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Internalization of pancreatic polypeptide Y4 receptors: correlation of receptor intake and affinity

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Abstract

Unlike neuropeptide Y receptors, the pancreatic polypeptide Y4 receptors display considerable differences in sequence and ligand-binding affinity across mammalian species. This could produce different receptor turnover rates in the same cellular membrane environment. Comparing rat, human and guinea-pig Y4 receptors expressed in Chinese hamster ovary (CHO) cells (K_d with human pancreatic polypeptide 14, 45 and 116 pM, respectively), we indeed found human pancreatic polypeptide internalization in the rank order of receptor affinities. A large fraction of the internalized human pancreatic polypeptide, similar across the Y4 species, was associated with secondary endosomes (density ≈ 1.05 in Percoll gradients) and lysosomes (density ≈ 1.11). For all Y4 receptors examined, this intake was potently and selectively inhibited by cholesterol-complexing polyene antibiotic filipin III and also by clathrin lattice formation inhibitor, phenylarsine oxide. Internalization differences found across Y4 receptor species to a degree compare with those observed for the cloned guinea-pig neuropeptide Y Y1 and human neuropeptide Y Y5 receptors and, generally, support ligand-binding affinities as important determinants of internalization for neuropeptide receptors.

Keywords: Buoyant density separation; Endocytosis rate; Endosomal sorting; Lysosomal sorting; Selective agonist endocytosis; Receptor affinity; Receptor conservation

1. Introduction

Several receptors for neuropeptide Y and related peptides ('Y receptors') are characterized by a high affinity of ligand binding (see, e.g. Lundell et al., 1996; Berglund et al., 1999; Parker et al., 2001a), which may mandate internalization as a regulatory mechanism to prevent oversignaling. In the case of neuropeptide Y Y1 receptors, a strong internalization was reported for both native (Fabry et al., 2000) and clonal (Gicquiaux et al., 2002; Parker et al., 2001c; Pheng et al., 2001) Y1 expressions. A significant internalization was also shown for cloned rat pancreatic polypeptide Y4 receptors (Parker et al., 2001c). The pancreatic polypeptide Y4 receptors poorly respond to neuropeptide Y and peptide YY (Gehlert et al., 1996; Balasubramaniam et al., 2001) and could be attuned to low extracellular levels of their polypeptide agonists, typically below 25 pM in blood of the rat (Akpan et al., 1992) and mouse (Havel et al., 1993) and below 50 pM in human or canine blood (e.g. Beglinger et al., 1981). The pancreatic polypeptides are known to be released in response to feeding, or even post-prandially (e.g. De Jong et al., 1986), and the activation of Y4 receptors could contribute to an inhibition of anabolic activities, e.g. via inhibition of adenylyl cyclase (Schober et al., 1998). This is especially of interest with respect to a strong association of pancreatic polypeptides with Y4 receptors (Balasubramaniam et al., 2001; Parker et al., 2001a), including the internalized receptor ligand complex (Parker et al., 2001c), which could lead to a protracted Y4 signaling.

Unlike the neuropeptide Y Y1 or Y2 receptors, the pancreatic polypeptide Y4 receptors show significant differences in sequence across mammalian species (Gehlert et al., 1996; Lundell et al., 1997; Eriksson et al., 1998; Wraith et al., 2000). Expressed in the same membrane environment of the Chinese hamster ovary (CHO) cells, the rat, human

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and guinea-pig Y4 receptors show affinities ranging from 10 to 150 pM (Parker et al., 2001a), which could lead to significant internalization differences. This study indeed confirms pancreatic polypeptide internalization in the rank order of respective affinities for the Y4 receptor expressions studied.

2. Experimental procedures

2.1. Chemicals

Human pancreatic polypeptide and human/rat neuropeptide Y were obtained from the American Peptide (San Diego, CA, USA). Cell culture media and supplements were obtained from Gibco BRL (Long Island, NY, USA). Percoll gradient-related chemicals were from Amersham-Pharmacia Biotech (Piscataway, NJ, USA). Molecular weight markers and gel chromatography supplies were from Bio-Rad (Hercules, CA, USA). All other chemicals were from Sigma (St. Louis, MO, USA). Filipin III (FIII) and phenylarsine oxide (PAO) were dissolved in dimethylsulfoxide and stored aliquoted at −80 °C. Cholesteryl hemisuccinate was also stored frozen as 10 mM emulsion in water.

2.2. Labeled compounds

Iodinations of human pancreatic polypeptide, rat pancreatic polypeptide and human neuropeptide Y were performed as described (Parker et al., 1998). After radiolabeling and separation by our procedure, the radioactive Y peptides were 75–90% monoiodinated and had specific activities in the range of 1500–1800 Ci/mmol (70–80% theoretical), as deduced by comparison in saturation assays with HPLC-purified monoiodinated [^{125}I]-labeled Y peptides supplied by Perkin-Elmer/NEN (Cambridge, MA, USA; specific activity 2170 Ci/mmol). Human transferrin was iodinated by the same protocol as the Y peptides. [^{35}S]guanosine-5′-*O*-(thiotriphosphate) (GTP-γ-S) (~ 1600 Ci/mmol) was also supplied by Perkin-Elmer/NEN.

