Published in final edited form as:
Peptides. 2012 September ; 37(1): 40–48. doi:10.1016/j.peptides.2012.06.008.

Surface masking shapes the traffic of the neuropeptide Y Y2 receptor

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Abstract

The neuropeptide Y (NPY) Y2 receptor shows a large masked surface population in adherent CHO cells or in forebrain cell aggregates, but not in dispersed cells or in particulates from these sources. This is related to adhesion via acidic motifs in the extracellular N-terminal domain. Masking of the Y2 receptor is lifted by non-permeabilizing mechanical dispersion of cells, which also increases internalization of Y2 agonists. Mechanical dispersion and detachment by EDTA expose the same number of surface sites. As we have already shown, phenylarsine oxide (PAO), a cysteine-bridging agent, and to a lesser extent also the cysteine alkylator N-ethylmaleimide, unmask the surface Y2 sites without cell detachment or permeabilization. We now demonstrate that unmasking by permeabilizing but non-detaching treatment with cholesterol-binding detergents digitonin and edelfosine compares with and overlaps that of PAO. The caveolar/raft cholesterol-targeting macrolide filipin III however produces only partial unmasking. Depletion of the surface sites by N-terminally clipped Y2 agonists indicates larger accessibility for a short highly helical peptide. These findings indicate presence of a dynamic masked pool including majority of the cell surface Y2 receptors in adherent CHO cells. This compartmentalization is obviously involved in the low internalization of Y2 receptors in these cells.

Keywords

G-protein-coupled receptor; CHO cells; Receptor compartmentalization; Cysteine redox; Cholesterol complexing

1. Introduction

Expressions of human and rodent neuropeptide Y (NPY) Y2 receptors in at least three cell lines show low internalization compared to other Y receptors [2, 13, 25, 27, 28, 46]. This should be primarily related to adhesive interactions of the N-terminal extracellular domain

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of this receptor (e.g. of the PDPEPE motif [32]), producing a masking that strongly reduces sites directly available for agonist attachment, and an effective compartmentalization. Similar to the Y2 receptor, most G-protein coupling receptors (GPCRs) that promote angiogenesis (and especially the chemokine receptors) have N-terminal extracellular regions with multiple acidic sidechains in large stretches without basic residues [29]. These acidic zippers serve in recognition, docking and binding of agonist peptides, but could also interact with other proteins to produce masking and reduce signal transduction and receptor traffic.

To enable comparisons of adhesive screening among the major Y receptors we examined Y2 receptors of four mammals that also have confirmed sequences for other canonical Y receptors (Y1, Y4 and Y5). With these species, we have reported a large masking of the guinea-pig Y2 receptor expressed in CHO cells [27, 28], of the human Y2 receptor in CHO cells [30], and of the native rat Y2 receptor in the forebrain [28].

No substantial masking was previously detected for the Y1 and the Y4 receptor, and this is confirmed and extended to the Y5 receptor. A survey of the constitution of extracellular domains of Y receptors indicates that the Y2 receptor has the largest adhesive potential; this is covered in supplementary tables S1-S3 , and in supplementary Fig. S1. Based on this, an objective was to examine the Y2 receptor masking in relation to the adhesion involved. This included comparisons of unmasking of surface Y2 sites by cell detachment (by mechanical disruption of the monolayer or via calcium chelation) with that by cysteine bridging or alkylation [27, 28]. Since cell adhesion is sensitive to depletion of cholesterol via disbanding of clusters of integrins [19, 22, 50] and other cell adhesion molecules [11], it was also of interest to compare effects upon Y2 receptor masking of cholesterol-complexing and cholesterol-neutral detergents, and of a cholesterol-aggregating macrolide [20, 41].

Another objective was to establish whether the access to the masked sites would importantly relate to size and structure of the agonist peptides. For the Y2 receptor this is facilitated by an agonism that is maintained at a considerable affinity upon clipping of any part of the acidic (1-17) sector of the NPY or PYY molecule [14, 23]; see [26] for affinity ranges). A high affinity could be achieved by minor modifications of the C-terminal tridecapeptide [37]. This highly helical peptide, hNPY(24-36) with acetylated leucines at positions 28 and 31 (abbreviated AcLeu), was compared in depletion of the CHO cell surface receptors with the canonical Y2 agonist hPYY(3-36) and the non-selective Y agonists (which all are much less structured).

