

Dipeptidyl Peptidase IV (DPIV/CD26) Degradation of Glucagon

CHARACTERIZATION OF GLUCAGON DEGRADATION PRODUCTS AND DPIV-RESISTANT ANALOGS*

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Over the past decade, numerous studies have been targeted at defining structure-activity relationships of glucagon. Recently, we have found that glucagon₁₋₂₉ is hydrolyzed by dipeptidyl peptidase IV (DPIV) to produce glucagon₃₋₂₉ and glucagon₅₋₂₉; in human serum, [pyroglutamyl (pGlu)³]glucagon₃₋₂₉ is formed from glucagon₃₋₂₉, and this prevents further hydrolysis of glucagon by DPIV (H.-U. Demuth, K. Glund, U. Heiser, J. Pospisilik, S. Hinke, T. Hoffmann, F. Rosche, D. Schlenzig, M. Wermann, C. McIntosh, and R. Pederson, manuscript in preparation). In the current study, the biological activity of these peptides was examined *in vitro*. The amino-terminally truncated peptides all behaved as partial agonists in cyclic AMP stimulation assays, with Chinese hamster ovary K1 cells overexpressing the human glucagon receptor (potency: glucagon₁₋₂₉ > [pGlu³]glucagon₃₋₂₉ > glucagon₃₋₂₉ > glucagon₅₋₂₉ > [Glu⁹]glucagon₂₋₂₉). In competition binding experiments, [pGlu³]glucagon₃₋₂₉ and glucagon₅₋₂₉ both demonstrated 5-fold lower affinity for the receptor than glucagon₁₋₂₉, whereas glucagon₃₋₂₉ exhibited 18-fold lower affinity. Of the peptides tested, only glucagon₅₋₂₉ showed antagonist activity, and this was weak compared with the classical glucagon antagonist, [Glu⁹]glucagon₂₋₂₉. Hence, DPIV hydrolysis of glucagon yields low affinity agonists of the glucagon receptor. As a corollary to evidence indicating that DPIV degrades glucagon (Demuth, *et al.*, manuscript in preparation), DPIV-resistant analogs were synthesized. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry was used to assess DPIV resistance, and it allowed kinetic analysis of degradation. Of several analogs generated, only [D-Ser²] and [Gly²]glucagon retained high affinity binding and biological potency, similar to native glucagon *in vitro*. [D-Ser²]Glucagon exhibited enhanced hyperglycemic activity in a bioassay, whereas [Gly²]glucagon was not completely resistant to DPIV degradation.

Glucagon is a 29-amino acid peptide hormone that is released from pancreatic α -cells and acts to raise blood glucose in the fasted state by increasing hepatic glycogenolysis and gluconeogenesis (1, 2). The circulating half-life of immunoreactive glucagon is estimated to be between 5 and 6 min in dogs and humans (3, 4). The tissues responsible for the clearance of glucagon from the circulation are somewhat controversial; however, it is generally accepted that the kidneys play the dominant role (reviewed in Refs. 5 and 6). Interestingly, there have been reports that glucagon degradation in blood or plasma is negligible (7, 8). Recently, evidence has been presented suggesting that dipeptidyl peptidase IV (DPIV)¹ is responsible in part for the inactivation of glucagon (9).² This finding is consistent with renal clearance of glucagon but conflicts with the absence of plasma degradation previously reported, as DPIV is found on the surface of lymphocytes and as a freely circulating enzyme in addition to the apical surface of the renal proximal tubules (10).

The glucagon receptor is a class B serpentine G-protein coupled receptor, belonging to the same family of hormone receptors as those for secretin, vasoactive intestinal peptide, glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide, glucagon-like peptide-1, calcitonin, parathyroid hormone, and pituitary adenylyl cyclase activating polypeptide (11). The ligand specificity of the glucagon receptor is primarily conferred by its extracellular amino terminus (12, 13); activation of the receptor results in activation of both the adenylyl cyclase/cyclic AMP and the phospholipase C/inositol trisphosphate intracellular cascades (14).

To date, a plethora of structure-activity studies on glucagon have been performed (the most recent comprehensive review in Ref. 15). These have generally consisted of rational and systematic investigations of ligand-receptor agonism and antagonism, resulting in an increased understanding of the charge-charge interactions between the hormone and receptor resulting in its ability to bind and activate the receptor (16). Other key findings include the importance of the amino terminus of glucagon in receptor activation (17), as well as important residues within the primary sequence of glucagon (16, 18).

Recently, it was discovered that purified pork kidney DPIV is

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¹ The abbreviations used are: DPIV, dipeptidyl peptidase IV; pGlu, pyroglutamyl; Fmoc, N-9-fluorenylmethyloxycarbonyl; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; EC₅₀, half-maximal effective concentration; (I/A)₅₀, ratio of inhibitor to agonist resulting in reduction of agonist alone 2-fold; CHO, Chinese hamster ovary; hGlucR cell, cell stably expressing the human glucagon receptor.

² H.-U. Demuth, K. Glund, U. Heiser, J. Pospisilik, S. Hinke, T. Hoffmann, F. Rosche, D. Schlenzig, M. Wermann, C. McIntosh, and R. Pederson, manuscript in preparation.

capable of hydrolyzing glucagon₁₋₂₉ to glucagon₃₋₂₉ and glucagon₅₋₂₉ *in vitro* and that, in human serum, it is converted first to glucagon₃₋₂₉, and subsequently its amino terminus is cyclized by a serum enzyme (possibly to pyroglutamyl-glucagon₃₋₂₉ ([pGlu³]glucagon₃₋₂₉)), thus preventing further DPIV degradation, as it does not fulfil the substrate requirements of the enzyme (*i.e.* it lacks a protonable amino terminus).² The specificity of DPIV was characterized to preferentially release dipeptides from the amino terminus of polypeptides with proline or alanine in the penultimate position (10). However, amino-terminal degradation of nontypical substrates has been reported, including sequential cleavage of amino-terminal dipeptides (19, 20).