2.3. Cell cultures and labeling

The cloned pancreatic polypeptide rat Y4 receptor (Lundell et al., 1996), human Y4 receptor (Eriksson et al., 1998), and guinea-pig Y4 receptor (Eriksson et al., 1998), and guinea-pig neuropeptide Y Y1 receptor (Berglund et al., 1999) were all expressed in CHO cells. The cloned human neuropeptide Y Y5 receptor was expressed in human embryonic kidney-293 (HEK-293) cells (Dumont et al., 2000). All cells were cultured in F-12/Dulbecco's modified Eagle medium (F-12/DMEM) at 250 μg/ml of geneticin and 2 mM L-alanyl–L-glutamine (GlutaMax1). At full confluence, the cell count was 190,000–235,000/cm^2. The number of receptors per cell in the above medium was stable over at least 20 passages with any of the cell lines used. The rat and human Y4 receptor-expressing CHO cells were each available at three receptor densities, produced by different inoculums of the transfecting complementary DNAs. Most kinetic and inhibition studies were done with 48-well (0.8 cm^2/well) plates, while the gradient characterization experiments were done with 6-well (9.4 cm^2/well) plates. The labeling with [^{125}I] peptides was done at 50 pM, using the corresponding nonlabeled peptides at 300 nM for the nonspecific binding correction. The labeling with [^{125}I]human transferrin was performed at 1 nM, with nonspecific binding correction at 10 μM unlabeled human transferrin. The [^{125}I]human neuropeptide Y tracer bound to cell-surface neuropeptide Y Y1 or Y5 receptors was extracted by 0.2 M CH_3COOH–0.5 M NaCl for 12 min at 0–4 °C. The [^{125}I]human pancreatic polypeptide tracer bound to surface Y4 sites was extracted either by the above procedure, or with 1 M potassium thiocyanate (KCNS; pH 7.4) for 15 min at 15 °C (for molecular size characterization of the internalized receptor ligand complexes). After solubilization of the 1 M KCNS residues at 10 mM cholate (10 min at 0–4 °C), the receptor ligand complex was precipitated by bovine γ-globulin and polyethyleneglycol (Parker et al., 1998). Free [^{125I}]Y ligands at levels up to 5 pM (which with the final precipitation volume of 0.9 ml exceeded the concentration found for internalized steady-state specific binding in all cases) essentially were not precipitated in the polyethyleneglycol procedure.

2.4. Receptor characterization

Particulate receptors were assayed as described (Parker et al., 2001a), terminating the assay by centrifugation for 15 min at 16,000 × g_{max}. The binding properties of the cell-surface receptors were characterized on monolayer cultures in Opti-Mem® medium (Gibco), in some cases after solubilizing the particulates with 10 mM sodium cholate at 0–4 °C over 10 min, with minimal shearing (see below). Sucrose gradient characterization of solubilized receptors and receptor G-protein complexes was done on linear 10–30% sucrose gradients (volume 9.2 ml) made in a buffer containing 10 mM HEPES·NaOH (pH 7.4), 4 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.05% BSA. The receptor-containing particulates, after labeling (90 min at 24 °C) with [^{125}I]-Y peptides at 50 pM or with [^{35}S]GTP-γ-S at 20 pM and surface washing to remove the unbound tracer, were resuspended in the above buffer and then brought to 10 mM sodium cholate at 0–4 °C, and the mixture slowly passed three times through a 23-gauge needle prior to loading onto gradients. The gradients were centrifuged for 12 h at 35,000 rpm. (218,000 × g_{max}) in the SW41 Ti rotor of the Beckman (Palo Alto, CA, USA) M-8 ultracentrifuge, at 5 °C, and then divided into 30 fractions prior to assessment of radioactivity distribution. Radioactivity in aliquots of the gradient fractions was measured after polyethyleneglycol precipitation (Parker et al., 1998). Immunoblotting of Gi1 and Gq α-

subunits was done using the SuperSignal chemiluminescent substrate and protocol (Pierce, Rockford, IL, USA).

2.5. Percoll gradient centrifugation experiments

For experiments employing Percoll gradients, the cells were incubated for indicated periods at 37 °C with appropriate drugs and radioactive peptides, then washed three times in ice with cold incubation medium, and once with 0.25 M sucrose–10 mM HEPES·NaOH (pH 7.4). The cells were then scraped into the sucrose solution (2 ml/well of 9.4 cm^2), and the suspensions homogenized by nitrogen cavitation, employing a stainless steel bomb (Kontes Glassware, Vineland, NJ, USA) immersed in ice, at an N_2 pressure of 900–950 kPa over 7 min. The disrupted cells were released dropwise and sedimented for 5 min at $500 \times g_{max}$ to remove nuclei and debris (which contained less than 2% of total cell-bound pancreatic polypeptide or neuropeptide Y receptor agonist peptide). This procedure produced essentially the same yields of plasma membranes and endosome-like particulates as the homogenization in a hypotonic medium (six passages through a 25-gauge hypodermic needle at flow rates below 10 ml/min in 10 mM HEPES·NaOH (pH 7.4) containing 0.05% bovine serum albumin). However, the cavitation procedure resulted in much lower solubilization and redistribution of a lysosomal marker enzyme, acid *N*-acetyl-β-D-hexosaminidase; hence, it was used in all experiments employing cell homogenates. The $500 \times g$ supernatants of individual homogenates (1.5 ml) were applied over progradients consisting of layers of 1 ml 60% sucrose, 5 ml 18% (v/v) Percoll (Amersham Pharmacia) and 4 ml 10% (v/v) Percoll (both Percoll solutions in 0.25 M sucrose–10 mM HEPES·NaOH, pH 7.4), precooled to 5 °C in 14 ml UltraClear® tubes for the SW41 Ti rotor. The tubes were centrifuged for 55 min at 5 °C in a Beckman M-80 ultracentrifuge using SW41 Ti rotor at 20,000 rpm. ($68{,}000 \times g_{max}$). The tube contents were divided into 20 fractions prior to determination of radioactivity (in polyethyleneglycol-precipitated aliquots) and enzyme activity. Density at the end of centrifugation was determined from distribution of Percoll density marker beads (Amersham-Pharmacia).

