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NOTE

The Positive Charge of the Imidazole Side Chain of Histidine\(^7\) Is Crucial for GLP-1 Action

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Abstract. Glucagon-like peptide-1(7-36)amide/(7-37) (GLP-1) is an incretin hormone which plays an important role in postprandial glucose homeostasis. Since GLP-1 potentiates glucose-induced insulin secretion, stimulates insulin biosynthesis and inhibits glucagon release, it is a potential tool for the treatment of diabetes mellitus. For this, an exact understanding of the structural/functional moieties of the peptide is mandatory. The present study investigates the importance of structural features of histidine\(^7\) at the N-terminus for GLP-1 action. Based upon binding and activity data obtained from ten different GLP-1 analogues we show that not the positive charge of the free α-amino group but the positive charge of the imidazole side chain of histidine is crucial for GLP-1 action. The presence of a ring structure and a basic function as well as the correct positioning of both seems to be decisive.

Key words: Glucagon-like peptide-1, Structure-activity relationship, Histidine

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GLUCAGON-like peptide-1(7-36)amide (GLP-1) is a post-translational product of the intestinal proglucagon processing [1] that is released from the L-cells of the gut in response to ingestion of nutrients. GLP-1 potently stimulates the insulin release from the endocrine pancreas in the presence of high blood glucose levels [2]. Furthermore, GLP-1 induces an increase of insulin biosynthesis, reduces circulating plasma glucagon levels and increases peripheral glucose utilization [2]. Because of these actions GLP-1 is considered as a promising tool in the treatment of diabetes mellitus.

Previously, specific receptors for GLP-1 have been identified and characterized on rat insulinoma-derived RINm5F cells and, subsequently, on several other insulinoma cell lines [3–5]. These findings together with the availability of the meanwhile recombinantly expressed GLP-1 receptor [2] allow exact molecular studies on the ligand, GLP-1. GLP-1 belongs to the glucagon/VIP/secretin-family of peptides all sharing a high sequence homology. In a previous study with glucagon, it was shown that the N-terminal histidine\(^1\) plays a crucial role in glucagon induced signal transduction [6]. Furthermore, there is evidence that histidine\(^1\) interacts with neighbouring aspartate\(^9\) or, if aspartate\(^9\) is absent, may utilize aspartate\(^15\) or aspartate\(^21\) [6]. Histidine\(^1\) is conserved in the amino acid sequences of GLP-1, Exendin-4, GLP-2, glucagon, VIP, secretin, PACAP-28 and peptide histidine isoleucine (PHI). GIP and growth hormone releasing factor (GRF) possess with tyrosine another aromatic residue at the same position. This suggests that for optimal biological function an aromatic structure is required at this position. While for glucagon it has been shown
that the positive charge of the imidazol ring of histidine is crucial for induction of signal transduction this does not seem to be important for GIP and GRF since tyrosin is not positively charged. In the present study, we investigated the role of the aromatic ring structure and the positive charge of the N-terminal histidine for GLP-1 binding and signal transduction.

Materials and Methods

Multiple peptide synthesis

The glucagon-like peptide-1 analogues were synthesized by solid-phase methodology on a SyRo II multiple peptide synthesizer (MultiSynTech, Bochum) applying Fmoc/tBu strategy. 5-(4'-aminomethyl-3',5'-dimethoxy-phenoxy)-pentanoic acid (ADPV) was used as an anchor molecule on aminomethylated polystyrene-(1%)-divinylbenzene (40 mg, 20 µmol/peptide) in order to obtain the peptide amides. The Fmoc-protected amino acids (Nova Biochem, Bad Soden) were coupled in a 10-fold excess for 40 min. Diisopropylcarbodiimide (1 eq) and 1-hydroxybenzotriazole (1 eq) were used as activating reagents. Deprotection was carried out in piperidine/dimethylformamide (1 : 1 v/v) for 2 x 10 min. Cleavage was performed in trifluoroacetic acid/ethanedithiol/dimethylsulfide/m-cresol (85 : 5 : 5 : 5) within 3 h. Yields of crude glucagon-like peptide-1 analogues from the resin were approximately 60%. The peptides were purified to purity of more than 95% by preparative HPLC on a nucleosil C18 column (5 µ; 25 mm x 250 mm; Grom, Ammerbuch). The peptides were characterized by means of electrospray mass spectrometry. Mass spectra were recorded on an AutoSpec T mass spectrometer equipped with an Electrospray interface (Fisons Instruments, Manchester). The mass values for all synthetic peptides were identical with the calculated values within the limits of the method.

