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N-terminal Acetylation Protects Glucagon-like Peptide GLP-1-(7-34)-amide from DPP-IV-mediated Degradation Retaining cAMP- and Insulin-releasing Capacity

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Abstract: Since its discovery glucagon-like peptide-1 (GLP-1) is investigated as a treatment for type II diabetes based on its major function as insulin secretagogue. A therapeutic use is, however, limited by its short biological half-life in the range of minutes, predominantly caused via degradation catalyzed by dipeptidyl peptidase IV (DPP-IV). Therefore, we aimed to design a GLP-1 analogue exhibiting resistance against DPP-IV-catalyzed inactivation while retaining its biologiocal activity. By means of matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) we have studied the stability of the N-terminally blocked new analogue Ac-GLP-1-(7-34)-amide against DPP-IV and compared it with both unblocked GLP-1-(7-34)-amide and the major naturally occurring form GLP-1-(7-36)-amide. GLP-1-(7-36)-amide and the C-terminally two amino acid residues shorter GLP-1-(7-34)-amide rapidly generated peptide fragments truncated by the N-terminal dipeptide. In contrast, the N-terminal blocked Ac-GLP-1-(7-34)-amide was not degraded in the presence of DPP-IV over a period of at least two hours. Ac-GLP-1-(7-34)-amide induced a concentration-dependent increase of intracellular cAMP production and insulin release from rat insulinoma RIN-m5F cells to an extent comparable to that found for the N-terminally unblocked peptides GLP-1-(7-34)-amide and GLP-1-(7-36)-amide. Ac-GLP-1-(7-34)-amide may thus have the potential to act as a new long-acting GLP-1 analogue with significant resistance against DPP-IV and retained biological activity *in vitro*. Further research is required to investigate whether Ac-GLP-1-(7-34)-amide also exhibits its characteristics in animal models and humans.

Key words: Glucagon-like peptide-1, GLP-1, dipeptidyl peptidase IV, acetylation, stability, MALDI-TOF MS

Introduction

Glucagon-like peptide-1 (GLP-1) is a neuroendocrine hormone reported to have strong insulinotropic actions. GLP-1 is generated from tissue-specific post-translational processing of preproglucagon in the intestinal L-cells. In man, most endogenous GLP-1 corresponds to the variants GLP-1-(7–37) and GLP-1-(7–36)-amide which are the biologically active forms of GLP-1. Following food intake it is released into the circulation [1-3]. Through binding to the G protein-coupled receptor GLP-1R [4-9] GLP-1 exhibits its biological activities such as stimulation of glucose-dependent insulin secretion [10, 11] and inhibition of glucagon secretion [12-14]. In addition, suppression of postprandial hyperglycemia and lowering blood glucose levels through GLP-1 have been shown in both, humans [15] and animals [16]. Since these anti-diabetogenic effects of GLP-1 are also seen in patients with type II diabetes [17-21] GLP-1 and related peptides may be promising candidates for the treatment of this disease.

However, due to its short plasma half-life the naturally occurring GLP-1 seems to be of limited use for the treatment of type II diabetic patients. The predominant enzyme responsible for the GLP-1 degradation in plasma is dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5) which removes a dipeptide from the N-terminus of the intact peptide. A half-life of GLP-1 in the range of 12–30 min has been reported [22, 23]. Several attempts have been undertaken to establish a structure-activity relationship of GLP-1 aiming to design peptide analogues exhibiting an increased *in vivo* stability, in particular against DPP-IV, with retained biological properties [24-27]. Recent research demonstrated improved proteolytic stability of PEGylated derivatives [28, 29], albumin conjugates [30] and fatty acid-modified analogues of GLP-1 [31], rather complex structured GLP-1 derivatives. A particular GLP-1 peptide denominated NN2211 and Liraglutide carrying a C18 fatty acid side chain is currently in clinical development [32]. Further reports deal with defined amino acid substitutions within the GLP-1 sequence. GLP-1 peptides containing a substitutition of position 8 (DPP-IV cleaves between residues in positions 8 and 9) by glycine [33], serine [34], valine [35] or an aminohexanoic acid spacer [36] were shown to represent stabilized derivatives compared to the native GLP-1. The biological activity of such variants in terms of receptor binding, cAMP production and insulin release remained mainly unchanged. However, the influence of a truncation of the C-terminus of GLP-1 was not yet examined in order to identify a biologically active

