Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 474 (2003) 31–42
www.elsevier.com/locate/ejphar

Ligand internalization by cloned neuropeptide Y Y_5 receptors excludes Y_2 and Y_4 receptor-selective peptides

Steven L. Parker[a,*], Michael S. Parker[b], Armin Buschauer[c], Ambikaipakan Balasubramaniam[d]

[a] Department of Pharmacology, University of Tennessee College of Medicine, 874 Union Avenue, Memphis, TN 38163, USA
[b] Department of Microbiology and Molecular Cell Sciences, University of Memphis, Memphis, TN 38152, USA
[c] Department of Medicinal Chemistry, Institute of Pharmacy, University of Regensburg, D-93040 Regensburg, Germany
[d] Division of GI Hormones, Department of Surgery, University of Cincinnati School of Medicine, Cincinnati, OH 45267, USA

Received 3 April 2003; received in revised form 20 June 2003; accepted 27 June 2003

Abstract

In human embryonic kidney-293 (HEK-293) cells, the cloned human neuropeptide Y Y_5 receptor saturably internalized agonists, with the rank order of neuropeptide Y-(19-23)-[Gly^1,Ser^3,Gln^4,Thr^6,Ala^{31},Aib^{32},Gln^{34}]human pancreatic polypeptide (neuropeptide Y-Aib-pancreatic polypeptide)>human neuropeptide Y>porcine peptide YY>[Pro^{34}]human peptide YY>[Leu^{31},Pro^{34}]human peptide YY≫human peptide YY-(3-36). Human pancreatic polypeptide competed [^{125}I]neuropeptide Y binding and internalization in neuropeptide Y Y_5 receptor-expressing cells, but itself showed no internalization. The internalization was strongly dependent on temperature. The surface binding, and especially the internalization, of human neuropeptide Y were highly sensitive to the clathrin network inhibitor phenylarsine oxide, and to the cholesterol-complexing antibiotic filipin III. The internalized ligands were present in particles corresponding to secondary endosomes in Percoll gradients, but especially in particles banding with the acid hexosaminidase lysosomal marker. At any temperature tested, internalization of the neuropeptide Y Y_5 receptor driven by human neuropeptide Y in HEK-293 cells was much slower than the internalization of the neuropeptide Y Y_1 receptor reported in the same cells, or in Chinese hamster ovary (CHO) cells. The neuropeptide Y Y_5 receptor subtype could be the metabotropic receptor responding to protracted challenges by neuropeptide Y-like peptides, and its density could be little sensitive to concentration of extracellular agonists.

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

1. Introduction

Two neuropeptide Y receptors most frequently identified as stimulators of feeding, the Y_1 and Y_5 subtypes (Kalra et al., 1999), share significant similarities of primary structure (Wraith et al., 2000), which may lead to a similar pattern of metabolic processing, including sequestration and internalization. The long third intracellular segment of the Y_5 receptor possesses large oligobasic sequences, similar to some ion channel-associated rhodopsin-like receptors, e.g. the muscarinic acetylcholine m_1 receptor (Allard et al., 1987), which is characterized by a high rate of agonist-induced internalization, and even of down-regulation linked to repeated processing (e.g. Vogler et al., 1999). The Y_1 receptor strongly internalizes the bound agonist peptides in several cell types, including native expression of the human receptor in neuroblastoma SK-N-MC cells (Fabry et al., 2000), clonal expression of the guinea-pig receptor in Chinese hamster ovary (CHO) cells (Parker et al., 2001c; Parker et al., 2002d), and clonal expression of the human receptor in human embryonic kidney-293 (HEK-293) cells (Gicquiaux et al., 2002).

While the muscarinic m_1 receptor possesses significant oligoacidic sequences in intracellular segments that could promote internalization by interaction with dynamin-like motors by analogy with SH3 sequences (Okamoto et al., 1997), or with phosphorylation promoters (Vogler et al., 1999), this is not encountered with the Y_5 receptor. Interaction with motor proteins and endocytotic sorting could be assisted by oligoaliphatic tracts in the first and second intracellular loops of the Y_1 receptor, by analogy with the EGF receptor (Kil et al., 1999). Such motifs are less

* Corresponding author. Tel.: +1-901-448-6019; fax: +1-901-448-7206.
E-mail address: StevenLeonardParker@msn.com (S.L. Parker).

0014-2999/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0014-2999(03)02039-9

prominent in intracellular segments of the Y_5 receptor. Also, the Y_5 binding site might use centrally placed short epitopes as well as the C-terminal segments of agonist peptides (see e.g. Keire et al., 2001). This can reduce or even prevent secondary associations that apparently follow the primary interaction of neuropeptide Y via N- and C-termini with the Y_1 site, and can support the inward vectorial drive that helps the massive internalization of the Y_1 receptor–ligand complex (Gicquiaux et al., 2002; Parker et al., 2002d). It should also be noted that the cytoplasmic 'tail' of the Y_5 receptors is short (about 18 residues in all reported Y_5 sequences, as opposed to 60 or 61 in the published Y_1 receptor sequences), which, by analogy with e.g. the gonadotropin releasing hormone receptor (Hislop et al., 2001), may serve to limit the internalization rate of the Y_5 receptor. In view of the above, one can expect a lower rate of internalization for the neuropeptide Y Y_5 receptor compared to the neuropeptide Y Y_1 receptor, and even to the pancreatic polypeptide Y_4 receptor. This study indeed finds a low rate of internalization for the cloned neuropeptide Y human Y_5 receptor expressed in HEK-293 cells.

2. Experimental procedures

2.1. Chemicals

The non-peptide Y_5 competitor Hu 296 (*N*4-phenyl-*N*2-(4-piperidin-1-yl-phenyl)quinazoline-2,4-diamine trihydrochloride) was synthesized according to Rueger et al. (1997). Hybrid Y_5-receptor selective Y peptide, neuropeptide Y-(19-23)-[Gly^1,Ser^3,Gln^4,Thr^6,Ala^{31},Aib^{32},Gln^{34}] human pancreatic polypeptide (Cabrele et al., 2000) and Y_1 receptor-selective non-peptide antagonist BIBP-3226 (diphenylacetyl-D-Arg-4-hydroxybenzylamide) were purchased from Bachem (Los Angeles, CA). The Y peptides human neuropeptide Y, porcine peptide YY, [Leu^{31},Pro^{34}] human peptide YY, [Leu^{31},Pro^{34}] human neuropeptide Y, [Pro^{34}] human peptide YY and human pancreatic polypeptide were obtained from the American Peptide (San Diego, CA), and [D-Trp^{34}] human neuropeptide Y was synthesized as described by Parker et al. (2000). Filipin III and phenylarsine oxide were purchased from Sigma (St. Louis, MO). Cholesteryl hemisuccinate was obtained from Calbiochem (La Jolla, CA). Filipin III and phenylarsine oxide were dissolved in dimethylsulfoxide and stored aliquoted at −80 °C. 'Filipin complex' (Sigma; a mixture of three isomers of filipin) was only about 25% as active as filipin III, and hence was not used. Cholesteryl hemisuccinate was prepared as a water emulsion and also stored at −80 °C.

2.2. Labeled peptides

All iodinations of Y peptides were performed as described (Parker et al., 1998b). Radiolabeled and separated by our procedure, the radioactive Y peptides were 85–90% monoiodinated and had specific activities in the range of 1800–1950 Ci/mmol (70–80% theoretical), as deduced by comparison in saturation assays with high performance liquid chromatography-purified monoiodinated [^{125}I]-labeled Y peptides human neuropeptide Y, [Leu^{31},Pro^{34}]human peptide YY and human peptide YY(3-36), supplied by PerkinElmer, Cambridge, MA (specific activity 2170 Ci/mmol). Human transferrin (Sigma) was iodinated by the same protocol as the Y_5 peptides.

2.3. Cell cultures and labeling

All cell types were cultured in F12/D-MEM medium (Gibco, Long Island, NY, USA) at 250 μg/ml of geneticin and 2 mM GlutaMax1 (Gibco).The cloned human Y_5 receptor was expressed in the human embryonic kidney-293 cells (human Y_5-HEK-293; Dumont et al., 2000) and also in human endometrial carcinoma cells (human Y_5-Hec-1B; Moser et al., 2000). Chinese hamster ovary (CHO) cells were used to express the neuropeptide Y guinea-pig Y_1 receptor (guinea-pig Y_1-CHO; Berglund et al., 1999), the neuropeptide Y guinea-pig Y_2 receptor (guinea-pig Y_2-CHO; Sharma et al., 1998) and the pancreatic polypeptide rat Y4 receptor (rat Y_4-CHO; Lundell et al., 1996). All cell lines used in this study had stable particulate receptor density, at the level of 6–12 fmol/100,000 cells, over up to 40 transfers in F12/Dulbecco's modified Eagle medium with 250 μg/ml of geneticin (Gibco). At full confluence, the cell count was 200,000–235,000/cm^2. Most kinetic and inhibition studies were done with 48-well (0.8 cm^2/well) plates, while the cells for gradient characterization experiments were cultured in six-well (9.4 cm^2/well) plates, or in individual 35-mm Petri dishes of similar surface area (supplied by Corning/Costar, Ithaca, NY, USA). The labeling with [^{125}I]Y peptides was done at 50 pM, using 1 μM non-labeled peptides for non-saturating (or non-specific) binding correction. The non-saturable binding was defined as the difference between the binding of 50 pM [^{125}I]-labeled peptide without and with 1 μM unlabeled peptide.