GLP-1 receptor binding assay

Chinese hamster lymphoblast (CHL) cells, stably transfected with the rat GLP-1 receptor cDNA in pTEJ-8 vector, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate and 1% non essential amino acids at 37 °C in 95% air and 5% CO2. 125I-GLP-1 was prepared as described previously [3]. The specific activity of the radiolabel was approximately 2000 Ci/mmol. Binding assays were performed as previously described [3]. Briefly, cells were detached from the culture bottles with phosphate-buffered saline (PBS; pH 7.3) containing 1.5 mM EDTA. The cells were centrifuged and resuspended in buffer (2.5 mM Tris-HCl, 120 mM NaCl, 1.2 mM MgSO4, 5 mM KCl, 15 mM CH3COONa; pH 7.4) containing 1% human serum albumin, 0.1% bacitracin and 1 mM EDTA. After incubation for 30 min at 37 °C with tracer (20,000 c.p.m.) in the absence and presence of a range of concentrations of unlabeled GLP-1, ice-cold incubation buffer was added and the samples were centrifuged; the radioactivity retained in the pellet was determined with a γ-counter. Binding in the presence of an excess of unlabeled GLP-1 (1 µM) was defined as unspecific and subtracted in each experiment to give specific binding. Analysis of the binding data was performed using the PC-program Prism (GraphPad, San Diego, USA).

Measurement of cytosolic Ca²⁺ concentration

Cytosolic calcium measurements were performed with the highly differentiated clonal rat β-cell line INS-1 that has retained typical properties of pancreatic β-cells [7, 8]. For measurements with cell suspensions, ISN-1 cells were grown in RPMI 1640 medium supplemented with 10 mM HEPES, 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin, as described [7]. The cells were detached with 0.27 mM EDTA/0.025% trypsin, and afterwards maintained in spinner culture in the same medium but with 25 mM HEPES and 5% fetal calf serum (spinner medium) for 3 h at 37 °C, to recover from the detachment procedure. Loading with the fluorescent calcium indicator fura-2 was then performed after resuspension in the same medium, with 1 µM fura-2/AM for 30 min at 37 °C. Measurements and calculation of [Ca²⁺]cyt were performed as described [9], with a Perkin Elmer LS 50B spectrophotometer in stirred cuvettes at 37 °C, with a modified Krebs-Ringer buffer containing 136 mM NaCl, 4.8 mM KCl, 2 mM CaCl₂, 1.2 mM
MgSO₄, 1.2 mM KH₂PO₄, 5 mM NaHCO₃, 10 mM glucose and 25 mM HEPES/NaOH, pH 7.4. Dye efflux was minimized by including 250 μM sulfinpyrazone. Extracellular dye-2 was determined by adding 250 μM MnCl₂ followed by 100 mM DTPA (diethylenetriaminepentaacetic acid) at the end of each experiment.

[Ca²⁺]ᵢ measurements in single attached cells with digital imaging fluorescence microscopy were performed with INS-1 cells grown on coverglasses in the medium described above under the conditions described previously [10]. An INS-1 cell carrying coverglass formed the bottom of a closed incubation chamber. The cells were superfused at 1 ml/min with a buffer containing 137 mM NaCl, 4.7 mM KCl, 0.56 mM MgCl₂, 1.28 mM CaCl₂, 1 mM Na₂HPO₄, 10 mM glucose and 10 mM HEPES/NaOH, pH 7.4, 37 ºC.

Results and Discussion

Histidine presents a positively charged α-amine at the N-terminus and additionally a positive charge and hydrogen bonding at the aromatic imidazole side chain. In order to investigate the role of histidine for binding and signal transduction of GLP-1, we extended GLP-1(7-36)amide with arginine creating GLP-1(6-36)amide. In another analogue we substituted histidine' by its D-isomer ((D-histidine')-GLP-1(7-36)amide). Extension by arginine at the N-terminus resulted in a reduced binding affinity (Kd: 216 ± 34.6 nM; native GLP-1 Kd: 0.3 ± 0.02 nM). While 10 nM GLP-1(7-36)amide induced an increase in cytosolic Ca²⁺ to 65 ± 4 nM, GLP-1(6-36)amide had no effect. When histidine’ was exchanged by its D-isomer, binding affinity was only slightly reduced (Kd: 7.42 ± 0.02 nM) whereby the effect on Ca²⁺ increase was only 18% (12 ± 7 nM) of the native peptide.

In further analogues, histidine was replaced by positively charged arginine ((R')-GLP-1(7-36)amide) or lysine ((K')-GLP-1(7-36)amide). The binding affinity of (R')-GLP-1(7-36)amide was reduced with a Kd of 383 ± 71 nM and the effect on cytosolic Ca²⁺ increase was reduced by 88% (8 ± 5 nM). The binding affinity of (K')-GLP-1(7-36)amide was only moderately reduced (Kd: 82.7 ± 15.2 nM) and the effect on cytosolic Ca²⁺ increase was reduced by 57% (28 ± 6 nM). These data suggest, that if the imidazol side chain is exchanged under preservation of the positive charge binding affinity and ability of signal transduction is only moderately reduced. It seems to be important that histidine is not replaced by an amino acid such as arginine.

It has been reported for glucagon, that histidine¹ might interact with aspartate⁹ [12]. Similarly, the histidine' of GLP-1 might interact with the negatively charged glutamate⁹. Such an interaction could be disturbed if the amino groups of the amino acid in position 7 are not in the correct position in relation to the peptide backbone which could be responsible for the reduced binding affinity and signal transduction of lysine and arginine. To further evaluate this hypothesis, histidine' was replaced by diamino butyric acid.

