Articles

Topographical Amino Acid Substitution in Position 10 of Glucagon Leads to Antagonists/Partial Agonists with Greater Binding Differences

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The role of position 10 in the β-turn region of glucagon was investigated by substituting chiral constrained amino acids and other modifications in the N-terminal region. A series of glucagon analogues have been designed and synthesized by incorporating β-methylphenylalanine isomers (2*S*,3*S*, 2*S*,3*R*, 2*R*,3*R*, and 2*R*,3*S*) at position 10 in order to explore the structural and topographical requirements of the glucagon receptor, and, in addition, utilizing previous studies which indicated that antagonism could be enhanced by modifications (des-His^1, Glu^9) and a bulky group at position 5. The structures of the new analogues are as follows: [des-His^1,-Tyr^5,Glu^9]glucagon-NH_2 **(II)**, [des-His^1,Tyr^5,Glu^9,Phe^{10}]glucagon-NH_2 **(III)**, [des-His^1,Tyr^5,Glu^9,-Ala^{10}]glucagon-NH_2 **(IV)**, [des-His^1,Tyr^5,Glu^9,(2*S*,3*R*)-β-MePhe^{10}]glucagon-NH_2 **(V)**, [des-His^1,-Tyr^5,Glu^9,(2*S*,3*S*)-β-MePhe^{10}]glucagon-NH_2 **(VI)**, [des-His^1,Tyr^5,Glu^9,D-Tyr^{10}]glucagon-NH_2 **(VII)**, [des-His^1,Tyr^5,Glu^9,D-Phe^{10}]glucagon-NH_2 **(VIII)**, [des-His^1,Tyr^5,Glu^9,D-Ala^{10}]glucagon-NH_2 **(IX)**, [des-His^1,Tyr^5,Glu^9,(2*R*,3*R*)-β-MePhe^{10}]glucagon-NH_2 **(X)**, and [des-His^1,Tyr^5,Glu^9,(2*R*,3*S*)-β-MePhe^{10}]glucagon-NH_2 **(XI)**. These analogues led to dramatically different changes in *in vitro* binding affinities for glucagon receptors. Their receptor binding potencies IC_{50} values (nM) are 2.3 **(II)**, 4.1 **(III)**, 395.0 **(IV)**, 10.0 **(V)**, 170.0 **(VI),** 74.0 **(VII)**, 34.5 **(VIII)**, 510.0 **(IX)**, 120.0 **(X),** and 180.0 **(XI)**. Analogues **II**, **III**, **V**, **VI**, and **XI** were found to be weak partial agonists/partial antagonists with maximum stimulation between 5%−9%, while the other compounds (**IV** and **VII**−**X**) were antagonists unable to activate the adenylate cyclase system even at concentrations as high as 10^{-5} M. In competition experiments, all of the analogues caused a right shift of the glucagon-stimulated adenylate cyclase dose−response curve. The pA_2 values were 6.60 **(II)**, 6.85 **(III)**, 6.20 **(IV)**, 6.20 **(V)**, 6.10 **(VI)**, 6.50 **(VII)**, 6.20 **(VIII)**, 5.85 **(IX)**, 6.20 **(X),** and 6.00 **(XI)**. Putative topographical requirements of the glucagon receptor for the aromatic side chain conformation in position 10 of glucagon antagonists are discussed.

Introduction

Glucagon is a 29-amino acid peptide hormone secreted by the α-cell in the pancreatic islets and plays a crucial role in glucose homeostasis, stimulating gluconeogenesis and glycogenolysis in the liver, leading to the production of glucose for release into the bloodstream.[1,2] Glucagon also causes lipolysis in the liver and fat cells.[3] The hormone binds to specific receptors in the liver plasma membrane followed by the activation of adenylate cyclase to increase the levels of the second messenger cAMP.

The role of glucagon in the pathogenesis of diabetes mellitus remains controversial. The bihormonal hypothesis[4−6] postulates that in type II diabetes the overproduction of glucose is due to excess circulating glucagon, while the underutilization of glucose is realized because of insulin abnormalities. Several researchers have tried to develop and design glucagon inhibitors (antagonists) that will provide direct evidence to test this hypothesis.[7] These glucagon receptor antagonists should bind to the plasma membrane receptor without signal transduction leading to activation of adenylate cyclase system or other second messengers.

Recently we have reported the synthesis and biological activity of a new glucagon analogue that was designed as a potent receptor antagonist with modifications in the N-terminal region. The structure of the new analogue is [des-His^1,des-Phe^6,Glu^9]glucagon-NH_2, and its binding potency IC_{50} value was 48 nM (glucagon's IC_{50} = 1.5 nM). This analogue was found to be the first "pure" glucagon antagonist[8] in a highly sensitive assay for glucagon-stimulated cAMP accumulation activity and showed a pA_2 value of 8.20. The glucagon receptor antagonist was tested *in vivo* (manuscript submitted), where it lowered the blood glucose levels to 60% in streptozotocin-induced diabetic rats. Two other glucagon antagonists [1-N^{α}-(trinitrophenyl)histidine,12-homoarginine]glucagon (THG)[9] and [des-His^1,Glu^9]glucagon-NH_2,[10] also were found to lower blood glucose in streptozotocin-induced diabetic rats.[11] These glucagon receptor antagonists reduced the blood glucose levels ∼55−60% in diabetic animals without the addition of exogenous insulin.