DPP-IV-resistant GLP-1 analogue. In this report, we describe (i) the stability of the N-terminally blocked and C-terminally truncated Ac-GLP-1-(7-34)-amide against DPP-IV and (ii) its biological activity *in vitro*, both compared with the unblocked truncated GLP-1-(7-34)-amide and full-length GLP-1-(7-36)-amide.

Material and Methods

Reagents

Peptides GLP-1-(7-34)-amide, Ac-GLP-1-(7-34)-amide and GLP-1-(7-36)-amide (Table 1) were synthesized using Fmoc chemistry by the Lower Saxony Institute for Peptide Research (Hannover, Germany) or PolyPeptide Laboratories (Wolfenbüttel, Germany) as peptide acetate and were purified by reversed-phase HPLC. Peptide purity was not less than 95%. Dipeptidyl peptidase IV (EC 3.4.14.5) from porcine kidney (10 U/mg) was purchased from Sigma-Aldrich (Deisenhofen, Germany). All other chemicals were of reagent or higher grade and were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich.

Incubation of GLP-1 Peptides with DPP-IV

Incubation of GLP-1 analogues (200 µg/ml) with DPP-IV (6 µg/ml) was carried out in sterilized 0.01 M Na_2HPO_4 at pH 7.6 and 37 °C in a total volume of 250 µl under gentle shaking for 2 h. Samples of 5 µl each were taken immediately after enzyme addition and after further 5, 10, 15, 30, 45, 60, and 120 min of incubation. Samples were diluted 1 : 40 with 0.1% (v/v) trifluoroacetic acid (TFA) in water prior to MALDI-TOF MS analysis.

Analysis by MALDI-TOF MS

Diluted samples obtained as described above were analyzed by MALDI-TOF MS in the linear positive ion mode following the common protocol of the dried-droplet technique mixing equal volumes (1 µl) of sample and MALDI matrix solution as described elsewhere [37]. The matrix consisted of α-cyano-4-hydroxycinnamic acid (5 mg/ml) dissolved in a 50 : 50 mixture of acetonitrile and 0.05% (v/v) TFA. Measurements were performed on a Voyager DE-Pro instrument (1.2 m flight tube, 337 nm laser, Applied Biosystems, Darmstadt, Germany) using an accelerating voltage of 20 kV controlled by the Biospectrometry Workstation 5.1 software. External mass calibration was carried out with the peptide standards bradykinin, secretin and ubiquitin (Sigma, St. Louis, MO, USA) supported by the Data Explorer 4.0 program for data analysis. Ten different positions of each sample spot were shot 100 times by the laser to accumulate ion intensities over the total of 1000 shots. Quantitative analysis was performed by calculating the percentage of the total signal area for each of the detected GLP-related signals.

Determination of Intracellular cAMP in Rat Insulinoma RIN-m5F Cells

Cyclic AMP levels were measured as described before [38, 39]. In brief, cells (RIN-m5F, rat insulinoma; ATCC, Manassas, VA, USA) were seeded into 24-well plates and grown to 90-95% confluency. Cells were washed three times with 100 µl medium (RPMI 1640, Invitrogen, Karlsruhe, Germany), and 500 µl assay medium (RPMI 1640 with 1 mM 3-isobutyl-1-methylxanthine, IBMX, Roche, Penzberg, Germany) were added for 15 min preincubation. GLP-1 peptides and control medium were added and incubated for 10 min. Incubation medium was removed and 100 µl 70% (v/v) ethanol were added to stop the reaction and lyse the cells. The plates were then frozen at –80 °C and subsequently dried at 40 °C to remove ethanol using a waterbath. Finally, 1 ml ELISA buffer (0.1 M NaH_2PO_4, 0.15 M NaCl, 0.005 M EDTA, 0.2 % bovine serum albumin, 0.01 % thimerosal, pH 7.0) was added to redissolve cAMP, which was then measured using a cAMP-ELISA-Kit (IHF, Hamburg, Germany) at a dilution of 1 : 200.