In the current study, the effects of the potentially physiologically relevant amino-terminally truncated glucagon fragments on the human glucagon receptor were examined. The glucagon fragments glucagon₃₋₂₉, glucagon₅₋₂₉, and [pGlu³]glucagon₃₋₂₉ were characterized on Chinese hamster ovary K1 (CHO-K1) cells transfected with the human glucagon receptor, with respect to agonist and antagonist activity as well as binding affinity, and compared with glucagon₁₋₂₉ and [Glu⁹]glucagon₂₋₂₉. Further studies using DPIV-resistant glucagon analogs were performed to support existing evidence for DPIV degradation of glucagon using *in vitro* methods and a bioassay.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—Glucagon analogs were synthesized with an automated synthesizer Symphony (Rainin) using a modified Fmoc protocol. Fmoc-protected amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, *N*-methylmorpholine, and NovaSyn resin were purchased from Novabiochem (Schwalbach, Germany). Dimethylformamide, dichloromethane, and high pressure liquid chromatography (HPLC) solvents were supplied by Roth (Karlsruhe, Germany) or J. T. Baker (Griesheim, Germany). The peptide couplings were performed by 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/*N*-methylmorpholine-activation using a 0.23-mmol NovaSyn TGR-resin at a 25 μ M scale. Cleavage from the resin was carried out by a cleavage-mixture consisting of 94.5% trifluoroacetic acid (Merck, Darmstadt, Germany), 2.5% 1,2-ethanedithiol (Merck) and 1% tris-isopropylsilane (Aldrich, Deisenhofen, Germany). Analytical and preparative HPLC were performed with a linear gradient (10–90% over 30 min) of acetonitrile with 0.1% trifluoroacetic acid on a 125-4 RP18 or a 250-20 RP8 column, respectively, using the LiChrograph HPLC system (Merck-Hitachi). To confirm peptide identity and verify purity, matrix-assisted laser-desorption/ionization-time of flight (MALDI-TOF) mass spectrometry was employed (described below).

Glucagon₁₋₂₉ was purchased from Peninsula Biolabs (Belmont, CA); [Glu⁹]glucagon₂₋₂₉ was obtained from Bachem (Torrance, CA).

Cell Culture—CHO-K1 cells were transfected with the human glucagon receptor (21) in pcDNA3 (Invitrogen, Carlsbad, CA) by the calcium phosphate co-precipitation method with a glycerol shock (22). Cells stably expressing the human glucagon receptor (hGlucR cells) were selected for with 800 μ g/ml Geneticin® (G418; Life Technologies, Inc.). Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Life Technologies, Inc.) supplemented with 10% newborn calf serum (Cansera, Rexdale, Ontario, Canada), antibiotics (50 units/ml each of penicillin G and streptomycin; Sigma), and maintained under high selection with G418. Cells were grown in 75 cm² T-flasks (Becton Dickinson, Mississauga, Ontario, Canada) at 37 °C and in a humidified 5% CO₂ atmosphere; they were harvested with trypsin/EDTA (Life Technologies, Inc.) and plated at a density of 50,000 cells per well into 24-well plates (Becton Dickinson). Forty-eight hours later, when cells had reached 1–5 \times 10⁵ cells/well, plates were used in cyclic AMP studies and binding experiments.

Cyclic AMP Studies—hGlucR cells were washed twice in serum-free, HEPES-buffered (15 mM; FisherBiotech, Fair Lawn, NJ) Dulbecco's modified Eagle's medium/Ham's F-12 medium, with 1% Trasylol® (aprotinin; Bayer, Etobicoke, Ontario, Canada) and 0.1% bovine serum albumin (radioimmunoassay fraction V; Sigma) and allowed to equilibrate in this medium for 1 h prior to stimulation. Cyclic AMP production was stimulated with concentrations of glucagon and glucagon analogs shown in the figures, in the above buffer with the addition of 0.5 mM 3-isobutyl-1-methylxanthine (Research Biochemicals International,

Natick, MA). Cyclic AMP stimulation proceeded for 30 min in the same medium described above, prior to lysis of cells in ice-cold 70% ethanol. Cellular debris was removed by centrifugation and intracellular contents were concentrated with a Speed Vac (Sorvall, Farmingdale, NY). Cyclic AMP content was measured by radioimmunoassay (Biomedical Technologies Inc., Stoughton, MA) using the manufacturer's protocol for nonacetylated samples. Antagonism of glucagon₁₋₂₉ action by glucagon analogs was determined by preincubating cells with various concentrations of analogs for 15 min, prior to 30 min of stimulation with 1 nM glucagon. The cyclic AMP stimulation and antagonism protocols used in this report are similar to those described previously (23).

Cell Binding Studies—Binding affinity of glucagon analogs was measured using competition binding experiments. Briefly, cells were incubated in the presence of 50,000 cpm 3-[¹²⁵I]iodotyrosyl¹⁰-glucagon (Amersham Pharmacia Biotech) in the presence or absence of glucagon or analogs at the concentrations shown in the figures, for 4 h at 4 °C in HEPES-buffered Dulbecco's modified Eagle's medium/Ham's F-12 medium with 0.1% bovine serum albumin and 1% Trasylol. Cells were washed twice in ice-cold buffer, followed by solubilization in 1 ml of 0.1 M NaOH and transfer to borosilicate tubes for counting of cell associated radioactivity. Nonspecific binding was defined as the cell-associated radioactivity measured in the presence of 1 μ M glucagon₁₋₂₉.

Surface Plasmon Resonance—Protein-protein interaction studies were performed using a BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden). This technology allows detection of biomolecules and monitoring of binding events between two or more molecules, in real-time, without the use of labels. The optical phenomenon of surface plasmon resonance, is based on the change in refractive index at the surface of a sensor chip. The refractive index (given in resonance units), is directly related to the mass concentration in the surface layer of the sensor chip and increases when analyte (interactant in free solution) binds to the immobilized ligand. Research grade CM5 chips, *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide, *N*-hydroxysuccinimide, ethanolamine, and P20 surfactant were obtained from BIAcore AB. Porcine kidney DPIV (specific activity, 31.2 units/mg) was purified as described previously (24) and immobilized onto the flow cell of the CM5 chip using amine coupling chemistry. One unit of DPIV activity is defined as the release of 1.0 μ M 4-nitroaniline per min from Gly-Pro-4-nitroaniline at 30 °C using a substrate concentration of 400 μ M in a HEPES buffer (40 mM, pH 7.6, I = 0.102 by KCl) (25). Release of 4-nitroaniline was measured spectrophotometrically at 390 nm. The immobilization steps were carried out at a flow rate of 20 μ l/min in buffer (20 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% P20 surfactant). The chip surface was activated for 12 min with a mixture of *N*-hydroxysuccinimide (50 mM) and *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide (200 mM). DPIV (200 nM in 10 mM acetate buffer, pH 4.0) was injected at a flow rate of 5 μ l/min, followed by a 7-min treatment of the chip with ethanolamine (1 M, pH 8.0) to block remaining activated groups. A baseline of 5000 resonance units was obtained following DPIV coupling to the sensor chip. Glucagon and analogs were dissolved in buffer (above) to cover a concentration range of 500 nM to 100 μ M and were injected at a flow rate of 30 μ l/min (25 °C). The binding profiles were analyzed with BIAevaluation software (version 3.0) to obtain dissociation constants (K_d).

