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Importance of a N-terminal aspartate in the internalization of the neuropeptide Y Y₂ receptor

Steven L. Parker^{a*}, Michael S. Parker^b, Ying Y. Wong^a, Renu Sah^c, Ambikaipakan Balasubramaniam^d, and Floyd Sallee^c

^aDepartment of Pharmacology, University of Tennessee Health Science Center, Memphis, TN 38163, USA

^bDepartment of Microbiology and Molecular Cell Sciences, University of Memphis, Memphis, TN 38152, USA

^cDepartment of Psychiatry, College of Medicine, University of Cincinnati, Cincinnati, OH 45267, USA

^dDepartment of Surgery, College of Medicine, University of Cincinnati, Cincinnati, OH 45267, USA

Abstract

With human neuropeptide Y Y₂ receptor expressed in the Chinese hamster ovary (CHO) cells, the Asp³⁵Ala mutation, and especially the change of Pro³⁴Asp³⁵ to Ala³⁴Ala³⁵, decrease the compartmentalization and strongly accelerate internalization of the receptor. These changes are not associated with alterations in agonist affinity, G-protein interaction, dimerization, or level of expression of the mutated receptors relative to the wildtype receptor. The proline-flanked aspartate in the N-terminal extracellular segment of the neuropeptide Y Y₂ receptor thus apparently has a large role in anchoring and compartmentalization of the receptor. However, the Pro³⁴Ala mutation does not significantly affect the embedding and cycling of the receptor.

Keywords

aspartate; compartmentalization; receptor cycling; receptor endocytosis; receptor masking

1. Introduction

The neuropeptide Y Y₂ receptor stands out in the rhodopsin family of G-protein coupling receptors by twin features of slow cycling (Parker et al., 2001; Gicquiaux et al., 2002; Berglund et al., 2003b) and large surface compartmentalization (Parker et al., 2001; Parker et al., 2002a; Parker et al., 2007c), thus far not shown for any other member of this receptor group. There also is a tenacious attachment of agonists to the neuropeptide Y Y₂ receptor in particulates from fragmented cells (Dautzenberg and Neysari, 2005) and on the surface of intact cells (Parker et al., 2007a). In conjunction with the low endocytosis of the neuropeptide Y Y₂ receptor (Parker et al., 2001; Gicquiaux et al., 2002) this could enable an extended signaling per agonist binding event. The above features could also be linked to the known participation of this receptor in angiogenesis (Zukowska-Grojec et al., 1998; Ekstrand et al., 2003), and to

*Corresponding author. Tel.: +1 901 850 7617. E-mail address: stevenleonardparker@msn.com (S.L. Parker).

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the negative regulation of transmitter release (Tu et al., 2005) and feeding (Batterham et al., 2002) via the receptor.

Structural features responsible for the generally low cycling of the neuropeptide Y Y₂ receptor are currently not defined. However, the multiple anionic sidechains in the N-terminal extracellular domain, including those in the unique PDPEPE motif (residues 34–39 in the sequence of the human receptor (Rose et al., 1995)), could significantly contribute to immobilizing interactions with the extracellular matrix as well as with cell-linking receptors, including integrins and cadherins. N-terminal sequences rich in acidic residues are also present in a number of other peptide receptors known to support angiogenesis (Parker et al., 2005b).

To clarify the possible importance of this type of structure for properties of the neuropeptide Y Y₂ receptor, we mutated to alanine the Pro³⁴ and Asp³⁵ residues in the human receptor. These choices were based on the known importance of aspartate for interactions of integrins and other receptors with the extracellular matrix (e.g. Ma et al., 1995 and Kamata et al., 1995). Our results indicate that the N-terminal Asp³⁵, especially in tandem with Pro³⁴, does strongly restrict the dynamism of the neuropeptide Y Y₂ receptor in the Chinese hamster ovary (CHO) cell membranes.