2.4. Receptor characterization

Particulate receptors were assayed as described (Parker et al., 2001a). Briefly, the assay buffer contained 8% sucrose, 0.2% proteinase-free bovine serum albumin, 0.025% bacitracin, 1 mM diisopropylfluorophosphate (Sigma), 4 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM HEPES. NaOH (pH 7.4) and 50 μM ATP, the particle concentration was 100–125 μg/ml, the assay volume was 0.2 ml, and the incubation time was 90 min at 23–24 °C, with or without competitors or inhibitors. The assay was terminated by centrifugation for 15 min at 16,000 × g_{max} at 4 °C, the supernatants were discarded, and the pellets surface-

washed by cold assay buffer prior to counting in a γ-scintillation counter. The binding properties of the cell-surface receptors were characterized on monolayer cultures in Opti-Mem® medium (Gibco). Iodinated Y peptides were input at 50 pM, and competed by up to nine concentrations of homologous or isologous peptides in the range of 3×10^{-11}–1×10^{-6} M. Polyethyleneglycol precipitation of particulates was done as described (Parker et al., 1998b), in some cases after solubilizing the particulates with 10 mM sodium cholate at 0–4 °C over 10 min. Binding parameter calculations were done in the LIGAND program (Munson and Rodbard, 1980), assuming a single specific component. Projections of the maximum specific binding of labeled peptides in the absence of unlabeled competitor were derived in the LIGAND program by hyperbolic fitting to the Michaelis–Menten equation (see Parker and Waud, 1971).

2.5. Percoll gradient centrifugation experiments

In experiments employing Percoll gradients to separate the endosomal and lysosomal particulates, the cells were incubated for indicated periods at 37 °C with appropriate drugs and radioactive Y peptides, then washed three times in ice with cold incubation medium, and once with cold 0.25 M sucrose–10 mM HEPES NaOH (pH 7.4). The cells were then scraped into the cold sucrose solution (2 ml per well of 9.4 cm^2), and the suspensions homogenized by nitrogen cavitation, and subjected to Percoll gradient fractionation as recently described (Parker et al., 2002a). Acid hexosaminidase assay was also performed as described in Parker et al. (2002a).

3. Results

3.1. Saturating and non-saturating human neuropeptide Y Y_5 receptor binding in HEK-293 cells compared to neuropeptide Y guinea pig Y_1 receptor binding in CHO cells

The agonist-competed surface binding (i.e. the peptide radioactivity extracted at pH 2.6 and 0–4 °C by 0.2 M CH_3COOH–0.5 M NaCl) of 50 pM [^{125}I]human neuropeptide Y or [^{125}I]neuropeptide Y-Aib-pancreatic polypeptide to human Y_5 receptor expressed in HEK-293 cells appeared to be more than 50% saturated within 90 min at 24 °C or 60 min at 37 °C (Table 1 and Fig. 1). Longer intervals of incubation especially at 37 °C resulted in significant degradation of the labeling peptides (>20% as detected by Bio-Gel P-4 chromatography Parker et al., 1998a), thus precluding longer kinetic comparisons. The cell-surface binding of human neuropeptide Y to Y_5 receptor-expressing cells was low even after 2 h at 15 °C, or over 8 h at 4 °C (in the absence of a significant loss of the [^{125}I]-labeled agonist peptide). Similar profiles were obtained with Hec-1B cells. The surface binding to guinea pig Y_1-CHO cells (used since the human Y_1-HEK-293 cells, which are similar in most of the binding parameters to this Y_1 receptor expression in CHO cells (compare Gicquiaux et al., 2002; Parker et al., 2002d) were not available for this study) reached >60% saturation within 4 h even at 4 °C, highlighting the large ligand-binding affinity and the relative constancy of cell-surface neuropeptide Y Y_1 receptor complement (Table 1). It is of interest to note that even at 37 °C the detected numbers of saturable surface Y_5 sites were less than 50% of the measured Y_1 sites (while the B_{max} values in competition assays using particulates were similar; legend of Table 1), which obviously relates to the difference in receptor affinity. No activation of the surface or internal (acid saline-resistant) Y_5 receptor binding could be achieved by pretreatment of the cells with digitonin, or by agents previously shown to activate the Y_2 (and to some extent also the Y_1 and the Y_4) receptors of CHO cells or rat brain cells (Parker et al., 2002c).

As expected, the cold acid saline-resistant specific or saturable human neuropeptide Y intake at 24 °C was much slower than at 37 °C (Table 1). The saturably internalized Y_5 tracer was quite low at 15 °C, and essentially absent at 4 °C (Table 1). At 15–37 °C, HEK-293 cell internalization of the Y_5 tracer was at least 10 times lower than the corresponding specific Y1-CHO cell intake of [^{125}I]human neuropeptide Y (Fig. 1 and Table 1), and did not saturate in 90 min at 37 °C (Fig. 1A). This is in a large contrast to

Table 1
Compared specific and non-specific binding of [^{125}I]human neuropeptide Y to human Y5-HEK-293 and Y_1-CHO cells at various temperatures

Cells and conditions	Surface non-saturable	Surface specific	Internal non-saturable	Internal specific
4 °C, 240 min				
Y_5 HEK-293	0.438 ± 0.027	0.327 ± 0.029	0.322 ± 0.086	0.025 ± 0.06
Y_1 CHO	0.477 ± 0.03	1.99 ± 0.07	0.362 ± 0.06	0.188 ± 0.03
15 °C, 180 min				
Y_5 HEK-293	0.420 ± 0.034	0.462 ± 0.021	0.395 ± 0.012	0.128 ± 0.011
Y_1 CHO	0.695 ± 0.012	2.07 ± 0.016	0.229 ± 0.01	6.24 ± 0.02
24 °C, 90 min				
Y_5 HEK-293	0.481 ± 0.09	1.12 ± 0.096	0.443 ± 0.09	0.247 ± 0.011
Y_1 CHO	0.639 ± 0.12	2.09 ± 0.09	0.43 ± 0.11	8.74 ± 0.16
37 °C, 60 min				
Y_5 HEK-293	0.601 ± 0.05	1.24 ± 0.048	0.277 ± 0.05	0.481 ± 0.021
Y_1 CHO	0.679 ± 0.09	2.44 ± 0.15	0.415 ± 0.032	5.22 ± 0.33

The data represent fmol [^{125}I]human neuropeptide Y (input at 50 pM) bound per 100,000 cells ± 1 S.E.M. ($n = 6$). The non-saturable binding was defined at 1 μM human neuropeptide Y, and subtracted from the binding at 50 pM in the absence of non-labeled peptide to obtain the specific binding shown. With particulate receptors, the K_d (nM) and B_{max} (fmol/mg particle protein) values were 0.157 ± 0.011 and 118 ± 22 for CHO-Y_1 cells, and 1.29 ± 0.26 and 132 ± 26 for human Y_5-HEK-293 cells ($n = 3$ for both lines). Under saturating conditions, 1 fmol/100,000 cells corresponds to about 6000 receptors per cell, or a binding of 7–7.5 fmol/mg total cell protein. The rates of Y_1 receptor internalization in CHO and HEK-293 cells are essentially similar (compare Parker et al., 2002d; Gicquiaux et al., 2002).

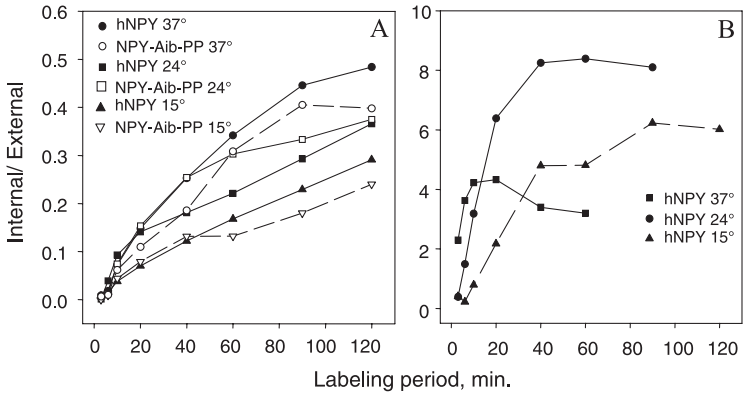

Fig. 1. Kinetics of receptor-linked internalization of Y_5 and Y_1 receptor ligands. The change in ratio of internalized to surface bound (external) ligand (cold acid saline-extracted and residual, respectively) was followed in human Y_5-HEK-293 cells and guinea-pig Y_1-CHO cells at 15, 24 and 37 °C. (A) Ratios of internal and external binding of [^{125}I]human neuropeptide Y ('hNPY') and [^{125}I]neuropeptide Y-Aib-pancreatic polypeptide ('NPY-Aib-PP') in human Y_5-HEK-293 cells. Profiles obtained with the above ligands in human Y_5-Hec-1B cells were quite similar. The estimates for human Y_5-HEK-293 half-periods of saturation (min) are presented below.