MALDI-TOF Mass Spectrometry and Kinetic Analysis of Degradation—Measurement of degradation of native glucagon and amino-terminally modified glucagon analogs was performed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry, as described previously (24, 25). Briefly, glucagon or analogs (25 μ M) were incubated in 0.04 M Tris/HCl (pH 7.6) with purified pork dipeptidyl peptidase IV (2.5 milliunits; 18.1 units/mg) or human serum (20%), obtained from healthy subjects. Kinetic analysis of results was performed as per previously published literature (24, 26, 27). MALDI-TOF mass spectrometry was used to quantify the amount of intact substrate *versus* time, and data were fitted to a first-order exponential decay equation to obtain the half-life ($t_{1/2}$) (26). To compare data from different peptides, it was necessary to use relative intensity on the ordinate axis rather than absolute intensity (μ V), as signal intensity varies from peptide to peptide at the same concentration (24, 26).

Bioassay—Synthetic glucagon analogs were tested *in vivo*, using a bioassay monitoring whole blood glucose concentration. Glucagon analogs were tested for bioactivity, degradation in normal rat plasma, and degradation in pure porcine DPIV. For biological activity, peptides were dissolved in saline and injected subcutaneously into unrestrained conscious fed male Wistar rats (~275 g). Animal work was in compliance with the guidelines set out by the National Institutes of Health (42). Analog dose was calculated by molar equivalence (7.1 nmol/kg), such that analog dose was equivalent to the dose of glucagon₁₋₂₉. Degradation

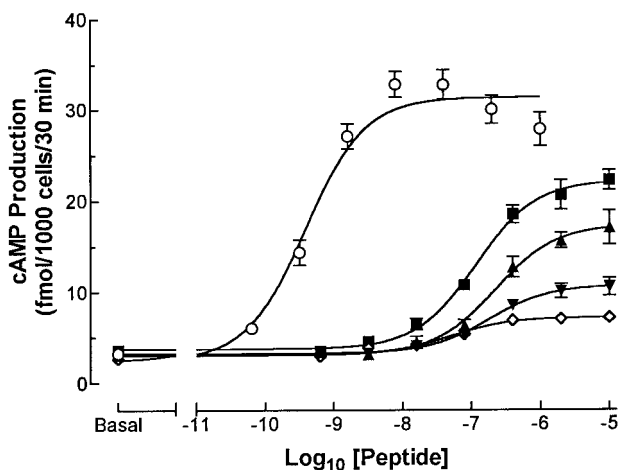


FIG. 1. Concentration-dependent stimulation of cyclic AMP production in CHO-K1 hGlucR cells by glucagon and synthetic fragments. Each data point represents the mean \pm S.E. of 3–4 experiments. Refer to Table I for potency and efficacy statistics. ○, glucagon₁₋₂₉; ▲, glucagon₃₋₂₉; ■, [pGlu³]glucagon₃₋₂₉; ▼, glucagon₅₋₂₉; ◇, [Glu⁹]glucagon₂₋₂₉.

tion by purified DPIV was assessed by incubation with 0.31 units of DPIV in phosphate-buffered saline (pH 7.4) at 37 °C for 3.25 h, followed by subcutaneous injection. Similarly, peptides were incubated in normal (male Wistar) rat plasma (1.0 ml) under the same conditions (37 °C, 3.25 h), prior to subcutaneous injection. Whole blood glucose concentration was measured using tail bleeds and the SureStep® glucose analyzer (LifeScan Canada Ltd., Burnaby, British Columbia, Canada).

Data Analysis—Data are presented as mean \pm S.E., with the number of experiments shown in the figure legends. Statistical significance was set at the 5% level and assessed using analysis of variance and Dunnett's multiple comparison test or the Newman-Keuls test as *post hoc* tests of significance where appropriate. Data analysis was done using the Prism software package (GraphPad, San Diego, CA). Cyclic AMP data are presented as fmol (10^{-15} mol) per 1000 cells and receptor binding data as percentage of binding in the absence of competitor (B/B_0). EC_{50} , IC_{50} , and $(1/A)_{50}$ values were obtained from nonlinear regression analysis using Prism. K_d values of DPIV for glucagon and analogs were measured using BIAevaluation software (BIAcore AB). Blood glucose data is presented as fold basal activity and the integrated glucose response over the measurement period, as determined by the trapezoidal method.

RESULTS

In Vitro Characterization of Amino-terminally Truncated Glucagon Fragments—Stimulation of cyclic AMP production in CHO-K1 hGlucR cells by glucagon₁₋₂₉, glucagon₃₋₂₉, [pGlu³]glucagon₃₋₂₉, and glucagon₅₋₂₉ is shown in Fig. 1. A summary of the statistical analysis is shown in Table I. Fragments were all partial agonists of the glucagon receptor, with the following rank of potency: [pGlu³]glucagon₃₋₂₉ > glucagon₃₋₂₉ > glucagon₅₋₂₉. [Glu⁹]glucagon₂₋₂₉ was included in antagonism experiments as a positive control because it is a well characterized antagonist (28); [Glu⁹]glucagon₂₋₂₉ exhibited a small but significant concentration-dependent increase in intracellular cyclic AMP content of hGlucR cells (2.7 times basal). Because the glucagon fragments were only partial agonists, they were also tested for possible antagonist activity (Fig. 2 and Table I). Glucagon₅₋₂₉ was found to antagonize cAMP stimulation by 1 nM glucagon₁₋₂₉ (2 μ M, $p < 0.05$; 10 μ M, $p < 0.01$), although to a lesser degree than [Glu⁹]glucagon₂₋₂₉. Glucagon₅₋₂₉ was an approximately 11-fold weaker antagonist (Table I). Neither glucagon₃₋₂₉ nor [pGlu³]glucagon₃₋₂₉ was found to antagonize glucagon₁₋₂₉ activity.

Competition binding experiments on hGlucR cells are shown in Fig. 3 and affinity statistics in Table I. Glucagon₁₋₂₉ and [Glu⁹]glucagon₂₋₂₉ exhibited approximately equal affinity for the glucagon receptor. All other truncated peptides showed

significantly lower affinity for the human glucagon receptor than glucagon₁₋₂₉ under the given assay conditions. [pGlu³]glucagon₃₋₂₉ and glucagon₅₋₂₉ both had approximately 5-fold lower affinity in binding competition experiments, whereas glucagon₃₋₂₉ had 18-fold lower affinity for the receptor.