2.6. Acid hexosaminidase assay

The activity of a lysosomal marker, acid β-*N*-acetyl-D-hexosaminidase (EC 3.2.1.52) was assayed using 50 μl aliquots of gradient fractions in 96-well plates, in a volume of 0.2 ml/assay, by the method of Burton and Lloyd (1976). The substrate was *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide at 2 mM final, in 0.1 M sodium acetate buffer (pH 5.0) containing 0.1% Triton X-100. After incubation of 2–4 h at 37 °C, the reactions were stopped by adding 1/4 volume of 0.5 M Na_2CO_3, and the absorbance of *p*-nitrophenol was quantitated at 420 nm in a SpectraMax 250 (Salt Lake City, UT, USA) microplate reader.

2.7. Data evaluation

Binding parameter calculations were done in the LIGAND program (Munson and Rodbard, 1980). Multiple comparisons following a positive analysis of variance (ANOVA) were done in Tukey's *t*-test (Zar, 1984).

3. Results

3.1. The internalized pancreatic polypeptide is largely found as a stable complex with the Y4 receptor, which may contain G-protein α-subunits

After short labeling (3 min), the 10 mM cholate-solubilized Y4-cell-bound [^{125}I]human pancreatic polypeptide not extracted by 1 M KCNS was 80–90% precipitated by polyethyleneglycol. In conditions of steady-state binding (40–60 min labeling), the polyethyleneglycol-precipitated fraction after cholate solubilization was still about 60% of the KCNS residue binding for the rat Y4 and about 40% for the guinea-pig Y4 receptor. The 1 M KCNS-defined internalized steady-state binding of the Y1/Y5 agonist human neuropeptide Y in guinea-pig Y1-CHO cells and human Y5-HEK-293 cells was about 35% and 20% precipitated by polyethyleneglycol, respectively. It should be noted that either the acid saline or the 1 M KCNS treatment dissociated more than 93% of peptide ligands specifically bound to

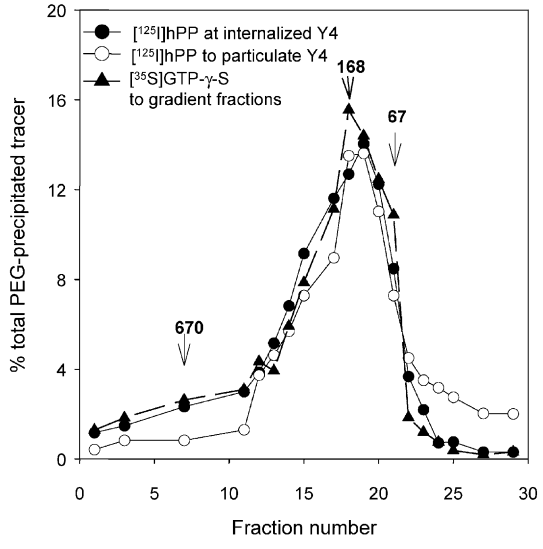

Fig. 1. Sedimentation profiles of [^{125}I]human pancreatic polypeptide-labeled human Y4 receptors and [^{35}S]GTP-γ-S-labeled G-proteins solubilized from CHO cell monolayers or cell particulates. Sodium cholate at 10 mM was used at 0–4 °C to solubilize monolayer residues after extraction of cell-surface-bound ligand and of [^{35}S]GTP-γ-S binding to G-protein receptor complexes from gradient fractions after cholate solubilization of particulates from disrupted cells. The '67', '168' and '670' labels mark the sedimentation positions of bovine serum albumin (Mr ~ 67,000), bovine γ-globulin (Mr ~ 168,000) and bovine thyroglobulin (Mr ~ 670,000), respectively. The sedimentation was performed for 12 h at $218{,}000 \times g_{max}$ and 5 °C. For further details, see Section 2.4.

digitonin-permealyzed monolayers of neuropeptide Y1 receptor-expressing CHO cells, to particulate pancreatic polypeptide Y4 receptors, or to particulate neuropeptide Y Y1 and Y5 receptors (data not shown).

A large fraction of the internalized human pancreatic polypeptide tracer (more than 70% following CHO monolayer labeling of 20 min at 37 °C) was detected after solubilization by cholate as 70–200 kDa material in sucrose gradients, as already found for the rat Y4 receptor (Parker et al., 2001c). The sedimentation of [^{125}I]human pancreatic polypeptide associated with the particulate rat Y4-CHO receptor solubilized after in vitro labeling was similar (Fig. 1). A similar molecular size distribution was found for the labeling by [^{35}S]GTP-γ-S of cholate-solubilized GTP-binding proteins in gradient fractions from rat Y4-CHO cells (Fig. 1). The steady-state internalized Y4 binding was less than 25% detached by GTP-γ-S at up to 100 μM. However, more than 50% of the binding could be dissociated by GTP-binding protein antagonist peptides melittin ($K_i = 13.7 \pm 2$ μM; $n = 3$) or mastoparan-7 ($K_i = 19.4 \pm 2.9$ μM; $n = 2$), indicating a significant association of GTP-binding proteins with the internalized 70–200 kDa rat Y4 receptor ligand complex. G-protein immunoblotting of gradient fractions found abundant Gi1α-subunit and also Gq α-subunit in the 70–200 kDa zone (data not shown).