	15 °C hNPY	15 °C Aib	24 °C hNPY	24 °C Aib	37 °C hNPY	37 °C Aib
External	58 ± 4.6	42 ± 8.5	18 ± 4.5	10.6 ± 0.9	9.1 ± 0.9	7.6 ± 0.6
Internal	37 ± 5.5	45 ± 5.9	63 ± 18	39 ± 1.3	36 ± 3.9	26 ± 1.6

(B) Ratios of internal to external binding of [^{125}I]human neuropeptide Y ('hNPY') in guinea pig Y_1-CHO cells. The estimated half-periods of saturation (in min) are presented below. The large periods of half-saturation for internal human neuropeptide Y at 15 and 24 °C reflect intracellular accumulation of undegraded neuropeptide Y.

	15 °C	24 °C	37 °C
External	3.4 ± 0.8	3.4 ± 1.0	11.7 ± 1.8
Internal	48 ± 14	28 ± 12	8.9 ± 3.4

guinea pig Y_1-CHO cell internalization of [^{125}I]human neuropeptide Y, which saturated even at 15 °C (Fig. 1B). Since the density of particulate receptors was quite similar in the cell lines compared (legend of Table 1), this difference obviously reflected a much lower rate of receptor-linked human neuropeptide Y internalization in Y_5 cells, probably connected to an about eight-fold lower binding affinity (legend of Table 1). The internalization of [^{125}I]human transferrin via its natively expressed receptor proceeded at quite similar rates in the HEK-293 and CHO lines used (with the respective half-periods of 5.8 ± 0.86 and 4.34 ± 0.65 min (n = 3 for each)).

The non-saturable acid saline-resistant binding of [^{125}I]human neuropeptide Y to either Y_5 or Y_1 cells did not differ much over the temperature range studied. No significant accumulation of intracellular neuropeptide Y was found in human Y_5-expressing cells at any temperature, as opposed to very significant accumulation of internalized peptide in Y_1-CHO cells at 15 or 24 °C (Fig. 1B and Table 1). In Y_1 receptor expressing cells, at least 30% of internalized human neuropeptide Y was physically associated with internal Y_1 receptors at any time point.

After 20 min of labeling at 37 °C, about 30% of the HEK-293 Y_5 ligand that was associated with an endosomal fraction in Percoll gradients (density 1.05–1.06; see Fig. 4) was precipitated by polyethyleneglycol following membrane solubilization at 10 mM cholate (at 0–4 °C), indicating presence of receptor-bound ligand in these particulates. After 20 min of labeling at 37 °C, close to 70% of neuropeptide Y associated with plasma membrane fraction (density ~ 1.015) was precipitated with polyethylene glycol following this solubilization (see Table 3).

3.2. Compared internalization of various [^{125}I]-labeled peptidic ligands of the neuropeptide Y Y_5 receptor

The neuropeptide Y Y_5 receptor is known to accept neuropeptide Y receptor ligands that non-selectively attach to various subtypes of the neuropeptide Y receptor, including neuropeptide Y and peptide YY, as well as the peptidic ligands which prefer Y_1, Y_2 and Y_4 receptors. It was therefore of interest to compare the extent of internalization of such Y receptor ligands in HEK-293 cells expressing the Y_5 receptor (Fig. 2). The interval of labeling at 37 °C was set to 60 min for all ligands (to avoid artifacts due to peptide degradation, which with the above length of incubation was less than 20% for any peptide tested). The internalized fraction of Y_5 receptor-selective agonist neuropeptide Y-Aib-pancreatic polypeptide and human neuropeptide Y was similar (35% and 39%, respectively; Table 2).

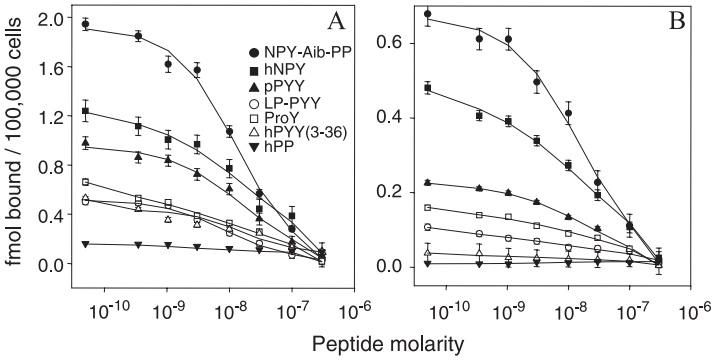

Fig. 2. Compared surface binding and internalization of seven [^{125}I]-labeled Y receptor agonists in human Y_5-HEK-293 cells over 60 min at 37 °C. Data are averages of four independent experiments. The [^{125}I]-labeled peptides were input at 50 pM, and the nonspecific binding correction was taken at 1 μM of the respective non-labeled peptides. The K_I and B_{max} values for the above competitions are presented in Table 2. See Moser et al. (2000) for K_I values with particulate receptors in competition of [^{3}H]human neuropeptide Y binding, and Table 2 and Parker et al. (2001a) for K_I values in competition of [^{125}I][Leu^{31},Pro^{34}]binding. Abbreviations in the graph: NPY-Aib-PP, hNPY: see the caption of Fig. 1; pPYY, porcine peptide YY; LP-PYY, [Leu^{31},Pro^{34}]human peptide YY; ProY, [Pro^{34}]human peptide YY; hPYY(3-36), human peptide YY-(3-36); hPP, human pancreatic polypeptide.

The internalized fraction for peptide YY-related 36 peptides was similar (21–24%), and significantly lower than for neuropeptide Y-aminoisobutuyrate-pancreatic polypeptide or human neuropeptide Y (Table 2). Among full-length peptide YY-related peptides, there was a clear labeling preference for porcine peptide YY over [Leu^{31},Pro^{34}]human peptide YY (which also internalized much less than porcine peptide YY, and even significantly less than [Pro^{34}]human peptide YY).

The Y_2-selective agonist human peptide YY-(3-36), which also acts as a Y_5 competitor (e.g. Parker et al., 2001b), displayed a strong surface binding of relatively high affinity (Table 2 and Fig. 2A), but internalized poorly, at less than 20% of the rate for [Leu^{31},Pro^{34}]human peptide YY, the most slowly internalized of the full-length peptide YY-like peptide that were tested (Fig. 2B). In a control experiment, the surface binding and internalization of 50 pM [^{125}I]human peptide YY-(3-36) with guinea-pig Y_2-CHO cells saturated in 40 min/37 °C at about 4.5 and 1.1 fmol/100,000 cells, respectively.

The Y_4 receptor agonist [^{125}I]human pancreatic polypeptide showed a low surface binding and essentially no internalization (Fig. 2 and Table 2), and this was confirmed with human Y_5-Hec-1B cells (data not shown). In a control experiment, surface binding and internalization of 50 pM [^{125}I]human pancreatic polypeptide with rat Y_4-CHO cells saturated in 60 min/37 °C at, respectively, 7.5 and 4.6 fmol peptide specifically bound/100,000 cells. At 37 °C and 50 pM of input, the non-saturable binding of [^{125}I]human pancreatic polypeptide in human Y_5-HEK-293 cells was (in fmol/100,000 cells) 0.058 ± 0.002 (cell-surface) and 0.0032 ± 0.0035 (internal), i.e. about an order of magnitude lower than found with an equimolar input of [^{125}I]human neuropeptide Y (see Table 1).

Table 2
Surface binding and internalization of peptides competing the Y_5 receptor of intact HEK-293 cells compared with particulate binding of the same peptides

[^{125}I] peptide	IC_{50}, nM surface	fmol bound/10^5 cells surface	IC_{50}, nM internal	fmol bound/10^5 cells internal	% Internalized at 60 min	K_I, nM particulate receptor	B_{max}, fmol particulate receptor
Neuropeptide Y-Aib-pancreatic polypeptide	1.28 ± 0.16	1.94 ± 0.05	1.57 ± 0.34	0.679 ± 0.033	35.0 ± 1.28	0.781 ± 0.14	146 ± 39
Human neuropeptide Y	2.19 ± 0.6	1.24 ± 0.09	2.06 ± 0.31	0.481 ± 0.017	38.8 ± 1.83	1.29 ± 0.26	132 ± 26
Porcine peptide YY	1.63 ± 0.41	0.979 ± 0.05	2.37 ± 0.42	0.226 ± 0.007	23.0 ± 1.07	1.10 ± 0.08	116 ± 15
[Pro^{34}]human peptide YY	2.23 ± 0.72	0.661 ± 0.026	7.06 ± 1.9	0.160 ± 0.025	24.2 ± 1.36	1.18 ± 0.11	202 ± 36
[Leu^{31},Pro^{34}]human peptide YY	0.887 ± 0.16	0.501 ± 0.024	1.25 ± 0.36	0.107 ± 0.009	21.4 ± 5.7	0.521 ± 0.08	111 ± 26
Human peptide YY-(3-36)	7.9 ± 2.1	0.533 0.01301	14,000 ± 3610	0.0374 0.027	7.1 ± 1.4	2.94 ± 0.16	76 ± 22
Human pancreatic polypeptide	9.1 ± 0.71	0.065 ± 0.017		<0.01			

All labeled peptides were input at 50 pM. The corresponding unlabeled peptides were input at seven concentrations in the range of 3×10^{-11}–3×10^{-7} M. The binding profiles are shown in Fig. 2. The K_I values were derived by logistic or exponential curve fitting from data corrected for the binding at 1 μM of human neuropeptide Y. The data for fmol bound/100,000 cells are the estimates of the maximum specific binding of the unlabeled peptide in the absence of unlabeled competitor, derived by hyperbolic fitting (Parker and Waud, 1971). The data for particulate Y_5 binding were derived from competition of 50 pM of the indicated [^{125}I]-labeled peptide by 8–10 different concentrations of the corresponding unlabeled peptide in the range of 0.03–100 nM (see Section 2). Both the binding to intact cells and particulate binding of [^{125}I]neuropeptide Y-Aib-human pancreatic polypeptide and [^{125}I]human neuropeptide Y were inhibited by non-peptidic Y_5 receptor competitor Hu 296 with a K_I of about 60 nM.

