The cytoplasmic domain close to the transmembrane region of the glucagon-like peptide-1 receptor contains sequence elements that regulate agonist-dependent internalisation

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Abstract

In order to gain better insight into the molecular events involved in the signal transduction generated through glucagon-like peptide-1 (GLP-1) receptors, we tested the effect of deletions and point mutations within the cytoplasmic tail of this receptor with a view to establishing relationships between signal transduction desensitisation and receptor internalisation. Wild-type and truncated (deletion of the last 27 amino acids (GLPR 435R) and deletion of 44 amino acids (GLPR 418R)) GLP-1 receptors bound the agonist with similar affinity. Deletion of the last 27 amino acids decreased the internalisation rate by 78%, while deletion of 44 amino acids containing all the phosphorylation sites hitherto described in this receptor decreased the internalisation rate by only 47%. Binding of the ligand to both receptors stimulated adenylyl cyclase. In

contrast, deletion of the region containing amino acids 419 to 435 (GLPR 419Δ435) increased the internalisation rate by 268%, and the replacement of EVQ^{408–410} by alanine (GLPR A^{408–410}) increased this process to 296%. In both receptors, the efficacy in stimulating adenylyl cyclase was decreased. All the receptors studied were internalised by coated pits, except for the receptor with a deletion of the last 44 amino acids, which also had a faster resensitisation rate. Our findings indicate that the neighbouring transmembrane domain of the carboxyl-terminal tail of the GLP-1 receptor contains sequence elements that regulate agonist-dependent internalisation and transmembrane signalling.

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Introduction

The internalisation of cell surface receptors is a process that mediates many functions, such as their own desensitisation, resensitisation, and down-regulation processes that modulate receptor responsiveness. The group of G-protein-coupled receptors, whose main function is to transduce information from the extracellular environment to the interior of the cell, initiates this process after a specific ligand has been bound to its receptor, thereby facilitating transmembrane signalling. Both clathrin-coated pits (Ghinea *et al.* 1992, Von Zastrow & Kobilka 1992, Hoxie *et al.* 1993, Von Zastrow *et al.* 1993, Roettger *et al.* 1995) and uncoated endocytic pathways (Raposo *et al.* 1989, Roettger *et al.* 1995) have been suggested to be involved in the internalisation of G-protein-coupled receptors. Clathrin-coated pits require cytoplasmic receptor motifs in order to interact with proteins of the endocytic machinery (Trowbridge *et al.* 1993, Clague 1998). Intracytoplasmic internalisation domains containing tyrosine have been identified for several receptors containing a single trans-

membrane domain (Chang *et al.* 1993, Sorkin & Carpenter 1993, Ohno *et al.* 1995). Endocytic domains have also been identified for several members of heptahelical G-protein-coupled receptors (Ferguson 2001), but no common endocytic motif has been identified. In some cases, sequences including aromatic amino acids are required (Thomas *et al.* 1995, Böhn *et al.* 1997a), while serine- and threonine-rich sequences in the third cytoplasmic loop or cytoplasmic tail are necessary for efficient internalisation to be achieved (Hausdorff *et al.* 1991, Benya *et al.* 1993, Widmann *et al.* 1997). Agonist binding induces the phosphorylation of serine and threonine residues of many G-protein-coupled receptors, and phosphorylated domains are recognised by β -arrestins, which act as adaptor proteins that also bind to clathrin (Ferguson *et al.* 1996, Goodman *et al.* 1996). Dileucine sequences have also been shown to bind adaptor proteins and to promote internalisation through clathrin-coated pits (Ferguson 2001). It is not clear whether both clathrin-coated pits and uncoated endocytic pathways play a similar role. The cholecystokinin receptor–ligand complex internalised by

uncoated pits has been purported to mediate rapid resensitisation of the receptor (Roettger *et al.* 1995).

Receptor desensitisation is a regulatory process through which cells attenuate their biological response (Lohse 1993, Freedman & Lefkowitz 1996, Böhn *et al.* 1997b). G-protein-coupled receptors are desensitised by two mechanisms: homologous desensitisation, which is an agonist-specific response, and heterologous desensitisation, which is a process dependent on the activation of a different receptor. Desensitisation is mediated by receptor phosphorylation and arrestins may bind to phosphorylated receptors and cause uncoupling from G-proteins (Böhn *et al.* 1997b). This process may be followed by internalisation in acidified endosomes (Ferguson *et al.* 1996, Goodman *et al.* 1996). Following this, receptors are dephosphorylated, recycling to the cell surface being necessary for resensitisation (Pipping *et al.* 1995, Garland *et al.* 1996).

Recently, the presence of anchoring and scaffold proteins bound to subcellular structures and associated with protein kinases, protein phosphatases and adaptor proteins has been reported. This may facilitate the action of these proteins involved in the signalling process (Pawson & Scott 1997).