2. Materials and methods

2.1 Mutation and expression of the neuropeptide Y Y₂ receptor

Oligonucleotides were constructed using the PrimerX program on the Bioinformatics website, and synthesized by Integrated DNA Technologies (Skokie, IL, USA). A³⁴: fw 5' CTAGAGGTGAACTGGTCGCTGACCCTGAGCCAGAG 3'; rv 5' CTCTGGCTCAGGGTCAGCGACCAGTTCACCTCTAG 3' A³⁴A³⁵: fw 5' CTCCTAGAGGTGAACTGGTCGCTGCCCCCTGAGCCAGAGCTTATAG 3'; rv 5' CTATAAGCTCTGGCTCAGGGGCAGCGACCAGTTCACCTCTAGGAG 3' A³⁵: fw 5' GAGGTGAACTGGTCCCTGCGCCTGAGCCAGAGCTTATAG 3'; rv 5' CTATAAGCTCTGGCTCAGGCGCAGGGACCAGTTCACCTC 3'

The oligonucleotides were used to induce mutations in the human neuropeptide Y Y₂ receptor wildtype cDNA packaged in the Invitrogen (Carlsbad, CA, USA) pc3.1+ vector. This cDNA was donated by the University of Missouri at Rolla (MO, USA). The mutations were done with Stratagene (La Jolla, CA, USA) QuickChange II mutagenesis kit, using the instructions supplied by the manufacturer. The successful mutations (as verified by sequencing performed by the Molecular Resources Center of the University of Tennessee Health Science Center at Memphis, TN, USA) were amplified and stably expressed in the Chinese hamster ovary (CHO) – K1 cells (American Type Culture Collection, Bethesda, MD, USA) at 400 µg/ml of geneticin. All peptides were from the American Peptide Co. (Sunnyvale, CA, USA). The radioactive tracers were supplied by PerkinElmer (Cambridge, MA, USA).

2.2 Cell culture

The cell culture was done essentially as described (Parker et al., 2005a). The K1 line of Chinese hamster ovary cells (CHO-K1; American Type Culture Collection, Baltimore, MD, USA) stably expressing the cloned non-mutated or mutated human Y₂ receptors were cultured at 400 µg/ml geneticin in D-MEM/F12 medium (Gibco, Long Island, NY, USA) containing 6% (v/v) of fetal calf serum.

2.3 The assay for internalization of the neuropeptide Y Y₂ receptor

This assay employed 25 – 200 pM of [¹²⁵I]-labeled human peptide YY(3–36) or 100 pM porcine peptide YY(1–36), over 1 to 15 min at 37 °C. After washing with cold saline / Ca²⁺ /

Mg²⁺ solution (140 mM NaCl + 3 mM CaCl₂ + 1 mM MgCl₂ + 10 mM NaHCO₃), the surface Y₂ binding was quantitated by extraction with cold 0.2 M acetic acid – 0.5 M sodium chloride (7 min at 0–4 °C), and the tracer internalized with the receptor was measured in residues following this extraction. The unextracted labeled peptide was considered as internalized with the receptor (as documented in (Parker et al., 2002b), esp. Fig. 1; also see Fig. 3C in this study.).

2.4 The assay of compartmentalization of the neuropeptide Y Y₂ receptor

The unmasking of surface sites was effected by pre-treatment with the permeabilizing detergent digitonin at 10 μM for 15 min at 25 °C, followed by washing and labeling with [¹²⁵I] peptide YY(3–36) over 4 or 10 min at 37 °C, or by labeling for 4 or 10 min at 37 °C in the presence of 10 μM phenylarsine oxide (added to the cells at 37 °C 5 min before the tracer). The labeling was terminated by dilution and washing with the cold saline solution, and extraction with 0.2 M CH₃COOH / 0.5 M NaCl as above. Pertussis toxin at 1 ng / ml in the culture medium over 18 hours was used to deplete the compartmentalized surface Y₂ sites (Parker et al., 2007b; Parker et al., 2007c).