3.3. Compared activity of non-selective and Y_5 receptor-selective agonists in Y_5 receptor-linked internalization of [^{125}I]human neuropeptide Y

In view of the results presented above, it was of interest to examine the competition of the binding and internalization of the natural ligand of the human Y_5 receptor, [^{125}I]human neuropeptide Y, by the mixed Y_2/Y_5 receptor ligand, human peptide YY-(3-36), and the mixed Y_4/Y_5 receptor ligand, human pancreatic polypeptide, as well as by high- and medium-affinity Y_5 receptor-selective agonists neuropeptide Y-Aib-pancreatic polypeptide (Cabrele et al., 2000) and [D-Trp^{34}]human neuropeptide Y (Parker et al., 2000).

The high-affinity agonist neuropeptide Y-Aib-pancreatic polypeptide competed [^{125}I]human neuropeptide Y with a larger efficacy, especially at higher molarities, resulting in about 40% lower IC_{50} for internalized counts. Both the surface binding and the internalization of [^{125}I]human neuropeptide Y were also competed by the Y_5-selective agonist [D-Trp^{34}]human neuropeptide Y (Parker et al., 2000) (IC_{50} 94 nM for surface binding, 164 nM for internalization). All agonists showed larger IC_{50} values for competition of the internalized binding relative to the cell-surface binding, indicating a significant agonist-driven acceleration of the intake.

As also expected (Michel et al., 1998; Moser et al., 2000; Parker et al., 2002b), and in a contrast to its low activity at the human Y_5-HEK-293 receptor (Fig. 2), the mixed Y_4/Y_5 receptor competitor human pancreatic polypeptide did inhibit the binding of [^{125}I]human neuropeptide Y to Y_5-HEK-293 cells, as well as the internalization of labeled human neuropeptide Y in these cells (Fig. 3). Similar results were obtained with [^{125}I]neuropeptide Y-Aib-pancreatic polypeptide in human Y_5-Hec-1B cells (not shown). However, the competition of [^{125}I]human neuropeptide Y internalization by human pancreatic polypeptide was shallow, with less than 54% inhibition at 1 μM pancreatic polypeptide, and the IC_{50} value was in excess of 100 nM. Also, not more than 80% of human neuropeptide Y surface binding was inhibited at 1 μM human pancreatic polypeptide. As also anticipated, the mixed Y_2/Y_5 receptor ligand human peptide YY-(3-36) inhibited the binding and internalization of [^{125}I]human neuropeptide Y. The surface binding was inhibited by about 85% at an IC_{50} close to 8 nM (legend of Fig. 3), very similar to the value obtained in the isologous inhibition (Table 2). The internalization of [^{125}I]human neuropeptide Y, however, was inhibited only about 50% (IC_{50} 82 nM for the affected portion of the intake), as also found with human pancreatic polypeptide.

3.4. Sensitivity of ligand attachment and internalization to inhibitors of receptor internalization

Among the internalization inhibitors tested, a large sensitivity was found for filipin III, a polyene antibiotic known to act in receptor internalization by complexing membrane cholesterol (Subtil et al., 1999). This compound produced more than 50% inhibition of Y_5 internalization at

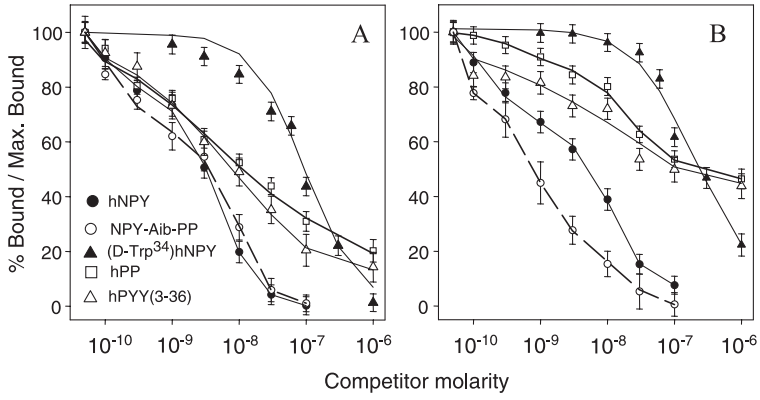

Fig. 3. Surface binding and internalization of [^{125}I]human neuropeptide Y in human Y_5-HEK-293 cells competed by three mixed Y receptor competitor peptides and two Y_5 receptor-selective peptide competitors. The binding of [^{125}I]human neuropeptide Y (50 pM) was done over 60 min at 37 °C in standard conditions (see Section 2.3), followed by extraction with cold acid saline to separate the surface-bound and internalized radioactive agonist. Non-labeled human neuropeptide Y, human peptide YY(3-36) and human pancreatic polypeptide were used at nine molarities from 3×10^{-11} to 1×10^{-6} M. Non-labeled [D-Trp^{34}]human neuropeptide Y was used at eight molarities between 1×10^{-9} and 1×10^{-6} M. The non-specific binding in all cases was defined at 1 μM cold human neuropeptide Y. All data are averages of six measurements in two independent experiments, shown ± S.E.M. See Moser et al. (2000) for K_I values with particulate receptors in competition of [^{3}H]human neuropeptide Y binding, and Parker et al. (2001a) for K_I values in competition of [^{125}I](Leu^{31},Pro^{34})binding. (A) Profiles of surface binding of [^{125}I]human neuropeptide Y. The IC_{50} values (nM, ± S.E.M.) are followed by percent specific binding displaced at 1 μM corresponding peptide (100 nM in the case of human neuropeptide Y): human neuropeptide Y, 3.01 ± 0.53 (99.8%); neuropeptide Y-Aib-pancreatic polypeptide, 2.18 ± 0.56 (99%); human pancreatic polypeptide, 4.73 ± 0.51 (79.7%); human peptide YY-(3-36), 7.73 ± 2.1 (85.7%); [D-Trp^{34}]human neuropeptide Y, 94.2 ± 6.7 (79.2%). Abbreviations in the graph: hNPY, NPY-Aib-PP: see the caption of Fig. 1; hPP, hPYY(3-36): see the caption of Fig. 2; (D-Trp^{34})hNPY, [D-Trp^{34}]human neuropeptide Y. (B) Profiles of internalization of [^{125}I]human neuropeptide Y. The IC_{50} values (nM, ± S.E.M.) listed below are followed by percent specific binding displaced at 1 μM corresponding peptide (100 nM in the case of human neuropeptide Y): human neuropeptide Y, 2.92 ± 0.96 (92.4%); neuropeptide Y-Aib-pancreatic polypeptide, 0.948 ± 0.28 (99%); human pancreatic polypeptide, 114 ± 20 (53.6%); human peptide YY-(3-36), 82 ± 9.6 (49.2%); [D-Trp^{34}]human neuropeptide Y, 164 ± 16 (77.9%).

3 μM (Fig. 4A), a molarity that did not cause a large decrease of the labeling of cell surface sites, or of the binding to the Y_5 receptor in isolated particulates (caption of Fig. 4A). The activity of filipin could be completely prevented by cholesteryl hemisuccinate at equimolar inputs (Fig. 4A). It should be noted, however, that filipin III also strongly reduced the binding of Y_5 ligands to either the cell-surface or the isolated particulate Y_5 sites, with an IC_{50} of about 6 μM. As expected, the macrolide also prevented internalization of [^{125}I]human transferrin, at molarities slightly above those inhibiting the intake of Y_5 receptor agonists (IC_{50} 4.3 μM; $n=4$). Effects of the polyene antibiotic upon Y_5 internalization could not be reversed by washing and reincubation without filipin for 20 min at 37 °C in the medium used (results not shown).