The glucagon-like peptide-1 (GLP-1) receptor is a member of the G-protein-coupled receptor subfamily (Dillon *et al.* 1993, Thorens 1993, Thorens *et al.* 1993, Alvarez *et al.* 1996), which includes secretin, calcitonin, parathyroid hormone (PTH), vasoactive intestinal peptide, glucose-dependent insulintrophic polypeptide, glucagon-like peptide-2 (GLP-2), and glucagon receptors. GLP-1 receptors mediate the effects of GLP-1(7–36)amide or GLP-1(7–37) on peripheral tissues, such as the stimulation of glucose-induced insulin secretion (Kreymann *et al.* 1987, Weir *et al.* 1989), arterial blood pressure, and pulmonary surfactant production (Barragán *et al.* 1994, Benito *et al.* 1998), and in the central nervous system they exert an inhibitory effect on food and water intake (Navarro *et al.* 1996, Tang-Christensen *et al.* 1996, Turton *et al.* 1996, Rodriguez de Fonseca *et al.* 2000). GLP-1 has a therapeutic potential in patients with type 2 diabetes. Treatment with this peptide lowers plasma glucose in diabetic subjects (Toft-Nielsen *et al.* 1999). It has been reported that the internalisation of the GLP-1 receptor is mainly mediated by clathrin-coated pits (Widmann *et al.* 1995), and that it can be prevented through use of a mutant receptor lacking 33 amino acids from the C-terminal tail containing three phosphorylation sites (Widmann *et al.* 1996, 1997). We attempted to use a GLP-1 receptor lacking the 44 amino acids from the C-terminal tail and we found only a 47% slower internalisation rate than with wild-type receptors. In an effort to gain a better understanding of the molecular events involved in the internalisation and signal transduction generated through GLP-1 receptors, here we characterised different mutants of GLP-1 receptors, allowing us to study

the relationship between signal transduction and receptor internalisation. Thus, we tested the effect of deletions and also point mutations of the amino acids E⁴⁰⁸, V⁴⁰⁹ and Q⁴¹⁰ within the cytoplasmic tail of the GLP-1 receptor. Our results indicate that the domain close to transmembrane region VII of the cytoplasmic tail of GLP-1 receptors also has sequence elements involved in the regulation of agonist-dependent internalisation and in the transmembrane signalling process.

Investigation of the signalling and desensitisation of the GLP-1 receptor is of considerable physiological and pathological importance. The activation of signalling through these receptors and the overexpression of the GLP-1 receptor have been proposed as therapeutic strategies for the treatment of neurodegenerative processes and to improve glucose-dependent insulin synthesis and secretion.

Materials and Methods

Mutagenesis of the GLP-1 receptor

A HindIII-XbaI fragment of rat GLP-1 receptor cDNA (Thorens 1993) was subcloned into PUC 18. Site-directed mutagenesis was carried out using the Transformer site-directed mutagenesis kit from Clontech (Palo Alto, CA, USA). A silent mutation strategy was used to create a BfrI restriction site at nucleotide 1322, using a mutagenic primer (5' CCCCTTAAGTGTCCCACCAGC 3') and a selection primer (5' GTGTATCTTATGCCAAGTACG 3') to eliminate a unique NdeI restriction site in the vector. A clone (BfrI) was selected and mutation was confirmed by sequencing, using an automated LKB ALF DNA sequencer (Amersham Pharmacia Biotech, Barcelona, Spain).

PUC18-GLPR BfrI was used to eliminate residues 436–463. The plasmid was cleaved with BfrI and PstI, followed by treatment with mung bean nuclease to generate blunt ends, after which the plasmid was religated. PUC 18-GLPR BfrI was also used to eliminate residues 419–435. In this case, the plasmid was cleaved with BfrI, followed by fill-in of 5' overhangs to generate blunt ends. After Eco47 III digestion, the plasmid was religated. The sequence was confirmed by sequencing, using an automated LKB ALF DNA sequencer. HindIII-XbaI mutant cDNAs were then cloned into the expression vector pcDNA3 (pcDNA3-GLPR 435R and pcDNA3-GLPR 419Δ435).

pcDNA3-GLPR was digested with Eco47 III and PstI, and this was followed by treatment with mung bean nuclease to eliminate single-stranded extensions. Finally, the plasmid was religated (pcDNA3-GLPR 418R). The sequence was confirmed by sequencing, using an automated LKB ALF DNA sequencer.

For substitution of amino acids E⁴⁰⁸, V⁴⁰⁹, and Q⁴¹⁰ by alanine, PCR site-directed mutagenesis was used, employing standard procedures. The following primers were used: two flanking primers – GLPR-1 oligonucleotide,

corresponding to nucleotides 969–993 and GLPR-2 oligonucleotide, corresponding to nucleotides 1384–1411 (Thorens 1993); the specific mutation primers GLPR-3 (5' TGTCAACAATGCGGCCGCGATGGAGTTTCG 3') and GLPR-4 (5' TCCATCGCGGCCGCATTGTT GACAAAG 3') were used to mutate EVQ into alanine residues. Mutation identity was verified by sequence analysis using an automated LKB ALF DNA sequencer. The fragments containing the mutated gene were subcloned into the SacI/PstI restriction sites of pcDNA3-GLPR to create (pcDNA3-GLPR A^{408–410}). GLP-1 receptors were expressed in CHO cells by transfection, using the calcium phosphate method (Chen & Okayama 1987). Transfected cells were selected by incubation with neomycin (400 mg/l) (geneticin, GIBCO, Life Technologies, Barcelona, Spain). Stable colonies were isolated using cloning rings.