### Table 1. GLP-1 replacement analogues

<table>
<thead>
<tr>
<th>GLP-1 analogues</th>
<th>Kₐ ± SEM (nM)</th>
<th>[Ca²⁺] relative activity (% of max.)</th>
</tr>
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<tbody>
<tr>
<td>GLP-1(7-36)NH₂</td>
<td>0.3 ± 0.02</td>
<td>100</td>
</tr>
<tr>
<td>GLP-1(6-36)NH₂</td>
<td>216 ± 34.6</td>
<td>0</td>
</tr>
<tr>
<td>GLP-1(8-36)NH₂</td>
<td>33.3 ± 5.4</td>
<td>18</td>
</tr>
<tr>
<td>(D-H7)-GLP-1-1NH₂</td>
<td>7.42 ± 1.0</td>
<td>66</td>
</tr>
<tr>
<td>(R7)-GLP-1-1NH₂</td>
<td>383 ± 71</td>
<td>12</td>
</tr>
<tr>
<td>(K7)-GLP-1-1NH₂</td>
<td>82.7 ± 15.2</td>
<td>43</td>
</tr>
<tr>
<td>(Y7)-GLP-1-1NH₂</td>
<td>52.1 ± 3</td>
<td>66</td>
</tr>
<tr>
<td>(Dab7)-GLP-1-1NH₂</td>
<td>452 ± 96</td>
<td>0</td>
</tr>
<tr>
<td>(N⁶-acetyl)-GLP-1-1NH₂</td>
<td>1.39 ± 0.81</td>
<td>96</td>
</tr>
<tr>
<td>(A7)-GLP-1-1NH₂</td>
<td>199 ± 53.9</td>
<td>18</td>
</tr>
<tr>
<td>(N⁶-acetyl-A7)-GLP-1-1NH₂</td>
<td>629 ± 280</td>
<td>0</td>
</tr>
</tbody>
</table>

To obtain relative activity the ratio (∝ 100) of the Ca²⁺ concentration obtained after stimulation with 10 nM of native GLP-1 or GLP-1 analogue was calculated. 10 nM GLP-1 increased Ca²⁺ to 65 ± 4 nM; n = 4. Abbreviations: H; histidine; R; arginine; K; lysine; Y: tyrosine; Dab; diamino butyric acid.
((Dab')-GLP-1(7-36)amide) which contains two amino groups with a reduced distance to the peptide backbone, but the binding affinity of (Dab')-GLP-1(7-36)amide was very low \((K_d: 452 \pm 96 \text{ nM})\) and no effect on cytosolic \(\text{Ca}^{2+}\) was detectable. Obviously, the imidazol side chain is required to ensure the optimal distance of the amino groups from each other and from the peptide backbone.

Aromatic substitution with tyrosine ((Y')-GLP-1(7-36)amide) resulted in an analog which had a reduced binding affinity \((K_d: 52.1 \pm 3 \text{ nM})\) and signal transduction \((66\% \text{ of native GLP-1; } 43 \pm 18 \text{ nM})\). This is in contrast to findings with glucagon where the replacement of histidine\(^1\) by tyrosine resulted in an improved binding but an almost total loss of signal transduction \([6]\). In order to determine whether the basic \(\alpha\)-amino group of histidine is more important than the positive charge in the imidazole side chain, we blocked the free \(\alpha\)-amino group by acetylation ((N\(^\text{acetyl}\)-GLP-1(7-36)amide). In another analogue, by exchanging histidine\(^1\) for alanine ((A7)-GLP-1(7-36)amide), we eliminated the basic function of the imidazole side chain. Furthermore, by acetylation of (A7)-GLP-1(7-36)amide ((N\(^\text{acetyl}\)-A7)-GLP-1(7-36)amide) we also blocked the free \(\alpha\)-amino group.

Compared to the native peptide, (N\(^\text{acetyl}\)-GLP-1(7-36)amide showed only a slightly reduced binding affinity and an unaltered signal transduction \((63 \pm 5 \text{ nM})\). In contrast hereto, (A7)-GLP-1(7-36)amide and (N\(^\text{acetyl}\)-A7)-GLP-1(7-36)amide showed an approximately 600-fold and 2000-fold reduction in binding affinity, respectively. Signal transduction of (A7)-GLP-1(7-36)amide was 18\% \((12 \pm 7 \text{ nM})\) of native GLP-1 while (N\(^\text{acetyl}\)-A7)-GLP-1(7-36)amide had no effect on the cytosolic \(\text{Ca}^{2+}\) concentrations. These data show that the amino group presented by the imidazole side chain is more important for GLP-1 binding than the \(\alpha\)-amino group.

In essence, our data show that the positive charge of the imidazole side chain of histidine is crucial for GLP-1 action. The presence of a ring structure and a basic function as well as the correct positioning of both seems to be decisive.

**Acknowledgement**

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**References**