2.5 The receptor and G-protein binding assays

To prepare particulates, the cells were homogenized in the receptor assay buffer (Parker et al., 2005a) using a Dounce homogenizer (8 strokes of the 0.1 mm-clearance pestle), the debris and nuclei were removed by sedimentation for 5 min at 600 g_{max}, and the supernatant sedimented for 12 min at 30,000 g_{max} to obtain pellets which were stored at –80 °C prior to use in assays. The affinity of the neuropeptide Y Y₂ receptor was assessed in particulates from the respective expressions by competition of 100 pM [¹²⁵I] peptide YY(3–36) by unlabeled peptide YY(3–36) or peptide YY(1–36) in the range of 3 pM to 100 nM (Parker et al., 2005a). The activation of the G_i α subunit nucleotide site by the neuropeptide Y Y₂ receptor agonist PYY(3–36) was measured at 0.2 nM [³⁵S]guanosine 5'-O-(3-thiotriphosphate) (GTPγS) using 0.01 – 100 nM peptide YY(3–36) (Parker et al., 2005a). The quantitation of the neuropeptide Y Y₂ receptor monomers and dimers, after labeling for 30 min at 25 °C with 20 pM [¹²⁵I] peptide YY(3–36) and solubilization by 10 mM each of cholate and digitonin at 4 °C, was done by the sucrose gradient procedure detailed in (Parker et al., 2008).

2.6 Data analysis and statistics

The receptor binding parameters were estimated in the LIGAND program (Munson and Rodbard, 1980). The half-periods and maxima of saturation in internalization studies were estimated by non-linear hyperbolic fitting in the SigmaPlot software (SPSS, Chicago, IL, version 8.02). The proportion of dimers in the gradient profiles was estimated using the ImageJ program, available at the website of the U.S. National Institutes of Health.

3. Results

3.1 Levels, affinity, G-protein activity and oligomerization of the neuropeptide Y Y₂ receptor following N-terminal mutations

The mutations to A³⁴, A³⁵ and A³⁴A³⁵ did not strongly affect the overall expression of the neuropeptide Y Y₂ receptor relative to the wildtype. Thus, using cDNA and lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) inputs of 1 μg/ml and 1.5 μl/ml, respectively, and 30 ml of F12/D-MEM medium per 75 cm² layer of confluent CHO cells, the total level of the receptors at the geneticin passage 8 was in the B_{max} range of 60–75 fmol/mg protein (Table 1), or 40–50 fmol/100,000 cells (Fig. 1A), compared to about 80 fmol/mg protein or 55 fmol/100,000 cells for the wildtype receptor at the same passage (Table 1 and Fig. 1A). The surface receptor unmasked by phenylarsine oxide was close to the wildtype level for the A³⁴ mutation, significantly lower than in the wildtype expression for the A³⁵ mutation, and strongly reduced

for the A³⁴A³⁵ mutation (Fig. 1B; also see Fig. 2C). The basal receptor levels, however, did not differ significantly (Fig. 1B; see also Fig. 2C).

With particulates isolated from homogenates of CHO cells expressing the wildtype and mutated neuropeptide Y Y₂ receptors, affinities for the Y₂ subtype-selective agonist peptide YY(3–36) and for the non-selective Y receptor agonist peptide YY(1–36) were quite similar (Table 1). The G-protein coupling ability, as evaluated by stimulation with 100 nM peptide YY(3–36) of the binding of [³⁵S]GTPγS to particulates, did not show significant affinity differences (Fig. 1C). The ratios of the binding at 200 pM [³⁵S]GTPγS and at 100 pM [¹²⁵I] peptide YY(3–36) were for any expression within 20% of the common average (the legend of Fig. 1C). The labeling of receptor monomers and dimers was also similar for the wildtype and the mutated receptors. More than 60% of the receptor-bound agonist was associated with the ~180 kDa complex of the Y₂ dimer with heterotrimeric G-proteins (Parker et al., 2007c) (Fig. 1D).

