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The positive charge at Lys-288 of the glucagon-like peptide-1 (GLP-1) receptor is important for binding the N-terminus of peptide agonists

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Abstract Lysine-288 in the glucagon-like peptide-1 receptor was predicted to be ideally positioned to play a role in hormone binding. Subsequent mutation of Lys-288 to Ala or Leu greatly reduced hormone affinity, while substitution with Arg had minimal effect. Compared to wild type, the Lys288-Ala receptor had a reduced affinity for three peptide ligands with complete N-terminal sequences but not for their N-truncated analogues. Hence, the role of this positively charged residue, which is conserved at the equivalent position in all other Family B receptors, was determined to be important for receptor interaction with the N-terminal eight residues of peptide agonists.

Key words: G protein-coupled receptor; Receptor; Agonist; Glucagon-like peptide-1; Antagonist; Exendin

1. Introduction

Glucagon-like peptide-1(7-36)amide (named GLP-1 throughout this paper) is a 30 amino acid hormone resulting from the post-translational processing of the proglucagon precursor in intestinal L cells [1]. Among the increasing number of physiological actions attributed to GLP-1, the best characterised is its role as an incretin, i.e. it potentiates glucose-dependent insulin release [2–4]. GLP-1 also promotes proinsulin gene transcription and insulin biosynthesis [5,6]; inhibits postprandial glucagon secretion [7,8], gastric motility and secretion [9]; and appears to act as a central anorexigenic hormone in rats [10]. Since the various GLP-1 effects collectively alleviate the diabetic phenotype, this hormone has attracted much interest as a therapeutic tool [11,12]. Unfortunately, GLP-1 is very rapidly inactivated by cleavage of two N-terminal residues by DPP IV (dipeptidyl peptidase IV) [13] and the design of more biostable agonists of the GLP-1 receptor (GLP-1R) is much needed.

Exendin-3 and exendin-4 are 39 amino acid hormones exclusively found in the venom of *Heloderma* lizards. They share approximately 50% sequence identity with GLP-1 and are potent GLP-1R agonists ([14,15]; Fig. 1C). Unlike for GLP-1, up to eight N-terminal residues can be removed from the exendin peptides without major loss of affinity [15,16], suggesting that the epitopes providing high-affinity binding are, in part, differentially distributed in the molecules. However, the first two N-terminal amino acids of the exendin peptides are also essential for biological activity and, indeed, the truncated exendin analogues lacking these residues behave as antagonists or inverse agonists at GLP-1R [14–17].

GLP-1R belongs to the Family B of G protein-coupled receptors (GPCRs), which amongst others includes receptors for glucagon, secretin and vasoactive intestinal peptide [18]. This receptor subclass is characterised by a relatively long N-terminal domain (~130 amino acids) that incorporates six conserved cysteine residues connected by three intramolecular disulphide bonds. Since the cysteine-pairing pattern appears to be conserved in all Family B GPCRs [19–21], this domain probably adopts a similar globular fold that is critical for peptide binding (e.g. [21–24]). The remaining region of Family B GPCRs is termed the 'core domain', which consists of the seven transmembrane helices (TM1–TM7) and interconnecting loops regions.

Our group have previously reported that Asp-198, a conserved residue at the extracellular end of the second TM helix (TM2), is specifically involved in binding the N-terminal region of GLP-1 [25]. In a search for other charged residues with functional importance, we utilised sequence alignments of Family B GPCRs to identify Lys-288, a conserved positive charge found at the equivalent position in all Family B GPCRs (Fig. 1A). A generic model of Family B GPCRs [26] suggested that Lys-288 lies on the buried face of the fourth TM helix (TM4) and, since it is close to the extracellular end, is ideally positioned to contribute to the formation of the hormone binding site. Using site-directed mutagenesis, we substituted Lys-288 with either Arg, Leu or Ala and analysed the consequences of the mutations via radioligand binding analyses and cyclic adenosine monophosphate (cAMP) accumulation assays.