In Vitro Characterization of Amino-terminally Modified Glucagon Analogs—Substitution or modification of amino acids 2 or 3 was used to generate DPIV-resistant glucagon analogs, given the substrate specificity of the enzyme. The affinity of DPIV for glucagon and amino-terminally modified analogs was determined by saturation binding experiments using surface plasmon resonance. Previous work has shown that immobilization of DPIV has no significant effects on catalytic efficiency relative to the soluble enzyme (29). The dissociation constant (K_d) of DPIV for native glucagon was in the μ M range (Table II). This value compares well with the K_m value for DPIV hydrolysis of glucagon using capillary zone electrophoresis and the K_i value obtained from spectrophotometric experiments using glucagon to inhibit DPIV hydrolysis of Gly-Pro-4-nitroaniline (data not shown).² Modification of the amino terminus about the scissile bond resulted in a 6–23-fold reduction in affinity for DPIV (K_d : glucagon < [D-Ser²]glucagon < [Gly²]glucagon < [Ser(P)²]glucagon < [D-Gln³]glucagon).

DPIV resistance was monitored using MALDI-TOF spectrometry of peptides incubated with purified porcine DPIV (Fig. 4). Quantitative kinetic analysis is given in Table III. Substituting the second amino acid of glucagon (L-serine) with its D-isomer completely blocked degradation by DPIV (Fig. 4A). [D-Gln³]glucagon was moderately resistant to DPIV (relative to native glucagon); altering the chirality of residue 3 also prevented further degradation of the analog to glucagon₅₋₂₉ by purified DPIV, as observed by MALDI-TOF mass spectrometry (Fig. 4C). In contrast, substitution of glycine for serine at position 2 did not render the peptide resistant to DPIV degradation (Fig. 4B). [D-Gln³]glucagon was similarly resistant to degradation in human serum: only a slight degradation was observed (Fig. 5C). [D-Ser²]glucagon showed an increased susceptibility to trypsin-like hydrolysis compared with native glucagon (Fig. 5A), indicated by generation of a glucagon fragment corresponding to amino acids 1–17 (cleavage between Arg¹⁷ and Arg¹⁸), followed by a carboxypeptidase-mediated release of Arg¹⁷. Modification of serine 2 with a phosphate group rendered the peptide resistant to purified DPIV (Fig. 4D), and in human serum, [Ser(P)²]glucagon showed retarded degradation, because the velocity of degradation was limited by the dephosphorylation of Ser(P)² (Fig. 5D). *In vitro* characterization of amino-terminally modified glucagon analogs on hGlucR cells is shown in Fig. 6 and summarized in Table II. [D-Ser²] and [Gly²] substitutions were the best tolerated, giving only slight reductions in receptor binding affinity (2–3-fold reduced) and cyclic AMP stimulating potency (approximately a 4-fold right shift in the concentration response curves) relative to native glucagon. Modifying the chirality of position 3 resulted in a peptide that had 19-fold lower binding affinity and 40-fold lower potency (EC_{50}) but an elevated maximal cyclic AMP production at high peptide concentrations (Fig. 6B and Table II). Phosphoserine at position 2 was not well tolerated, giving only an 8-fold reduction in binding affinity, yet a 76-fold greater EC_{50} .

Bioassay of Amino-terminally Modified Glucagon Analogs—The basal fed blood glucose concentrations of rats from each test group did not significantly differ from one another, and the mean fed glycemia was 7.5 ± 0.2 mM ($n = 60$). The characteristic glucagon₁₋₂₉ effect on glycemia over the course of 1 h showed a rapid rise in circulating glucose over the first 20 min, to a maximum of 11.7 ± 0.4 mM ($n = 8$), followed by a return to

TABLE I

Summary of molecular weights, agonism, antagonism and binding statistics of amino-terminally truncated glucagon fragments on CHO-K1 hGlucR cells

Data represent mean \pm S.E. ($n \geq 3$). See text for specific methods.

	Expected molecular weight	Measured molecular weight ^a	Adenylyl cyclase activity		Antagonism ((I/A) ₅₀)	Receptor binding (IC ₅₀)
			EC ₅₀	Maximum cAMP		
			nM	fmol/1000 cells		nM
Glucagon ₁₋₂₉	3482.8	3482.9 ^b	0.398 \pm 0.058	32.8 \pm 1.4		8.83 \pm 0.60
Glucagon ₃₋₂₉	3258.6	3256.7	239 \pm 20 ^c	17.0 \pm 1.9 ^c	N/A ^d	155 \pm 29 ^c
[pGlu ³]Glucagon ₃₋₂₉	3241.6	3242.2	131 \pm 34 ^c	22.3 \pm 1.1 ^c	N/A	48.7 \pm 4.1 ^c
Glucagon ₅₋₂₉	3073.4	3074.9	182 \pm 33 ^c	10.5 \pm 1.0 ^c	2159	45.3 \pm 9.4 ^c
[Glu ⁹]Glucagon ₂₋₂₉	3359.7	ND ^e	59.8 \pm 19.2 ^c	7.2 \pm 0.5 ^c	192	6.37 \pm 0.74

^a Molecular weight measured using MALDI-TOF mass spectrometry.

^b Synthetic glucagon₁₋₂₉ was obtained from Peninsula Biolabs (Belmont, CA).

^c $P < 0.05$ relative to glucagon₁₋₂₉.

^d N/A = not applicable.

^e ND, not determined (synthetic [Glu⁹]glucagon₂₋₂₉ was obtained from Bachem (Torrance, CA).

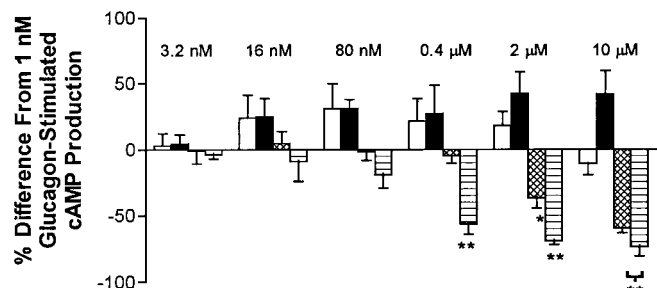


FIG. 2. Characterization of antagonist properties of amino-terminally truncated forms of glucagon on hGlucR cells. Bars represent the mean \pm S.E. of the percentage of difference above or below the cyclic AMP stimulated by 1 nM glucagon₁₋₂₉ ($n = 4$). Cells were preincubated with synthetic glucagon analogs at the concentrations shown for 15 min prior to challenge with 1 nM glucagon, as described under "Experimental Procedures." Refer to Table I for antagonist potencies. Open bars, glucagon₃₋₂₉; filled bars, [pGlu³]glucagon₃₋₂₉; cross-hatched bars, glucagon₅₋₂₉; horizontally striped bars, [Glu⁹]glucagon₂₋₂₉. *, $p < 0.05$; **, $p < 0.01$.