3.2 Compartmentalization of the neuropeptide Y Y₂ receptor following N-terminal mutations

A general consideration of the compartmentalization of the neuropeptide Y Y₂ receptor in a cell line and in rat brain is presented in two recent communications (Parker et al., 2007c; Parker and Balasubramaniam, 2008). Unmasking by 10 μM phenylarsine oxide increased the agonist-accessible surface receptor to a large and similar degree for the wildtype receptor and the A³⁴ mutation (Fig. 2A). However, relative to the basal level, the unmasked receptor was significantly less for the A³⁵ mutation, and very much lower with the change to A³⁴A³⁵ (Fig. 2A). A similar decrease in compartmentalization of the receptor for the A³⁴A³⁵ and the A³⁵ mutation was found by unmasking with the detergent digitonin at 10 μM (Fig. 2B). It should be emphasized that the pretreatment with this concentration of digitonin at 25 °C did not result in a loss of cell protein or a detachment of cells. Pretreatment with pertussis toxin at 1 ng/ml for 18 h did not substantially reduce the basal cell surface binding of the agonist [¹²⁵I] peptide YY(3–36) in either the wildtype or the A³⁴A³⁵ expression. However, the surface sites that could be unmasked by the arsenical, as expected, were strongly reduced by the toxin in the wildtype (Parker et al., 2007b), and almost eliminated in the mutant expression, as observed at either 4 min or 10 min of labeling with the agonist (Fig. 2C).

3.3 Internalization of the neuropeptide Y Y₂ receptor following N-terminal mutations

Effects of mutations on the rate of internalization of the neuropeptide Y Y₂ receptor were first examined with the A³⁴A³⁵ mutant using four concentrations of [¹²⁵I] peptide YY(3–36) in the range producing a substantial saturation (as based on the K_{diss} values in Table 1) of the binding of this agonist (Fig. 3A, B). The internalization was found to be accelerated significantly in the mutant over the entire input range of the labeled agonist, at either 4 or 10 min of labeling. The increase relative to the wildtype was similar at 4 min (Fig. 3A) and 10 min (Fig. 3B) of labeling. As shown in Fig. 3C, at an input of 100 pM the recovered internalized peptide is intact at a constant ~80% between 2 and 15 min of incorporation. The intact agonist is tenaciously bound and should be largely associated with the receptor (Dautzenberg and Neysari, 2005; Parker et al., 2007c). We found no accumulation of either the principal product of peptide YY processing, the C-terminal hexapeptide VTRQRY (Frerker et al., 2007), or of the C-terminal tyrosine, both of which would be detected by the [¹²⁵I] label (which in peptide YY (3–36) labeled via the chloramine-T procedure is localized by more than 85% in the C-terminal tyrosine).

The agonist internalization, as measured by the uptake of 100 pM [¹²⁵I] peptide YY(3–36), was much increased by the A³⁴A³⁵ mutation, and also strongly increased by the A³⁵ mutation (Fig. 4A). The A³⁴ mutation, however, showed a low degree of internalization, similar to the wildtype receptor (Fig. 4A and Table 2). Internalization was also compared using as the tracer the non-selective Y receptor agonist [¹²⁵I] peptide YY(1–36) (the parent peptide of peptide

YY(3–36)), which at the Y₂ sites has binding properties very similar to peptide YY(3–36) (Table 1). The full-length peptide YY internalized somewhat faster than peptide YY(3–36) in the wildtype expression, and especially in the A³⁴A³⁵ expression (Fig. 4B and Table 2). The difference in internalization between the A³⁴A³⁵ mutation and the wildtype expression was especially pronounced at lower temperatures, being about fourfold between 4–30 min at 15 °C.

We also tested the anionic polysaccharide heparin as a possible promoter of internalization of the neuropeptide Y Y₂ receptor. At 5 μM, heparin (6.5 kDa) indeed increased the internalization of 100 pM [¹²⁵I] peptide YY(3–36) significantly at either 4 min or 10 min in the A³⁴A³⁵ expression (by 43.5 ± 3.5 and 25.1 ± 5%, respectively), but only marginally in the wildtype expression (19.4 ± 2 and 4.8 ± 1.8%, respectively; n = 12 for both). At up to 30 μM, heparin did not affect the affinity and capacity of the binding of peptide YY(3–36) to either the cell surface or the particulate neuropeptide Y Y₂ receptors.