2. Materials and methods

2.1. Materials

GLP-1, exendin-4 and exendin-4(9-39) were from Bachem (Saffron Walden, UK) while all other peptide ligands were custom synthesised by Genosphere Biotechnologies (Paris, France). ^{125}I-exendin-4(9-39), labelled via Bolton–Hunter reagent at Lys-12, was purchased from NEN-Perkin Elmer (Boston, MA, USA). ^{125}I-GLP-1 was a kind gift from Novo Nordisk (Copenhagen, Denmark). [^{3}H]adenine and [^{14}C]cAMP were obtained from Amersham. Dowex 50W-X4 and alumina were purchased from Bio-Rad. Cell culture reagents were ob-

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Abbreviations: GLP-1, glucagon-like peptide-1(7-36)amide; GLP-1R, glucagon-like peptide-1 receptor; TM, transmembrane; ECL, extracellular loop

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tained from Gibco-Invitrogen and Sigma-Aldrich (Poole, UK). General chemicals were from BDH-Merck (Poole, UK) and Sigma-Aldrich.

2.2. Mutagenesis

Lys-288 was mutated to alanine, leucine and arginine using the QuickChange® Site-Directed Mutagenesis kit (Stratagene, Amsterdam Zuidoost, The Netherlands). The template vector was pcDNA3 (Invitrogen) containing the cDNA sequence encoding the rat GLP-1R cDNA [18].

2.3. Cell culture and transfection

HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were stably transfected using the SuperFect® Transfection Reagent (Qiagen Ltd., Crawley, UK) and transfected clones were selected by addition of 800 μg ml^{-1} G418 antibiotic.

2.4. Membrane preparations

HEK-293 cells cultured to confluence on five ≈85 cm^2 Petri dishes (pre-coated with poly-D-lysine) were washed with phosphate-buffered saline (PBS). Lysis was achieved by addition of 15 ml ice-cold sterile water and incubation on ice for 5 min. Cells were thoroughly washed with ice-cold PBS and scraped from the plates. Membranes were pelleted by centrifugation and resuspended in 1 ml binding buffer (BB: 2 mM HEPES pH 7.4, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mg L^{-1} bacitracin) and homogenised through a 23G needle. Aliquots were snap-frozen in liquid N_2 and stored at −80°C.

2.5. Radioligand binding assays

Specific binding of 50 pM ^{125}I-GLP-1(7-36)amide or 50 pM ^{125}I-exendin(9-39) to membrane preparations was calculated in the absence and presence of a range of concentrations of unlabelled peptides. Components were mixed in a total volume of 300 μl in BB and incubated for 1 h at room temperature. Unbound ligand was removed by washing with cold PBS through Whatman GF/C fibre-glass paper presoaked in 5% non-fat powdered milk. Filters were cut, allowed to dry and radioactivity was counted using a gamma counter.

2.6. cAMP accumulation assays

Transfected cells were seeded into six-well plates and grown to confluence. On the day of the assay, cells were incubated in DMEM with 2 μCi ml^{-1} [^{3}H]adenine for 2 h at 37°C. After several washes with pre-warmed (37°C) PBS, cells were stimulated for 12 min with various concentrations of pre-warmed (37°C) agonist made up in DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine. The supernatant was aspirated and the cells lysed using 5% trichloroacetic acid containing 2.5 nCi ml^{-1} [^{14}C]cAMP. The intracellular [^{3}H]cAMP produced was purified (alongside the [^{14}C]cAMP internal column standard) by the sequential use of Dowex and alumina columns and counted using a liquid scintillation counter calibrated for dual isotope counting [27].

2.7. Data analysis

IC_{50} and EC_{50} values were calculated using non-linear regression with the aid of the GraphPad Prism® Version 3.0 software (San Diego, CA, USA). Means and standard errors were calculated from −Log IC_{50} (pIC_{50}) and −Log EC_{50} (pEC_{50}) values. Statistical analysis of significance was calculated using a two-tailed unpaired Student's *t*-test.

3. Results and discussion

The importance of the positive charge at position 288 is highlighted by its conservation across Family B GPCRs (Fig. 1A). In addition, its predicted position on the interior face of TM4 ([26]; Fig. 1B) places it at a position that is ideal for an interaction with juxtaposed residues on other TM helices, or indeed with the peptide ligand itself. In order to examine whether the positive charge is required for correct receptor