2. Experimental procedures

2.1 Materials

Digitonin and edelfosine ((7R)-4-hydroxy-7-methoxy-N,N,N-trimethyl-3,5,9-trioxa-4-phosphaheptacosan-1-aminium-4-oxide; frequently abbreviated as ET18OCH3) were from Calbiochem (La Jolla, CA). Triton X-100 (polyethyleneglycol mono [4-(1,1,3,3-tetramethylbutyl)phenyl] ether), Tween 80 (polyoxyethylene(20)sorbitan monooleate) and CHAPS 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) were from Fisher Scientific. [^{125}I] human peptide YY(3-36) (hPYY(3-36)) and [^{125}I human neuropeptide Y (hNPY) were supplied by Phoenix Pharmaceuticals (Shadyvale, CA, USA). ^{125}I-porcine / rat peptide YY(pPYY), ^{125}I human pancreatic polypeptide (hPP) and [^{35}S] GTPγS were from PerkinElmer (Billerica, MA). The corresponding non-labeled peptides were from Bachem Bioscience (King of Prussia, PA). The selective Y2 antagonist BIIE0246, N-[(1S)-4-[(aminoiminomethyl)amino]-1-[([2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino)carbonyl]butyl]-1-(2-[4-(6,11-dihydro-6-oxo-5H-dibenz[b,e]azepin-11-yl)-1-piperazinyl]-2-oxoethyl)cyclopentaneacetamide and the selective Y1 antagonist BIBP3226, R-N 2-(diphenylacetyl)-N-(4-hydroxyphenyl)methyl-argininamide, were from Tocris

(Ellisville, MD). Collagenase C-0255, filipin III and other chemicals were from Sigma (St. Louis, MO).

cDNAs for human Y receptors were provided by University of Missouri at Rolla (MO), and for guinea pig Y receptors by Dr. Magnus Berglund (Department of Neuroscience, University of Uppsala, Sweden).

Animal experimentation was supervised and approved by the Animal Care and Use Committee of the University of Tennessee at Memphis (protocol #1255).

2. 2 Comparisons of sequence alignments, motifs and tracts

Alignments of receptor sequences were performed in *clustal* program (*expasy* website), using the Gonnet matrix, and in SSEARCH3 program [33], using BLOSUM50 matrix. Sequence correspondences for domains in receptors of the same type (Table S1) were calculated for the minimal domain length ranges, with factors of 1 for identical residues, 0.5 for residues unconditionally correspondent, and 0.25 for residues that correspond in sequence context. Three-type (helical, coiled and sheet) structures of Y receptors were evaluated in *porter* program (http://distill.ucd.ie/distill/ ; [35]. Correspondences for the structures were calculated from assignations in the *porter* program as percent of the structural matches within domains of the Y receptors (tables 1 and S1).

2. 3 Cell culture and experimental treatments

CHO cells stably expressing human Y1, Y2 and Y4 receptors and HEK-293 cells stably expressing the Y5 receptor were grown to 80-90% confluence in 48-well plates (Corning, Ithaca, NY), using 1 ml per 0.95 cm^2 well (48-well plates; Corning, Ithaca, NY) of F12 Ham/DMEM medium (Gibco/Invitrogen, Carlsbad, CA) with 6% fetal bovine serum, 400 μg/ml geneticin, penicillin/streptomycin and standard vitamin supplement. The same medium without antibiotics and with 0.2% protease-free BSA instead of FBS was used at 0.25 ml per well as the cell incubation medium for short-term experimental treatments.

Pretreatment with surfactants was for 20 min at 37 °C in 0.25 ml medium, using up to 30 μM digitonin, 60 μM edelfosine, or 100 μM of cholate, CHAPS or Tween 80. With CHO cells, these concentrations did not induce significant cell detachment or protein loss. Permeabilization by digitonin reached a plateau at 20 μM (as judged by labeling by ^{35}S-GTPγS). The macrolide filipin III induced permeabilization in the range of 0.6 - 3 μM, with cell lysis starting at about 10 μM.

2.4 Cell detachment and binding assays with cell suspensions

Unmasking of Y2 receptor surface sites by various agents and treatments was compared with cysteine-bridging agent phenylarsine oxide (PAO), which at 30 μM enables access of large Y2 agonists (34- or 36-residue peptides) to most surface sites (as revealed in comparisons with unmasking by detergents or by mechanical lifting, see figures 2-4), and below 200 μM does not inhibit agonist binding to particulates from any of the Y2 expressions tested. At up to 30 μM, this trivalent arsenical did not induce permeabilization of Y2-CHO cells to Trypan Blue, or to ^{35}S-GTPγS.