Recent isolation of the glucagon receptor by Jelinek *et al.*[12] has demonstrated that it belongs to a family of G-protein-coupled hormone receptors and is related to those of calcitonin (CT), parathyroid hormone (PTH), secretin, and vasoactive intestinal peptide (VIP). The cloned receptor-bound glucagon caused an increase in the intracellular concentration of cAMP and also transduced a signal that led to an increase in calcium concentration. It has been shown that glucagon recep-

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Table 1. Structure of Glucagon and Its Analogues

peptide	structure
I, glucagon	His-Ser-Gln-Gly-Thr[5]-Phe-Thr-Ser-Asp-Tyr[10]-Ser-Lys-Tyr-Leu-Asp[15]-Ser-Arg-Arg-Ala-Gln[20]-Asp-Phe-Val-Gln-Trp[25]-Leu-Met-Asn-Thr-COOH
II	[des-His[1],Tyr[5],Glu[9]]glucagon-NH_2
III	[des-His[1],Tyr[5],Glu[9],Phe[10]]glucagon-NH_2
IV	[des-His[1],Tyr[5],Glu[9],Ala[10]]glucagon-NH_2
V	[des-His[1],Tyr[5],Glu[9],(2*S*,3*R*)-β-MePhe[10]]glucagon-NH_2
VI	[des-His[1],Tyr[5],Glu[9],(2*S*,3*S*)-β-MePhe[10]]glucagon-NH_2
VII	[des-His[1],Tyr[5],Glu[9],D-Tyr[10]]glucagon-NH_2
VIII	[des-His[1],Tyr[5],Glu[9],D-Phe[10]]glucagon-NH_2
IX	[des-His[1],Tyr[5],Glu[9],D-Ala[10]]glucagon-NH_2
X	[des-His[1],Tyr[5],Glu[9],(2*R*,3*R*)-β-MePhe[10]]glucagon-NH_2
XI	[des-His[1],Tyr[5],Glu[9],(2*R*,3*S*)-β-MePhe[10]]glucagon-NH_2

tor activation involves not only the adenylate cyclase system but also inositol phosphate. Wakelam *et al.*[13] demonstrated that glucagon at low concentration causes the production of inositol phosphates and the breakdown of phospholipids.

The major goal in this research has been to design and synthesize potent glucagon analogues that will enable us to separate the transduction message of the hormonal signal from the structural components that are necessary for binding the hormone to its receptor. From structure−activity relationship studies, it has been demonstrated that the N-terminal region of glucagon, in particular residues 1−6, 9, and 10, is important for the transduction of glucagon's physiological message, whereas the C-terminal region is important for binding to the receptor.[14,15]

In this paper we describe the total synthesis of 10 new glucagon analogues by solid-phase methodology. This investigation examined modifications in the N-terminal region of glucagon, in particular residues 1, 5, 9, and 10 (histidine, threonine, aspartic acid, and tyrosine, respectively), and in addition β-MePhe isomers were introduced in the Tyr[10] position of glucagon to study the effects of constraint or bias conformational preferences of the side chain moiety[16] on biological activities. Since methods of asymmetric synthesis of β-methyl aromatic amino acids have been developed,[16−18] serial substitutions of biologically important aromatic amino acids with four β-methylated stereoisomers became an important tool for investigating topographical requirements of peptide receptors for the side chain conformations.[19] The β-methyl derivatives of Phe, Tyr, and Trp have been incorporated into various biologically active peptides[20−23] to better understand structure−activity relationship. The importance of residue 1 to transduction was noted by Lin *et al.*[24] who indicated that des-His[1] was a weak partial agonist. It has been demonstrated that the position 9 aspartic acid is crucial for transduction of the message and operates in conjunction with His[1] for activation of glucagon's action.[25] The potent antagonist [des-His[1],Glu[9]]glucagon-NH_2 with a binding affinity of 40% clearly indicates the roles of positions 1 and 9,[26,27] whereas Krystenansky *et al.*[28] have reported the importance of the phenolic hydroxyl group of the position 10 tyrosine. Finally, the replacement of threonine by tyrosine at position 5 has been studied in this laboratory and was found to produce glucagon antagonists.[29,30] In this study we have evaluated the possible synergy of these positions for enhancing the antagonist activity of glucagon analogues.