Determination of Secreted Insulin in Rat Insulinoma RIN-m5F Cells

Prior to determination of insulin the corresponding protocol for GLP-1 stimulation of RIN-m5F cells as outlined above was used. Insulin in cell culture supernatants was directly measured using a commercially available insulin-RIA kit (Linco Research; St. Louis, MO, USA).

Table 1. GLP-1 analogues and their metabolites produced by dipeptidyl peptidase IV.

Peptide	Sequence	MW_{av} [Da]
GLP-1-(7-34)-amide	HAEGTFTSDVSSYLEGQAAKEFIAWLVK-$CONH_2$	3084.4
GLP-1-(9-34)-amide	EGTFTSDVSSYLEGQAAKEFIAWLVK-$CONH_2$	2876.2
Ac-GLP-1-(7-34)-amide	Ac-HAEGTFTSDVSSYLEGQAAKEFIAWLVK-$CONH_2$	3126.4
GLP-1-(7-36)-amide	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-$CONH_2$	3297.7
GLP-1-(9-36)-amide	EGTFTSDVSSYLEGQAAKEFIAWLVKGR-$CONH_2$	3089.4
GLP-1-(11-36)-amide	TFTSDVSSYLEGQAAKEFIAWLVKGR-$CONH_2$	2903.3

Ac, acetyl; MW_{av} ,averaged molecular weight; $CONH_2$, amide.

Results and Discussion

As shown by Pauly et al. [40] DPP-IV is the major circulating proteolytic enzyme cleaving the N-terminal dipeptidyl residue from the incretin hormones glucose-dependent insulinotropic polypeptide (GIP-1-42) and GLP-1-(7-36)-amide. Thereby, these natural peptides are converted into biologically inactive products unable to elicit glucose-dependent insulin secretion [41]. Several attempts including truncations, amino acid exchanges, introduction of non-natural amino acids, and side chain modifications were undertaken to modify the GLP-1 molecule aiming to prevent degradation by DPP-IV and other enzymes such as the neutral endopeptidase NEP 24.11 also likely to degrade GLP-1. Some of these GLP-1 analogues exhibit increased stability towards proteolytic cleavage, but, however, are associated with a loss of bioactivity. It is hypothesized that through N-terminal acetylation the C-terminally reduced GLP-1 may exhibit stronger resistance against proteolytic degradation by DPP-IV. Therefore, we tested the stability and biological activity of Ac-GLP-1-(7-34)-amide in comparison to its underivatized analogues GLP-1-(7-34)-amide and GLP-1-(7-36)-amide.

Stability of GLP-1 Peptides in the Presence of DPP-IV

For quantitative interpretation of the mass spectra we made two assumptions: (i) all degradation products obtained from GLP-1 exhibiting a truncated peptide backbone are detectable by MALDI-TOF MS, and (ii) GLP-1 substrates and their corresponding degradation products have similar ionization properties resulting in similar MS signal intensities and areas when present in equal concentrations. Based on these assumptions quantitative interpretation of the mass spectra obtained during incubation allowed to monitor the relative concentration-time profiles of GLP-1 degradation.

GLP-1-(7-34)-amide was N-terminally truncated by DPP-IV-mediated cleavage of the His-Ala dipeptide generating its fragment GLP-1-(9-34)-amide (Fig. 1A) which was unambiguously identified by MALDI-TOF MS analysis (Fig. 1B). No additional degradation products were detected within the investigated incubation period of two hours. Following an exponential decay the concentration of GLP-1-(7-34)-amide decreased yielding an apparent period of half-change of 35 min (Fig. 1A). Slightly different results were obtained from the DPP-IV treatment of the longer GLP-1-(7-36)-amide. As shown in Fig. 1D two truncation products were generated from consecutive dipeptidyl cleavage: GLP-1-(9-36)-amide (m/z 3090.4) resulting from cleavage of the N-terminal His-Ala dipeptide and GLP-1-(11-36)-amide (m/z 2904.4) produced from additional cleavage of Glu-Gly corresponding to positions 9-10. The corresponding concentration-time profile (Fig. 1C) indicates an exponential decay of the substrate with an apparent period of half-change of 23 min. Accordingly, the detectable major degradation product GLP-1-(9-36)-amide increased exponentially, and GLP-1-(11-36)-amide was produced to a much smaller extent of approximately 11% after 2 h. Production of the latter metabolite was not reported by Pauly et al. which may be due to the shorter incubation period applied of only 16 min during which GLP-1-(7-36)-amide was degraded only to nearly 50% by DPP-IV [40]. Table 1 provides an overview on tested GLP-1 substrates and detected cleavage products.