For mechanical dispersion, 80-90% confluent cells were washed three times with Salts buffer (0.14 M NaCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes.NaOH pH 7.4). The cells were gently dislodged (at 0-4 °C) using cut-tipped polypropylene 0.5 ml bulb pipettes (to avoid smearing by rounded tips), and the collected suspension sedimented for 5 min at 400 × g. The pellets were gently resuspended by plastic stirring rods in the cell incubation medium (see above), followed by slow passing through 21-gauge injection needle.

Detachment by EDTA was effected by incubation for 10 min at 25 °C in buffer containing 0.14 M NaCl, 0.01 M Na phosphate and 1 mM EDTA (final pH 7.5). The cells were then collected and resuspended as above.

Aliquots of cell suspensions were mixed with the appropriate agents and incubated for 30 min a 37 °C. After sedimentation for 5 min at 400 × g and surface washing, the pellets were resuspended and labeled with 50 pM [^{125}I] hPYY(3-36) for 20 min at 37 °C in a final volume of 0.25 ml, in polypropylene tubes. The control adherent cells were labeled under the same conditions in wells. The incubation of cell suspensions was terminated by adding excess cold Salts buffer and sedimentation for 10 min at 10,000 × g / 4 °C, followed by double surface wash with the cold Salts buffer. For adherent cells, the incubations were terminated by adding an excess of cold Salts buffer in ice, removal by aspiration, and double washing with 0.8 ml/well of cold Salts buffer. To extract the surface receptor -associated agonist, the washed cell pellets were resuspended by 3-fold passage through 22-gauge needle in 0.50 ml / tube of cold 0.2 M CH_3COOH-0.5 M NaCl (pH 2.7), adding the 0.25 ml wash of the syringe, followed by incubation for 7 min in ice. The extraction was terminated by sedimentation for 10 min at 10,000 × $g_{max,}$ the extracts collected, and the pellets and collecting pipette washed with 0.25 ml cold acid saline, adding that to the extract. The monolayer cells in wells were extracted by 0.75 ml of acid saline for 7 min in ice, the extracts collected, and the cells and collecting pipette then washed by 0.25 ml cold acid saline, adding that to the extract. The pellets were then solubilized in 1.0 ml 0.1 M NaOH for 30 min at 80 °C. Radioactivity was counted in a γ-scintillation spectrometer ([^{125}I] agonists) or in a liquid scintillation counter (^{35}S-GTPγS). Protein was assayed by the micro-Bradford procedure.

2.5 Receptor and G-protein binding assays

In situ assay of agonist binding in 48-well chambers was done in the incubation medium without antibiotics and containing 0.2% protease-free BSA and proteinase inhibitors (10 μg/ml each of aprotinin, chymostatin, leupeptin and pepstatin and 1 mM diisopropylfluorophosphate) and 50 pM [^{125}I] hPYY(3-36) tracer, for 20 min at 37 °C in the final volume of 0.25 ml/well, followed by removal by suction on an ice bath and washing by ice-cold Salts buffer.

For assays with particulates, Y2-expressing cells were homogenized (12 strokes at 800 rev/min in a teflon/glass homogenizer with 0.1 mm clearance) at 0-4 °C in 0.25 M sucrose containing 3 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM Hepes.NaOH (pH 7.4) and the above proteinase inhibitors, applying 12 strokes of 0.1 -mm clearance teflon pestle. The homogenates were sedimented for 10 min at 100 × g_{max} (CHO cells or brain) , and the supernatants sedimented for 15 min at 30,000 × g_{max} to get particulate fractions. These were resedimented 15 min at 30,000 × g_{max} from the above medium and stored at −80 °C for not more than 14 days before assays. The assay was performed in 0.10 ml of the Salts buffer with proteinase inhibitors, using [^{125}I] hPYY(3-36) tracer at 50 pM for 20 min at 25 °C, with termination by centrifugation (15 min at 31,000 × g_{max} and 4 °C). The supernatants were removed by suction, the pellets surface-washed with the assay buffer and counted. The particulate binding of 200 pM ^{35}S-GTPγS was followed as described [30].