3.2. A comparison of the internalization of Y4 receptor from three mammalian species with guinea-pig Y1 and human Y5 receptors

After 40 min at 37 °C, tracer depletion by degradation was less than 20% for either human pancreatic polypeptide or human neuropeptide Y with any cell line used (data not shown); that is, the observed binding was little influenced by loss of the available tracer. The time course of tracer binding saturation was not radically different across the compared Y4 and Y1 receptor species for either the internalized or the surface-bound fraction (Table 1). Among pancreatic polypeptide Y4 receptors, binding to CHO cells expressing the rat Y4 receptor saturated somewhat faster than that to the human or the guinea-pig species (Table 1). It should be noted that the kinetic parameters in Table 1 reflect multiple binding, internalization and recycling components, which could be dissected by a panel of inhibitors of specific steps or events for each receptor, supported by mathematical modeling. The above kinetic comparisons are in good agreement with our previous report (Parker et al., 2001c) and indicate a sufficient saturation of the cell surface as well as the internalized binding for the Y4 and the Y1 receptors over 40 min at 37 °C. Very similar internalization patterns were observed for rat pancreatic polypeptide in rat Y4 cells

Table 1
Parameters of internalization at 37 °C of Y receptors expressed in cell lines

Receptor and cell type	$T/2$ external	B_{max} external	$T/2$ internal	B_{max} internal
Rat Y4-CHO	6.9 ± 0.75	2.91 ± 0.18	7.2 ± 0.6	2.38 ± 0.14
Human Y4-CHO	8.8 ± 1.4	1.73 ± 0.08	12.3 ± 4.9	0.47 ± 0.04
Guinea-pig Y4-CHO	9.5 ± 2.5	0.61 ± 0.04	11.1 ± 1.3	0.23 ± 0.02
Guinea-pig Y1-CHO	11.7 ± 0.82	1.21 ± 0.08	8.9 ± 1.7	2.25 ± 0.12
Human Y5-HEK-293	6.9 ± 0.4	2.15 ± 0.15	37.3 ± 4.2	0.26 ± 0.02

$T/2$ is the half-period of saturation of the binding (in min), and B_{max} is the estimate of the maximum fmol ligand bound per 100,000 cells. The binding parameters were derived by nonlinear hyperbolic curve fitting (in Rodbard's implementation (Munson and Rodbard, 1980) of the Marquardt–Levenberg algorithm) based on Michaelis–Menten equation for enzyme kinetics. The labeling was done with 50 pM of a [^{125}I] agonist (human pancreatic polypeptide for the Y4 receptors and human neuropeptide Y for the Y1 and Y5 receptors), for 40 min at 37 °C in the OptiMem® buffer. The internalized and surface-bound tracers were separated as described in Section 2.5.

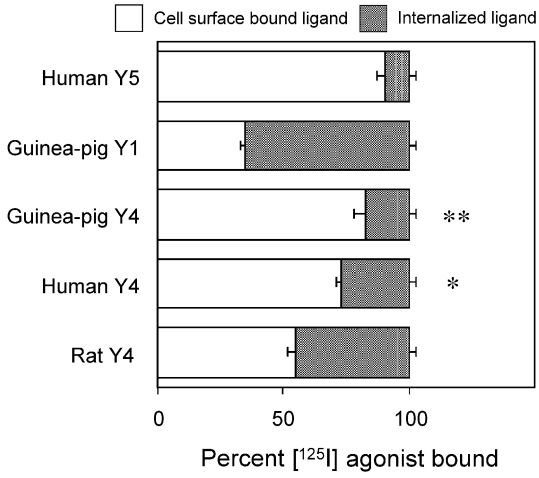

Fig. 2. Steady-state internalization of the cloned Y4, Y1 and Y5 receptors expressed in mammalian cell lines. The Y4 receptors in CHO cell monolayers were labeled with 50 pM [^{125}I]human pancreatic polypeptide for 40 min at 37 °C, at receptor densities of 59,000, 48,000 and 58,200 per cell for the rat Y4, human Y4 and guinea-pig Y4 receptors, respectively (corresponding to respective B_{max} values of 73.5, 59.2 and 72.3 fmol per mg cell protein). The guinea pig Y1 and human Y5 receptors in monolayers of CHO cells (61,000 receptors/cell or 75.6 fmol/mg cell protein) and HEK-293 cells (50,500 receptors/cell or 63 fmol/mg cell protein), respectively, were labeled with [^{125}I]human neuropeptide Y for 40 and 60 min, respectively, at 37 °C. (All receptor density estimates are based on single-specific component hyperbolic fitting, not on least-squares Scatchard linearization). The removal of tracers bound to the cell-surface receptors is described in Section 2.3. The tracers were input at 50 pM. The results represent averages of quadruplicate assays in three separate experiments, shown ± 1 S.E.M. Significance at the level of 95% and 99% confidence in Tukey multiple comparisons following a positive ANOVA (Section 2.7) for human and guinea-pig Y4 receptors vs. the rat Y4 receptor is indicated by single and double asterisks, respectively. All Y4 receptors and the human Y5 receptor were significantly less internalized than the guinea-pig Y1 receptor at the level of 99% confidence. The steady-state internalization of rat Y4 receptor labeled by [^{125}I]-rat pancreatic polypeptide was 46.8 ± 1.4% at the receptor density of 59,000/cell. The internalization of human transferrin in rat Y4-CHO cells and human Y5-HEK-293 cells showed the respective half-periods of 4.34 ± 0.65 and 5.8 ± 0.86 min ($n = 3$).