As indicated by the shorter period of half-change the larger GLP-1-(7-36)-amide appears to be less stable than GLP-1-(7-34)-amide. In contrast to both N-terminally unblocked GLP-1 peptides the acetylated variant Ac-GLP-1-(7-34)-amide did not show any degradation within 2 h (Fig. 1E, F), thereby clearly proving the protective effect of the N-terminal blocking group. Corresponding results were obtained when incubating GLP-1 analogues in human EDTA plasma underlining the predominant role of DPP-IV in GLP-1 processing (results not shown).

cAMP Production and Insulin Secretion by GLP-1 Peptides

In the DPP-IV-free rat pancreatic beta cell line RIN-m5F GLP-1-(7-34)-amide elevated cAMP production with an EC_{50} of about 40 nM, whereas the other two GLP-1 analogues tested were less efficient (EC_{50} ~ 200 nM) (Fig. 2A). However, it is well accepted that cAMP elevation is not the sole indicator of GLP-1 bioactivity. For this reason we also determined the ability of the GLP-1 analogues to influence insulin secretion. Our data show that with respect to the induction of insulin secretion from RIN-m5F cells acetylated and non-acetylated GLP-1-(7-34)-amide are of equal efficiency (Fig. 2B), indicating that introduction of the acetyl group does not disrupt the biological activity. Insulin secretion was significantly elevated over control levels starting at 100 nM for GLP-1-(7-34)-amide and GLP-1-(7-36)-amide (both $p < 0.05$) but already significantly elevated at 10 nM for Ac-GLP-1-(7-34)-amide. Although these differences are small they clearly indicate that a loss of bioactivity does not occur by introduction of an acetyl group into the smallest tested GLP-1 derivative.

Summary and Conclusion

In the present report we have tested the chemically synthesized GLP-1 analogue, Ac-GLP-1-(7-34)-amide, characterized by an N-terminally blocking acetyl group and a C-terminal truncation by two amino acid residues. Despite these structural modifications Ac-GLP-1-(7-34)-amide retains its biological activity. Moreover, the degree of activity was comparable to that of non-acetylated GLP-1-(7-34)-amide and the larger GLP-1-(7-36)-amide. These findings are consistent with earlier reports describing that despite C-terminal truncation GLP-1 peptides still retain full biological activity [42, 43]. In addition, the N-terminal acetylation may cause an almost complete protection against DPP-IV cleavage, a finding also reported for the correspondingly blocked GLP-1-(7-36)-amide [25]. However, to what extent the lacking two amino acid residues may add an additional contribution concerning DPP-IV resistance still remains to be shown.

In summary, we have demonstrated that following incubation with DPP-IV the new peptide Ac-GLP-1-