The non-specific binding was defined at 2 μM BIIE0246 for the Y2, at 10 μM BIBP3226 for the Y1, at 100 nM hPP for the Y4, and at 1 μM hNPY for the Y5 receptor. In nucleotide site assays this binding was defined at 10 μM unlabeled GTPγS, and the incubation buffer contained 0.14 M NaCl, 4 mM $MgCl_2$,3 μM GDP, 0.2% BSA, 50 μM EDTA and 1 mM Hepes.NaOH, pH 7.4. The binding to digitonin-permeabilized adherent cells was however done in the cell incubation medium with 0.2% BSA (see above).

2.6 Statistics and curve fitting

Multiple comparisons in Scheffé *t* test following analysis of variance were done in prostat 5.0 program (Poly Software, Pearl River, NY). Student's *t* tests were done in Microsoft Excel.

SigmaPlot program (SPSS, Chicago, IL) was used for curve fitting. Two-component exponential fits produced lower residual variance than single-component linear and exponential fits. Sigmoid fits assuming one specific and one asymptotic component in some cases could not provide reliable regression parameters. A model assuming one exponential and one linear component however produced consistently low errors on parameters (see Table 4) and was implemented as an add-on fit. The equation used was $y_{exp} = a * e^{-bx} + c + d * x$, where y_{exp} = the predicted y value, × = the current independent variable (e.g. a molarity of the Y receptor agonist), a = the ordinate intercept of the exponential component, b = slope of the exponential component, c = the ordinate intercept of the linear component, d = slope of the linear component.

2.7 Supplementary sequence and structural data

Constitution of exocellular parts in Y receptors indicates a significant adhesive character only in the Y2 receptor. This is summarized in the supplement, section S1.1. Also, the adhesion of the Y2 receptor could principally depend on acidic zippers in the N-terminal domain, which is compared with other Y receptors in section S1.2 of the supplement. The corresponding data for mammalian and other vertebrate Y2 receptors are illustrated in Fig. S1 and tabulated in supplementary tables S1-S4.

3. Results

3.1 Among Y receptors, only Y2 receptors show significant surface masking

A large population of masked surface Y2 receptors (made accessible to agonist peptides through cysteine-bridging trivalent arsenical phenylarsine oxide (PAO)) was first reported for guinea pig Y2 expression in CHO cells, in the absence of similar activation of Y1 or Y4 surface sites [27]. A similar masking of the human Y2-CHO expression was first reported in [31]. Masking of the Y2 site was also found for the native rat Y2 expression in forebrain [28], and is also found for the native rabbit brain Y2 expression (data not shown).

With receptors in particulates isolated by homogenization and sedimentation as described in Methods, pretreatment with 30 μM digitonin, 30 μM PAO, or 1 mM EDTA had no significant effect on the binding parameters of the human and guinea pig Y2-CHO receptors, or the native rat or rabbit forebrain Y2 receptors.

PAO at 30 μM did not induce significant permeabilization or detachment of CHO cells in monolayers, and at up to 1 mM did not change the binding of hPYY(3-36) to particulate Y2 sites; to our knowledge, these findings were not previously reported. The mechanical dispersion of intact cells or detachment by EDTA however both allow access to more surface Y2-CHO sites than *in situ* unmasking by the arsenical (see Fig. 6). We now find that a similar increase of the surface Y2 binding in CHO expressions can be induced by cholesterol-binding detergent digitonin at concentrations that produce permeabilization but do not detach CHO cells. As seen in Fig. 1, pretreatment with 30 μM digitonin unmasked similar numbers of Y2-CHO surface sites as PAO, and again did not increase the Y1-CHO or Y4-CHO surface sites. We were not able to stably express the hY5 receptor in CHO cells. Agonist binding to the hY5-HEK293 expression was blocked by PAO with an ic_{50} of 4.9 ± 0.5 μM (probably linked to bridging of the CC pair at m7.46-47), and was not increased

above control levels by PAO or digitonin in the range of 0.1-30 μM, or by mechanical detachment .

After treatment with 30 μM PAO or digitonin, the labeling of adherent confluent cells increased about 4-fold for human and guinea pig Y2-CHO expressions (Fig. 1). An up to 3-fold increase of the Y2 binding by PAO pretreatment is also found with cell aggregates from rat forebrain [28]. With *particulates* from human and guinea pig CHO-Y2 cells or from rat brain, the Y2 receptor density detected after treatment by 30 μM PAO fluctuated within 15% of the control value, without changes in affinity.