Binding of ¹²⁵I-GLP-1(7–36)amide to cell surface receptors

GLP-1(7–36)amide was radiolabelled with ¹²⁵I by the Chloramine T method and mono ¹²⁵I-GLP-1(7–36)amide was isolated by reverse-phase HPLC (Calvo *et al.* 1995). Specific activity was between 3500 and 4000 d.p.m./fmol. Cells grown in 24-well plates (2 × 10⁵/well) were washed with serum-free medium and incubated for 30 min at 37 °C in KRH (118 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 25 mM HEPES, pH 7.4) with 0.5% bovine serum albumin (BSA). Then, cells were chilled on ice, and ¹²⁵I-GLP-1(7–36)amide (0.1 nM) and unlabelled GLP-1(7–36)amide (0–500 nM) were added for 16 h at 4 °C. Following this, cells were washed three times with cold medium and solubilised with 1 M NaOH. Non-specific binding of ¹²⁵I-GLP-1(7–36)amide was estimated in the presence of 500 nM unlabelled ligand, and this value was subtracted from the total binding values. For covalent cross-linking analysis, ¹²⁵I-GLP-1(7–36)amide was bound as described above. The cells were washed twice with cold medium, washed in the same medium without BSA, and cross-linking was initiated by the addition of 0.5 mM disuccinimidyl suberate (DSS) for 15 min at 4 °C. The reaction was quenched with 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA and solubilised with Laemmli sample buffer (Laemmli 1970), and samples were analysed by SDS-PAGE and autoradiography.

cAMP production assays

cAMP levels were measured with the cAMP Biotrack enzyme immunoassay system (Amersham Pharmacia Biotech) following the manufacturer's instructions. Briefly, untransfected CHO cells and cells expressing wild-type and mutant GLP-1 receptors were grown in 12-well plates in F-12 medium supplemented with 5% FBS. CHO cells were washed and incubated in F-12 medium containing 0.1% FBS overnight. Cells were then washed with HBS

medium (130 mM NaCl, 0.9 mM NaH₂PO₄, 0.8 mM MgSO₄, 5.4 mM KCl, 1.8 mM CaCl₂, 20 mM HEPES, pH 7.4, plus 25 mM glucose) and incubated in HBS containing 1 mM IBMX and 0.1% BSA in the presence or absence of different concentrations of GLP-1(7–36)amide (0.001 – 100 nM) for 8 min. Then, cells were washed with HBS, treated with lysis buffer, and immediately processed for immunoassay. In parallel experiments, the binding of ¹²⁵I-GLP-1(7–36)amide to cell surface receptors was determined at 4 °C. Agonist-stimulated cAMP production was normalised by ¹²⁵I-GLP-1(7–36)amide bound to cell surface receptors.

Detection of phospho ERK1/ERK2

CHO cells expressing wild-type and mutant GLP-1 receptors were washed and incubated in F-12 medium containing 0.2% FBS overnight. Cells were treated in the absence or presence of 10 nM GLP-1(7–36)amide for different times and were subsequently lysed in 25 mM HEPES (pH 7.4), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5 mM EDTA, 500 mM NaCl, 50 mM NaF, 5 mM pyrophosphate, 1 mM orthovanadate, 10 μM leupeptin and 1 mM PMSF. Proteins from each sample (50 μg) were resolved by SDS-PAGE and electrotransferred onto a nitrocellulose filter. After blocking in TBS (20 mM Tris, pH 7.4, and 150 mM NaCl) containing 0.1% Tween-20 and 5% non-fat dry milk overnight at 4 °C, the filter was incubated in TBS 2% non-fat dry milk 0.1% Tween-20 with a polyclonal (1: 1000) phospho-specific anti-ERK antibody obtained from New England Biolabs (Beverly, MA, USA) for 1–2 h at room temperature. After washing off excess antibody, the filters were incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1: 3000) (Amersham Pharmacia Biotech) for 1 h at room temperature. Chemiluminescence detection was carried out using the ECL reagents.

Internalisation of the GLP-1 receptor

The internalisation rate was measured as previously described (Alvarez *et al.* 1990). Briefly, CHO cells were incubated in KRH with 0.5% BSA at 37 °C for 30 min. Then, cells were incubated with 0.1 nM ¹²⁵I-GLP-1(7–36)amide, and ¹²⁵I-GLP-1(7–36)amide binding was determined at different times. Intracellular ¹²⁵I-GLP-1(7–36)amide was measured as cell-associated radioactivity resistant to acid wash by incubation of the cells for 3 min at 4 °C with 50 mM NaCl, 150 mM glycine (pH 3.0). Cell surface-bound ¹²⁵I-GLP-1(7–36)amide was calculated by subtraction of the intracellular bound ¹²⁵I-GLP-1(7–36)amide from the total binding.