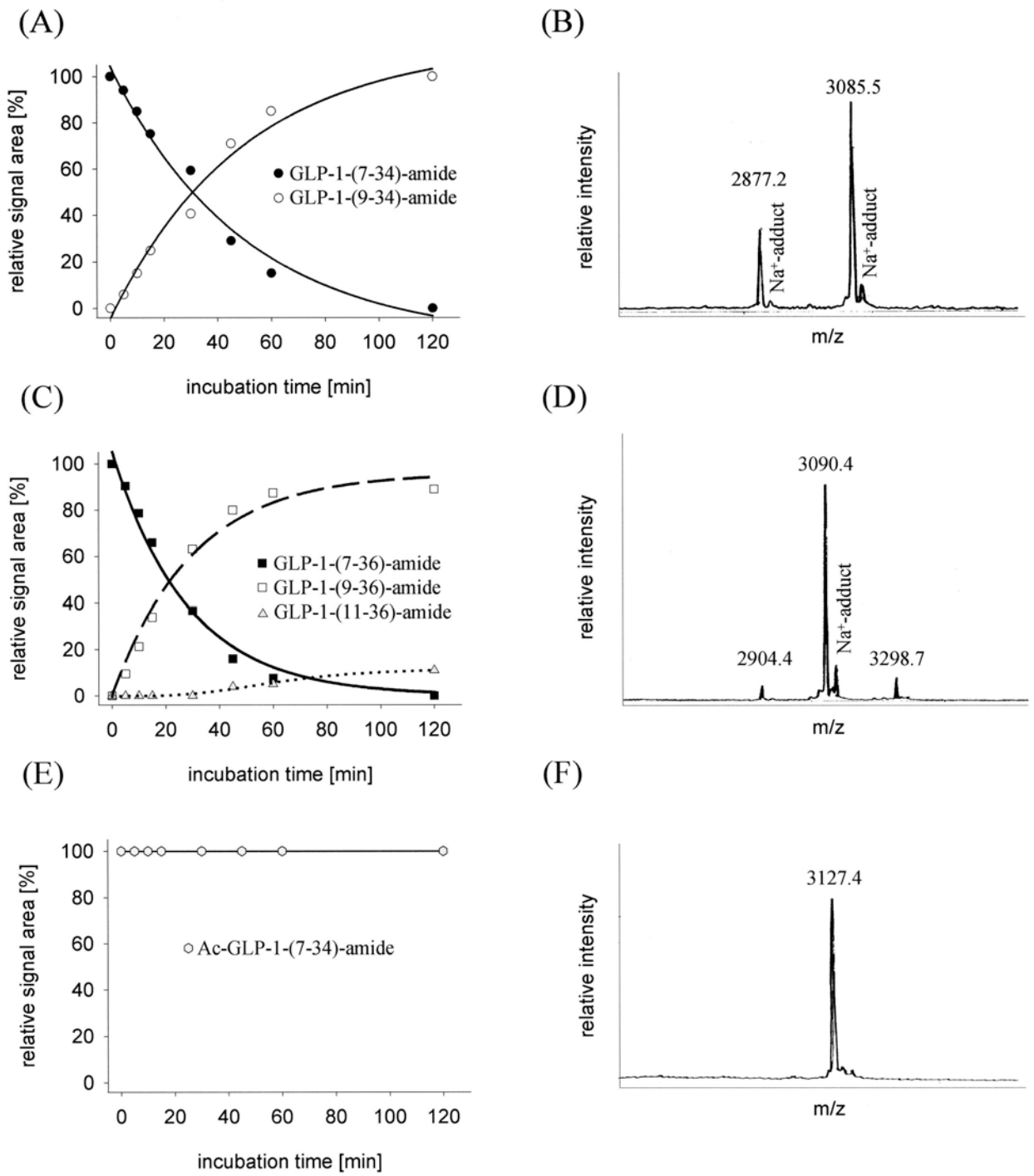

Fig. 1. Degradation of GLP-1 analogues by incubation in dipeptidyl peptidase IV-buffered solution. Representative concentration-time profiles (A, C, E) were obtained from MALDI-TOF MS measurements in the linear positive ion mode. Mass spectra for metabolite profiling (B, D, F) were recorded after 60 min (B, F) and 15 min (D) of incubation. A, B: Degradation of GLP-1-(7-34)-amide. m/z 3085.5: GLP-1-(7-34)-amide; m/z 2877.2: GLP-1-(9-34)-amide. C, D: Degradation of GLP-1-(7-36)-amide. m/z 3298.7: GLP-1-(7-36)-amide; m/z 3090.4: GLP-1-(9-36)-amide; m/z 2904.4: GLP-1-(11-36)-amide. E, F: Stability of Ac-GLP-1-(7-34)-amide. m/z 3127.4: Ac-GLP-1-(7-34)-amide, no degradation products were detected. m/z: mass/charge ratio.