3.2 A comparison of Y2 receptor unmasking by PAO, surfactants and filipin III

Digitonin (a non-ionic steroid glycoside surfactant and a classic precipitant for cholesterol, e.g. [7]) at up to 30 μM unmasks surface Y2-CHO receptors without cell detachment or significant protein leaking, but with permeabilization to nucleotides (Fig. 2). PAO at 30 μM induces no protein loss or cell detachment, and does not cause permeabilization of attached CHO cells (Fig. 2). In 80-90% confluent hY2-CHO cells, the agonist-accessible cell surface levels of the receptor similar to PAO-unmasked levels are achieved at 20-30 μM digitonin. This also results in saturation of the access of ^{35}S-GTPγS to rapidly activated GTPase nucleotide sites (in CHO cells mostly those of the G_{i3} α subunit [39]) that can be reached by the nucleotide without protein loss. Protein leakage is observed above 30 μM, and cell detachment starts at about 50 μM of digitonin. The detachment is also found above 100 μM PAO. With mechanically lifted cells, PAO causes some loss of surface Y2 sites at 30 μM (Fig. 3).

Fig. 2 compares unmasking of surface Y2-CHO sites by PAO, surfactants and macrolide filipin III in adherent cells. Digitonin and PAO at 30 μM produce a similar unmasking of binding sites for both agonists, and this does not increase significantly by using the agents together at 30 μM each. It should be noted that treatment with up to 100 μM PAO did not permeabilize the attached cells. Edelfosine ("Et18OCH3"), a phospholipid ether surfactant (and phospholipase C inhibitor [38]) which also strongly interacts with cholesterol (e.g. [34]) produced Y2 unmasking similar to digitonin, however saturating at about 60 μM. As with digitonin at 30 μM, there was no significant cell detachment or loss of cell protein, and edelfosine also did not change the number of surface sites exposed by the arsenical. Cholate, a weak anionic steroid surfactant, is inactive at 100 μM in hY2-CHO unmasking, and the same applies to the zwitterionic detergent CHAPS (Fig. 2), and to emulsifier Tween-80 (not shown). These agents also did not permeabilize CHO cells at 100 μM. Cholate and CHAPS however lyse CHO cells and particulates above 1 mM, as does digitonin.

With macrolide filipin III, permeabilization was difficult to separate from loss of about 40% of mainly cytosolic protein (in the range of 6-10 μM; see Fig. 3), occurring without cell detachment Extensive cell detachment was found above 15 μM filipin. The binding of ^{35}S-GTPγS to cells after filipin treatment was highly variable, but permeabilization was achieved at 1-3 μM, based on Trypan Blue admission. (With Y2-CHO *particulates*, filipin inhibited agonist binding with an ic_{50} of about 100 μM). At 3 μM there was no loss of either particulate or surface Y2 sites. With mechanically detached cells, filipin at up to 3 μM did not affect cell protein or increase the already maximized accessible sites (Fig. 3). With attached cells, the largest unmasking by filipin was twice less than with digitonin or PAO (Fig. 2).

The binding of ^{35}S-GTPγS to intact CHO cells is very low, and typically increases 10-15 -fold in conditions of saturated unmasking of the surface Y2 receptor by digitonin (figures 2 and 4), The binding of ^{35}S-GTPγS to Y2-CHO cells saturates in a concentration-dependent fashion in the range of 3-30 μM of digitonin (Fig. 4), and is highly sensitive to cell

pretreatment with pertussis toxin ([30] and Fig. 4). As expected [30], the effect of the toxin is suppressed by co-treatment with NH_4Cl (Fig. 4). There is a large activation of the nucleotide sites over the basal level by agonist NPY (Fig. 4). The pertussis toxin-sensitive nucleotide sites detected by short-term binding of a low molarity (200 pM) of GTPγS mainly belong to G_{i3} α subunit, the most abundant G_i-type α subunit expressed in CHO cells [39].

