, in accord with previous observations that PEGylation may improve protease resistance.[6]

The stability of Ac-VPAC2P-PEG to DPPIV proteolysis suggests that the acetylated peptide may have a longer in vivo lifetime than the unacetylated analog. The concentration of active peptide in rat plasma was assessed with an ex vivo bioassay of plasma from rats administered VPAC2P-PEG or Ac-VPAC2P-PEG. The assay reports on cAMP production by CHO cells expressing the VPAC2 receptor treated with ex vivo plasma extracts. Activity ascribable to VPAC2P-PEG is not detected 24 h after administration, suggesting that VPAC2P-PEG is cleared in vivo within 24 h (Figure 4). In contrast, plasma from rats treated with the acetylated peptide Ac-

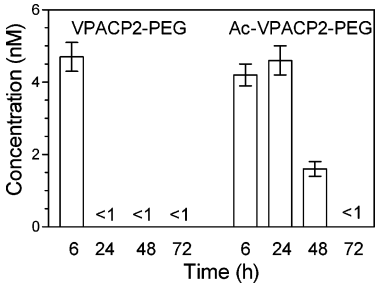

Figure 4. The concentration of VPAC2P-PEG in ex vivo rat plasma falls below 1 nM within 24 h. In contrast, Ac-VPAC2P-PEG is present at 48 h. The limit of detection of the assay is 1 nM.

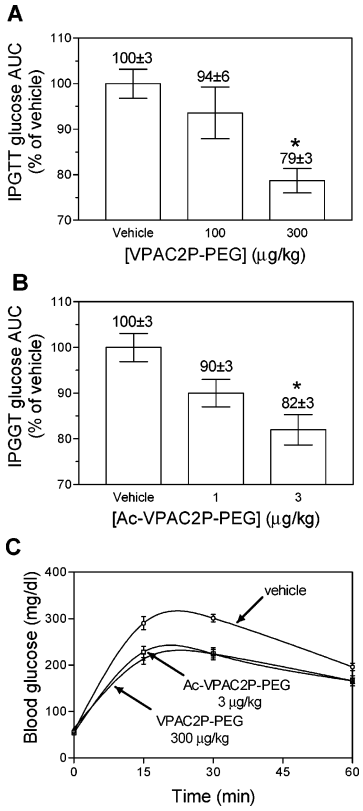

Figure 5. Acetylation increases in vivo efficacy in rats. (A) The unacetylated peptide VPAC2P-PEG causes significant glucose disposal. (B) Ac-VPAC2P-PEG is as efficacious as the unacetylated analog at 100-fold lower doses. (C) Time courses for glucose disposal. Fasting blood glucose was measured 24 h after dosing. Statistical significance is indicated with an asterisk ($p < 0.01$).

VPAC2P-PEG exhibits significant VPAC2 receptor stimulation after 48 h (Figure 4). These results suggest that acetylation imparts a significantly enhanced in vivo lifetime to Ac-VPAC2P-PEG.

The enhanced stability in rat plasma of Ac-VPAC2P-PEG suggests the possibility of an improved in vivo efficacy relative to the unacetylated peptide. VPAC2P-PEG enhances glucose disposal in the intraperitoneal glucose tolerance test (IPGTT; Figure 5A), in accord with previous results.[7] Remarkably, Ac-VPAC2P-PEG induces comparable glucose disposal to VPAC2P-PEG at 100-fold lower doses (Figure 5B). The biochemical studies of protease resistance suggest that the dramatic increase of in vivo efficacy imparted by acetylation arises at least in part from the enhanced protease stability of Ac-VPAC2P-PEG.

We conclude that N-terminal acetylation of the VPAC2 receptor agonist peptide VPAC2P-PEG[7] to produce Ac-VPAC2P-PEG imparts in vitro resistance to DPPIV proteolysis without affecting in vitro VPAC2 receptor activation or selectivity. Our findings reinforce the notion that N-terminal acetylation of DPPIV substrates provides a general approach to the in vitro stabilization of peptide therapeutics that are susceptible to inactivation by N-terminal proteolysis[13] and demonstrate that DPPIV stabilization by N-terminal acetylation can translate to improved in vivo efficacy. Strikingly, the modification imparts a remarkable improvement of in vivo lifetime and efficacy, thereby opening up the possibility of a novel long-acting peptide-based glucose-dependent insulin secretagogue for the management of type II diabetes.

Materials and Methods

Peptide Synthesis. Ac-VPAC2P was synthesized with an Applied Biosystems 433A peptide synthesizer using Fmoc chemistry with HBTU activation on Rink amide resin, and the N-terminus was acetylated with acetic anhydride. The peptide was cleaved with 84.6% TFA, 4.4% phenol, 4.4% water, 4.4% thioanisol, and 2.2% ethanedithiol and precipitated from the cleavage cocktail with cold *tert*-butyl methyl ether. The precipitate was washed with the cold ether and dried under argon. Purification was performed with reversed-phase C_{18} chromatography with linear water/acetonitrile gradients containing 0.1% TFA. Peptide identity was confirmed with electrospray mass spectrometry, and purity was confirmed with C_{18} (Waters Atlantis 186001331) and cation-exchange (TosaHass SP-5PW) HPLC.

