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[des His^1, des Phe^6, Glu^9]GLUCAGON AMIDE: A NEWLY DESIGNED "PURE" GLUCAGON ANTAGONIST

Bassem Y. Azizeh, Brian A. Van Tine, Noel S. Sturm, Ann Marie Hutzler, Clinton David, Dev Trivedi and Victor J. Hruby*

Department of Chemistry, University of Arizona, Tucson, AZ 85721, U. S. A.

Abstract: We report the synthesis and biological activity of a new glucagon analog that was designed as a glucagon receptor antagonist by appropriate modifications in the N-terminal region of glucagon. The structure of the new analog is **[des His^1, des Phe^6, Glu^9]glucagon amide**, and its binding potency IC_{50} value of 48 nM. The compound was found to be a pure antagonist in a new much more sensitive assay for glucagon stimulated cAMP accumulation activity and showed a pA_2 value of 8.20 in this assay.

Introduction

Glucagon, a 29 amino acid peptide hormone, is secreted by cells in the islets of Langerhans in the mammalian liver and plays an important role in the regulation of blood glucose levels. The bihormonal hypothesis[1,2] postulates that in Type II diabetes the overproduction of glucose is due to excess circulating glucagon whereas the underutilization of glucose is observed because of insulin abnormalities. Our aim has been to design and synthesize glucagon antagonists and to separate the transduction message of the hormonal signal from the binding message of glucagon to its receptor.[3] Therefore, potent glucagon antagonists with no partial agonist activity are our most valuable tool for testing this hypothesis and hopefully leading to a therapeutic approach for the treatment of Type II diabetes.

Two of the most potent antagonists to date are [1-N$^{\alpha}$-trinitrophenyl histidine, 12-homoarginine]glucagon (THG)[4] and [des His^1, Glu^9]glucagon amide.[5] These two compounds were shown to reduce blood glucose in streptozoticin-induced diabetic rats.[6,5] As a result, these glucagon receptor antagonists reduced the blood glucose levels ~50-65% in diabetic animals without the addition of exogenous insulin.

The major focus in this research for glucagon antagonists is to design analogs that are pure antagonists with no partial agonist activity. From structure-function studies, it has been shown that the C-terminal region is important for receptor recognition and binding, while the N-terminal region of glucagon is critical for the transduction of glucagon's physiological message.[7] In general, a good glucagon receptor antagonist should bind to the plasma membrane receptor without activating the adenylate cyclase system or other second messengers systems.[8]

In order to obtain potent glucagon antagonists, the approach we are following is based on previous results from this laboratory[9] as well as that of Merrifield.[10] It has been reported that [des His^1, Glu^9]glucagon amide was a glucagon antagonist with a binding affinity 40% that of native glucagon and a pA_2 value of 7.25.[5] It also has been suggested that Phe^6, a highly conserved residue in the glucagon hormone family, was part of a hydrophobic patch and involved in receptor binding. The deletion of Phe^6 in the N-terminal region was a key

approach in obtaining potent glucagon antagonists.[9] Combining these two studies, we describe the design, synthesis, characterization and biological activity of a new glucagon analog [des His^1, des Phe^6, Glu^9]glucagon amide. This compound was found to be a pure antagonist that binds to the glucagon receptor with an IC_{50} of 48 nM, and which does not activate the adenylate cyclase system even at high concentrations. The pA_2 value was found to be 8.20. The amino acid sequence of [des His^1, des Phe^6, Glu^9]glucagon amide is:

H-Ser-Gln-Gly Thr^5-Thr-Ser-Glu^9-Tyr-Ser-Lys-Leu-Asp^{15}-Ser-Arg-
Arg-Ala-Gln^{20}-Asp-Phe-Val-Gln-Trp^{25}-Leu-Met-Asn-Thr-NH_2

Experimental Procedure

Synthesis and Purification: The synthesis of [des His^1, des Phe^6, Glu^9]glucagon amide was carried out on an Applied Biosystems (ABI) 431A peptide synthesizer on a 0.25 mmol scale. Using an Fmoc strategy, the glucagon was synthesized by solid-phase methodology using a 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin. The following protecting groups were used: Arg (2,2,5,7,8-pentamethyl chroman-6-sulfonyl, Pmc); Asn (trityl); Asp (t-butyl); Gln (trityl); Glu (t-butyl); Lys (Boc); Ser (t-butyl); Thr (t-butyl); and Tyr (t-butyl). The analog was cleaved from the resin using standard techniques and a cleavage mixture of 90% trifluoroacetic acid, 5% anisole, 2.5% methyl sulfide, and 2.5% 1,2-ethanedithiol. The purification was carried out on Perkin-Elmer Model 410-BIO instrument by preparative high-performance liquid chromatography (HPLC) with a VYDAC 218 TBP-16 column (16 x 250 mm). The sample was eluted by applying a gradient of 10-90% acetonitrile in 0.1% aqueous TFA over 30 min period using a flow rate of 5 mL/min. The product containing the eluent was collected and lyophilized. Homogeneity was checked by HPLC on an analytical VYDAC 218 TBP-16 column (4.6 x 250 mm). The analog was characterized by amino acid analysis, TLC and by electrospray mass spectroscopy (ES-MS) where the calculated mass was 3211.5 and the experimental value was 3212.0.