3.3 Masked Y2 sites are exposed by cysteine bridging much more than by cysteine alkylation

Pretreatment of adherent hY2-CHO cells by the potent cysteine alkylator N-ethylmaleimide (NEM) at 30 μM unmasked less than 50% of sites made accessible by PAO (Fig. 5), similar to previous findings with the guinea pig Y2 expression [28]. The NEM ic_{50} with particulate Y2 receptor was 146 ± 14 μM with [^{125}I] hPYY(3-36) (as previously reported [28]), and 303 ± 24 μM with ^{35}S-GTPγS (as newly measured; n = 3 for both). MTSET (2-[trimethylammonium)ethyl] methanethiosulfonate), the membrane-excluded alkylator with a bulky sidechain [17]) at 30-100 μM did not unmask any surface Y2 receptors (Fig. 5). The MTSET ic_{50} value for [^{125}I] hPYY(3-36) binding to Y2-CHO particulates was 264 ± 27 μM (n = 3; [28]) , which apparently was connected to the inactivation of G_i α subunits in the particulates (ic_{50} with [^{35}S] GTPγS 13.1 ± 1.29 μM, n = 2). The permeant compound 2-aminoethyl methanethiosulfonate (MTSEA) could not be used, since it inhibited both Y2 agonist binding (ic_{50} 26.2 ± 3.8 μM, n = 2) and GTPγS binding (ic_{50} 23.6 ± 3.4 μM), both apparently as linked to G-protein inactivation.

The above findings indicated that cysteine alkylation is less efficacious than cysteine bridging in unmasking the Y2 receptor. It should be noted that both NEM and MTSET at 30 μM did not cause a significant decrease in internalization of [^{125}I] hPYY(3-36), while PAO reduced the intake by 88% at that molarity. This is in accord with the known low permeation of NEM and MTSET, and with high permeation of PAO (e.g. [12]).

As expected from studies on reduction of disulfides in other receptors [1, 9, 43], the disulfide breaker dithiothreitol (DTT) did not unmask surface Y2 receptors at levels not affecting Y2 agonist binding with adherent CHO cells (≤ 3 mM). However, DTT at ≥300 μM (Fig. 5) fully prevented effects of 30 μM PAO (see also [28]).

The above results indicate that formation of stable disulfides from cellular and ECM cysteines unmasks considerably larger fraction of Y2-CHO surface sites than alkylation of cysteines accessible on cell surface or ECM. The masking of more than 70% of the CHO-Y2 surface receptors could be strongly dependent on a dynamic bridging of cysteines in cell surface proteins and ECM proteins.

3.4 Mechanical unmasking equals that by PAO or EDTA, but strongly increases internalization of the Y2 receptor

Unmasking of Y2 sites by PAO (Fig. 2) suggested that access of the large Y2 agonists to the masked sites could be regulated by adhesive packing that can involve numerous proteins. As related to cell detachment caused by metal ion chelation, the masking also should be stabilized by calcium. EDTA at 1 mM induced cell detachment with permeabilization to GTPγS and a significantly reduced internalization of agonists, in agreement with studies on other epithelial-type cells (e.g. [42]. This detachment increased the surface Y2 sites to levels observed with mechanical dispersion (Fig. 6). The surface sites unmasked by PAO were found to also be exposed by mechanical cell detachment without disruption (as judged by Trypan Blue exclusion and low labeling by ^{35}S-GTPγS).

From the difference in accessible Y2 sites for mechanically dispersed cells and PAO-treated adherent cells (Fig. 6), sticking to ECM / substratum apparently masks some Y2 sites that in attached cells cannot be made accessible by the arsenical. In hY2-CHO cells these sites amount to about 25% of all sites that could be unmasked without cell disruption.

As seen in Fig. 6, mechanical lifting activated approximately equal numbers of surface sites as the detachment by 1 mM EDTA, and in either case pretreatment with 20 μM PAO did not significantly alter numbers of the detected surface sites. However, in-assay 30 μM PAO significantly reduced the sites unmasked by mechanical lifting alone (Fig. 3), and filipin III caused loss of sites already at 6 μM (Fig. 3). Intake of the agonist with mechanically detached cells saturated at 38 ± 3.8 % of the surface binding vs. 31 ± 2.7% with adherent cells (Fig. 6). This amounts to about fivefold total increase in receptor-linked agonist intake.

The above findings indicate that non-disruptive shear, removal of divalent cations and stable bridging of cysteines unmask a similar and large fraction (>70% of total) of surface Y2 receptors in CHO cells.