PEGylation was performed at the unique C-terminal Cys residue, with branched 43 kDa methoxy-PEG-maleimide (Nektar mPEG2-MAL). A 2-fold molar excess of PEG reagent was added to peptide in 10 mM sodium phosphate, pH 6, for 2 h at room temperature. The PEGylated peptide was purified with cation exchange (TosaHass SP-5PW) to remove residual PEG and free peptide, and the product was dialyzed against water. Purity and PEGylation was confirmed with C_{18} (Waters Atlantis 186001331) and cation-exchange (TosaHass SP-5PW) HPLC and with SDS-PAGE.

The preparation of VPAC2P-PEG was described elsewhere.[7]

Mass Spectrometry Analysis of DPPIV Proteolysis. Peptides at 20 μM were incubated with 842 pM human DPPIV in 100 mM HEPES, pH 7.4, at 37 °C for 0 or 24 h. Reaction aliquots were quenched with 200 nM of the DPPIV inhibitor **2** (NVP-DPP728)[18] and frozen. Aliquots (20 pmol of peptide) diluted 5-fold with water were desalted with a conditioned Millipore C_{18} ZipTip. Sample was eluted with matrix (10 mg/mL α-cyanohydroxycinnamic acid in 50% acetonitrile, 0.1% TFA) directly onto the MALDI plate. Samples were analyzed on an Applied Biosystems Voyager DE-PRO MALDI, operated in the reflector ion mode.

Edman Analysis of DPPIV Proteolysis. Peptides at 20 μM were incubated with 842 pM DPPIV in 100 mM HEPES, pH 7.4, at 37 °C for 0−24 h. Reaction aliquots (0.4 nmol peptide) were quenched with 200 nM of the DPPIV inhibitor **2** and frozen. Samples were diluted 10-fold with 0.1% TFA, desalted, and washed with 0.1% TFA with an Applied Biosystems ProSorb sample cartridge. Edman degradation was carried out on an Applied Biosystems Procise 494HT protein sequencing system using the pulsed-liquid method, according to manufacturer instructions.

PAC1, VPAC1, and VPAC2 Receptor Activation Assay. cAMP accumulation assays were performed as described previously.[4] Briefly, CHO cells transfected with the human PAC1, VPAC1, or VPAC2 receptor and test peptide (Ac-VPAC2P, Ac-VPAC2P-PEG, or PACAP27) were incubated at 37 °C for 15 min in 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1% BSA, 0.5 mg/mL bacitracin, and 100 μM 3-isobutyl-1-methyl-xanthine. cAMP content was determined with a competitive ^{125}I-cAMP scintillation proximity assay (Amersham Pharmacia Biotech). Data were fit to a single-site binding model with Prism 3.0.3.

PAC1, VPAC1, and VPAC2 Receptor Competition Binding Assays. Assays were performed as described previously.[4] Briefly,

10 μg membrane prepared from CHO cells transfected with the human PAC1, VPAC1, or VPAC2 receptor, 0.1 nM ^{125}I-PACAP27 (New England Nuclear, NEX294) and test peptide (Ac-VPAC2P, Ac-VPAC2P-PEG, or PACAP27) were incubated at 37 °C for 20 min in 20 mM HEPES (pH 7.4), 150 mM NaCl, 0.5% BSA, 2 mM $CaCl_2$, and 0.1 mg/mL bacitracin. The reaction was stopped by cooling on ice for 20 min. Bound and unbound radioligands were separated by filtration through filters pretreated with 0.1% polyethylenimine and washed several times with a BSA-based wash solution. The filter plate was dried, scintillant added, and radioactivity quantified with a MicroBeta counter. Data were fit to a single-site binding model with Prism 3.0.3.

Plasma Concentration Bioassay. Concentrations of active Ac-VPAC2P-PEG in plasma derived from rats dosed subcutaneously with Ac-VPAC2P-PEG (30 μg/kg) were determined by measuring luciferase reporter gene expression under control of the CRE promoter in response to cellular cAMP. Ex vivo plasma aliquots were mixed with an equal volume of acetonitrile, vortexed, centrifuged to precipitate protein, and evaporated to dryness under vacuum. The sample was resuspended in water, rehydrated at 4 °C overnight, and sonicated for 10 min. Sample (30 μL) and buffer (70 μL 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.5 mg/mL bacitracin, and 100 μM 3-isobutyl-1-methylxanthine) were incubated with CHO cells coexpressing VPAC2 and luciferase for 4 h. Bright-Glo luciferase assay system (Promega) reagents were added, and the luciferase signal was detected with a Packard TopCount. The results were calibrated with a standard curve made from control plasma containing Ac-VPAC2P-PEG of known concentrations.

IPGTT. The IPGTT was performed in male Wistar rats (250−275 g) as described previously.[19] Briefly, fasting tail blood glucose was measured 24 h after dosing with vehicle (phosphate-buffered saline) or peptide, the animals were administered 2 g/kg glucose intraperitoneal, and tail blood glucose was measured again after 15, 30, and 60 min. The AUC was calculated using the trapezoidal method, and statistical significance was evaluated using ANOVA.

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Supporting Information Available: Structure of Ac-VPAC2P-PEG and receptor activation and binding curves for data are reported in Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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