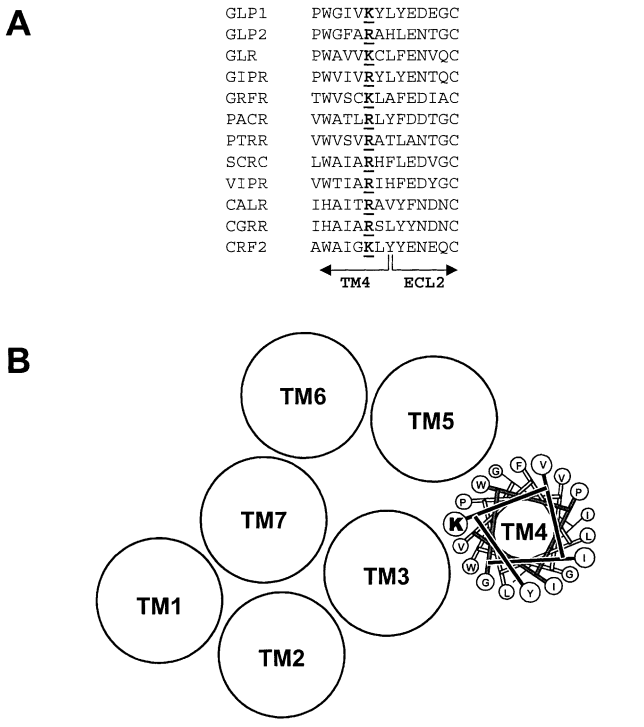

Fig. 1. A: The aligned sequences of various human Family B GPCRs at the junction of TM4 and ECL2. The residues aligned with Lys-288 are shown underlined and all are positively charged Lys or Arg residues. The receptors are for the following ligands: GLP1, glucagon-like peptide-1; GLP2, glucagon-like peptide-2; GLR, glucagon; GIPR, gastric intestinal peptide; GRFR, growth hormone-releasing hormone; PACR, pituitary adenylate cyclase activating polypeptide; PTRR, parathyroid hormone; SRCR, secretin; VIPR, vasoactive intestinal peptide; CALR, calcitonin; CGRR, calcitonin gene-related peptide; CRF2, corticotropin releasing factor. B: A view, from the extracellular side of the membrane, of a model for the arrangement of the helices in rGLP-1R based upon the prediction methods by Donnelly [26]. A helical wheel showing the predicted orientation of TM4 relative to the remaining helices highlights the position of Lys-288 (bold, large font) as being on the interior helical face and ideally placed to interact with the hormone. C: An alignment of the full-length and truncated peptides used in this study. In order to facilitate direct comparison with exendin-4 (EX-4), the numbering of GLP-1 has been modified from the conventional system so that its first residue is His-1. All the peptides were C-terminally amidated.

Table 1
pEC_{50} and pIC_{50} values for GLP-1 at the wild-type rGLP-1R and three mutant receptors

	rGLP-1R	Lys288-Arg	Lys288-Ala	Lys288-Leu
pIC_{50}	8.6 ± 0.13 (3)	8.1 ± 0.02 (3)	Not detectable	Not detectable
pEC_{50}	9.4 ± 0.11 (3)	9.2 ± 0.23 (3)	7.0 ± 0.16 (3)	7.5 ± 0.13 (3)

pIC_{50} values are from homologous competition binding assays using ^{125}I-GLP-1 agonist tracer.

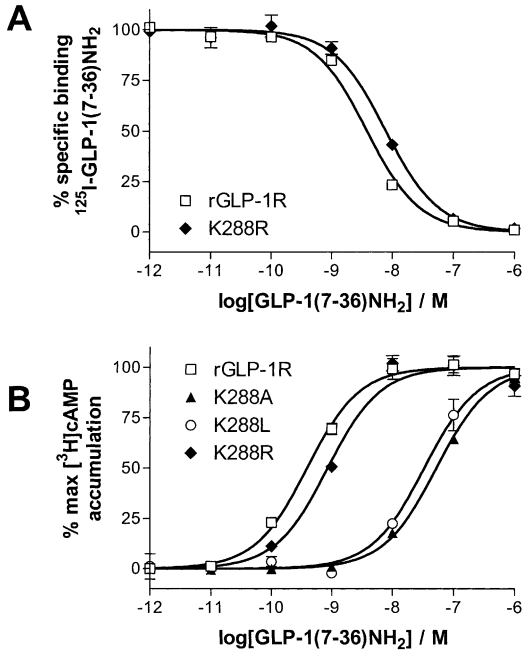

Fig. 2. A: Homologous competition binding curves for rGLP-1R (squares) and Lys288-Arg (diamonds) receptors. Both curves represent one of three independent experiments for which each point represents the mean of triplicate values with S.E.M. displayed as error bars. Counts were normalised to the maximal specific binding within each data set. The Lys288-Ala and Lys288-Leu mutant receptors displayed no specific binding for ^{125}I-GLP-1(7-36)amide and hence are not shown. B: cAMP accumulation curves for GLP-1 stimulation of rGLP-1R (squares), Lys288-Arg (diamonds), Lys288-Leu (circles) and Lys288-Ala (triangles). Each curve represents one of three independent experiments where each data point represents the mean of triplicates with S.E.M. displayed as error bars.