The vicinal cysteine-bridging arsenical phenylarsine oxide was an even more potent inhibitor of Y_5 internalization. The IC_{50} for phenylarsine oxide inhibition of [^{125}I]human neuropeptide Y internalization was only 0.8 μM, with little change in surface binding, or the binding to isolated particulates (Fig. 4B). However, a significant drop in surface or particulate binding was already apparent at 6 μM phenylarsine oxide. The internalization of [^{125}I]human transferrin in Y_5 cells was significantly inhibited above 10 μM of phenylarsine oxide (IC_{50} 12.4 μM; $n=3$). Inhibition of the Y_5 internalization at 3 μM phenylarsine oxide could be counteracted by 300 μM of a disulfide disruptor, dithiothreitol (Fig. 4B). Effects of phenylarsine oxide were not reversed by washing and reincubation without phenylarsine oxide for up to 60 min at 37 °C in the medium employed.

Sucrose, a known inhibitor of clathrin network formation (e.g. Fire et al., 1991), inhibited the human neuropeptide Y internalization in Y_5-HEK-293 cells above 0.3 M (Fig. 4C), again similar to the inhibition of internalization of transferrin (which showed an IC_{50} of 380 ± 56 mM; $n=4$). The blockade of internalization by sucrose was quite selective relative to the surface binding and apparently developed over a narrow span of sucrose molarity, being essentially complete at 0.44 M. The effect of sucrose, however, could be completely reversed by washing and reincubation of 20 min at 37 °C in the OptiMem® medium (data not shown).

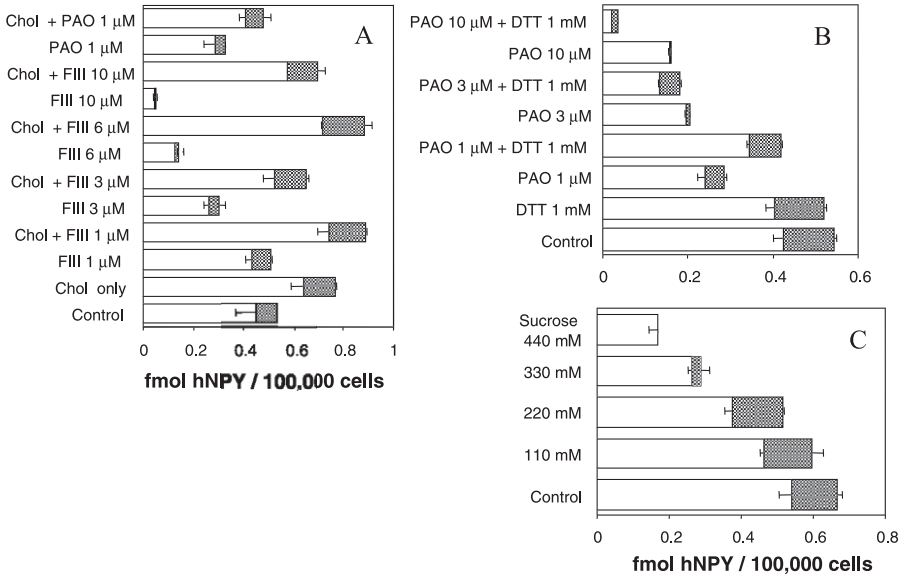

Fig. 4. Inhibition of [^{125}I]human neuropeptide Y labeling in human Y_5-HEK-293 cells by three commonly employed inhibitors of receptor-linked neuropeptide internalization. The indicated agents were in all cases added to the cells 10 min before starting the labeling. The length of labeling at 37 °C was 60 min in all cases. (A) Inhibition by polyene antibiotic filipin III and its prevention by cholesterol. 'Chol' refers to cholesteryl hemisuccinate used at 10 μM. 'FIII' = filipin III; 'PAO' = phenylarsine oxide. The IC_{50} values (μM filipin III) were 3.7 ± 0.23 and 2.9 ± 0.12 for external and residual [^{125}I]human neuropeptide Y, respectively ($n=3$). The binding of [^{125}I]human neuropeptide Y to particulate HEK-293 Y_5 receptors was inhibited by filipin III with an IC_{50} of 6.4 ± 2 μM ($n=3$). Inhibition of Y_5 internalization by filipin III could not be reversed by reincubation for up to 60 min in filipin III-free medium. In guinea pig Y_1-CHO cells, filipin III inhibited internalization of human neuropeptide Y by 86% with an IC_{50} of 0.95 ± 0.3 μM ($n=6$), without a clear inhibition of surface binding below 20 μM, while the binding of human neuropeptide Y to particulate guinea pigY_1-CHO receptors was inhibited with an IC_{50} of 36 ± 5.4 μM (68.2 ± 2% inhibition at 100 μM; $n=3$). (B) Inhibition by the vicinal cysteine-bridging arsenical phenylarsine oxide and its prevention by disulfide-breaking agent dithiothreitol. The IC_{50} values (μM phenylarsine oxide) were 5.9 ± 2 and 0.749 ± 0.07 for external and internal [^{125}I]human neuropeptide Y, respectively ($n=3$). The binding of [^{125}I]human neuropeptide Y to particulate HEK-293 Y_5 receptors was inhibited by phenylarsine oxide with an IC_{50} of 14.6 ± 4.3 μM ($n=3$). The arsenical also inhibited the binding of [^{125}I]human neuropeptide Y to particulates from Y_5-HEK-293 cells, with an IC_{50} of 11 ± 3 μM. In guinea pig Y_1-CHO cells, phenylarsine oxide inhibited internalization of human neuropeptide Y with an IC_{50} of 2.4 ± 0.5 μM (91.1% inhibition at 30 μM; $n=6$), without clear inhibition of surface binding below 200 μM, while the binding of human neuropeptide Y to particulate guinea-pig Y_1-CHO receptors was inhibited with a K_I of 377 ± 25 μM (72 ± 4% inhibition at 1 mM; $n=3$). Above 1 mM, dithiothreitol was also inhibitory to Y_5 binding, with either cell monolayers or particulates. Inhibition of Y_5 internalization by phenylarsine oxide was not reversed by reincubation for up to 60 min in arsenical-free OptiMem® medium. (C) Inhibition by the clathrin network formation inhibitor sucrose. Inhibition of Y_5 receptor internalization by sucrose was fully reversed by reincubation without the sugar for 30 min in OptiMem® medium."DTT" = dithiothreitol.

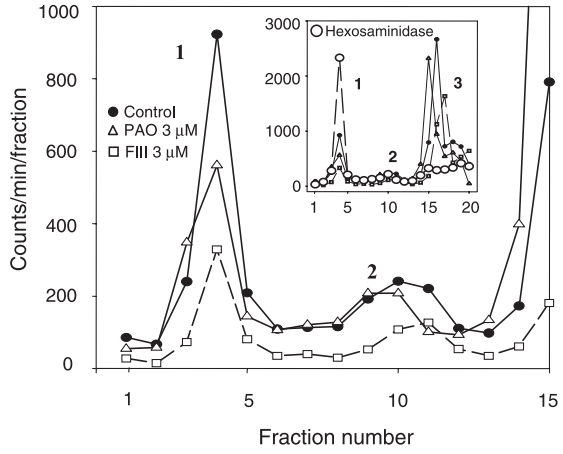

Fig. 5. Inhibition of the short-term incorporation of [^{125}I]neuropeptide Y-Aib-pancreatic polypeptide into endosomal and lysosomal particulates from human Y_5-HEK-293 cells by filipin III and phenylarsine oxide. The cells were labeled with [^{125}I]neuropeptide Y-Aib-pancreatic polypeptide for 20 min at 37 °C, with pretreatment (10 min at 37 °C) and cotreatment with 3 μM filipin III or 1 μM phenylarsine oxide over the interval of labeling. The cells were washed, lysed by N_2 cavitation, loaded on Percoll pro-gradients and sedimented as described in Section 2.5. Acid hexosaminidase assay was also done as described in Section 2.5. Aliquots of the gradient fractions were precipitated by polyethyleneglycol protocol (without and with lysing at 10 mM cholate; see Section 2.5) prior to radioactivity counting. The data in the graphs are from polyethyleneglycol precipitation without detergent lysis of particulates. The comparison of the binding without and with cholate lysis is given in Table 3. Abbreviations in the graph are explained in the legend of Fig. 4.

3.5. Association of the internalized neuropeptide Y Y_5 receptor–ligand complex with endosome-like elements

Endosomal specializations of plasma membrane are known to carry many internalized receptor–ligand peptide complexes (for reviews see e.g. Mukherjee et al., 1997; Ceresa and Schmid, 2000), and can be separated from the bulk of plasma membrane fragments by density gradient centrifugation (e.g. Urade et al., 1988; Tjelle et al., 1996). We have developed a Percoll gradient procedure (see Section 2.5) enabling a reproducible single-step isolation of an endosome-like fraction from homogenates of cell lines expressing Y_1, Y_2, Y_4 and Y_5 receptors, as well as transferrin receptors. With all receptors and cell types, these particulates (density 1.05–1.06 in Percoll gradients formed in 0.25 M sucrose) could be shown to carry up to 25% of short-term labeling of the cells by the respective [^{125}I] ligands, and this fraction could be significantly reduced by inhibitors of receptor internalization, including filipin III and phenylarsine oxide.