Redistribution of cell surface GLP-1 receptors

CHO cells were seeded in 24-well plates and incubated with 10 nM GLP-1(7–36)amide for different times (3, 6,

PRIMARY STRUCTURE

Figure 1 Primary structure of the C-terminal tail from the wild-type and mutants of the GLP-1 receptor. The 56 C-terminal amino acids of the wild-type rat GLP-1 receptor and their mutants are shown.

10, 15, 30 and 60 min). Then, cells were washed at 0 °C and subsequently incubated for 3 min at 0 °C with 50 mM NaCl, 150 mM glycine (pH 3.0) to remove ligand bound to the cell surface and washed with KRH containing 0.5% BSA. Cell surface receptors were then measured as described above.

Effect of metabolic inhibitors and hypertonic medium on the internalisation process

For metabolic depletion, CHO cells were washed twice with glucose-free HBS buffer and incubated in the same buffer containing 20 mM 2-deoxyglucose and 10 mM sodium azide. Hypertonic treatment was performed as previously described (Heuser & Anderson 1989, Hansen *et al.* 1993). Briefly, cells were washed twice with HBS containing 0.45 M sucrose and incubated in the same medium for 30 min at 37 °C. Then, cells were incubated with 0.1 nM ¹²⁵I-GLP-1(7–36)amide and after a 6-min incubation cell-associated radioactivity and acid wash-resistant radioactivity were measured as previously described.

Data analysis

Data for competition experiments were analysed by non-linear regression with the GraphPad Prism computer program (GraphPad Software, San Diego, CA, USA). Dissociation constants (K_d) and concentrations of receptor sites (B_{max}) were calculated from IC_{50} values using the method of Cheng and Prusoff (1973). Data are expressed as means ± S.E.M. Statistical comparisons were performed

using the Student's *t*-test and Newman-Keuls test with at least three separate experiments performed in duplicate.

Results

Kinetics of ¹²⁵I-GLP-1(7–36)amide internalisation

A series of mutant receptors containing deletions and point mutations within the cytoplasmic tail of GLP-1 receptors was constructed (Fig. 1).

The rate of GLP-1 receptor internalisation was measured in cells expressing wild-type and GLPR 435R and GLPR 418R mutant receptors after the determination of total cell-associated and acid wash-resistant radioactivity as a function of time. A linear rate of ¹²⁵I-GLP-1(7–36)amide internalisation was observed up to 8 min in cells expressing wild-type and mutant receptors (Fig. 2). The apparent first-order endocytic rate constant was $0.196 \pm 0.016 \text{ min}^{-1}$ for wild-type receptors: i.e. quite different from that obtained with mutant receptors (Table 1). As compared with the cells with the wild-type receptor, the rate of internalisation decreased by 78% in cells expressing receptors with the deletion of 27 amino acids in the C-terminal tail (GLPR 435R), while the deletion of 44 amino acids in the same region (GLPR 418R) produced a 47% decrease. The rate of internalisation of GLP-1 receptors was also similar in a pool of clones expressing the wild-type and mutant GLP-1 receptors.

Binding studies

CHO cells expressing wild-type or mutant receptors were used to analyse their binding properties with

Figure 2 Endocytosis of ^{125}I -GLP-1(7–36)amide by CHO cells transfected with wild-type and mutant GLP-1 receptors. The rate of ^{125}I -GLP-1(7–36)amide endocytosis was measured in CHO cells expressing wild-type, GLPR 435R, GLPR 418R, GLPR A $^{408-410}$ and GLPR 419 Δ 435 GLP-1 receptors. Cells were incubated with 0.1 nM ^{125}I -GLP-1(7–36)amide and total and acid wash-resistant (intracellular) cell-associated radioactivity was measured at different times. The data are represented in the form of an IN/SUR plot, means $\pm$ S.E.M. ($n=6$). IN, intracellular ^{125}I -GLP-1(7–36)amide; SUR, cell surface-bound ^{125}I -GLP-1(7–36)amide. The first-order endocytic rate constants calculated from this data are shown in Table 1.