(Fig. 2). The internalization of agonist via the Y5-HEK-293 receptor was not fully saturated after 60 min at 37 °C, and longer intervals of incubation led to a significant fragmentation of the tracer peptide.

As expected (Parker et al., 2001c), all pancreatic polypeptide Y4 receptors expressed in CHO cells in steady-state conditions internalized considerably less than the guinea-pig neuropeptide Y Y1-CHO receptor, but much more than the neuropeptide Y human Y5-HEK-293 receptor (Fig. 2). The steady-state fraction internalized was little dependent on the density of rat Y4 receptor expression in CHO cells (23,000–114,000 receptors/cell), or that of human Y4-CHO expression (3000–48,000 receptors/cell), ranging from 44% to 48% of total cell-associated ligand for three rat Y4 receptor expressions in CHO cells and from 27% to 28% for three human Y4 receptor expressions in CHO cells (Fig. 3). Also, the binding of [^{35}S]GTP-γ-S to cell particulates was little affected by human pancreatic polypeptide for particles derived from CHO cells expressing the rat Y4 receptor, or by human neuropeptide Y for particles from CHO cells expressing the guinea-pig Y1 receptors (about 50,000 receptors/cell), at up to 1 μM of either Y peptide. The above findings indicate that only a minor fraction of active membrane-bound CHO cell GTP-binding proteins was associated with the respective receptors in the range of receptor densities examined.

3.3. Ligand peptide affinity and the extent of receptor internalization

The peptidic ligand affinity and the observed extent of receptor internalization were significantly correlated (r>0.85) across CHO expressions of pancreatic polypeptide

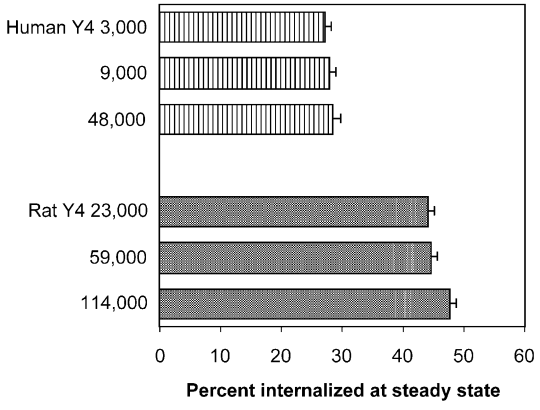

Fig. 3. Steady-state internalization of [^{125}I]human pancreatic polypeptide in CHO cells stably expressing different densities of rat and human Y4 receptors. The labeling with [^{125}I]human pancreatic polypeptide was for 40 min at 37 °C (Section 2.4). The number of receptors per cell is shown in the respective bar labels and corresponds (in order of decreasing receptor densities) to B_{max} values of 143, 74.2 and 28.9 fmol per mg protein for rat Y4 receptor expressions, and 60.4, 11.3 and 3.77 fmol per mg cell protein for human Y4 receptor expressions. The data represent averages of four to nine separate experiments, ± S.E.M. Within Y1 or Y4 groups, no difference was significant at or above the level of 95% confidence (Section 2.7).

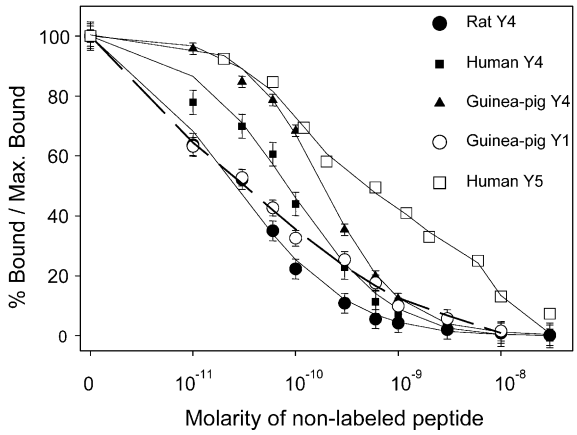

Fig. 4. Profiles of competition of [^{125}I]-labeled agonists by the corresponding nonlabeled agonist peptides in particulates from CHO cells expressing Y4 or Y1 receptors and from HEK-293 cells expressing Y5 receptors. The Y4 receptors were labeled with [^{125}I]human pancreatic polypeptide, and the Y1 and Y5 receptors with [^{125}I]human neuropeptide Y, all at 50 pM, in the presence of 1×10^{-11} to 3×10^{-8} M of the cold agonists, for 90 min at 24 °C (Section 2.4). The K_d values for the above competitions (n=3) are presented in Table 2.