3.5 Size and structure of agonists vs. accessibility to masked cell surface Y2 sites

Accessibility of an agonist to a screened binding site would depend on the size as well as structure of the agonist molecule. All Y2 agonists are expected to have largely helical C-terminal dodecapeptide (which contains the primary binding motifs for attachment to the Y2 receptor (e.g. [21]); the predicted structures are shown in Table 1. The C-terminal fragments should have a higher degree of structuring. The 85% helical 13-peptide hNPY(24-36), acetyl-Leu(28, 31) (here abbreviated AcLeu), which has a high binding affinity [37], could be expected to access the Y2 binding site more frequently than the only 44% helical hPYY(3-36), which has a large coiled N-terminal section (Table 1).

Treatment of intact CHO-Y2 monolayers with the 36-peptides hNPY and pPYY and the Y2-selective 34-peptide hPYY(3-36) resulted in similar profiles of decrease of either the sites accessible without unmasking, or the sites exposed by phenylarsine oxide (results with hNPY and pPYY not shown). The latter sites were depleted to a similar level by the 13-peptide. Depletion of the hY2-CHO receptor with all tested peptides saturated between 30 and 100 nM within 30 min at 37 °C (Fig. 7). (Treatment with 300 nM of any of the agonists did not produce reduction relative to 100 nM, possibly due to a stimulation of cycling and *de novo* synthesis of the Y2 receptor at very high agonist inputs.) However, pretreatment with AcLeu produced a significantly larger reduction of the sites accessible to the long agonists without unmasking. [^{125}I] hPYY(3-36) was used as tracer for experiments in Fig. 7 and Table 2; similar profiles were obtained with [^{125}I] pPYY.

In the conventional comparison of fitting models for residual variance and errors on parameters, the single linear, bi-linear and single exponential models were much worse than the sigmoid (which however in some cases did not yield rational binding parameters) or the bi-exponential model. The best estimates were obtained assuming one exponential and one linear component of the binding (see Methods). The high-affinity exponential K1 values were below 13 nM in all two-component fits. The above experiments thus indicate two components (also clearly discernible in Fig. 7) in the Y2 receptor depletion driven by agonists. These components were observed in both non-masked and arsenical-unmasked surface receptors (Table 2). The depletion of non-masked receptors by the 13-peptide was significantly larger relative to the 34-peptide, while the apparent depletion efficacy for this component was 2.5 times higher for the 34-peptide (Table 2), compatible with its 3.8-fold higher affinity at the Y2 receptor (see Table 2 footnote).

4. Discussion

Masking of the Y2 receptor should significantly depend on enrichment in acidic residues and proline at the N-terminus. Asp35 in the second acidic zipper of this domain is known to be involved in the low internalization of the hY2-CHO expression [32]. Primate, rodent and lagomorph Y2 receptors at this position have PD**P**EPE ec1 motif, and bovine and porcine Y2 receptors have PD**S**EPE (Table S4), and it is of interest to examine if this difference may influence masking and adhesion. The rabbit and bovine ec1 domains also differ from primate / rodent in the first N-terminal acidic zipper (tables S1 and S4). The bird and fish Y2 receptors lack both the PDP(S)EPE motif and the second acidic zipper in the ec1 domain (Table S4) and could have lower adhesion and masking.

The adhesion-supporting extracellular parts of the four mammalian Y2 receptors compared in this work would include the two large acidic zippers (Table S2) and the PDPEPE motif [32] in the ec1 domain, which are much more exposed than extracellular loops, and also are not high-affinity targets for Y peptides. The adhesion may also engage the acidic zipper at the N-terminus of the ec3 domain (tables S2-S3), which however is a high-affinity target for Y agonists [21, 37]. Other exocellular domains of mammalian Y2 receptors show very little difference among species, and high correspondence in these parts is maintained across the vertebrate classes (tables S3-S4). The ec1 and ec3 domains in the Y1 and Y4 receptors have no large homoacidic tracts and in CHO expressions do not support masking.

Based on Y2-CHO activation by mechanical dispersion, a significant portion of the masking could be due to tight contacts with ECM proteins and substratum. The partner complement could strongly differ across cell types in relation to expression of the various adhesion molecules. However, the conserved ec1 EADENQTVE and PDP(S)EPE motifs of mammalian Y2 receptors (Table S4) could reflect clade -related preferences for specific groups of adhesive partners, and even for specific individual partners.