Results

Chemical Synthesis and Characterization. The 10 new glucagon analogues **II**−**XI** (Table 1) were synthesized by solid-phase methods using either the N^{α}-Fmoc or the N^{α}-Boc strategy. For the N^{α}-Fmoc strategy, compounds **II**−**IV**, and **VII**−**XI** were prepared using 4-[(2′,4′-dimethoxyphenyl)-Fmoc-(aminomethyl)]phenoxy resin so as to obtain the carboxamide terminal peptide. Most coupling reactions employed 0.5 M HOBt/0.45 M HBTU in NMP. After drying the resin, the analogues were cleaved from the resin by using a cleavage mixture of 90% TFA, 5% anisole, 2.5% methyl sulfide, and 2.5% 1,2-ethanedithiol. In the N^{α}-Boc strategy, analogues **V** and **VI** were synthesized using a *p*-methylbenzhdrylamine resin substituted at the level of 0.51 mmol of NH_2/g. Briefly, most coupling reactions employed 1,3-diisopropylcarbodiimide (DIC) and *N*-hydroxybenzotriazole (HOBt) and (benzotriazolyloxy)-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) in NMP in the presence of DIEA as coupling reagents. The reactions were monitored by the Kaiser test.[31] A high/low-HF technique was used to cleave the peptide from the resin. The crude peptides were isolated and purified by reversed-phase high-pressure liquid chromatography (RP-HPLC). The purity of the peptides was characterized by electrospray mass spectroscopy, thin-layer chromatography (TLC) in three different solvent systems, and amino acid analysis (see the Experimental Section).

Biological Studies. The agonist and antagonist properties of the peptides were investigated in the adenylate cyclase assay, and the results obtained are shown in Table 2. The binding potency for these compounds was obtained using liver plasma membranes in which the displacement of ^{125}I-labeled glucagon was measured, and the results are shown in Table 2.

In compound **II**, where Tyr[10] was retained, binding nearly equipotent (IC_{50} = 2.3 nM) with that of glucagon was obtained as a result of introducing a bulkier side chain in position 5, where Tyr was substituted for Thr in conjunction with the des-His[1] and Glu[9] modifications. However, this compound turned out to be a partial agonist with a maximum stimulation of only 5%. The glucagon analogue **III** in which Phe[10] replaced Tyr[10] also was found to be a partial agonist with a maximum stimulation of 6% and a pA_2 value of 6.85. The binding potency IC_{50} value for this analogue was 4.1 nM (Table 2). Compounds **IV** and **IX** were found to be antagonists with less than 1% binding potency to the glucagon receptor. The results were consistent with our previous findings[28] and strongly support the hypothesis that

Table 2. Biological Activities of Glucagon Analogues

	receptor binding		adenylate cyclase		
peptide[a]	IC_{50} (nM)	rel binding potency (%)[b]	EC_{50} (nM)	max stimulation (%)	pA_2
I	1.5 ± 0.20	100	8 ± 1.25	100	
II	2.3 ± 0.31	65	−	5	6.60
III	4.1 ± 1.15	37	−	6	6.85
IV	395. ± 48.0	0.4	ia[c]	0	6.20
V	10. ± 2.5	15.0	−	7	6.20
VI	170. ± 26.	1.0	−	9	6.10
VII	74. ± 20.	2.0	ia	0	6.50
VIII	34.5 ± 7.0	4.3	ia	0	6.20
IX	510. ± 81.	0.3	ia	0	5.85
X	120. ± 19.	1.3	ia	0	6.20
XI	180. ± 23.	0.8	−	5.4	6.15

[a] See Table 1 for structures. [b] Relative binding potency = [(receptor binding IC_{50} for glucagon)/(receptor binding IC_{50} for glucagon analogue)] × 100. [c] ia, inactive at 10^{-5} M.

Tyr^{10} is essential for potent binding in glucagon, since the removal of the phenolic ring reduces the binding over 2 orders of magnitude (Table 2). In compounds **V** and **VI**, we have substituted the (2*S*,3*S*)- and (2*S*,3*R*)-β-MePhe isomers at position 10. The effect of substitution of the β-chiral center in this residue led to dramatically different binding potencies to the glucagon receptor. The binding potencies for **V** and **VI** were found to be 10 and 172 nM, respectively. The 17-fold difference in binding between these two analogues undoubtedly is due to the topographical differences caused by incorporating these asymmetric amino acids into position 10 and demonstrates the critical importance of the 10 position in glucagon interaction with the glucagon receptor. Analogues **V** and **VI** were found to be partial agonists with maximum stimulation of 7% and 9% and pA_2 values of 6.20 and 6.10, respectively.