After 20 min of labeling with [^{125}I]neuropeptide Y-Aib-pancreatic polypeptide, sedimentation of human Y_5-HEK-293 cell homogenates in self-generating Percoll gradients resulted in definition of three particulate zones containing the labeled ligand (Fig. 5). The particles banding at density of 1.10–1.11 (labeled as zone 1) contained most of the acid hexosaminidase activity associated with total homogenate, and a significant fraction of the internalized ligand (up to 25% after labeling of 20 min at 37 °C). Somewhat heterogenous membrane material banding at density of 1.05–1.06 (buoyant zone 2) contained about a sixth of total particle-attached radioactivity. The plasma membrane/light endosome zone, buoyant at a low density (1.015; zone 3), contained most of the ligand in short-term labeling. With guinea pig Y_1-CHO receptor, the radioactivity in zone 2 was much more prominent than that associated with zone 1. Solubilization of particulates in the cold by 10 mM sodium cholate followed by precipitation with polyethyleneglycol recovered up to 60% of radioactivity from zone 3, and about a third of radioactivity from zone 2, but essentially no radioactivity from zone 1 with human Y_5-HEK-293 cells (Table 3). Lack of tracer precipitation by polyethyleneglycol indicates presence of the ligand physically separated from the receptor (as less than 5% of free [^{125}I]neuropeptide Y-Aib-pancreatic polypeptide or [^{125}I]human neuropeptide Y,

Table 3
Recovery of [^{125}I]human neuropeptide Y bound to hY_5-HEK-293 cells or to guinea-pig Y_1-CHO cells in Percoll gradient zones as related to treatment with inhibitors

Density zone	Control		Filipin III		Phenylarsine oxide	
	Y_5	Y_1	Y_5	Y_1	Y_5	Y_1
Percent of radioactivity recovered from zones 1–3 by polyethyleneglycol without solubilization						
1.10 (3)	24.5 ± 1.9	6.7 ± 0.4	13 ± 1.1	3.8 ± 0.4	14.5 ± 0.8	4.7 ± 0.7
1.05 (2)	13 ± 0.7	16.2 ± 0.25	10.2 ± 1	6.5 ± 1.4	10.4 ± 0.8	4.3 ± 0.8
1.015 (1)	62.5 ± 2.4	77.1 ± 0.72	76.8 ± 1.4	89.7 ± 0.8	75.1 ± 3.1	91 ± 4.3
Percent recovery by polyethyleneglycol after solubilization at 10 mM cholate						
1.10 (3)	< 1	37.7 ± 1.7	< 1	19.9 ± 2.6	< 1	20.1 ± 1.2
1.05 (2)	26.6 ± 3.7	69.6 ± 3.6	19.5 ± 6	63.7 ± 4.3	23 ± 4	77.5 ± 5.5
1.015 (1)	67.6 ± 4.5	82.9 ± 3.4	64.1 ± 6	76.7 ± 3.9	58 ± 5.1	86.1 ± 1

The cells were pretreated by 1 μM (Y_5) or 3 μM (Y_1) filipin III, and by 3 μM (Y_5) or 30 μM (Y_1) phenylarsine oxide for 15 min at 37 °C, labeled with 50 pM [^{125}I]neuropeptide Y-Aib-pancreatic polypeptide for 20 min (Y_5) or with 50 pM [^{125}I]human neuropeptide Y for 10 min (Y_1) at 37 °C in the presence of inhibitors, washed, cavitated and sedimented through self-generated Percoll gradients for 55 min at 68,000 × g_{max} (see Section 2.5). Aliquots of the gradient fractions were precipitated with polyethyleneglycol without or with prior lysis by 10 mM cholate (10 min at 0–4 °C) to assess the total and the receptor-associated radioactivity. The results are averages of four gradients (in two experimental runs) for each receptor.

input at up to 5 pM, was precipitated in the polyethyleneglycol procedure).

Filipin III at 3 μM produced a large decrease in labeling especially of zone 2 (Table 3; presumably secondary endosome material, on which see e.g. Sako et al., 1990; Tjelle et al., 1996). This could also be shown at 3 μM of phenylarsine oxide (Fig. 5). Filipin III also strongly decreased the labeling of zone 1 material (dense endosomes and primary lysosomes), which, however, was less pronounced with 3 μM phenylarsine oxide (Table 3).

Profiles of the labeling by [^{125}I]human neuropeptide Y of guinea pig Y_1-CHO receptor showed a very strong inhibition by either filipin III (at 3 μM) or phenylarsine oxide (at 30 μM) in lysosomal and endosomal bands, as well as a strong increase of plasma membrane labeling due to accumulation of surface receptors (Table 3 and Parker et al., 2002d). These results were reported in detail elsewhere (Parker et al., 2002d).

4. Discussion

Our results show saturable internalization of full-length neuropeptide Y or peptide YY-like peptides in HEK-293 cells expressing the human Y_5 neuropeptide Y receptor. This intake is highly dependent on the length of incubation and temperature, and is competed by isologous and homologous peptides. The binding and especially the internalization of Y_5 ligands also show a large sensitivity to a cysteine-bridging agent and to a membrane fluidity modifier, which strongly favors assumption of a receptor-linked entry. Also, a substantial portion of the Y_5 ligand associated with endosome-like particulates is, after solubilization, precipitated in the polyethyleneglycol/bovine γ-globulin procedure, indicating association with the Y_5 receptor. This fraction is much larger for the Y_1 receptor, which has a more stable steady-state association with neuropeptide Y (Parker et al., 2002d), and comprises up to 90% of endosome-associated internalized Y_4 ligand (Parker et al., 2002a).

The rates of human Y_5 receptor-linked internalization of neuropeptide Y in HEK-293 cells were much lower than found with the guinea pig Y_1-CHO receptor, while the rate of internalization of human transferrin was similar in the two lines. Also, human Y_1 receptors expressed in HEK-293 cells show a fast internalization and recycling (Gicquiaux et al., 2002), similar to our results with guinea-pig Y_1 expression in CHO cells (Parker et al., 2001c, 2002d, and this study). This is also similar to the known fast recycling of another neuropeptide receptor, the V_{1A} vasopressin receptor (Innamorati et al., 1999), and of the β_2-adrenoceptor (Barak et al., 1994).

The internalization rate difference between neuropeptide Y Y_5 and Y_1 receptors could principally result from a much lower affinity of Y_5 binding to the surface sites (also apparent with particulate receptors; compare e.g. Borowsky et al., 1998; Criscione et al., 1998; Moser et al., 2000; Statnick et al., 1998 for the Y_5, and Berglund et al., 1999; Gehlert et al., 1997; Parker et al., 1998a for the Y_1). This type of mechanistic causality could be relatively little dependent on the cell strain. Recently, we were able to show that the rank order of human pancreatic polypeptide affinities is reflected in the internalization rates of three mammalian pancreatic polypeptide Y_4 receptors in CHO cells (Parker et al., 2002a).

Our results indicate a substantially higher internalization rate for neuropeptide Y and a hybrid neuropeptide Y and pancreatic polypeptide-related Y_5 receptor agonist (Cabrele et al., 2000) compared to peptide YY and related full-length Y peptides. This is similar to our recent findings with the Y_1 receptor, which also preferentially internalizes neuropeptide Y-related peptides over peptide YY and analogues (Parker et al., 2002d). The Y_5 receptor gene apparently arose through endoreduplication of the Y_1 receptor gene (Herzog et al., 1997), probably prior to the emergence of the peptide YY subfamily of Y peptides (Larhammar et al., 1998), and its protein product could be structurally attuned to handle neuropeptide Y rather than peptide YY. Also, most of the evolution of the Y_1/Y_5 receptor gene tandem could be tied to neural environments, which in species now extant mainly express and process neuropeptide Y.

The low internalization of N-terminally truncated peptide YY derivative human peptide YY-(3-36) strongly contrasts the relatively high-affinity attachment of the peptide to surface Y_5 sites. This may indicate participation of N-terminal amino acids of neuropeptide Y in attachment(s) to endocytotic vehicles internalizing the Y_5 receptor–ligand peptide complex. The clipped peptide YY derivative could act as an antagonist (or a partial agonist) at the human Y_5-HEK-293 site, in line with recent experiments indicating its ability to inhibit feeding (Batterham et al., 2002).

The Y_4 receptor ligand human pancreatic polypeptide was efficacious only as a competitor of neuropeptide Y, and not as an independent agonist-like ligand, and it also did not internalize via the Y_5 site, or non-saturably. The pancreatic polypeptide may act similar to certain opioid agonists (Whistler et al., 1999), however, presenting an exceptionally high relative [agonist] activity versus endocytosis (RAVE) value at the Y_5 receptor.