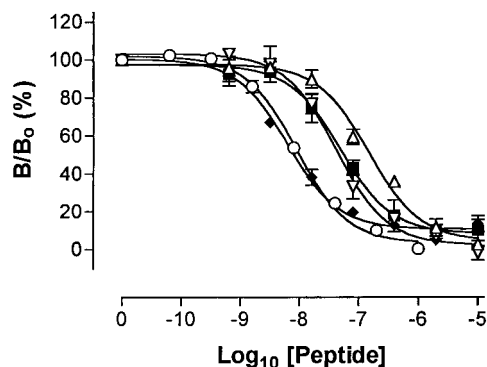


FIG. 3. Competitive binding displacement curves of synthetic glucagon fragments on CHO-K1 hGlucR cells. Data are the mean \pm S.E. of 3–4 experiments. Refer to Table I for binding affinities (IC₅₀). \circ , glucagon₁₋₂₉; Δ , glucagon₃₋₂₉; \blacksquare , [pGlu³]glucagon₃₋₂₉; ∇ , glucagon₅₋₂₉; \blacklozenge , [Glu⁹]glucagon₂₋₂₉.

preinjection levels within 60 min after injection. The amino-terminally truncated peptides glucagon₃₋₂₉, [pGlu³]glucagon₃₋₂₉, and glucagon₅₋₂₉ yielded no change in circulating glucose levels, when administered at a concentration 10 times higher than that of native glucagon (data not shown).² Modification of the amino terminus to generate DPIV-resistant analogs either resulted in enhanced bioactivity ([D-Ser²]glucagon) or reduced bioactivity ([Ser(P)²], [Gly²], and [D-Gln³] substituted glucagon analogs) *in vivo*, relative to equimolar doses of native glucagon (Fig. 7 and Table II). Incubation of modified peptides with porcine kidney DPIV showed that all analogs except [Gly²]glucagon retained bioactivity after the incubation, rela-

tive to glucagon₁₋₂₉ incubated under the same conditions (Table III). The *in vivo* biological potency of [Ser(P)²]glucagon was too weak to draw any conclusions about resistance to serum degradation using a bioassay.

DISCUSSION

Several lines of evidence have resulted in the necessity for reassessment of glucagon degradation *in vivo*. Controversy in the past regarding glucagon degradation, with respect to specific enzymes and organs involved, needs to be clarified. Evidence presented here indicates that dipeptidyl peptidase IV is a prime candidate for enzymatic inactivation of glucagon. The recent finding demonstrating DPIV in the secretory granules of the pancreatic islet α -cell compels one to question how much of the pancreatic glucagon enters the circulation intact (30). Grondin *et al.* (30) further argue that the low pH of the secretory granule would not permit activity of DPIV, and thus DPIV would not be active until granule contents are secreted. The discovery that glucagon is successively hydrolyzed by dipeptidyl peptidase IV into amino-terminally truncated peptides² raises a number of questions. The first question is the role of the hydrolyzed peptides: are they simply degradation products, or do they have a physiological role? The emergence of the "mini-glucagon" story in the pancreas suggests the hypothesis of a local action of amino-terminally truncated glucagon. Processing of glucagon by miniglucagon-generating endopeptidase to glucagon₁₉₋₂₉, results in a peptide having differential effects on cardiac myocytes (31) and having the ability to inhibit insulin release in the picomolar range (32). This report forms the foundation for further work on glucagon degradation products and their possible function *in vivo*.

Fragments similar to those described here have been tested for agonism and antagonism in other biological systems. Glucagon₅₋₂₉ was found to have <0.001% of the potency of native glucagon in the rat hepatocyte membrane adenylyl cyclase activity assay (33), and it was found that this fragment also acted as an antagonist in this tissue ((I/A)₅₀ \approx 71). In the current study, using cells overexpressing the human glucagon receptor, it was found that glucagon₅₋₂₉ has 28.5% of the potency of glucagon₁₋₂₉ (Fig. 1 and Table I), and indeed, it does act as a weak antagonist on these cells (Fig. 2). Similar glucagon analogs to those tested here, [Glu⁹]glucagon₃₋₂₉ and [Glu⁹]glucagon₅₋₂₉, have also been previously characterized (34). The binding affinities reported on the Glu⁹ substituted analogs (34) are consistent with the trend observed with native fragments on transfected cells (Fig. 3); however, the amino acid substitution at position 9 alone ([Glu⁹]glucagon₁₋₂₉) was also shown to have dramatic effects on binding affinity (34). Similarly, [Glu⁹]glucagon₁₋₂₉ had significantly reduced potency, and amino-terminally truncated (desHis¹) peptides showed

TABLE II

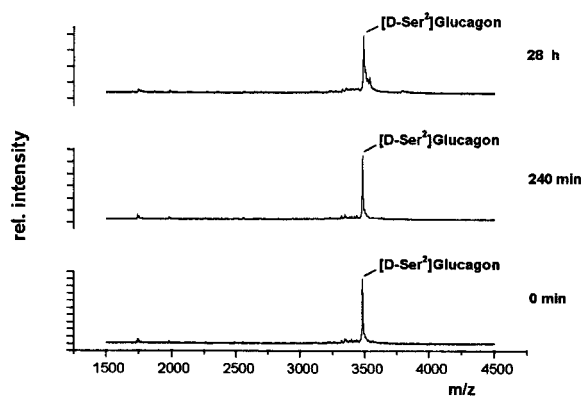
Summary of molecular weights, agonism and binding statistics of amino-terminally modified glucagon analogs on CHO-K1 hGlucR cells or dextran-immobilized DPIV

Data represent mean \pm S.E. ($n \geq 4$). See text for specific methods.