Compounds **VII** and **VIII** that include the des-His^1, Glu^9, and Tyr^5 modifications in addition to a D-Tyr^{10} or a D-Phe^{10} substitution turned out to be somewhat weak antagonists with binding potency IC_{50} values of 74.0 and 34.5 nM, respectively, but both were unable to activate the adenylate cyclase system even at concentrations as high as 10^{-5} M. Introducing a D-Phe and its (2*R*,3*R*)- and (2*R*,3*S*)-β-methylphenylalanine derivatives at position 10 decreases considerably the binding affinities. The glucagon analogue **VIII** turned out to be a weak antagonist unable to activate the adenylate cyclase system even at concentrations as high as 10^{-5} M with a pA_2 value of 6.20. The other two β-MePhe isomers, 2*R*,3*R* and 2*R*,3*S*, compounds **X** and **XI** (Table 2), were found to have binding potencies of 120.0 and 180.0 nM, respectively. Analogue **X** was found to be an antagonist with a pA_2 value of 6.20, whereas analogue **XI** was found to be a partial agonist/antagonist with maximum stimulation of 5.4% and a pA_2 value of 6.15.

Discussion

In this report we have focused on using the des-His^1 and Glu^9 modifications[26] in conjunction with modifications in position 5 and 10 that are known to effect the binding and transduction processes of glucagon with its receptor.[10,29,30] Threonine in position 5 was replaced by tyrosine which earlier had been shown to aid in producing glucagon antagonists[32] with increased potency.[33] In addition, the aromatic amino acid residue Tyr^{10} was substituted with Phe^{10} and Ala^{10} as well as with their configurational isomers D-Phe^{10} and D-Ala^{10} to examine what effect these changes in structure and overall orientation of the N-terminal region of glucagon would have on glucagon binding and transduction processes with respect to the glucagon receptor. It has been suggested earlier by Gratzer and Beaven[34] that Tyr^{10} has a concentration-dependent pK_a which led Korn and Ottensmeyer[35] to propose a role for this residue in glucagon action. According to their proposal the Tyr^{10} hydroxyl group is hydrogen-bonded to serine in position 8. The losses in binding in going from analogues **II** to **III** and then to **IV** (Table 2) may be due in part to the removal of the phenolic hydroxyl group at that position, but it appears that steric effects of the aromatic ring in position 10 play a major role (Table 2).

According to NMR studies of the glucagon conformation in the presence of lipid micelles,[36] the 10−14 region can contain an α-helical turn in this medium. The nature of this turn, where a hydrophobic patch is created by the Phe^6, Tyr^{10}, and Tyr^{13} side chains, is consistent with the X-ray crystallographic structure of glucagon.[37] Such a continuous hydrophobic cluster may play an important role in the hormone−receptor interaction. In the case of analogues **VII** and **VIII**, though the aromatic ring has been retained, D-substitution should alter the alignment of the Phe^6, Tyr^{10}, and Tyr^{13} side chains, which may result in the loss of the continuous hydrophobic region of the hormone. The reduced binding potencies for analogues **VII** and **VIII** could be explained by this change in hormone−receptor interaction. For analogues **IV** and **IX**, where the aromatic ring has been removed, the binding potency is further decreased due to the reduced hydrophobic interaction with the receptor. It is important to note that most of the analogues which contain L-Ala or a D-amino acid in position 10 have no apparent agonist activity, whereas analogues with L-Phe^{10} or L-Tyr^{10} do have weak partial agonist activity. These results suggest that the presence of an aromatic side chain in position 10 and its proper orientation are important for biological transduction. Analogues **II**, **III**, **V**, and **VI** were found to be partial agonists, and their maximal stimulation ranged between 5% and 10% (Table 2). This clearly indicates that agonists favor the L-configuration in position 10, while D-substitutions generally produce pure antagonism. The only D-Phe^{10}-containing analogue which shows weak stimulation of adenylate cyclase activity is **XI**, which actually has a D-β-MePhe^{10} substituent.

The β-methyl amino acids (Figure 1) were initially designed as tools to favor one of three possible staggered rotamers of the side-chain torsion angles χ^1 (Figure 2). It was presumed that the β-methyl substituent, due to its steric repulsion with the adjacent amide and carbonyl groups, will favor the rotamer which places the much less bulky β-proton between the NH and CO groups (Figure 3). Recent NMR and theoretical studies[38−40] revealed a more complicated rotamer equilibrium for the β-methyl-substituted phenylalanine side chains. The side-chain rotamer populations obtained for the β-MePhe stereoisomers in [β-MePhe^3]CCK-8[38] and [β-Me-*p*-NO_2Phe^4]DPDPE[39] analogues showed that the β-methyl substituent does not always favor one particular χ^1 rotamer but rather always eliminates the χ^1 rotamer which places both bulky β-substituents in a

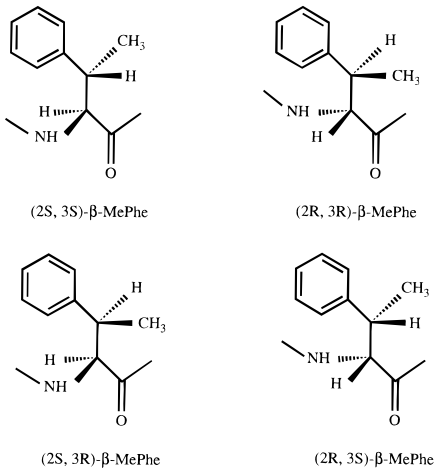

Figure 1. Structures of the four isomers of β-methylphenylalanine which were incorporated at position 10 of glucagon.