The low non-saturable binding found for human pancreatic polypeptide probably results from a low affinity for membrane lipids, and helps explain why this peptide does not pass the hematoencephalic barrier (Whitcomb et al., 1990), in contrast to neuropeptide Y (Kastin and Akerstrom, 1999). It is of interest that the related rat pancreatic polypeptide (which, however, displays a significant sequence difference with either the human or the bovine pancreatic polypeptide; see Parker et al., 2002b) was shown in several studies to poorly contest the Y_5 receptor binding of agonists such as neuropeptide Y or peptide YY (Gerald et al., 1996; Moser et al., 2000; Parker et al., 2002a), which in the present study were found to internalize significantly via the Y_5

receptor. Both human pancreatic polypeptide and rat pancreatic polypeptide differ from full-length neuropeptide Y/peptide YY peptides by substitution of Ala for Tyr as the N-terminal residue, and by numerous other non-conservative substitutions in the N-terminal portion of their molecules (see Parker et al., 2002a). These substitutions obviously reduce both the binding of pancreatic polypeptides to the Y_5 site and the incidence of their internalization. As predictable from binding studies with particulate Y_5 receptors, our results with intact cells confirm the ability of human pancreatic polypeptide to modulate both the surface binding and the internalization of neuropeptide Y. This could indicate a concentration-related ability of pancreatic polypeptides to serve as partial antagonists of Y_5 receptors.

Internalization differences reported here could also reflect differences in receptor environment and organization, including membrane fluidity and cholesterol disposition (see e.g. Gimpl et al., 1997). The large sensitivity of cell surface or particulate Y_5 receptors to the cholesterol-complexing polyene filipin III underlines the conformational fragility of Y_5 binding site(s). This could further point to an important regulation of Y5 receptor activity by osmotic changes and ion fluxes, especially in neuronal surroundings.

Both the cell-surface and particulate binding to Y_5 receptors is exceptionally sensitive to the cysteine-bridging arsenical, phenylarsine oxide. This could result from cross-linking of vicinal cysteines in the seventh transmembrane segment (paired in this domain of all Y_5 receptors, but not of any other Y receptor type). Bridging or alkylation of these residues might impart a large ligand dynamics to the binding site of the Y_5 receptor.

Evolution of Y_5 receptor function toward ligand sharing and a lower affinity of binding relative to other Y receptors might have added functionality also seen in the glucagon/secretin family of G-protein coupling receptors, which all accommodate numerous peptidic ligands (see e.g. Lundberg et al., 2001). The versatile utilization of ligand peptides across these receptors could provide a dynamic reserve necessary for coping with multiple metabolic demands. A binary Y_1/Y_5 receptor functionality can help explain the long feeding stimulation induced by intracerebroventricular neuropeptide Y (Kalra et al., 1999).

From experiments presented in this study, the limited internalization or sequestration of Y_5 receptor ligands can be viewed as a tool for accommodating or containing the agonist potential. A large accumulation seen for the Y_5 receptor ligand in the lysosome/dense endosome fraction is not found with the Y_1 receptor ligand in CHO cells. This is reminiscent of differences e.g. between the endothelin-B receptor, metabolically consigned to processing via a lysosomal compartment (Oksche et al., 2000), and the faster-recycling endothelin-A receptor (Bremnes et al., 2000). Our results indicate separation of the Y_5 receptor from the internalized agonist prior to, or at the lysosomal stage, and the undamaged receptor might escape degradation. Also, the lysosomal sorting for a Y receptor could be somewhat cell type-specific (although the available studies with the Y_1 receptor show no significant difference between the HEK-293; Gicquiaux et al., 2002; and the CHO expressions; Parker et al., 2001c, 2002d). The slow internalization of the Y_5-HEK-293 receptor may largely reflect a non-cycling, degradative removal, which represents a much lower fraction of internalized traffic for the Y_1-HEK-293 (Gicquiaux et al., 2002), or for the Y_1-CHO receptor.

Acknowledgements

This investigation was supported in part by NIH grant HD 13703, and by the State of Tennessee Educational Funds. We gratefully acknowledge the synthesis of the Y_5 receptor competitor Hu 296 by Dr. Christoph Hutzler (University of Regensburg, Germany).

References

Allard, W.J., Sigal, I.S., Dixon, R.A., 1987. Sequence of the gene encoding the human M1 muscarinic acetylcholine receptor. Nucleic Acids Res. 15, 10604.

Barak, L.S., Tiberi, M., Freedman, N.J., Kwatra, M.M., Lefkowitz, R.J., Caron, M.G., 1994. A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated beta 2-adrenergic receptor sequestration. J. Biol. Chem. 269, 2790–2795.

Batterham, R.L., Cowley, M.A., Small, C.J., Herzog, H., Cohen, M.A., Dakin, C.L., Wren, A.M., Brynes, A.E., Low, M.J., Ghatei, M.A., Cone, R.D., Bloom, S.R., 2002. Gut hormone PYY(3-36) physiologically inhibits food intake. Nature 418, 650–654.

Berglund, M.M., Holmberg, S.K., Eriksson, H., Gedda, K., Maffrand, J.P., Serradeil-Le Gal, C., Chhajlani, V., Grundemar, L., Larhammar, D., 1999. The cloned guinea pig neuropeptide Y receptor Y1 conforms to other mammalian Y1 receptors. Peptides 20, 1043–1053.

Borowsky, B., Walker, M.W., Bard, J., Weinshank, R.L., Laz, T.M., Vaysse, P., Branchek, T.A., Gerald, C., 1998. Molecular biology and pharmacology of multiple NPY Y5 receptor species homologs. Regul. Pept. 75–76, 45–53.

Bremnes, T., Paasche, J.D., Mehlum, A., Sandberg, C., Bremnes, B., Attramadal, H., 2000. Regulation and intracellular trafficking pathways of the endothelin receptors. J. Biol. Chem. 275, 17596–17604.

Cabrele, C., Langer, M., Bader, R., Wieland, H.A., Doods, H.N., Zerbe, O., Beck-Sickinger, A.G., 2000. The first selective agonist for the neuropeptide YY5 receptor increases food intake in rats. J. Biol. Chem. 275, 36043–36048.

Ceresa, B.P., Schmid, S.L., 2000. Regulation of signal transduction by endocytosis. Curr. Opin. Cell Biol. 12, 204–210.

Criscione, L., Rigollier, P., Batzl-Hartmann, C., Rueger, H., Stricker-Krongrad, A., Wyss, P., Brunner, L., Whitebread, S., Yamaguchi, Y., Gerald, C., Heurich, R.O., Walker, M.W., Chiesi, M., Schilling, W., Hofbauer, K.G., Levens, N., 1998. Food intake in free-feeding and energy-deprived lean rats is mediated by the neuropeptide Y5 receptor. J. Clin. Invest. 102, 2136–2145.

Dumont, Y., Cadieux, A., Doods, H., Fournier, A., Quirion, R., 2000. Potent and selective tools to investigate neuropeptide Y receptors in the central and peripheral nervous systems: BIB03304 (Y1) and CGP71683A (Y5). Can. J. Physiol. Pharmacol. 78, 116–125.

Fabry, M., Langer, M., Rothen-Rutishauser, B., Wunderli-Allenspach, H., Hocker, H., Beck-Sickinger, A.G., 2000. Monitoring of the internal-

ization of neuropeptide Y on neuroblastoma cell line SK-N-MC. Eur. J. Biochem. 267, 5631–5637.

Fire, E., Zwart, D.E., Roth, M.G., Henis, Y.I., 1991. Evidence from lateral mobility studies for dynamic interactions of a mutant influenza hemagglutinin with coated pits. J. Cell Biol. 115, 1585–1594.

Gehlert, D.R., Schober, D.A., Gackenheimer, S.L., Beavers, L., Gadski, R., Lundell, I., Larhammar, D., 1997. [^{125}I]Leu31, Pro34-PYY is a high affinity radioligand for rat PP1/Y4 and Y1 receptors: evidence for heterogeneity in pancreatic polypeptide receptors. Peptides 18, 397–401.

Gerald, C., Walker, M.W., Criscione, L., Gustafson, E.L., Batzl-Hartmann, C., Smith, K.E., Vaysse, P., Durkin, M.M., Laz, T.M., Linemeyer, D.L., Schaffhauser, A.O., Whitebread, S., Hofbauer, K.G., Taber, R.I., Branchek, T.A., Weinshank, R.L., 1996. A receptor subtype involved in neuropeptide-Y-induced food intake. Nature 382, 168–171.

Gicquiaux, H., Lecat, S., Gaire, M., Dieterlen, A., Mely, Y., Takeda, K., Bucher, B., Galzi, J.L., 2002. Rapid internalization and recycling of the human neuropeptide Y Y(1) receptor. J. Biol. Chem. 277, 6645–6655.

Gimpl, G., Burger, K., Fahrenholz, F., 1997. Cholesterol as modulator of receptor function. Biochemistry 36, 10959–10974.

Herzog, H., Darby, K., Ball, H., Hort, Y., Beck-Sickinger, A., Shine, J., 1997. Overlapping gene structure of the human neuropeptide Y receptor subtypes Y1 and Y5 suggests coordinate transcriptional regulation. Genomics 41, 315–319.

Hislop, J.N., Everest, H.M., Flynn, A., Harding, T., Uney, J.B., Troskie, B.E., Millar, R.P., McArdle, C.A., 2001. Differential internalization of mammalian and non-mammalian gonadotropin-releasing hormone receptors. Uncoupling of dynamin-dependent internalization from mitogen-activated protein kinase signaling. J. Biol. Chem. 276, 39685–39694.