4. Discussion

The internalization of agonists of the neuropeptide Y Y₂ receptor is prevented by common inhibitors of clathrin-linked endocytosis (Parker et al., 2001; Parker et al., 2002a), and thus should be coupled with endocytosis of the receptor. One should note that the estimated rate of internalization of the human neuropeptide Y Y₂ receptor in CHO cells at 100 pM of the agonist peptide YY(3–36) (wildtype half-period of ~ 6 min) is much higher than the rate we previously found for the guinea-pig neuropeptide Y Y₂ receptor at 50 pM of the same agonist (half-period ~15 min; (Parker et al., 2001). This difference is connected primarily to the twice larger concentration of the labeled agonist used in the current study, since the internalization of unmodified Y receptors is substantially accelerated by increasing concentration of the agonists (Parker et al., 2005a). We found a sustained increase in the rate of internalization of the receptor-linked agonist with concentrations up to 200 pM.

Our current estimates differ even more from the value obtained with the *Renilla* luciferase tagged rhesus neuropeptide Y Y₂ receptor in the human embryonic kidney-293 (HEK-293) cells (t_{1/2} ~23 min; (Berglund et al., 2003b). It should be noted that the studies of internalization of tagged Y receptors in HEK-293 cells used very high concentrations of the driving agonists (100 nM (Gicquiaux et al., 2002) or 1 μM (Berglund et al., 2003b)), possibly pointing to low rates of cycling of the constructs. Also, the above studies with fluorescently tagged Y receptors used suspensions of HEK-293 cells, and our work with unmodified receptors employed CHO cell monolayers. The receptor-linked internalization of agonist peptides by monolayer cultures could be substantially faster than in suspensions of the same cells (e.g. Blanchard et al., 1983). The apparent differences could be evaluated by comparing simultaneously the kinetics of internalization of the fluorescent neuropeptide Y Y₂ receptor constructs and of the [¹²⁵I]-labeled agonists.

The fraction of the cycled neuropeptide Y Y₂ receptor is small compared to other neuropeptide Y receptors, in accord with previous observations (Parker et al., 2001; Gicquiaux et al., 2002; Berglund et al., 2003a). This again points to an immobilization through compartmentalization of a large part of the surface neuropeptide Y Y₂ receptors. However, most importantly, the internalization of both labeled agonists is very much faster and larger with the A³⁴A³⁵ mutation, indicating a significant reduction in the rate of internalization by the native P³⁴D³⁵ motif.

We utilized internalization of the agonist peptides to assess the surface dynamics of the wildtype and the mutated neuropeptide Y Y₂ receptors. The approach based on cDNA constructs containing receptors and fluorescent molecules (for an in-depth review see Bohme

et al., 2007) was not used, since the covalent additions, especially of long sequences, could significantly influence the cycling of the G-protein coupling receptors, as already noted for human β -adrenergic receptors (McLean and Milligan, 2000), and as seems likely from the available studies with the Y receptors (Gicquiaux et al., 2002; Berglund et al., 2003a). Also, with a protein examined for effects of point mutations, as in the present study, an additional large mutation (such as attachment of the 314-residue *Renilla reniformis* luciferase (The U.S. National Library of Medicine access CAA01908), or the 228-residue *Aequorea victoria* green fluorescent peptide (Swiss-Protein access P42212) to the 381-residue human neuropeptide Y Y₂ receptor protein (Rose et al., 1995) may have differential effects on the receptor properties that can be disproportionately affected by the mutations under scrutiny. In the case of the neuropeptide Y Y₂ receptor, co-internalization of the agonist with the receptor is not significantly compromised by dissociation of the agonist, since the intact Y₂ agonist is quasi-irreversibly associated with the receptor (Dautzenberg and Neysari, 2005; Parker et al., 2007c), and our results show no accumulation of the degraded agonist over the studied length of endocytosis. As is clear from our experiments, a large fraction of the internalized neuropeptide Y Y₂ receptor agonist, essentially constant over short intervals, should represent receptor-agonist complexes.