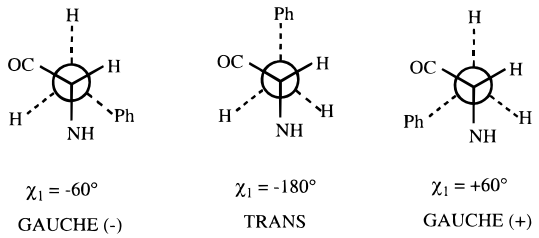

Figure 2. *Gauche* (−), *trans*, and *gauche* (+) conformations of α-amino acids.

gauche orientation to the carbonyl group, *i.e.*, a *trans* rotamer ($\chi^1 \approx 180°$) of both *erythro*-isomers 2*S*,3*S* and 2*R*,3*R*, a *gauche* (+) rotamer ($\chi^1 \approx 60°$) of the (2*S*,3*R*)-isomer, and a *gauche* (−) rotamer ($\chi^1 \approx -60°$) of the 2*R*,3*S*-isomer, while allowing the other two conformers in each case.

Probable "bioactive" conformations of the phenylalanine side chains were recently suggested for two classes of β-MePhe-substituted cyclic opioids[39,40] based on comparative analysis of binding affinities and rotamer preferences of β-MePhe stereoisomers. Although an extensive conformational analysis has not been performed for the glucagon analogues in this study, some preliminary conclusions may be drawn from their relative binding potencies, assuming that the rotamer preferences of β-MePhe[10] stereoisomers are qualitatively similar to that found for β-MePhe side chains in the CCK-8[38] and DPDPE[39] analogues. The rationale for this assumption is that, despite different positions and, perhaps, different backbone conformations of the β-MePhe residues, very similar rotamer populations were found for the same stereoisomers in [β-MePhe[3]]CCK-8[38] and [β-Me-*p*-NO_2Phe[4]]DPDPE analogues.[39] In both series of peptides, *gauche* (−) and *gauche* (+) rotamers were most populated for (2*S*,3*S*)-β-MePhe, and *gauche* (−) and *trans* rotamers were most populated for (2*S*,3*R*)-β-MePhe. For the glucagon series we shall relate binding potencies of β-MePhe[10]-containing analogues to the L-Phe[10] substitution, analogue **III**, which possesses almost the same binding affinity as the parent antagonist [des-His[1],Tyr[5],Glu[9]]glucagon amide containing tyrosine in position 10. The (2*S*,3*R*)-β-MePhe[10] stereoisomer **V** was only 2 times less potent than the reference analogue **III**, while the 2*S*,3*S*-stereoisomer **VI** was 37 times less potent. This indicates that the additional β-methyl group does not significantly affect the receptor

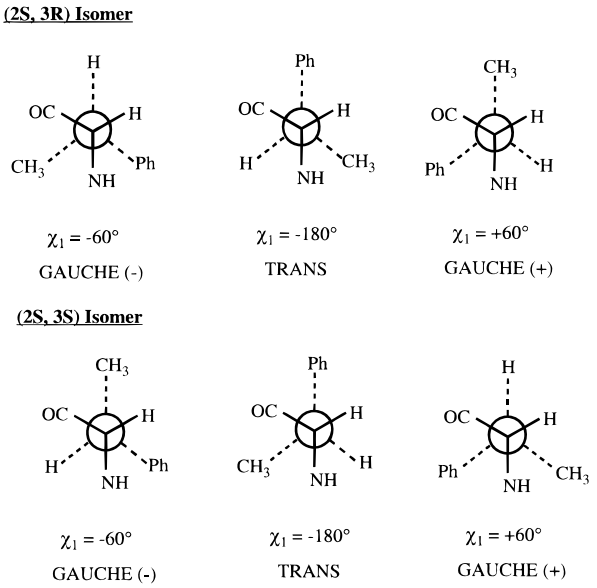

Figure 3. Conformational analysis of the β-methylphenylalanine 2*S*,3*R*- and 2*S*,3*S*-diastereomers.

binding of the 2*S*,3*R*-stereoisomer but considerably affects the binding of the 2*S*,3*S*-stereoisomer. The *gauche* (+) rotamer of β-MePhe[10], according to previous results,[38,39] is expected to be more populated for the less potent 2*S*,3*S*-stereoisomer **VI** than for the more potent 2*S*,3*R*-stereoisomer **V**. Therefore, this rotamer population seems to be irrelevant in the receptor binding of the glucagon antagonists. In contrast, the *trans* rotamer, which was shown to be more populated for the 2*S*,3*R*-stereoisomer than for the 2*S*,3*S*-stereoisomer, seems to be the most probable "binding" rotamer of phenylalanine side chains in the L-Phe[10]-containing analogues **III**, **V**, and **VI**. At present we cannot exclude the alternative possibility that the phenylalanine side chains bind to the receptor in a *gauche* (−) orientation, which is expected to be quite populated for all three L-Phe[10]-containing analogues (**III**, **V**, and **VI**), and that the low affinity of the 2*S*,3*S*-stereoisomer is caused by direct interference of its β-methyl group with the receptor binding site.