GLP-1(7–36)amide. The data from the competition binding assays are shown in Fig. 3A. The K_d and B_{max} calculated from the IC_{50} values are shown in Table 2 and revealed a similar pattern of displacement of the radioactive peptide by increasing amounts of the unlabelled peptide in the wild-type, GLPR 435R- and GLPR

Table 1 Kinetic rate constant for GLP-1 receptor endocytosis. The first-order rate constant for the endocytosis of GLP-1 receptors was estimated from the data of Fig. 2. The results represent the means $\pm$ S.E.M. of six independent experiments

Receptor	Endocytosis rate (min^{-1})	% control
Wild-type	0.196 ± 0.016	100
GLPR 435R	0.0426 ± 0.003	21.73
GLPR 418R	0.104 ± 0.011	53.06
GLPR A $^{408-410}$	0.58 ± 0.079	296
GLPR 419 Δ 435	0.458 ± 0.023	233.6

B

Figure 3 Analysis of ^{125}I -GLP-1(7–36)amide binding to wild-type and mutant GLP-1 receptors and cross-linking of ^{125}I -GLP-1(7–36)amide bound to GLP-1 receptors. (A) CHO cells expressing wild-type, GLPR 435R, GLPR 418R, GLPR A $^{408-410}$ and GLPR 419 Δ 435 GLP-1 receptors were incubated with 0.1 nM ^{125}I -GLP-1(7–36)amide and increasing concentrations of unlabelled peptide. The competition binding curves are shown. Data are means $\pm$ S.E.M. ($n=3-6$) and were expressed as percent of maximal specific binding. (B) Covalent cross-linking analysis of ^{125}I -GLP-1(7–36)amide binding to CHO cells expressing wild-type and mutant GLP-1 receptors. The cells were incubated with ^{125}I -GLP-1(7–36)amide in the presence (+) or absence (–) of 0.5 μM unlabelled GLP-1(7–36)amide for 16 h at 4 $^{\circ}\text{C}$. The cells were washed and cross-linking was initiated by treatment with 0.5 mM DSS for 15 min at 4 $^{\circ}\text{C}$. Autoradiograms of ^{125}I -GLP-1(7–36)amide–receptor complexes as indicated by the arrow are shown.

418R- expressing cell lines. The competition binding data of wild-type and mutant receptors best fitted a single-site model.

Table 2 GLP-1(7–36)amide binding parameters and adenylyl cyclase stimulation for wild-type and mutant GLP-1 receptors expressed in CHO cells. The GLP-1(7–36)amide binding parameters were determined by competitive binding assays using CHO cells stably transfected with wild-type and mutant GLP-1 receptors. The data were analysed by non-linear regression and the dissociation constant (K_d) and the concentrations of receptor sites (B_{max}) of these receptors were calculated from IC_{50} values as described in Materials and Methods. The data on adenylyl cyclase activity were analysed using non-linear least squares regression. The agonist potency EC_{50} and maximal GLP-1(7–36)amide-stimulated adenylyl cyclase activity (R_{max}) are shown. Data represent the means $\pm$ S.E.M. of three–six independent experiments

Receptor	¹²⁵ I-GLP-1 binding parameters		Adenylyl cyclase stimulation	
	K_d (nM)	B_{max} (fmol/ 2×10^5 cells)	EC_{50} (nM)	R_{max} (pmol/ 2×10^5 cells)
Wild-type	1.46 $\pm$ 0.08	12 $\pm$ 1.65	0.18 $\pm$ 0.043	6.3 $\pm$ 1.4
GLPR 435R	1.62 $\pm$ 0.17	32.22 $\pm$ 1.91	0.06 $\pm$ 0.024	10.7 $\pm$ 0.6
GLPR 418R	0.35 $\pm$ 0.068	11.83 $\pm$ 3.14	0.11 $\pm$ 0.036	6.6 $\pm$ 0.29
GLPR 419Δ435	0.28 $\pm$ 0.07	0.46 $\pm$ 0.24	0.2 $\pm$ 0.13	3.57 $\pm$ 1.3
GLPR A ^{408–410}	0.25 $\pm$ 0.006	3.19 $\pm$ 0.1	0.073 $\pm$ 0.0125	2.19 $\pm$ 0.016*

* $P < 0.05$ compared with cells expressing wild-type receptors (Newman–Keuls test).

The identification of ¹²⁵I-GLP-1(7–36)amide–receptor complexes using a cross-linking assay is shown in Fig. 3B. In all cases, a single labelled band with a molecular mass of 64–68 kDa in cells expressing the wild-type receptor (68 kDa) and mutant truncated receptor was found; labelling of the ligand–receptor complex disappeared in the presence of unlabelled GLP-1(7–36)amide.

Activation of adenylyl cyclase

Agonist binding to the GLP-1 receptor resulted in the stimulation of adenylyl cyclase, as determined by the increase in intracellular cAMP contents. The dose–response curve after GLP-1(7–36)amide treatment of CHO cells expressing wild-type receptors is shown in Fig. 4. The agonist stimulated cAMP production in a dose-dependent manner in CHO cells expressing wild-type receptors; the EC_{50} of GLP-1(7–36)amide was 0.18 ± 0.04 , maximal stimulation (R_{max}) being observed at 10 nM dose of agonist (Table 2). CHO cells expressing GLPR 435R and GLPR 418R showed a similar potency and efficacy in stimulating cAMP production (Fig. 4, Table 2). Similar results were obtained using a pool of clones expressing the wild-type and mutant GLP-1 receptors.