We selected for this initial study the PD aspect of the PDPEPE sequence in the N-terminal extracellular segment of the human Y₂ receptor, based on the known importance of aspartate sidechains in the association of integrins and other connector proteins with the extracellular matrix (Ma et al., 1995; Golbik et al., 2000). Among G-protein coupling receptors of all known families, this N-terminal extracellular sequence is found only in rodent and primate neuropeptide Y Y₂ receptors. While thus far there are no specific reports on the use of a PD motif in specific interactions with juxtacellular or extracellular partners, it is noteworthy that multiple sequences rich in PD and PE motifs are found in the large extracellular domains of the secretin group adhesion receptors ontogenetically associated with neuronal streams (McMillan et al., 2002). The bovine and porcine neuropeptide Y Y₂ receptors have a modified, and also unique, PDSEPE motif, in which the hydroxyl sidechain sandwiched between Asp and Glu could serve in association with intercellular adhesion molecules and collagens, as in the case of integrin β 2 (Kamata et al., 1995). Flexibility due to the flanking proline residues should enable multiple electrostatic interactions of the acidic residues with ionic partners, to a considerable binding strength (e.g. Jackson et al., 2006), and these interactions would involve spatially non-contiguous partners. There are no G-protein coupling receptors with PDPD sequence in the N-terminal extracellular domain, and the N-terminal extracellular PEPE sequence is, beside the neuropeptide Y Y₂ receptors, found only in the orphan GPCR12. Single acidic residues flanked by proline in this domain are more frequent, with the PDP motif found in 11, and the PEP motif in 21 receptors in addition to the Y₂ receptor. The above, however, adds to only about 40 molecules among the ~800 GPCRs with confirmed sequences, and could point to association of the carrier receptors with specific types of adhesion partners. The receptor internalization can be controlled by specific proteins of the extracellular matrix (e.g. (Boura-Halfon et al., 2003) for the insulin receptor), via multiple electrostatic interactions (Kauf et al., 2001). The stimulation of the neuropeptide Y Y₂ receptor internalization by heparin that we observed in the A³⁴A³⁵ mutant should relate to inhibition of contacts of the residues 36–39 (PEPE), possibly weakened due to the absence of residues P³⁴ and D³⁵. The neuropeptide Y Y₂ receptor partners affected by heparin could include fibronectins in the extracellular matrix (Naito et al., 2000).

The strongly acidic N-termini found in a number of G-protein coupling receptors are frequently used as primary or auxiliary agonist-binding sites, e.g. in chemokine receptors (Blanpain et al., 1999; Proudfoot et al., 2001) and in the apelin receptor (Zhou et al., 2003). These domains can also have a role in the promotion of angiogenesis by the receptors (see e.g. Parker et al., 2005b). With the neuropeptide Y Y₂ receptor, the N-terminal domain is not involved in the

ligand association (as is obvious from our finding the absence of sensitivity to heparin in the binding of a receptor agonist). The acidic / aromatic residue-rich N-terminal extracellular domain of the neuropeptide Y Y₂ receptor can therefore serve principally in anchoring and embedding interactions, and the PDPEPE motif appears to be importantly involved in these activities. This can also involve intermolecular bridges to metal ion-dependent adhesion sites of integrins (Rieu et al., 1996). Further studies should deal with effects upon the receptor cycling of modifications of other parts of the PDPEPE motif and of overlapping or adjacent sequences, as well as mutation to neutral residues of the entire motif, or deletion of the motif.

It is noteworthy that tissues rich in tubular epithelia, e.g. the kidney cortex, can contain the neuropeptide Y Y₂ receptor in excess of 1 pmol/mg membrane protein (Sheikh et al., 1989). Interactions of the receptor via the N-terminal extracellular domain with the extracellular matrix, or with other cells, could conceivably induce a transition of the receptor to the status of a structural constituent, even preventing detection by the conventional labeling techniques. Participation of the neuropeptide Y Y₂ receptor in angiogenesis (Zukowska-Grojec et al., 1998; Ekstrand et al., 2003), tubulogenesis, and generally in the cell-cell and the cell-matrix (Kuo et al., 2007) association may need to be further addressed especially by cytochemical and immunochemical methods.

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Fig. 1.