The relatively low binding potencies of the D-Phe[10]-containing analogues **VIII**, **X**, and **XI** may be attributed in the first place to a local change in backbone conformation imposed by the D-residue substitution. Note that the D-Phe[10] analogue **VIII**, which has no modifications at the β-carbon, is 9 times less potent than the reference L-Phe[10]-containing analogue **III** (Table 2). The possible changes in hydrophobic stacking of Phe[6], Tyr[10], and Tyr[13] upon a D-amino acid substitution have been discussed above. These changes combined with a reduced side-chain mobility may cause a bad fit of analogues **X** and **XI** to the glucagon receptor, which resulted in their poor binding affinities.

In summary, 10 new glucagon analogues have been designed and synthesized in order to explore structural and topographical requirements of the glucagon receptor to the amino acid in position 10 of the glucagon sequence. The substitution of L-Phe for L-Tyr shows that the hydroxyl group in position 10 has only a weak influence on the receptor binding. However, introducing a D-Phe and its β-methyl derivatives into position 10 considerably decreases the binding affinity, presumably because of an influence on conformation preferred by

Table 3. Physical Data for Glucagon Analogues

peptide	MW calcd	MW found[a]	HPLC k' [b]	TLC,[c] R_f A	TLC,[c] R_f B	TLC,[c] R_f C
II	3420.79	3420.60	4.15	0.65	0.63	0.62
III	3404.80	3404.90	4.18	0.66	0.61	0.64
IV	3328.70	3328.30	4.01	0.66	0.73	0.69
V	3418.80	3419.20	4.37	0.49	0.55	0.47
VI	3418.80	3418.60	3.89	0.53	0.53	0.50
VII	3420.79	3420.50	4.56	0.63	0.64	0.59
VIII	3404.80	3404.90	4.05	0.60	0.62	0.64
IX	3328.70	3329.10	4.61	0.58	0.61	0.63
X	3418.80	3419.20	4.21	0.51	0.49	0.54
XI	3418.80	3418.10	4.07	0.58	0.59	0.58

[a] MW found by electrospray mass spectroscopy. [b] k' values were determined under the following conditions: gradient, 10−90% acetonitrile in 0.1% TFA over 30 min; flow rate, 1.5 mL/min. [c] TLC: silica gel 60 F-245, 0.25 mm layer thickness; solvent systems: A, 1-butanol/acetic acid/pyridine/water (5:4:1:4); B, 1-butanol/acetic acid/pyridine/water (4:1:1:3); C, ethyl acetate/pyridine/acetic acid/water (12:4:4.2:2.2).

the glucagon receptor. The 15-fold difference between binding potencies of the (2*S*,3*R*)- and (2*S*,3*S*)-β-MePhe[10] analogues reveals strong topographical requirements of the glucagon receptor to the orientation of the phenyl ring in position 10. Presently we cannot discriminate between the *trans* and *gauche* (−) rotamers as the possible phenylalanine orientations at the receptor. Further structure−activity and conformational studies are necessary to understand the glucagon receptor requirements.