Effect on mitogen-activated protein kinase pathways

The stimulation of mitogen-activated protein (MAP) kinase pathways by the GLP-1 receptor has been reported. We therefore investigated the ability of wild-type and mutant receptors to interact with this pathway by determining the level of ERK1/ERK2 phosphorylation after GLP-1(7–36)amide treatment (Fig. 5). Stimulation of CHO cells expressing wild-type receptors with 10 nM agonist produced an increase in the phosphorylated forms

of ERK1/ERK2, the maximum effect being observed after 5-min treatment, thereafter decreasing progressively after longer exposure times.

CHO cells expressing GLPR 435R and GLPR 418R receptors showed an increase in phosphorylated ERK1/ERK2 proteins; the maximum effect persisted for 15 min, thereafter decreasing progressively at longer times of exposure to the agonist.

Figure 4 cAMP production after stimulation with GLP-1(7–36)amide. CHO cells expressing wild-type, GLPR 435R, GLPR 418R, GLPR 419Δ435 and GLPR A^{408–410} GLP-1 receptors were stimulated with different concentrations (0.1 nM–1 μM) of GLP-1(7–36)amide for 8 min and the production of cAMP was determined by enzyme immunoassay. The data obtained were normalised by the number of cell surface receptors measured in parallel by binding assays. The data presented are means $\pm$ S.E.M. of three separate experiments performed in duplicate.

Characterization of GLP-1 receptors with a deletion of amino acids 419–435 and effect of the substitution of E⁴⁰⁸, V⁴⁰⁹, and Q⁴¹⁰ by alanine

The effect of the deletion of amino acids 419–435 on the rate of GLP-1 receptor internalisation was measured after the determination of total cell-associated and acid wash-resistant radioactivity as a function of time. As shown in Fig. 2 the apparent first-order endocytic rate constant was $0.46 \pm 0.023 \text{ min}^{-1}$ and the substitution of E⁴⁰⁸, V⁴⁰⁹, and Q⁴¹⁰ by alanine also caused an increase in the rate of endocytosis: $0.58 \pm 0.08 \text{ min}^{-1}$ compared with $0.196 \pm 0.016 \text{ min}^{-1}$, the value for wild-type receptors. In contrast, deletion of amino acids 419–435 and substitution by alanine at residues 408–410 did not modify the potency in stimulating cAMP production (Fig. 4, Table 2). However, the mutant receptors showed a decreased efficacy as compared with the wild-type receptor (Table 2). The data from studies designed to analyse the agonist-binding properties are shown in Fig. 3A. The K_d and B_{max} calculated from the IC_{50} values are shown in Table 2. These data indicate that both mutations produce minor effects on agonist affinity, but the number of cell surface receptors is dramatically reduced as compared with wild-type receptors (Table 2).

Redistribution of GLP-1 receptors in the presence of agonist

The continued exposure to the agonist produces desensitisation, with a progressive decrease in the cell surface numbers of GLP-1 receptors. Accordingly, the effect of 10 nM GLP-1(7–36)amide pretreatment on the number of cell surface GLP-1 receptors was measured (Fig. 6A). In cells expressing wild-type receptors, receptor numbers at the cell surface decreased to $46.6 \pm 4.5\%$ of the initial values during the first 15 min of incubation, thereafter continuing to fall slowly to $36 \pm 3.9\%$ after 1 h. However, the number of cell surface receptors in cells expressing GLPR 435R and GLPR 418R decreased much more slowly, with only a 15–20% reduction after 15 min, while treatment with GLP-1(7–36)amide for only 3 min decreased cell surface receptors to 37% in cells expressing GLPR 419 Δ 435 and to 20% in cells expressing GLPR A^{408–410}.

Effect of metabolic inhibitors and hypertonic medium on the internalisation process

To study whether wild-type and mutant GLP-1 receptors are internalised by clathrin-coated pits, the rate of internalisation was also measured on metabolically depleted cells to analyse whether this process was energy-dependent, and also in the presence of hypertonic medium, which prevents membrane-associated clathrin lattices from being formed and the formation of clathrin-coated vesicles. Under both experimental conditions – metabolic depletion

Figure 5 Effect of GLP-1(7–36)amide on phosphorylation of ERK1/ERK2 in CHO cells expressing wild-type and mutant GLP-1 receptors. CHO cells were treated with 10 nM GLP-1(7–36)amide for the indicated periods of time. Then, cell extracts were processed by SDS-PAGE and Western blot. Immunoblots against phospho-ERK1/ERK2 for cells expressing wild-type (A), GLPR 435R (B), and GLPR 418R (C) GLP-1 receptors are shown. The positions of immunoreactive phosphorylated forms of MAP kinase (MAPK) proteins 44 and 42 kDa are indicated by the arrows. Similar results were obtained in three separate experiments. (D) Relative percentage of MAPK phosphorylation after stimulation with 10 nM GLP-1 at different times. * $P < 0.05$ compared with cells expressing wild-type receptors (Newman-Keuls test).

and hypertonic treatment – a similar degree of inhibition of internalisation in cells expressing wild-type (86.5%, 88%), GLPR 419 Δ 435 (75.4%, 80.7%) and GLPR A^{408–410} (75%, 72%) receptors was observed (Fig. 6B). The effect of

metabolic inhibitors and hypertonic medium was lower in cells expressing GLPR 435R receptors (41.5% and 46.5%), while in cells expressing GLPR 418R receptors

the inhibitory effect of metabolic depletion was 54.3% and the presence of hypertonic medium elicited a reduction of 31%.