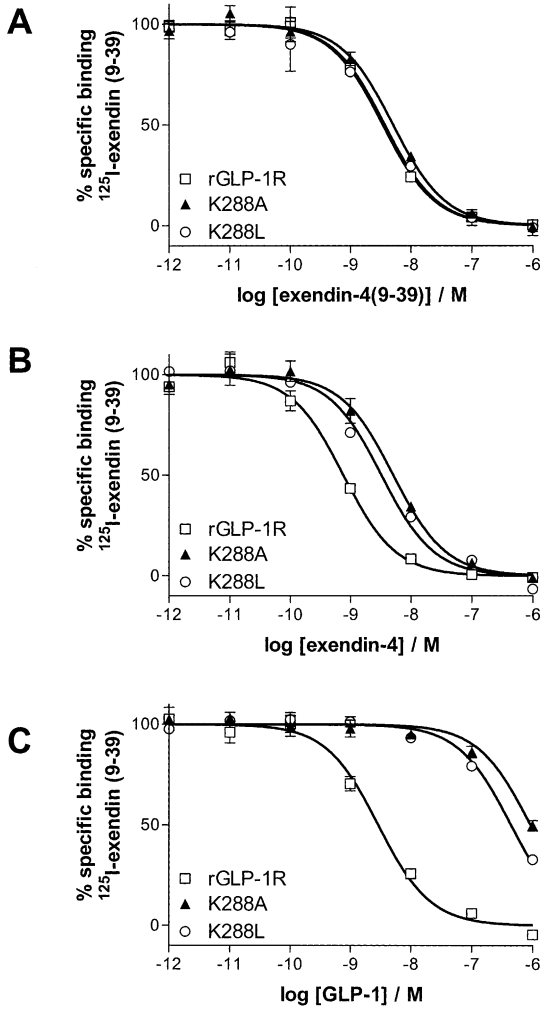

Fig. 3. Competition binding curves, using ^{125}I-exendin(9-39) radioligand, for rGLP-1R (squares), Lys288-Leu (circles) and Lys288-Ala (triangles), each using three unlabelled peptides. A: Exendin(9-39); B: xendin-4; C: GLP-1. Curves represent one of three independent experiments for which each point represents the mean of triplicate values with S.E.M. displayed as error bars. Counts were normalised to the maximal specific binding within each data set.

function, we replaced Lys-288 with the aliphatic uncharged residues leucine and alanine. As a positive control, we also replaced Lys-288 with arginine, a residue with a positively charged side chain similar to the native lysine and which is found at the equivalent position in other closely related receptors (Fig. 1A).

As can be seen in Table 1 and Fig. 2, removal of the positive charge at residue 288 had a dramatic effect upon receptor function. While the Lys288-Arg receptor maintains properties similar to the wild-type rGLP-1R with respect to both affinity and potency, both the Lys288-Ala and Lys288-Leu mutants displayed no detectable binding of ^{125}I-GLP-1, and also a >75-fold increase in the EC_{50} for agonist-induced cAMP accumulation. The ability of GLP-1 to activate the Lys288-Ala and Lys288-Leu receptors demonstrated that the receptor's structure was intact and that the hormone still bound to the receptors, albeit with reduced affinity. Hence, the absence of detectable ^{125}I-GLP-1 binding was likely due to the necessarily low concentration (50 pM) of radioligand used in the binding assays, which results is very low receptor occupancy at the Lys288-Ala and Lys288-Leu receptors.

In order to assess whether the effect caused by the removal of the positive charge at Lys-288 was specific to the binding of the agonist GLP-1, we employed an alternative radioligand – the antagonist ^{125}I-exendin-4(9-39). Remarkably, the Lys288-Ala and Lys288-Leu receptors bound exendin-4(9-39) with unaltered affinity compared with rGLP-1R, demonstrating that the mutations had not adversely affected the structure or trafficking of the mutant receptors (Fig. 3A, Table 2). Heterologous competition binding assays using the antagonist tracer also highlighted a modest reduction (approximately

Table 2
pIC_{50} values for wild-type rGLP-1R and the two mutant receptors lacking a positive charge at residue 288

	Wild type	Lys288-Ala	Lys288-Leu
Exendin(9-39)	8.2 ± 0.08 (5)	8.3 ± 0.04 (3)	8.3 ± 0.06 (3)
Exendin-4	9.2 ± 0.09 (3)	8.2 ± 0.07 (3)	8.3 ± 0.14 (3)
GLP-1	8.5 ± 0.07 (4)	6.4 ± 0.23 (3)	6.5 ± 0.14 (3)

pIC_{50} values are from competition binding assays using antagonist ^{125}I-exendin(9-39) as the tracer and the three unlabelled peptides shown.