Y4 receptors (Fig. 4 and Tables 1 and 2). The high internalization rate of the guinea-pig Y1 receptor in CHO cells was also positively linked to the binding affinity of human neuropeptide Y ($\leq$0.1 nM). The human Y5 receptor expressed in HEK-293 cells, with human/rat neuropeptide Y binding affinity above 1 nM (Table 2), was internalized slowly (Fig. 2). The parameters of internalization of human transferrin were similar in human Y5 receptor-expressing HEK-293 cells and guinea-pig Y4 receptor-expressing CHO cells (see the caption of Fig. 2); the low intake of Y5 receptor ligand complex in human Y5 receptor-expressing

Table 2
Compared affinities of cellular and particulate Y receptors

Receptor and cell type	Cell surface K_i (pM)	Internal K_i (pM)	Particulate K_d (pM)
Rat Y4-CHO	17.6 ± 2.5	26.4 ± 4.4	13.5 ± 3
Human Y4-CHO	61 ± 7.2	41 ± 7.7	45 ± 7
Guinea-pig Y4-CHO	174 ± 14	158 ± 17	116 ± 8
Guinea-pig Y1-CHO	192 ± 21	81 ± 12	94 ± 12
Human Y5-HEK-293	3012 ± 268	2922 ± 160	1556 ± 168

The data represent averages of three assays employing 8–10 concentration points in the range of 1×10^{-11} to 1×10^{-7} M of human pancreatic polypeptide (for Y4 receptors) or human neuropeptide Y (for Y1 and Y5 receptors). The respective [^{125}I]-labeled peptides were input at 50 pM. The cellular receptors were labeled for 40 min at 37 °C, followed by separation of the cell-surface tracer by acid saline (Section 2.3); the particulate receptors were labeled for 90 min at 24 °C, followed by centrifugation and surface washing of the pellets containing the labeled receptors. For further details, see Sections 2.3–2.4. The K_i and K_d estimates were obtained by nonlinear fitting in the LIGAND program (Munson and Rodbard, 1980), assuming a single specific binding component.

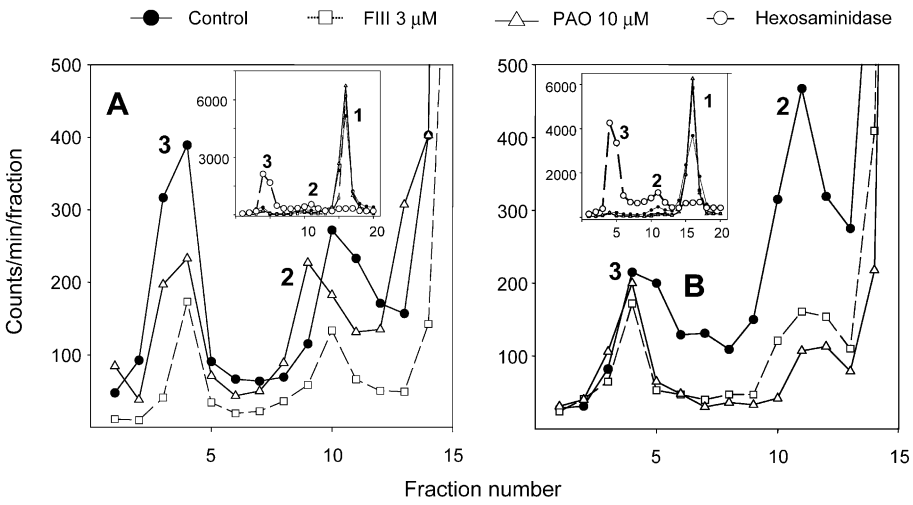

Fig. 5. Distribution of [^{125}I]human pancreatic polypeptide and acid hexosaminidase activity in subcellular membrane fractions from rat Y4-CHO and guinea-pig Y1-CHO cells separated in Percoll density gradients. The labeling at 50 pM [^{125}I]human pancreatic polypeptide (Y4 receptor expressing CHO cells) or [^{125}I]human neuropeptide Y (Y1 receptor expressing CHO cells) was for 20 min at 37 °C with or without 3 μM filipin III (FIII) or 10 μM PAO (after a pretreatment of 10 min at 37 °C with the same agents). Following cell lysis by cavitation, the lysates were sedimented for 55 min at 28,000 × g_{max} through self-generating Percoll gradients (Section 2.5). The hexosaminidase activity represents *p*-nitrophenol absorbance at 420 nm after 2 h (graph A) or 4 h (graph B) of assay incubation (Section 2.6), multiplied by 5000 to allow the use of axis units numerically similar to counts/min for the [^{125}I]human pancreatic polypeptide tracer.

HEK-293 cells should relate to the low affinity and poor tenacity of Y5 ligand attachment.

The competitive inhibition of [^{125}I] agonist binding compared between the particulate and the cell-bound receptor did not differ radically for any receptor species examined (Table 2), indicating a similarity of receptor affinities in intact bilayer and fragments produced by homogenization. The internalized binding was also competed at similar relative affinities for all receptors (Table 2), indicating a direct dependence of ligand internalization on the cell-surface attachment of the agonists used.

3.4. Distribution of Y4 receptors across endosomal/lysosomal compartments and the sensitivity to internalization inhibitors

Following internalization, both the rat and the guinea-pig pancreatic polypeptide Y4 receptors were significantly associated with endosome-like particulates in Percoll gradients (density ≈ 1.05; Fig. 5 and Table 3) after 20 min of labeling. A larger portion of the internalized polyethyleneglycol-precipitable tracer was present, however, in the gradient's $d \approx 1.11$ fraction (found by acid beta-hexosaminidase assay to contain the bulk of lysosomal material; insets of Fig. 4). This is in contrast with sorting of the internalized polyethyleneglycol-precipitable neuropeptide Y tracer in Y1-CHO cells, which was mainly associated with endosome-like particles (Fig. 5 and Table 3).