Discussion

Previous studies have indicated that the internalisation of GLP-1 receptors is dependent on phosphorylation in the 33 amino acid C-terminal tail. Our results show that the region in the neighbourhood of the VII transmembrane domain also contains additional sequence elements that regulate receptor internalisation. Thus, the results obtained in cells expressing C-terminal truncated mutants showed that deletion of the last 27 amino acids (cytoplasmic tail of 29 amino acids) decreased the internalisation rate by 78%. These results are in agreement with previous findings using a mutant receptor lacking 33 amino acids in the C-terminal tail (Widmann *et al.* 1997). However, deletion of the 44 amino acids (cytoplasmic tail of 12 amino acids) elicited only a 47% slower internalisation rate than wild-type receptors. In an attempt to gain better insight into this process, we also studied the relationship between internalisation and other functional properties of GLP-1 receptors.

Truncated GLPR 435R and GLPR 418R mutant receptors bound the agonist with similar affinity to that of the wild-type receptors, but in cells expressing GLPR 435R the number of cell surface receptors was increased. The cross-linking assay also revealed that GLP-1(7–36)amide was specifically bound to those receptors.

To investigate the relationship between internalisation and signal transduction, we explored the ability of the different mutants of the GLP-1 receptor to stimulate adenylyl cyclase as well as the MAP kinase pathway. Our data revealed that receptors with a deletion of the last 27 or 44 amino acids of the cytoplasmic tail conserved a similar potency and efficacy in stimulating adenylyl cyclase. These data agree with the results reported using a mutant

Figure 6 Cell redistribution of wild-type and mutant GLP-1 receptors induced by their agonist, and the effect of metabolic inhibitors and hypertonic medium on endocytosis of wild-type and mutant GLP-1 receptors. (A) The effect of pretreatment with 10 nM GLP-1(7–36)amide for different periods of time on cell surface expression of GLP-1 receptors was investigated. Cells were incubated in the presence of agonist for different times. They were then washed and the ligand bound to the cell surface was removed by acid wash. Finally, [¹²⁵I]-GLP-1(7–36)amide binding to cell surface receptors was measured. Results are means ± S.E.M. (*n*=6). (B) CHO cells expressing wild-type or mutant GLP-1 receptors were preincubated in the absence (control) or presence of either hypertonic media or metabolic inhibitors for 30 min. Then, 0.1 nM [¹²⁵I]-GLP-1(7–36)amide was added and after 6 min of incubation total and acid wash-resistant cell-associated radioactivity was measured. The percentage of the rate of endocytosis was determined. Data represent means ± S.E.M. of three independent experiments performed in triplicate.

receptor lacking 33 amino acids from the C-terminal tail (Widmann *et al.* 1996) and similar results have been reported for glucagon receptors with C-terminal deletions (Buggy *et al.* 1997). The effect on the MAP kinase pathway was observed as an increase in phosphorylated forms of ERK-1/ERK-2 promoted by agonist stimulation in cells expressing wild-type GLP-1 receptors. Similar results have been reported previously in both RIN and CHO-GLPR cells (Montrose-Rafizadeh *et al.* 1999). Other members of this family of G-protein-coupled receptors, such as PTH receptors, also undergo increases in the phospho-ERK-1 form following agonist stimulation (Verheijen & Defize 1997). The effect observed in cells expressing GLPR 435R and GLPR 418R mutant receptors was similar, but the increase in phosphorylated forms of ERK-1/ERK-2 persisted longer. It has previously been reported that stimulation of the MAP kinase pathway by β -adrenergic receptors requires the interaction of the ligand-receptor complex with adaptor proteins involved in the endocytic process, such as β -arrestins (Barlic *et al.* 2000). In our studies, we failed to observe any correlation between the phosphorylation of MAP kinases and the rate of internalisation.