Levels of the CHO cell-expressed wildtype and N-terminally mutated human neuropeptide Y Y₂ receptors, and a comparison of the activity of the G_i nucleotide site and the receptor binding site in particulates from these expressions. A. The overall binding of the agonist [¹²⁵I] peptide YY(3–36) for the wildtype (WT) and the N-terminally mutated receptors bearing the indicated mutations. The measurements were done on particulates from the respective expressions and the corresponding binding parameters are shown in Table 1. B. The basal and phenylarsine oxide (PAO; 10 μM) - unmasked surface binding of [¹²⁵I] peptide YY(3–36). The acid saline-extracted binding of 100 pM [¹²⁵I] peptide YY(3–36) was measured after 12 min of labeling at 37 °C, without or with the arsenical in the medium. Significant differences in *post hoc* Tukey tests with the wildtype expression for the unmasked binding are indicated by asterisks (* for 95% confidence; ** for 99% confidence). C. Stimulation of the binding of [³⁵S]GTPγS (200 pM) by peptide YY(3–36) at eight concentrations in the range of 10 pM to 100 nM (using the protocol detailed in Parker et al., 2005a). The results are averages of three separate binding experiments. The EC₅₀ values, in pM peptide YY(3–36) ± 1 S.E.M.: wildtype, 43 ± 4.8; A³⁴, 46 ± 5.5; A³⁵, 48 ± 8; A³⁴A³⁵, 55 ± 9. The ratios of fmol bound at 200 pM [³⁵S]GTPγS

and 100 pM [125 I] peptide YY(3–36) for the same samples ($n = 6$): wildtype, 0.43 ± 0.04 ; A 34 , 0.49 ± 0.02 ; A 35 , 0.54 ± 0.03 ; A 34 A 35 , 0.61 ± 0.015 . D. The binding of [125 I] peptide YY (3–36) (20 pM, 30 min at 25 °C) to the neuropeptide Y Y $_2$ receptor monomer/G $_i$ α complex (90 kDa) and to the receptor dimer / G-protein heterotrimer complex (180 kDa) separated in 5–20% sucrose gradients (18 h at $218,000 \times g_{\max}$ and 5 °C) after solubilization at 10 mM each of cholate and digitonin (using the protocol detailed in Parker et al., 2008). The profiles shown are representative of at least two runs. The proportion of dimers as estimated in the ImageJ program was above 60% in all cases.

Fig. 2.

Compartmentalization of the cell surface binding sites is strongly decreased in the A³⁵ and especially in the A³⁴A³⁵ mutation. [¹²⁵I] peptide YY(3–36) (100 pM) was used to label the neuropeptide Y₂ receptor sites on the surface of cells in wells. All results are averages of at least six wells, $\pm$ 1 S.E.M. Differences in *post hoc* Tukey tests significant vs. the wildtype expression (A, B) or vs. the corresponding basal binding (C) are indicated by asterisks (* for 95%, ** for 99% confidence). A. Unmasking by phenylarsine oxide (PAO; pretreatment for 5 min and co-treatment during the labeling with [¹²⁵I] peptide YY(3–36) in wells for 10 min at 37 °C). B. Unmasking by pretreatment with 10 μ M digitonin (15 min at 25 °C) followed by labeling of cells with [¹²⁵I] peptide YY(3–36) in wells for 10 min at 37 °C. C. The basal and PAO-unmasked cell surface binding of [¹²⁵I] peptide YY(3–36) to cells pretreated with pertussis toxin (PTX) for 18 h in the culture medium. The labeling was for 4 or 10 min at 37 °C, and PAO (10 μ M) was added 5 min before the tracer.

Fig. 3.

Binding of [¹²⁵I] peptide YY(3–36) shows a consistently increased internalization of the Y₂ agonist in cells expressing the A³⁴A³⁵ receptor in the range of 20–200 pM, and an equilibration of receptor intake and processing / degradation within short intervals. A. Receptor-linked internalization at 20, 50, 100 and 200 pM of the tracer over 4 min at 37 °C. B. Receptor-linked internalization at 20, 50, 100 and 200 pM of the tracer over 10 min at 37 °C. C. Bio-Gel P-4 gel filtration (using 35 ml columns in 1 M CH₃COOH) shows about 80% of intact internalized agonist at 2 and at 15 min of internalization of [¹²⁵I]PYY(3–36) (at 100 pM) with the A³⁴A³⁵ receptor. Very similar profiles were obtained with the wildtype receptor, and also with labeling periods of 4, 7 and 10 min. The elution positions at arrows: V₀, the void volume; VTRQRY, the C-terminal hexapeptide of peptide YY(3–36), Y, the C-terminal tyrosine of peptide YY(3–36), both detected by [¹²⁵I] labeling and verified by HPLC. The total column volume is at the position of the free tyrosine.