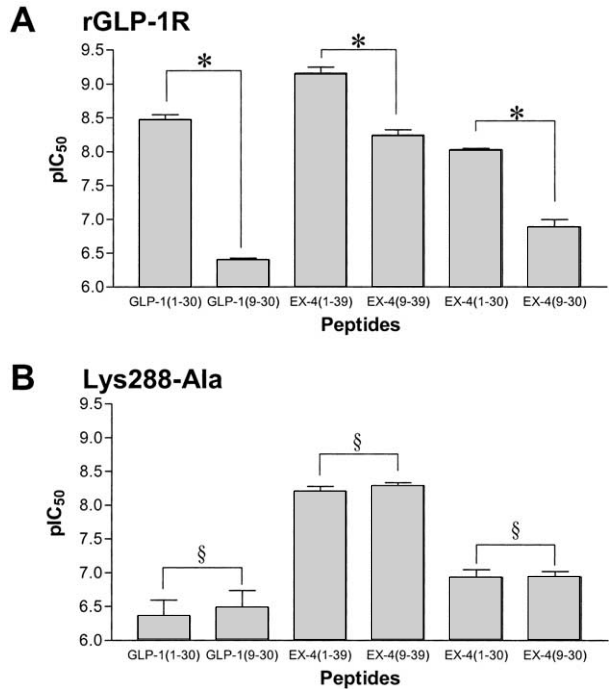

Fig. 4. Comparison of pIC_{50} values, each with six peptides, for (A) rGLP-1 and (B) Lys288-Ala. At rGLP-1R, the full-length peptides display significantly different pIC_{50} values (*, $P < 0.003$) when compared with their N-terminally truncated analogues. However, at Lys288-Ala the full-length and truncated peptides have pIC_{50} values that are not significantly different (§, $P < 0.3$). The affinities of the truncated peptides at rGLP-1 (A) were very similar to their affinities at Lys288-Ala (B).

10-fold; Fig. 3B) in the affinity of the agonist exendin-4 but, as expected from Fig. 2, a more dramatic reduction (approximately 100-fold; Fig. 3C) in GLP-1 affinity (Table 2).

As discussed in the introduction, the N-terminus of exendin-4 can be cleaved without significant loss of affinity while the affinity of GLP-1 is highly sensitive to N-terminal cleavage [16]. Hence, since the role of the positive charge at residue 288 was clearly selective for GLP-1 affinity, we hypothesised that it may be involved in the receptor's interaction with the N-terminus of GLP-1. To test this, we analysed the affinity of rGLP-1R and Lys288-Ala for three GLP-1R agonists and their N-terminally truncated analogues (Table 3, Fig. 4). The affinity profile at the wild-type receptor shows a significant difference ($P < 0.0003$) between the affinity of the full-length and truncated peptides (Fig. 4A), while the mutant receptor is non-selective for peptide truncation with no significant difference ($P < 0.3$) in the affinity of any peptide compared with its truncated analogue (Fig. 4B). The reduction in affinity at the wild-type receptor resulting from the removal of the N-terminal peptide sequence was approximately 100-fold for GLP-1 but only 10-fold for exendin-4 and exendin-4(1-30).

As can be observed from Table 3 ('fold change' row), the Lys288-Ala mutation significantly reduces ($P < 0.005$) the affinity of only those peptides with complete N-terminal sequences, while the truncated peptides bind to the mutant with equivalent affinity as to the wild-type receptor. In all three cases, the magnitude of the reduction in the affinity of each full-length peptide, caused by the mutation of Lys-288 to alanine, was equivalent to the reduction observed at rGLP-1R with the N-terminally truncated analogue. These two observations of reduced affinity, one caused by peptide truncation and the other by Lys-288 mutation, are not additive when the truncated peptides are tested against the mutant receptor, suggesting that the same interaction has been broken in both cases. Since the affinity-enhancing interaction between the N-terminus of GLP-1 and the receptor is greater than that for exendin-4, the effect of the removal of the positive charge at residue 288 causes a larger reduction in GLP-1 affinity compared to exendin-4.