Experimental Section

Materials. N^{α}-Fmoc- and N^{α}-Boc-protected amino acids, pMBHA resin (0.51 mmol of NH_2/g substitution), and 4-[(2′,4′-dimethoxyphenyl)-Fmoc-(aminomethyl)]phenoxy resin were purchased from Bachem (Torrence, CA). Other chemicals and solvents were purchased from the following sources: trifluoroacetic acid (TFA) (Halocarbon Products, NJ); anisole, 1,2-ethanedithiol, 1-hydroxybenzotiazole (HOBt), diisopropylethylamine (DIEA), diisopropylcarbodiimide (DIC), and 1-methyl-2-pyrrolidinone (NMP) (Aldrich, Milwaukee, WI); dichloromethane (DCM) (Mallinckrodt Specialty Chemicals, Paris, KY); HPLC-quality acetonitrile (Burdick & Jackson, Muskegon, MI); (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP reagent) (Peptide International, Louiville, KY); 2-(1*H*-benzotrizol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Richelieu Biotechnologies Inc., Saint Hyacinthe, Canada); bovine serum albumin, cAMP, GTP, ATP, chromatographic alumina (type WN3, neutral), and all enzymes (Sigma Chemicals, St. Louis, MO); [^{125}I]glucagon, [^{3}H]cAMP, and α-^{32}P (New England Nuclear, Boston, MA); *N*,*N*-dimethylformamide (DMF) (Fischer Scientific, Pittsburgh, PA); and Dowex AG 50-W4 cation exchange resin (BioRad, San Diego, CA). TLC was performed using Merck silica gel 60 F-254 plates (0.25 mm layer thickness), and the following solvents were used (Table 3): (A) 1-butanol/acetic acid/water/pyridine (5:4:4:1); (B) 1-butanol/acetic acid/water/pyridine (4:1:3:1); (C) ethyl acetate/pyridine/acetic acid/water (12:4:4.2:2.2). The peptides were detected on the TLC plates using iodine vapor. The purification was achieved on a Perkin-Elmer Model 410-BIO instrument by a preparative high-performance liquid chromatography (HPLC) C_{18}-bonded silica gel column (VYDAC 218 TBP-16, 16 × 250 mm). The peptides were eluted with a linear gradient of acetonitrile in 0.1% aqueous TFA (10−90%) over 30 min at a flow rate of 5.0 mL/min. The separations were monitored at 280 nm and integrated with a Perkin-Elmer LC-235 diode array detector. The amino acid analyses were done at the University of Arizona Biotechnology Core Facility. The system used was an Applied Biosystems Model 420A amino acid analyzer with automatic hydrolysis (vapor phase at 160 °C for 1 h 40 min using 6 N HCl) and precolumn phenylthiocabamyl-amino acid (PTC-AA) analysis. The purity of the peptides was checked by analytical RP-HPLC using a VYDAC 218 TBP-16 column (4.6 × 250 mm) and by TLC in three different solvent systems (Table 3). The structures of the pure peptides were confirmed by electrospray mass spectroscopy (Table 3) and amino acid analysis.

Synthesis of the N^{α}-*tert*-Butyloxycarbonyl Derivatives of (2*S*,3*S*)- and (2*S*,3*R*)-β-Methylphenylalanine. The syntheses of N^{α}-(*tert*-butyloxycarbonyl)-β-methylphenylalanine isomers were carried out by slight changes of previously published experiments.[41] Briefly, a solution of 2.0 g (11.2 mmol) of (2*S*,3*S*)-β-MePhe or 2.0 g (11.2 mmol) of (2*S*,3*R*)-β-MePhe in 100 mL of dioxane−water (2:1) was cooled in an ice bath, and 2.70 g (12.4 mmol) of di-*tert*-butyl dicarbonate was added slowly. The pH was adjusted to 10.5 with 5 N sodium hydroxide, and the solution was continuously stirred and adjusted to 10.5. The mixture was stirred at room temperature overnight. The solvents were removed under vacuum, the solid was dissolved in water and cooled to 0 °C, and 70 mL of ethyl acetate was added. The aqueous solution was cooled to 0 °C, acidified with 10% citric acid, and extracted with 4 × 50 mL portions of ethyl acetate. The collected organic phases were washed twice with water and brine, dried over sodium sulfate, and evaporated to dryness. The white crystal product finally gave 2.25 g of N^{α}-Boc-(2*S*,3*S*)-β-MePhe (71%) and 2.18 g of N^{α}-Boc-(2*S*,3*R*)-β-MePhe (69%) whose properties are the same as those previously reported.

Peptide Synthesis. (A) Using an N^{α}-Fmoc strategy, eight of the glucagon analogues were synthesized by solid-phase methodology using a 4-[(2′,4′-dimethoxyphenyl)-Fmoc-(aminomethyl)]phenoxy resin.[42] The synthesis of the glucagon analogues [des-His[1],Tyr[5],Glu[9]]glucagon-NH_2 **(II)**, [des-His[1],Tyr[5],Glu[9],Phe[10]]glucagon-NH_2 **(III)**, [des-His[1],Tyr[5],Glu[9],Ala[10]]-glucagon-NH_2 **(IV)**, [des-His[1],Tyr[5],Glu[9],D-Tyr[10]]glucagon-NH_2 **(VII)**, [des-His[1],Tyr[5],Glu[9],D-Phe[10]]glucagon-NH_2 **(VIII)**, [des-His[1],Tyr[5],Glu[9],D-Ala[10]]glucagon-NH_2 **(IX)**, [des-His[1],Tyr[5],Glu[9],-(2R, 3R)-β-MePhe[10]]glucagon-NH_2 **(X)**, and [des-His[1],Tyr[5],-Glu[9],(2*R*,3*S*)-β-MePhe[10]]glucagon-NH_2 **(XI)** were carried out on an Applied Biosystems (ABI) 431A automated synthesizer on a 0.25 mmol scale. The following side-chain-protecting groups were used Arg(2,2,5,7,8-pentamethylchroman-6-sulfonyl,Pmc), Asn(trityl), Asp(*t*-Bu), Gln(trityl), Glu(*t*-Bu), Lys-(Boc), Ser(*t*-Bu), Thr(*t*-Bu), and Tyr(*t*-Bu). N^{α}-Fmoc-protected amino acids (4 equiv) were added sequentially, using HBTU with HOBt as coupling reagents in NMP. The analogues were 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.