Innamorati, G., Sadeghi, H., Birnbaumer, M., 1999. Phosphorylation and recycling kinetics of G protein-coupled receptors. J. Recept. Signal Transduct. Res. 19, 315–326.

Kalra, S.P., Dube, M.G., Pu, S., Xu, B., Horvath, T.L., Kalra, P.S., 1999. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocr. Rev. 20, 68–100.

Kastin, A.J., Akerstrom, V., 1999. Nonsaturable entry of neuropeptide Y into brain. Am. J. Physiol. 276, E479–E482.

Keire, D.A., Bowers, C.W., Solomon, T.E., Reeve, J.R., 2001. Structure and receptor binding of PYY analogs. Peptides 23, 305–321.

Kil, S.J., Hobert, M., Carlin, C., 1999. A leucine-based determinant in the epidermal growth factor receptor juxtamembrane domain is required for the efficient transport of ligand–receptor complexes to lysosomes. J. Biol. Chem. 274, 3141–3150.

Larhammar, D., Soderberg, C., Lundell, I., 1998. Evolution of the neuropeptide Y family and its receptors. Ann. N.Y. Acad. Sci. 839, 35–40.

Lundberg, P., Lundgren, I., Mukohyama, H., Lehenkari, P.P., Horton, M.A., Lerner, U.H., 2001. Vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide receptor subtypes in mouse calvarial osteoblasts: presence of VIP-2 receptors and differentiation-induced expression of VIP-1 receptors. Endocrinology 142, 339–347.

Lundell, I., Statnick, M.A., Johnson, D., Schober, D.A., Starback, P., Gehlert, D.R., Larhammar, D., 1996. The cloned rat pancreatic polypeptide receptor exhibits profound differences to the orthologous receptor. Proc. Natl. Acad. Sci. U. S. A. 93, 5111–5115.

Michel, M.C., Beck-Sickinger, A., Cox, H., Doods, H.N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., Westfall, T., 1998. XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. Pharmacol. Rev. 50, 143–150.

Moser, C., Bernhardt, G., Michel, J., Schwarz, H., Buschauer, A., 2000. Cloning and functional expression of the hNPY Y5 receptor in human endometrial cancer (HEC-1B) cells. Can. J. Physiol. Pharmacol. 78, 134–142.

Mukherjee, S., Ghosh, R.N., Maxfield, F.R., 1997. Endocytosis. Physiol. Rev. 77, 759–803.

Munson, P.J., Rodbard, D., 1980. LIGAND: a versatile computerized approach for characterization of ligand-binding proteins. Anal. Biochem. 107, 220–239.

Okamoto, P.M., Herskovits, J.S., Vallee, R.B., 1997. Role of the basic, proline-rich region of dynamin in Src homology 3 domain binding and endocytosis. J. Biol. Chem. 272, 11629–11635.

Oksche, A., Boese, G., Horstmeyer, A., Furkert, J., Beyermann, M., Bienert, M., Rosenthal, W., 2000. Late endosomal/lysosomal targeting and lack of recycling of the ligand-occupied endothelin B receptor. Mol. Pharmacol. 57, 1104–1113.

Parker, R.B., Waud, D.R., 1971. Pharmacological estimation of drug–receptor dissociation constants. Statistical evaluation. I. Agonists. J. Pharmacol. Exp. Ther. 177, 1–12.

Parker, S.L., Parker, M.S., Crowley, W.R., 1998a. Characterization of Y1, Y2 and Y5 subtypes of the neuropeptide Y (NPY) receptor in rabbit kidney. Sensitivity of ligand binding to guanine nucleotides and phospholipase C inhibitors. Regul. Pept. 75–76, 127–143.

Parker, S.L., Parker, M.S., Sweatman, T., Crowley, W.R., 1998b. Characterization of G-protein and phospholipase C-coupled agonist binding to the Y1 neuropeptide Y receptor in rat brain. Sensitivity to G-protein activators and inhibitors and to inhibitors of phospholipase C. J. Pharmacol. Exp. Ther. 286, 1–12.

Parker, E.M., Balasubramaniam, A., Guzzi, M., Mullins, D.E., Salisbury, B.G., Sheriff, S., Witten, M.B., Hwa, J.J., 2000. [D-Trp(34)] neuropeptide Y is a potent and selective neuropeptide Y Y(5) receptor agonist with dramatic effects on food intake. Peptides 21, 393–399.

Parker, M.S., Berglund, M.M., Lundell, I., Parker, S.L., 2001a. Blockade of pancreatic polypeptide-sensitive neuropeptide Y (NPY) receptors by agonist peptides is prevented by modulators of sodium transport. Implications for receptor signaling and regulation. Peptides 22, 887–898.

Parker, M.S., Lundell, I., Berglund, M.M., Parker, S.L., 2001b. Internalization of pancreatic polypeptide Y4 receptors: correlation of receptor intake and affinity. Society for Neuroscience 2001 Annual Meeting, San Diego, CA. Abstract 461.468.

Parker, S.L., Kane, J.K., Parker, M.S., Berglund, M.M., Lundell, I.A., Li, M.D., 2001c. Cloned neuropeptide Y (NPY) Y1 and pancreatic polypeptide Y4 receptors expressed in Chinese hamster ovary cells show considerable agonist-driven internalization, in contrast to the NPY Y2 receptor. Eur. J. Biochem. 268, 877–886.

Parker, M.S., Lundell, I., Parker, S.L., 2002a. Internalization of pancreatic polypeptide Y4 receptors: correlation of receptor intake and affinity. Eur. J. Pharmacol. 452, 279–287.

Parker, M.S., Lundell, I., Parker, S.L., 2002b. Pancreatic polypeptide receptors: affinity, sodium sensitivity and stability of agonist binding. Peptides 23, 291–302.

Parker, S.L., Parker, M.S., Kane, J.K., Berglund, M.M., 2002c. A pool of Y2 neuropeptide Y receptors activated by modifiers of membrane sulfhydryl or cholesterol balance. Eur. J. Biochem. 269, 2315–2322.

Parker, S.L., Parker, M.S., Lundell, I., Balasubramaniam, A., Buschauer, A., Kane, J.K., Yalcin, A., Berglund, M.M., 2002d. Agonist internalization by cloned Y1 neuropeptide Y (NPY) receptor in Chinese hamster ovary (CHO) cells shows strong preference for NPY, endosome-linked entry and fast receptor recycling. Regul. Pept. 107, 49–62.

Rueger, H., Schmidlin, T., Rigollier, P., Yamaguchi, Y., Tintelnot-Blomley, M., Schilling, W., Criscione, L., Mah, R., 1997. Preparation of 2-aminoquinazolines as neuropeptide Y subtype Y5 receptor antagonists. Chem. Abstr. 127, 108941.

Sako, Y., Sato, S.B., Ohnishi, S., 1990. Subpopulations of endosomes generated at sequential stages in the endocytic pathway of asialoganglioside-containing ferrite ligands in rat liver. J. Biochem. (Tokyo) 107, 846–853.

Sharma, P., Holmberg, S.K., Eriksson, H., Beck-Sickinger, A.G., Grundemar, L., Larhammar, D., 1998. Cloning and functional expression of the guinea pig neuropeptide Y Y2 receptor. Regul. Pept. 75–76, 23–28.

Statnick, M.A., Schober, D.A., Gackenheimer, S., Johnson, D., Beavers, L., Mayne, N.G., Burnett, J.P., Gadski, R., Gehlert, D.R., 1998. Characterization of the neuropeptide Y5 receptor in the human hypothalamus: a

lack of correlation between Y5 mRNA levels and binding sites. Brain Res. 810, 16–26.

Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M.A., Keen, J.H., McGraw, T.E., 1999. Acute cholesterol depletion inhibits clathrin-coated pit budding. Proc. Natl. Acad. Sci. U. S. A. 96, 6775–6780.

Tjelle, T.E., Brech, A., Juvet, L.K., Griffiths, G., Berg, T., 1996. Isolation and characterization of early endosomes, late endosomes and terminal lysosomes: their role in protein degradation. J. Cell Sci. 109, 2905–2914.

Urade, R., Hayashi, Y., Kito, M., 1988. Endosomes differ from plasma membranes in the phospholipid molecular species composition. Biochim. Biophys. Acta 946, 151–163.

Vogler, O., Nolte, B., Voss, M., Schmidt, M., Jakobs, K.H., van Koppen, C.J., 1999. Regulation of muscarinic acetylcholine receptor sequestration and function by beta-arrestin. J. Biol. Chem. 274, 12333–12338.

Whistler, J.L., Chuang, H.H., Chu, P., Jan, L.Y., von Zastrow, M., 1999. Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron 23, 737–746.

Whitcomb, D.C., Taylor, I.L., Vigna, S.R., 1990. Characterization of saturable binding sites for circulating pancreatic polypeptide in rat brain. Am. J. Physiol. 259, G687–G691.

Wraith, A., Tornsten, A., Chardon, P., Harbitz, I., Chowdhary, B.P., Andersson, L., Lundin, L.G., Larhammar, D., 2000. Evolution of the neuropeptide Y receptor family: gene and chromosome duplications deduced from the cloning and mapping of the five receptor subtype genes in pig. Genome Res. 10, 302–310.