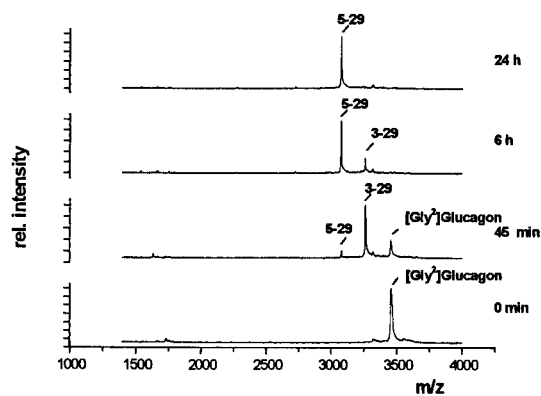
	Calculated molecular weight	Measured molecular weight	Adenylyl cyclase activity		Receptor binding (IC_{50})	DPIV binding (K_d)
			EC_{50}	Maximum cAMP		
			<i>nM</i>	<i>fmol/1000 cells</i>	<i>nM</i>	μM
Glucagon	3482.8	3482.9	0.398 ± 0.058	32.8 ± 1.4	8.83 ± 0.60	2.97 ± 0.01
[D-Ser ²]Glucagon	3482.8	3485.2	1.41 ± 0.41	38.1 ± 3.8	23.6 ± 6.4^a	18.5 ± 0.1^a
[(P)Ser ²]Glucagon	3562.8	3564.1	30.3 ± 7.2^a	31.8 ± 2.0	69.1 ± 5.7^a	19.2 ± 0.2^a
[Gly ²]Glucagon	3451.8	3452.0	1.70 ± 0.67	31.9 ± 1.3	16.6 ± 5.5	22.2 ± 0.1^a
[D-Gln ³]Glucagon	3482.8	3483.9	15.9 ± 10.1^a	45.5 ± 3.2^a	164 ± 79^a	68.0 ± 4.8^a

^a $P < 0.05$ relative to glucagon₁₋₂₉.

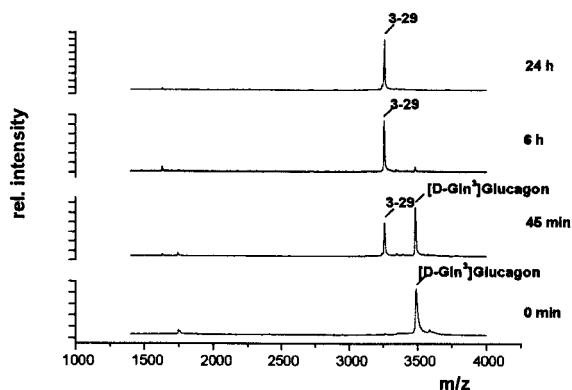
A [D-Ser²]Glucagon



B [Gly²]Glucagon



C [D-Gln³]Glucagon



D [Ser(P)²]Glucagon

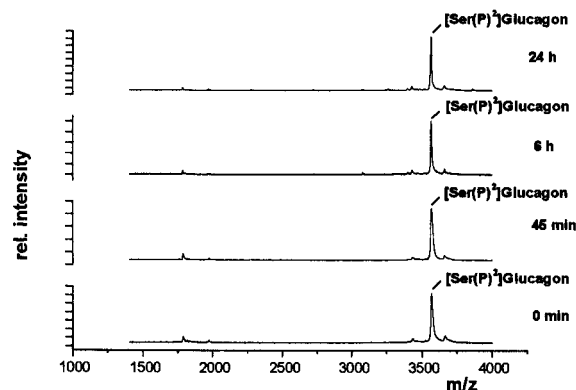


FIG. 4. Degradation of amino-terminally modified glucagon analogs by purified porcine dipeptidyl peptidase IV monitored by MALDI-TOF mass spectrometry. Kinetic analysis of degradation can be found in Table III. Refer to text for specific methods.

negligible adenylyl cyclase stimulating activity (34). In light of the finding that only glucagon₅₋₂₉ showed antagonism on hGlucR cells, it is likely that the Glu⁹ substitution was responsible for the antagonism observed for [Glu⁹]glucagon₃₋₂₉, and resulted in $(I/A)_{50}$ ratios similar to [Glu⁹]glucagon₂₋₂₉, as was also the case for [Glu⁹]glucagon₅₋₂₉ (34). Native glucagon₅₋₂₉ showed only weak antagonism compared with [Glu⁹]glucagon₂₋₂₉ (Fig. 2). The $(I/A)_{50}$ value for [Glu⁹]glucagon₂₋₂₉ obtained in the current study was higher than that reported previously (34); however, it is likely that this is simply due to overexpression of glucagon receptors in the system used for the current study. The activities of the fragments tested support the importance of the amino terminus in glucagon signal transduction. Cyclization of the side chain of Gln³ to form [pGlu³]glucagon₃₋₂₉ increased both binding affinity and potency as compared with glucagon₃₋₂₉ (Fig. 1 and 3). Glucagon₅₋₂₉ also retained high affinity binding (greater than glucagon₃₋₂₉) but showed lower potency when compared with

either [pGlu³]glucagon₃₋₂₉ or glucagon₃₋₂₉.

Surprisingly, the use of heterologous expression systems for the testing of glucagon antagonists have not been reported in the literature, and the hepatocyte adenylyl cyclase assay is the most widely used assay system. The only exception was Hjorth *et al.* (35), who examined the possible inverse agonism of [Glu⁹]glucagon₂₋₂₉ using constitutively active glucagon receptor mutants [H178R] transfected into COS-7 cells. Recently, potent phosphodiesterase inhibitors have been used to characterize "pure" glucagon antagonists (18). The overexpression of the glucagon receptor in CHO-K1 cells has also proven to be highly sensitive to partial agonism and thus may also serve the same purpose.

Characterization of DPIV-resistant, amino-terminally modified glucagon analogs is consistent with published literature. The general conclusion from random molecular mutagenesis screening was that modification of the amino terminus of glucagon reduces biological activity, implicating it as an important

TABLE III

Degradation of glucagon analogs assessed by MALDI-TOF spectrometry and *in vivo* (integrated glucose responses)Data represent mean \pm S.E. ($n \geq 4$). See under "Experimental Procedures" for specific protocols.