Fig. 4.

Kinetics of internalization of the wildtype, A³⁴, A³⁵ and A³⁴A³⁵ neuropeptide Y Y₂ receptors in CHO cells followed by two labeled agonists. The results are averages of 6–8 wells. A. The kinetics of internalization at 100 pM [¹²⁵I] peptide YY(3–36), the selective agonist of the neuropeptide Y Y₂ receptor. The binding parameters are reported in Table 2. The difference between the wildtype and the A³⁴A³⁵ mutation was significant in the Student's *t* test. B. The kinetics of internalization at 100 pM [¹²⁵I] peptide YY(1–36), a non-selective agonist of the neuropeptide Y Y₁, Y₂ and Y₅ receptors. The binding parameters are reported in Table 2. The difference between the wildtype and the A³⁴A³⁵ mutation was significant in Student's *t* test.

Table 1The binding of two agonist peptides to wildtype and mutated neuropeptide Y Y₂ receptors

Agonist and receptor expression	K _{diss} , pM	B _{max} , fmol/mg protein
[¹²⁵ I] peptide YY(3–36)		
Wildtype (P ³⁴ D ³⁵)	85.1 ± 12	81.2 ± 4.4
A ³⁴	83.5 ± 13.3	69.2 ± 3
A ³⁵	82.5 ± 12.4	75.1 ± 5
A ³⁴ A ³⁵	78.9 ± 11.9	62.6 ± 5.8
[¹²⁵ I] peptide YY(1–36)		
Wildtype	80.3 ± 11.5	84.8 ± 8.8
A ³⁴ A ³⁵	73.4 ± 11.6	67.6 ± 4.5

Competition of either [¹²⁵I]-labeled agonist (input at 100 pM) was done by eight concentrations of the corresponding unlabeled peptide in the range of 3 pM to 10 nM, and the non-specific binding was defined at 100 nM of the respective non-labeled peptide. The results are averages of at least three determinations for each agonist and paradigm.

Table 2Internalization of [125 I] peptide YY(3–36) and [125 I] peptide YY(1–36) with neuropeptide Y Y₂ receptors

Agonist and expression	Half-period, min. $\pm$ S.E.	Max. internal tracer as % surface binding
[125 I] peptide YY(3–36)		
Wildtype (P ³⁴ D ³⁵)	5.55 $\pm$ 1.57	117 $\pm$ 14
A ³⁴	3.67 $\pm$ 0.77	93 $\pm$ 7
A ³⁵	4.21 $\pm$ 0.78	196 $\pm$ 14 ^b
A ³⁴ A ³⁵	3.28 $\pm$ 0.46 ^a	212 $\pm$ 10 ^b
[125 I] peptide YY(1–36)		
Wildtype (P ³⁴ D ³⁵)	3.82 $\pm$ 0.14	130 $\pm$ 21
A ³⁴ A ³⁵	2.02 $\pm$ 0.51 ^a	234 $\pm$ 26 ^b

The results are averages of six measurements using 100 pM of [125 I]-labeled agonists and time points of 2, 4, 7, 10 and 15 min. The corresponding data are shown in graphs A (for peptide YY(3–36)) and B (for peptide YY(1–36)) of Fig. 5. The half-periods (min) and maximum internalized binding as percentage of the corresponding surface binding were estimated in nonlinear hyperbolic fits. Significance in Student's *t* tests of parameters against the corresponding wildtype average:

^a at 95%

^b at 99% confidence. With the wildtype expression, the internalization of [125 I] peptide YY(3–36) increased almost four-fold over the input range of 20–100 pM at time points of 4 and 10 min (*n* = 8), and with a good saturation between 100 and 200 pM (see Fig. 3).