Hence, the data are consistent with the removal of the affinity-enhancing interaction between the N-terminus of the peptide agonists and the receptor. However, despite their reduced agonist affinity, the mutant receptors can nevertheless be activated by GLP-1 (Fig. 2, Table 1) and exendin-4 (pEC_{50} is 8.5 ± 0.13 and 7.6 ± 0.24 for rGLP-1 and Lys288-Ala respectively, $n = 3$). This suggests that the efficacy-generating N-terminal region of the peptide agonists still interacts with the mutant receptors. For both GLP-1 and exendin-4, the increase in their pEC_{50} at Lys288-Ala was consistent with the increase in their pIC_{50}. Hence, the effect of the removal of the positive charge at residue 288 was to specifically abolish the affinity-enhancing interaction between the agonist's N-terminus and the receptor rather than the activity-generating interaction required for efficacy. The interaction of the N-terminal region of Family B peptide ligands with the core domain of their receptors has been highlighted before (e.g. [23,25,30]). For example, we have shown that Asp-198 at the extracellular end of TM2 provides a hydrogen bond that is required for the affinity-enhancing interaction between the N-terminus of GLP-1 and its receptor [25]. Indeed, the characteristics of the Asp198-Ala mutant receptor are very similar to the Lys288-Ala mutant described here.

The role of the positive charge at position 288 has not been examined to date in any Family B GPCR. However, the role of charged residues in the first extracellular loop (ECL) of GLP-1R has been investigated previously in a study that included the examination of another conserved positive charge at position 227, close to the boundary of the first ECL and TM3 [23]. This charged residue was shown to play a role in GLP-1 affinity but not in receptor activation. In addition, the second ECL and proximal regions of TM4 and TM5 have been implicated in peptide binding in the related glucagon receptor [28]. Substitution of Lys-12 of glucagon by Ser, the equivalent residue in GLP-1, results in a >70-fold reduction

Table 3
pIC_{50} values for wild-type rGLP-1R and Lys288-Ala

	GLP-1(1-30)	GLP-1(9-30)	EX-4(1-39)	EX-4(9-39)	EX-4(1-30)	EX-4(9-30)
rGLP-1R	8.5 ± 0.07 (4)	6.4 ± 0.02 (5)	9.2 ± 0.09 (3)	8.2 ± 0.08 (5)	8.0 ± 0.02 (3)	6.9 ± 0.11 (4)
Lys288-Ala	6.4 ± 0.23 (3)	6.5 ± 0.25 (3)	8.2 ± 0.07 (3)	8.3 ± 0.04 (3)	6.9 ± 0.08 (3)	6.9 ± 0.11 (3)
Fold change	126*	0.8	10*	0.8	13*	1

pIC_{50} values are from competition binding assays using antagonist ^{125}I-exendin(9-39) as the tracer. The asterisk * refers to a comparison between rGLP-1R and Lys288-Ala which was significantly different ($P < 0.005$). EX-4, exendin-4.

in affinity, which was rescued by the substitution of the second ECL and proximal regions of TM4 and TM5 of the glucagon receptor with that of GLP-1R. This rescue implicated Lys-12 of glucagon in a direct interaction with a region of the glucagon receptor within ECL2 and the regions at the top of TM4 and TM5. However, this interaction may not be applicable to GLP-1 binding at the GLP-1R since the equivalent position in GLP-1 itself is remarkably tolerant to alteration: for example, Ser-12 in GLP-1 can be mutated to Lys and substituted with a bulky γ-L-glutamoyl(Nα-hexadecanoyl) group without adverse effects [29]; while position 12 in exendin-4 is naturally a Lys residue which furthermore can be modified by Bolton–Hunter reagent without losing its high receptor affinity. Hence, despite the sequence conservation present in both the peptides and their receptors, it appears that the role of residue 12 in receptor binding is different for GLP-1 and glucagon.

The proposed interaction between the N-terminal region of the peptide agonists and the core domain of the receptor is compatible with other generic binding models for Family B GPCRs [24,28–33]. In summary, therefore, we have shown that a conserved positive charge at position 288 of rGLP-1R is required for the affinity-enhancing interaction between the N-terminal eight residues of peptide agonists and the GLP-1R.

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