(B) Using an N^{α}-Boc strategy, the other two glucagon analogues, [des-His[1],Tyr[5],Glu[9],(2*S*,3*S*)-β-MePhe[10]]glucagon-NH_2 **(V)** and [des-His[1],Tyr[5],Glu[9],(2*S*,3*R*) β-MePhe[10]]glucagon-NH_2 **(VI)**, were synthesized using a Vega Coupler 250 or a Vega Coupler 1000 synthesizer. These two compounds were prepared using *p*-methylbenzhydrylamine resin substituted at the level of 0.51 mmol/g of resin. The following amino acid side-chain-protecting groups were used Arg(N^G-Tos), Asp-(O-Bzl), Glu(γ-O-Bzl), Lys(N^{ϵ}-2,4-diCl-Cbz), Ser(O-Bzl), Thr-(O-Bzl), Trp(N^{in}-For), and Tyr(2,6-diCl-Bzl). A sample of pMBHA resin was neutralized with 10% DIEA in DCM (2 × 50 mL) followed by a DCM wash (4 × 50 mL). The amino acid N^{α}-Boc-Thr(Bzl) was coupled to the resin using DIC and HOBt as coupling reagents for 2 h. N^{α}-Boc amino acids were added to the growing peptide chain as their preformed symmetrical anhydrides in 3-fold excess. The synthetic protocol consisted of the following steps: (a) 50% TFA in DCM with 1% methionine (1 × 2 min); (b) same as step a (1 × 2 min); (c) DCM (3 × 2 min); (d) 10% DIEA in DMF (3 × 2 min); (e) DCM (3 × 2 min); (f) coupling by preformed symmetric anhydride in DCM for 30 min or by DIC/HOBt for 2 h; (g) 10% DIEA in DCM (1 × 2 min); (h) DCM (1 × 2 min); (i) coupling by preformed symmetric anhydride in DMF for 30 min or by DIC/HOBt for 2 h; (k) DCM (3 × 2 min); (l) ethanol (3 × 2 min). The reactions were monitored by the Kaiser test.[31] After the last synthetic cycle, the N-terminal Boc group was removed (steps a−e) and the resin was dried *in vacuo*. The low/high-HF technique was used to cleave the peptide from the resin to avoid any side reactions.[43]

Isolation of Liver Plasma Membrane. The liver plasma membranes were obtained from Sprague−Dawley rats weighing 200−250 g as described by Neville[44] and modified by Pohl.[45] The protein concentration was determined by the method of Markwell *et al*.[46]

Receptor Binding Assay. The binding assay was performed according to Wright and Rodbell[47] in which competition for glucagon receptors between [^{125}I]glucagon and the glucagon analogue was measured. Briefly, an incubation medium had a volume of 500 μL consisting of liver plasma membrane containing 50 μg of protein, 15×10^4 cpm of [^{125}I]glucagon, and unlabeled glucagon or glucagon analogues at a desired concentration (range from 0 to 10 μM), all in 25 mM Tris-HCl with 0.5% BSA (pH 7.4 at 25 °C). The mixture was incubated for 10 min at 30 °C followed by immediate cooling in an ice bath and filtered through a 0.45 μm cellulose acetate filter previously soaked for 12 h in a Tris−BSA buffer; 4 mL of ice cold Tris−BSA buffer was used for washing, and the amount of radioactivity remaining on the filter was quantitated using a LKB1275 mini-gamma counter. Nonspecific binding, measured in the presence of excess unlabeled peptide (1024 nM), was 15−20% of the total binding and substracted in each case to give the specific binding. Results are expressed as the percent 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 3′,5′-cyclic AMP as described by Lin *et al*.[21] Labeled cAMP was determined by the method of Solomon *et al*.[48] using Dowex 50 and alumina columns. Briefly, 0.1 mL of incubation medium consisted of 1 mM [α-^{32}P]ATP (∼50 cpm/pmol), 5 mM $MgCl_2$, 10 μM GTP, 1 mM EDTA, 1 mM cAMP, containing 10^4 cpm of [^{3}H]cAMP, 25 mM Tris-HCl (pH 7.4 at 25 °C), 1% BSA, 35 μg of membrane protein, and an ATP regeneration system that had 20 mM phosphocreatine and 0.72 mg/mL (100 units/mL) creatine phosphokinase. Results are expressed as the percent of stimulation of cAMP production over basal. Assays were done twice in triplicate.

For the pA_2 values, a dose−response plot was obtained by determining the response when the concentration of glucagon was varied while the concentration of the antagonist was kept the same. The plots were generated by using different concentrations ranging between 1 and 10 μM antagonist. The dose−response curves exhibit shifts in the EC_{50} values which were used in calculating the pA_2 values as described by Schild.[49]

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