A further proof for the involvement of the $d \approx 1.05$ and the $d \approx 1.11$ particulates in pancreatic polypeptide Y4 receptor ligand internalization is the large sensitivity to PAO and to filipin III, which both produced large decreases of the receptor-bound tracer in these fractions, also observed with the neuropeptide Y Y1 receptor (Fig. 5). In contrast, these agents produced an increase of the surface binding of human pancreatic polypeptide (1 M KCNS or acid saline-detached) as well as of the binding to predominantly plasma membrane-associated sites in the lightest gradient component ($d \approx 1.015$; Fig. 5 and Table 3). The sensitivity to filipin III of [^{125}I]human pancreatic polypeptide binding to rat or guinea-pig pancreatic polypeptide Y4 receptors, or of [^{125}I]human neuropeptide Y binding to guinea-pig neuropeptide Y Y1 receptors, could be entirely eliminated by inclusion of double molar excess (6 μM) cholesteryl hemisuccinate in the incubation mixture (see also Parker et al., 2002b). The sensitivity to 10 μM PAO was likewise

Table 3
Recovery of [^{125}I]-labeled agonists bound to Y receptors in Percoll gradient zones as related to treatment of cells with filipin III

Density zone	Rat Y4 control	Rat Y4 filipin III	Guinea-pig Y4 control	Guinea-pig Y4 filipin III	Guinea-pig Y1 control	Guinea-pig Y1 filipin III
1.11 (3[a])	8.9 ± 0.12	4.2 ± 0.53	6.7 ± 0.27	3.46 ± 0.5	6.7 ± 0.4	3.8 ± 0.4
1.05 (2[a])	10.8 ± 1.2	5.0 ± 0.5	10.8 ± 0.36	3.5 ± 1.0	16.2 ± 0.25	6.5 ± 1.4
1.015 (1[a])	80.3 ± 1.5	90.8 ± 2.9	82.4 ± 1.4	93 ± 2.7	77.1 ± 0.72	89.7 ± 0.8

[a] Numbers in parentheses correspond to zone numbers in Fig. 5. The treatment with filipin III cell labeling and processing are described in the legend of Fig. 5. Similar results were obtained with 10 μM PAO (see Fig. 5). All receptors were expressed in CHO cells.

abolished by 100 μM of dithiothreitol, as already reported (Parker et al., 2001c).

4. Discussion

As indicated in the Introduction, the main hypothesis tested in this study was that at least for heptahelical rhodopsin-like receptors of similar structure and in a similar membrane environment, the extent of ligand receptor complex internalization would strongly depend on the affinity of ligand binding. This could be shown for three pancreatic polypeptide Y4 receptors and is corroborated by the profiles found for neuropeptide Y Y1 and Y5 receptors. The internalization of pancreatic polypeptides could be shown to proceed by endocytosis of Y4 receptor complex with the agonist peptide, very likely also containing G-protein α-subunits, and in intact cells protected from ligand stripping by chaotropic agents, as different from cell surface or particulate receptors. This is similar to our previous findings on neuropeptide Y Y1 receptors employing chaotrope differentiation of cell surface and internalized binding (Parker et al., 2001c) as well as to findings with fluorescent ligand-tagged Y1 receptors (Fabry et al., 2000) and green fluorescent protein-tagged Y1 receptor (Gicquiaux et al., 2002).

In CHO cells expressing pancreatic polypeptide Y4 receptors, the internalization of human pancreatic polypeptide could be shown to significantly correlate with the receptor-binding affinity in terms of saturating intake. The importance of receptor affinity as a determinant of the extent of internalization is also seen from comparisons with neuropeptide Y Y1 receptors expressed in the same cell type (CHO cells) and with neuropeptide Y Y5 receptors expressed in HEK-293 cells (which, however, were found to have a transferrin internalization dynamics close to that in CHO cells and also were recently reported to rapidly internalize and recycle the human neuropeptide Y Y1 receptor (Gicquiaux et al., 2002)). The relatively low internalization of the rat and human pancreatic polypeptide Y4 receptors compared to guinea-pig neuropeptide Y Y1 receptor of a somewhat lower affinity (see Table 2), however, indicates the importance in the endocytotic process of features not directly connected to the binding affinity. The lack of substantial internalization for the human neuropeptide Y Y5 receptor may rest mainly on low affinity of ligand binding, but could also be connected to shortness of the C-terminal cytoplasmic portion in all Y5 receptors. The absence of cytoplasmic 'tail' is known to be responsible for the low internalization rates of all mammalian luteinizing hormone-releasing hormone (LHRH) receptors (see Hanyaloglu et al., 2001). Since the agonist internalization via neuropeptide Y Y1 receptors follows a similar dynamics in CHO cells (Parker et al., 2001c) and HEK-293 cells (Gicquiaux et al., 2002) as well as via neuropeptide Y Y5 receptors in Hec-1b cells (S.L. Parker, M.S. Parker and J.K. Kane, in preparation) and HEK-293 cells (this work), the patterns found for Y4 receptors expressed in CHO cells will probably be similar at least with the epithelial-derived cell lines mentioned above.