(7-34)-amide is not proteolytically degraded while still exhibiting its agonistic functions on the cellular level. In contrast to more complex structured GLP-1 analogues designed to be resistant against DPP-IV degradation, Ac-GLP-1-(7-34)-amide has a reduced chain length with the native amino acid sequence and a chemically changed N-terminal structure. Thus, Ac-GLP-1-(7-34)-amide may not only have the potential to exert high resistance to DDP-IV resulting in an improved exposure after administration while retaining biological activity. Due to its simple structure it also may have pharmaceutical advantages, in particular regarding the chemical production, over more complex GLP-1 analogues such as the hydrophobically modified Liraglutide

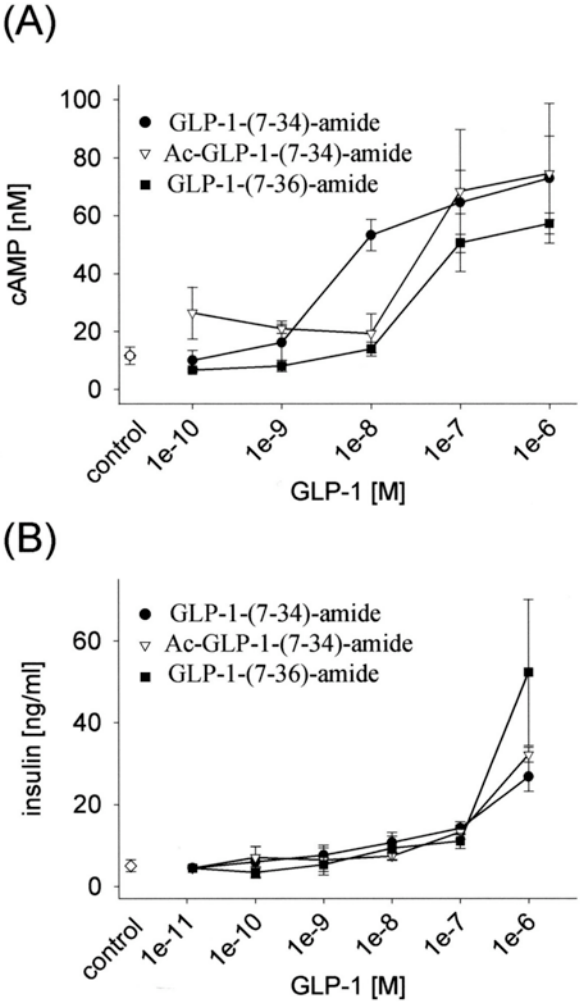

Fig. 2. Cyclic AMP and insulin production of rat insulinoma RIN-m5F cells after incubation with GLP-1 analogues. A: Comparison of the potency to elevate cAMP of the three GLP-1 analogues GLP-1-(7-36)-amide, GLP-1-(7-34)-amide and Ac-GLP-1-(7-34)-amide in rat insulinoma RIN-m5F cells. All three analogues elevated intracellular cAMP levels dose-dependently. GLP-1-(7-34)-amide was the most potent analogue (EC_{50} ~ 40 nM) whereas the other two analogues were about five times less potent (EC_{50} ~ 200 nM). The values represent the means ± SD obtained from 3-5 replicates. B: Comparison of the potency to elevate insulin secretion of the three GLP-1 analogues GLP-1-(7-36)-amide, GLP-1-(7-34)-amide and Ac-GLP-1-(7-34)-amide in rat insulinoma RIN-m5F cells. All three analogues elevated insulin levels dose-dependently. GLP-1-(7-36)-amide was the most potent analogue whereas the other two analogues were slightly less potent. The values represent the means ± SD obtained from 3-5 replicates. Note that the insulin levels do not reach saturation in the tested range of concentrations.

or albumin and PEG conjugates of GLP-1. Further research applying experimental animals and clinical studies are necessary to investigate whether Ac-GLP-1-(7-34)-amide also exerts its characteristics *in vivo*.

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