Our data suggest an inhibitory role of the region containing amino acids 419–435 in the internalisation process. In further experiments, we analysed the effect of deletion of amino acids 419–435 and we also checked the effect of substitution by alanine of the three amino acids, E⁴⁰⁸, V⁴⁰⁹ and Q⁴¹⁰, close to transmembrane region VII, which are conserved in several members of this G-protein-coupled receptor subfamily. Mutant GLPR 419 Δ 435 and GLPR A^{408–410} receptors internalised faster (between 2.3 and 2.96 times) and showed a reduced cAMP production as compared with wild-type receptors. Deletion of residues 419–435 and substitution of EVQ by alanines in the GLP-1 receptors decreased the efficacy in stimulating adenylate cyclase activity by approximately 1.8- and 3-fold respectively. These results are in conflict with the notion that the production of this second messenger constitutes the switch to initiate the internalisation process (Widmann *et al.* 1995). Also, mutant receptors that fail to stimulate G-proteins show internalisation deficiencies (Behya *et al.* 1994, Moro *et al.* 1994). Our data are consistent with the results found for mutant receptors that interact with Gs and Gq but that show deficiencies in the internalisation process or those that do not interact with Gs and Gq and are internalised correctly (Cheung *et al.* 1990, Hunyady *et al.* 1994). Our data also suggest that endocytosis of the ligand-receptor complex favours its uncoupling from G proteins. Mutant receptors that internalised faster had a reduced cAMP production with a similar EC₅₀ potency. Our data also indicate a correlation between the rate of internalisation and the number of cell surface receptors. Thus, the highest level of GLP-1 receptors at the cell surface was found in cells expressing receptors deficient in internalisation (GLPR 435R), and cells expressing

receptors that internalised faster than the wild-type (GLPR A^{408–410} and GLPR 419 Δ 435) expressed a reduced number of cell surface receptors.

The effects observed in the internalisation and signalling of mutant GLPR 419 Δ 435 and GLPR A^{408–410} receptors might be due to conformational changes in the C-terminal region of the receptor, promoting the interaction of these mutant receptors with adaptor proteins and a faster internalisation. A conformational change of the C-terminal tail has also been proposed to explain the results obtained after mutation of a small region in the proximal portion of the carboxyl tail in β -adrenergic receptors (Hausdorff *et al.* 1991). However, in that case the substitution of residues in this region blocked the sequestration and phosphorylation of these receptors. The region of receptor amino acids from 419–435 contains Ser^{431/432}, the consensus sequence for protein kinase C. The absence of these residues might regulate the phosphorylation of three doublets involved in the internalisation process: Ser^{441/442}, Ser^{444/445} and Ser^{451/452}. However, we have previously reported that substitution of Ser^{431/432} by alanine did not modify either the rate of endocytosis or the potency or efficacy of these receptors in stimulating adenylate cyclase (Vázquez *et al.* 2005).

The differences in the rate of internalisation also affected the redistribution promoted by agonist exposure. After 15-min exposure with GLP-1(7–36)amide, cells expressing wild-type receptors conserved 45–50% of their cell surface receptors. These data are comparable to the results reported for glucagon receptor internalisation: 40–50% of cell surface receptors were internalised in 10 min (Buggy *et al.* 1997). Cells expressing the truncated mutant receptors GLPR 435R and GLPR 418R, in contrast to cells expressing the wild-type, preserved 80–85% of the initial cell surface receptor numbers. However, cells expressing GLPR 419 Δ 435 and GLPR A^{408–410} conserved only 37% and 20% of their cell surface receptors respectively after 3-min exposure to the agonist. All these data indicate that, after agonist exposure, cell surface receptors are essentially dependent on the rate of endocytosis, although our findings also suggest differences in the recycling time. Cells expressing GLPR 418R receptors maintained a number of cell surface receptors after exposure to the agonist, similar to cells expressing GLPR 435R receptors; however, the rate of endocytosis was twofold slower for the latter type of receptor.

The data concerning internalisation for receptors lacking the 44 amino acids of the C-terminal tail led us to investigate whether these receptors were internalised through coated pits or through a different pathway. This region contains all the phosphorylation sites so far identified for GLP-1 receptors and previously described to mediate the process of endocytosis by coated pits. The results obtained after hypertonic medium treatment revealed that GLP-1 receptors are mainly internalised by coated pits in cells expressing wild-type and mutant

receptors, except in those expressing GLPR 418R, and that 15–25% of the rate of endocytosis measured was not dependent on metabolic energy. In the endocytosis experiments, intracellular-associated radioactivity was measured after an acid wash, a procedure that in all cases eliminated 80–90% of the radioactivity bound to the cell surface. The percentage of internalisation not dependent on metabolic energy was higher for cells expressing truncated receptors. It is possible that some of these receptors could enter the cell through a pathway that is not dependent on metabolic energy. The presence of hypertonic medium in cells expressing GLPR 418R receptors did not disturb the internalisation of 30% of the receptors, suggesting that these receptors might be internalised by both coated and uncoated pits. Similar results have been reported previously for cells expressing truncated PTH receptors (Huang *et al.* 1995). These data could also explain the faster rate of resensitisation in cells expressing GLPR 418R receptors. The cholecystokinin A receptor has been reported to be internalised through coated pits and uncoated pits and the authors of that paper suggested that the uncoated pit pathway might be involved in the rapid resensitisation of those receptors (Roettger *et al.* 1995).

Our results show that the proximal portion of the carboxyl tail of GLP-1 receptors contains sequence elements that regulate agonist-dependent internalisation. Mutations in this GLP-1 receptor region might change the conformation of the cytoplasmic tail and hence alter its functional activity.

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