Isolation of Liver Plasma Membrane: The liver plasma membranes were obtained from Sprague-Dawley rats weighing 200 to 250 g as described by Neville[11] and modified by Pohl.[12] The protein concentration was determined by the method of Markwell et al.[13]

Receptor Binding Assay: The binding assay was performed according to Wright and Rodbell[14] where competition for glucagon receptors between [^{125}I]-glucagon and the glucagon analog was measured. Results are expressed as the % inhibition of [^{125}I]-glucagon specific binding. Assays were performed in triplicate and repeated twice.

Adenylate Cyclase Assay: The adenylate cyclase activity was measured by the conversion of [α-^{32}P]ATP to cAMP as described by Lin et al.[15] Labeled cAMP was determined by the method of Solomon et al.[16] using Dowex 50 and alumina column. Results were expressed as the % of stimulation of cAMP production over basal. Assays were done twice and in triplicate.

cAMP Accumulation Assay: Hepatocytes were isolated by the method of Berry and Friend[17] with slight modification by Heyworth and Houslay.[18] The isolated hepatocytes were challanged with the hormone in the presence of 25 µM Rolipram for 5 min before precipitation with 2% perchloric acid and neutralization with 0.5 M triethanolamine and 2 M KOH. Determination of the cAMP accumulation in cells was then performed using a methodology similar to the competitive method of Brown et al.[19] Results were expressed as a percent of maximal

glucagon stimulation over basal, and all determinations were made with at least three experiments done in triplicate.

Results and Discussion

The new glucagon analog was synthesized by solid phase methodology using an Fmoc strategy on 4-(2'-4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin. The peptide was cleaved from the resin, and the side protecting groups were removed by the use of trifluoroacetic acid in the presence of scavengers. The crude product was purified by reversed-phase high-performance liquid chromatography (RP-HPLC). The peptide was obtained in a highly purified form (>98%) with an average yield of 19%. The amino acid analysis for this analog was: Asx 3.34(3); Glx 4.03(4); Ser 3.65(4); Gly 1.20(1); Arg 2.12(2); Thr 2.93(3); Ala 1.06(1); Tyr 1.83(2); Val 0.91(1); Met 0.86(1); Leu 2.04(2); Phe 0.94(1) and Lys 1.00(1). The final product was checked for its purity by TLC in three solvent systems: I, 1-butanol-acetic acid-water-pyridine (15:3:10:12); II, 1-butanol-acetic acid-water-pyridine (60:10:50); III, ethyl acetate-pyridine-acetic acid-water (12:4:4.2:2.2). The values from these three solvents were 0.33, 0.38, and 0.53 respectively.

For this new analog, [des His[1], des Phe[6], Glu[9]]glucagon amide, the receptor affinity was determined using a standard glucagon binding assay that measured the displacement of [^{125}I]-labeled glucagon.[20] The IC_{50} value for this analog was 48 nM. In addition, the analog was also tested for its ability to transduce the biological message in the adenylate cyclase assay. In this assay, the analog was found to be a pure antagonist, unable to activate the system up to a concentration of 10^{-5} M. The compound was further examined in competition experiments where a shift in the dose response curve for glucagon's adenylate cyclase activity was obtained in the presence of different concentrations of antagonists ranging from 100 nM to 10 μM. The pA_2 value of 8.20 was calculated by the method Schild.[21]

Furthermore, this analog was tested for its cAMP accumulation with rat hepatocytes in the presence of a specific type IV phosphodiesterase (PDE) inhibitor Rolipram. We found in this assay system (results from our laboratory) that this was the most "pure" glucagon receptor antagonist, compared to the ones reported in the literature[4,9,22] (which also were measured) with no agonist activity at any concentration, whereas the others showed activity at high concentrations.

It has been shown that [des His[1], Glu[9]]glucagon amide is a potent glucagon antagonist.[10] In this analog, aspartic acid in position 9 was shown to be a critical residue for glucagon activity, and the importance of His[1] in signal transduction was already known.[15] The C-terminal amide gave an analogue with 40% binding relative to glucagon, and with no adenylate cyclase activity in the liver plasma membrane assay. It has been suggested from structure-function studies[9] and NMR[23,24] that the phenylalanine at position 6 is part of a hydrophobic patch which also includes Tyr[10] and Leu[14]. Phe[6] is a highly conserved residue throughout the glucagon hormone family and has been shown to be an important residue for the glucagon binding receptor interaction.

Combining these previous results, a glucagon analog [des His[1], des Phe[6], Glu[9]]glucagon amide was designed using the template [des His[1], Glu[9]]glucagon amide and the deletion of Phe[6]. The analog was found to be a pure antagonist in the adenylate cyclase assay, unable to activate the system up to a concentration of 10^{-5} M. In the receptor binding assay, the IC_{50} value for this analog was 48 nM compare to glucagon's 1.5 nM. The binding results were consistent with our previous studies[9] where the removal of Phe[6] affected the binding because of its inclusion in the hydrophobic patch in the N-terminal.

In conclusion, the pA_2 value of 8.20 for [des His^1, des Phe^6, Glu^9]glucagon amide and our results on cAMP accumulation with rat hepatocytes in the presence of a PDE inhibitor make this analog the purest highly potent antagonist known so far.

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