	Half-time of degradation: MALDI-TOF spectrometry		Bioactivity Peptide in saline	Bioactivity: Subcutaneous Injection	
	Purified DPIV	20% Human serum		Purified DPIV	Normal rat plasma
	<i>min</i>				
Control ^a			39.3 \pm 13.1	39.3 \pm 13.1	30.2 \pm 10.5
Glucagon	18.8 \pm 0.5	336 \pm 36	144.5 \pm 17.8	27.7 \pm 10.5 ^b	27.2 \pm 8.9 ^b
[D-Ser ²]Glucagon	ND ^c	46.7 \pm 4.0 ^d	187.5 \pm 31.1 ^b	113.0 \pm 10.2 ^c	27.6 \pm 8.5
[(P)Ser ²]Glucagon	ND	584 \pm 72 ^{f,g}	55.2 \pm 10.2 ^b	79.6 \pm 25.4 ^e	19.2 \pm 3.0
[Gly ²]Glucagon	27.7 \pm 2.1 ^g	355 \pm 37	90.5 \pm 15.4 ^b	40.9 \pm 12.7	52.7 \pm 15.2
[D-Gln ³]Glucagon	118 \pm 8.2 ^g	801 \pm 79 ^g	77.3 \pm 11.3 ^b	78.8 \pm 24.8 ^c	35.3 \pm 14.9

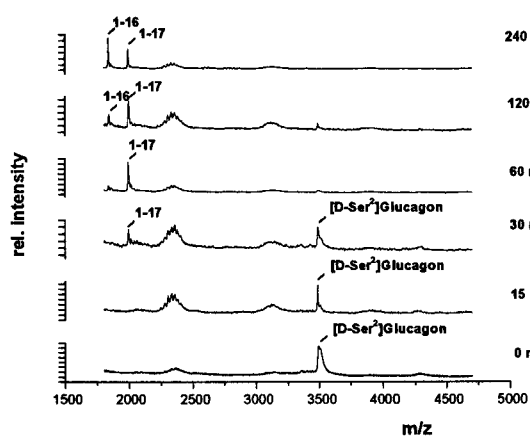
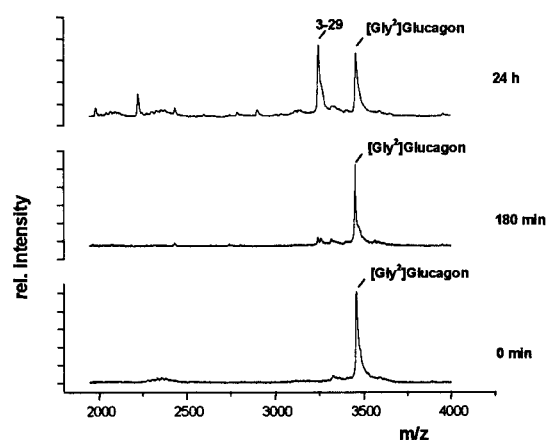
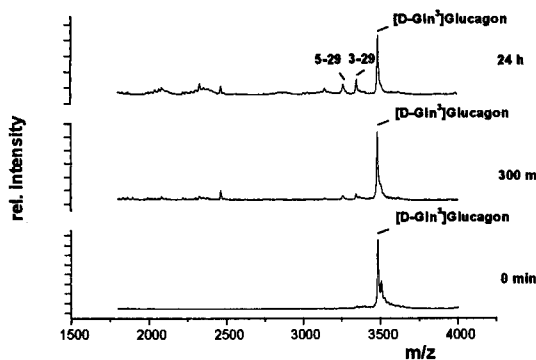
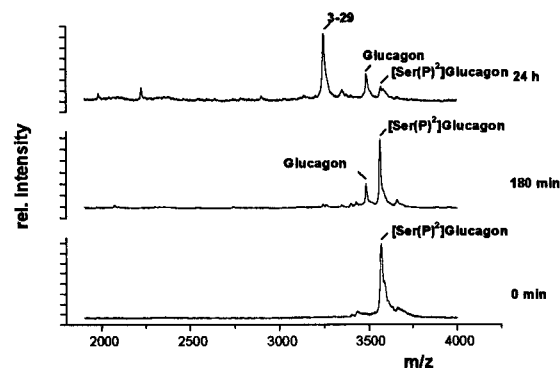
^a Injection or incubation solution without peptide.^b $P < 0.05$ relative to glucagon in saline.^c ND, not detectable.^d Value represents half-time of degradation by trypsin-like enzyme (*i.e.* not DPIV).^e $P < 0.05$ relative to response to glucagon in phosphate-buffered saline + purified DPIV.^f Value represents half-time of dephosphorylation, then degraded as glucagon₁₋₂₉.^g $P < 0.05$ relative to native glucagon degradation.A [D-Ser²]GlucagonB [Gly²]GlucagonC [D-Gln³]GlucagonD [Ser(P)²]Glucagon

FIG. 5. Degradation of amino-terminally modified glucagon analogs by human serum monitored by MALDI-TOF mass spectrometry. Kinetic analysis of degradation can be found in Table III. Refer to text for specific methods. Trypsin-like degradation of [D-Ser²]glucagon is suggested by the generation of fragments corresponding to residues 1–16 and 1–17; the primary sequence of glucagon has arginine residues at positions 17 and 18.

domain necessary for receptor activation (17). Robberecht *et al.* (36) found that altering the chirality at positions 2 and 3 of glucagon has minor effects on potency in the hepatocyte adenylyl cyclase assay; [D-Ser²]glucagon was equivalent to native glucagon in terms of cAMP formation and binding affinity, whereas reversing the chirality of position 3 had significant effects on both parameters. Unson and Merrifield (37) also substituted the D-isomer of serine in position 2; however, they found that it dramatically reduced affinity and potency of this analog *in vitro*. Our work using cells transfected with the

human glucagon receptor is consistent with the earlier studies using hepatocyte membranes (36). The [D-Ser²] substitution was better tolerated than [D-Gln³], when looking at *in vitro* cAMP stimulatory activity and receptor binding affinity (Fig. 6 and Table II).