Internalization of the pancreatic polypeptide Y4 receptors as well as of the neuropeptide Y Y1 receptor was sensitive to a trivalent arsenical crosslinker of vicinal cysteines and especially to a cholesterol-complexing polyene. This may indicate participation of both clathrin- (Mukherjee et al., 1997) and caveolin (Anderson, 1998)-containing plasma membrane structures in the internalization of these receptors. Removal especially of liganded Y4 receptors, which all show both a high binding affinity and a considerable stability of the receptor agonist complex (Parker et al., 2001a,b), could serve to limit excessive signaling. Receptors that enter the clathrin-coated pit pathway are detected in secondary endosomes (Mukherjee et al., 1997), as also shown for two pancreatic polypeptide Y4 receptors and a neuropeptide Y Y1 receptor in this study. However, a fraction especially of the Y4 receptors could be associated with cholesterol-rich rafts and consigned to a form of transcytosis, as found for bradykinin receptors (de Weerd and Leeb-Lundberg, 1997; Sabourin et al., 2002). This may aid signal termination as well as redelivery of receptors, G-proteins and effectors such as phospholipase-Cβ1 (Chidiac and Ross, 1999).

The molecular size distribution of detergent-solubilized GTP-γ-S binding proteins essentially corresponds with that of the liganded internalized (or at least sequestered in a form inaccessible to external chaotropic ions at low temperatures) Y4 receptor. The bulk of the solubilized internalized material (as well as of the ligand bound to Y4 receptors in particulates from disrupted cells) sedimented in the Mr 70,000–200,000 region. This zone should contain complexes of the liganded receptor with α-subunits of heterotrimeric G-proteins, possibly even in oligomeric formations (Dean et al., 2001). Dynamin, a large GTPase (Mr ≈ 100,000) known to participate in clathrin-linked endocytosis of rhodopsin-like receptors (Chu et al., 1997; Li et al., 2000), and capable of physical interactions at least with the βγ components of heterotrimeric G-proteins (Liu et al., 1997), should also be present in this zone.

The lack of activation by Y peptides of GTP-γ-S binding to particulates from the receptor expressions used in this study indicates that only a small fraction of plasma membrane G-protein is coupled to the receptors at the densities used (< 150 fmol/mg cell protein with any pancreatic polypeptide Y4 receptor expression and only about 60 fmol/mg protein with the neuropeptide Y Y1 receptor expression used). The modeling systems used to study the activation of GTP analog binding to Gα-nucleotide site by agonist binding to rhodopsin-like receptors typically employ receptor densities of 1 pmol/mg cell protein or above (e.g. Newman-Tancredi et al., 1997; Waelbroeck, 2001). The binding of ligands to rhodopsin-like receptors is considered to be promoted by complexing of the receptor by nucleotide-free Gα (Seifert et al., 2001), which results in an increased affinity of the receptor for its cognate ligand. Liganding, in

turn, should promote attachment of GTP to the Gα, followed by its hydrolysis to GDP and dissociation of the receptor. The presence of C–X–C motifs in the cytoplasmic tail of rat (Lundell et al., 1996) and mouse (Gregor et al., 1996) pancreatic polypeptide Y4 receptors may point to a preference for G14α- or G16α-subunits in signal transduction (Kuang et al., 1996). Complexes of these G-proteins with liganded pancreatic polypeptide Y4 receptors could be quite stable, as indicated by a low sensitivity to GTP-γ-S and only a moderate sensitivity to wasp toxins found in this work, and by the large ionic chaotrope tenacity of steady-state pancreatic polypeptide binding to Y4 receptors (Parker et al., 2001a, 2002a).

Transfer from endosomes to lysosomes, or at least the fraction of the receptor-bound ligand held in lysosomes, was larger for the rat and guinea-pig Y4 receptors relative to the guinea-pig Y1. This might indicate a sorting difference that is observed for differentially internalized subtypes of several rhodopsin-like peptide receptors (including neuropeptide Y Y1 and Y2 (Parker et al., 2001c), endothelin A and B (Bremnes et al., 2000), opioid δ and κ (Chu et al., 1997), somatostatin sst2 (Mentlein et al., 2001) and sst4 (Kreienkamp et al., 1998), and chemokine CXCR1 and CXCR2 receptors (Richardson et al., 1998). The intracellular segments of all Y4 receptors differ from those of any mammalian Y1 receptor by a lower fraction of acidic residues and by larger numbers of cysteine and proline residues. All known pancreatic polypeptide Y4 receptors contain C–X–C and C–X–X–C (X = any amino acid residue) motifs in intracellular and transmembrane segments, while these motifs are absent from structure of any of the mammalian neuropeptide Y Y1 receptors. These features could contribute toward lysosomal sorting of the Y4 receptors, possibly by way of disulfide isomerase activity (Wunderlich et al., 1995) especially as related to C–X–X–C thioredoxin boxes (Langenbach and Sottile, 1999), which are also found in endosomal and lysosomal membrane proteins (Moriwaki et al., 2001; Origasa et al., 2001; Suzuki et al., 2001). The C-terminal sequences of the pancreatic polypeptide Y4 receptors also contain a conserved bulky hydrophobic motif L–V–L, which may help a lysosomal sorting by analogy with the L–L motif (see, e.g. Gabilondo et al., 1997). These features could result in a larger late endosomal/lysosomal localization of the internalized pancreatic polypeptide Y4 compared to the neuropeptide Y Y1 receptor. The ultimately recycled fraction of the internalized receptor would not necessarily be lower for the Y4 relative to the Y1 receptor, as the sufficiently preserved receptor molecules may not be condemned by proteasomal/lysosomal chaperones (Horwich et al., 1999) and could be returned to the plasma membrane after removal of the ligand at a late endosomal/lysosomal stage (Paasche et al., 2001). However, at least in CHO cells, the intracellular processing could be much slower for any known pancreatic polypeptide Y4 receptors relative to mammalian neuropeptide Y Y1 receptors.

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