Due to the difficulty in obtaining second order rate constants from substrates that are hydrolyzed so slowly, and because some of the peptide analogs undergo different degradation fates, the more general parameter of half-life ($t_{1/2}$) was chosen as a general means of comparison for degradation of glucagon

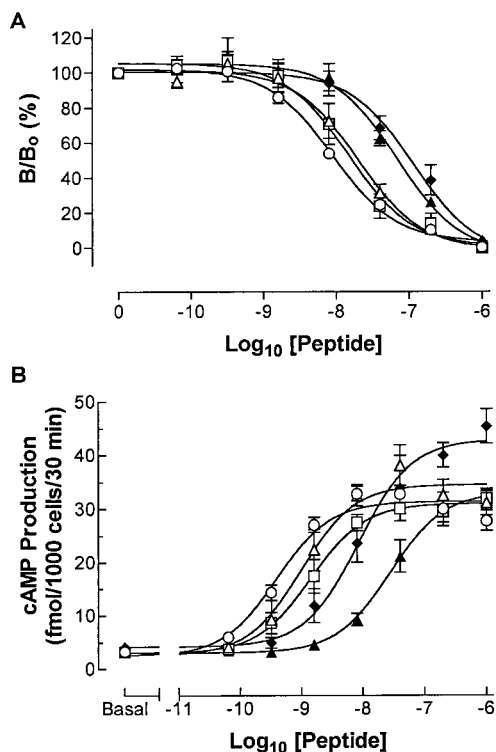


FIG. 6. *In vitro* functional characterization of amino-terminally modified glucagon analogs on CHO-K1 hGlucR cells. *A*, competitive binding displacement curves. *B*, concentration-dependent stimulation of cyclic AMP. Each data point represents the mean \pm S.E. of at least four experiments. Refer to Table II for binding affinities (IC_{50}), potency, and efficacy statistics. \circ , glucagon₁₋₂₉; \triangle , [D-Ser²]glucagon₁₋₂₉; \blacktriangle , [Ser(P)²]glucagon₁₋₂₉; \square , [Gly²]glucagon₁₋₂₉; \blacklozenge , [D-Gln³]glucagon₁₋₂₉.

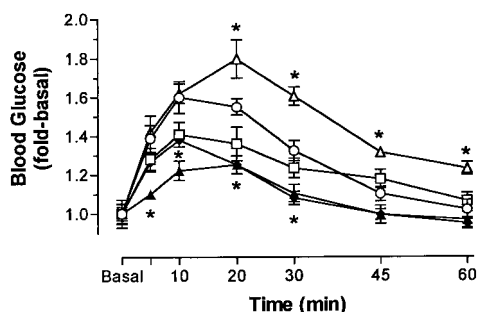


FIG. 7. **Bioactivity of glucagon analogs *in vivo*.** Equimolar doses (7.1 nmol/kg) of glucagon or analog were injected subcutaneously into unrestrained, conscious rats. Each data point represents the mean \pm S.E. of at least four experiments. Whole blood glucose was measured using samples obtained from the tail vein on a SureStep blood glucose analyzer. \circ , glucagon₁₋₂₉; \triangle , [D-Ser²]glucagon₁₋₂₉; \blacktriangle , [Ser(P)²]glucagon₁₋₂₉; \square , [Gly²]glucagon₁₋₂₉; \blacklozenge , [D-Gln³]glucagon₁₋₂₉. *, $p < 0.05$.

and amino-terminally modified peptides (Table III). Using this method, a measure of degradation in human serum could be obtained; however, as only 20% serum was used, values are underestimates of the true degradation by serum. Furthermore, serum DPIV activity represents only a fraction of the DPIV activity *in vivo*, as DPIV is found on endothelial cells and the surface of lymphocytes, among other tissues (10). Using surface plasmon resonance measurements, binding constants (K_d) of amino-terminally modified glucagon analogs and purified DPIV could be obtained, and all showed a significant reduction in affinity (6–23-fold; Table II). However, despite their binding kinetics, analogs exhibited variable resistance to purified DPIV (Table III). [D-Ser²]glucagon had the greatest

affinity for DPIV of the modified peptides but was completely resistant to DPIV degradation; in contrast, [Gly²]glucagon had a K_d similar to that of [D-Ser²]glucagon but was degraded at a rate only slightly slower than native glucagon by purified DPIV. Hence, it appears that a moderate reduction in substrate-enzyme affinity by amino-terminal modification does not necessarily affect the rate-limiting step of DPIV-mediated hydrolysis to a great degree.

Previous studies on [D-Ser²] and [D-Gln³] were limited to *in vitro* structure-function studies. With the objective of generating DPIV-resistant glucagon analogs, to support the hypothesis of DPIV degradation of glucagon, an *in vivo* assay system was necessary. The [D-Ser²] substitution was the only analog that possessed enhanced ability to increase circulating glucose levels relative to native glucagon. The greater potency *in vivo* can be attributed to the lack of degradation by DPIV, as the *in vitro* potency was found to be moderately reduced (Fig. 6). However, this substitution rendered the peptide more susceptible to degradation by trypsin-like enzymes (Fig. 5). Other amino-terminally modified glucagon analogs were not suitable to demonstrate the contribution of DPIV to the degradation of glucagon, as they possessed reduced biological activity *in vitro*, and *in vivo*, or were susceptible to DPIV degradation.

The question remains as to the physiological relevance of DPIV-mediated glucagon hydrolysis. In general, K_d , K_m , or K_i values of peptide substrate/inhibitor-enzyme binding are mainly of theoretical interest, as they are usually 1–5 orders of magnitude greater than circulating levels of peptide. However, if the second-order rate constant of a protease-catalyzed reaction can be determined (e.g. K_{cat}/K_m), values obtained from different peptides can be helpful in interpreting biological significance. The second order rate constant for DPIV-catalyzed glucagon hydrolysis was $2.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, which is comparable to the rate constants for the incretins glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide and glucagon-like peptide-1 and other known DPIV substrates (data not shown)² (20, 24, 26, 38). Notably, the incretins were found to be DPIV substrates *in vitro* (38), prior to demonstration of *in vivo* relevance (39). Pauly *et al.* (24) hypothesized that *in vivo* inhibition of DPIV would enhance the incretin effect, a hypothesis that was later shown to be correct (25, 40, 41). Similar studies investigating the physiological importance of DPIV-mediated glucagon degradation will undoubtedly be forthcoming.

In summary, several structure-activity relationships of glucagon have been assessed *in vitro* and *in vivo*, with specific reference to degradation of glucagon by dipeptidyl peptidase IV. Amino-terminally truncated glucagon fragments were all weak partial agonists of the human glucagon receptor and showed no glycemic effect *in vivo*. The role of DPIV degradation in glucagon metabolism was also studied using amino-terminally modified glucagon analogs. Of these peptides, [D-Ser²] and [Gly²]glucagon were the best tolerated modifications, as assessed by cAMP production and competitive binding studies on hGlucR cells. [D-Ser²] was the only peptide suitable for *in vivo* studies, as [Gly²] was not DPIV-resistant; [D-Ser²] exhibited enhanced biological activity relative to native glucagon in a bioassay. We have thus provided further evidence that DPIV is likely a primary enzyme involved in glucagon degradation. With the foundation of research set, the physiological roles of potentially biologically relevant amino-terminally truncated glucagon peptides remain to be elucidated.

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