The two extracellular cysteines in ec2 and ec3 domains of the Y2 receptor (Fig. S1 and Table S1) should form a stable bridge (present in both opsins [44] and non-visual A-GPCRs [6]. This bridge in the Y2 receptor is obviously not affected, because PAO shows K_I above 2 mM with the particulate Y2 binding [28]. PAO thus would act on cysteines in other molecules, including adhesive partners of the Y2 receptor. Cysteine bridging could involve a number of adhesion proteins (ECM proteins, cell-linking integrin and cadherin receptors and other (e.g. scavenger) receptors) and could also involve intracellular motifs, since this arsenical is highly permeant (e.g. [12]). PAO may directly affect a large spectrum of intracellular proteins possessing CC motifs, including components of the clathrin / dynamin system, small GTPases and GEFs, and some components of G-protein heterotrimers (G_q class α, and $G_{β1}$ subunits). Bridging the adjacent cysteines in intracellular domains of rod opsins and many A-GPCRs, or of extracellular cysteines in ECM proteins and cell-linking receptors, could lower adherence by preventing acylation of cysteine [24]. PAO could also “take over” the reactive cysteine bridges [47]. In all of the above cases, stable changes in protein reactivity and structure could reduce the dynamic adhesion [10]. The difference in unmasking between NEM and MTSET points to involvement of cysteine(s) that could be accessed by ethylated maleimide, but not by the more bulky methanethiosulfonate.

Detergents digitonin and edelfosine modify the masking adhesion without acting directly on cysteine redox, achieving unmasking similar to PAO. At non-micellating levels that are effective in unmasking of the Y2 receptor in CHO cells, digitonin (critical micellar concentration ≥100 μM) should tightly complex cholesterol *in situ* to effect pore formation, and similar could be expected for edelfosine [34]. The involved cholesterol might be mainly in the outer leaflet [49]. In terms of adhesion, the main effect of these surfactants could be a

loosening of the adhesive network, allowing agonist access to normally shielded sites. The lower unmasking found with filipin however could relate to formation of large aggregates with cholesterol especially in caveolar rafts [41] and away from the substratum [5] and junctions [4]. Filipin preference for such rafts could be important in the partial Y2 unmasking by this agent. The lack of effect of non-micellating concentrations of zwitterionic CHAPS and weakly anionic cholate, both without preference for cholesterol, underline the importance of direct detergent association with cholesterol for the unmasking.

Unmasking by mechanical dispersion in the absence of cell permeabilization and selective cholesterol perturbation or removal is larger than found with the above agents, which again argues for involvement of adhesive networks. The dispersion also strongly increases Y2-linked agonist internalization relative to adherent cells, indicating that the unmasked sites are functional and situated at, or very close to, surface rafts. The stability of Y2 agonist attachment in the absence of internalization or transduction [8] in conjunction with mechanical and other unmasking offers opportunities especially for studying the export of receptor dimers.

Our findings indicate that the surface masking of the Y2 receptor could to a degree reflect restricted access of the large (34-36-residue) physiological agonists. The depletion difference between the 13-peptide and the 34-peptide was significant only for the non-masked receptors, although AcLeu increased the exponential component in the PAO-unmasked receptors as well (Table 2). Reduction of the inaccessible sites saturated to very similar levels with both agonists, and obviously lagged compared to that of the sites accessible to 34-peptide, which indicates a precursor role. The depletion component fitted as linear should be a composite of contributions of externalization of the dimeric receptor and of different stages in surface activation of the dimer and its monomeric product [48]. In any case, in CHO monolayers about a half of the Y2 surface receptor complement seems to be maintained by a balance of adhesion, unmasking, recycling, and insertion of newly made receptors.

The regulation of internalization of the agonist-accessed Y2 sites appears to occur mainly at the C-terminal “tail” [46]. This domain has lower phosphorylation potential and arrestin reactivity than those of Y1 or Y4 receptors [2, 16, 25]. This difference should importantly relate to the large reported kinetic differences between the Y2 and other Y receptors [2, 25, 27]. However, masking and compartmentalization of surface sites would strongly affect the apparent rates, depending on cell type and also the culture conditions. Much of the above work was done with expressions in HEK293 cells, which (as different from CHO or COS7 cells) adhere poorly and need enhancement of adhesion e.g. by basic polyaminoacid coating [46], by introduction of adhesive proteins [40], or by sub-line selection (anecdotally, a frequent practice). Comparisons of cycling could be difficult among expressions in various cell types and without or with adhesive additions. The use of poly-lysine to improve cell attachment may significantly affect the cycling rate for the Y2 receptor.

Rates of the Y2-linked internalization of the selective agonist hPYY(3-36) in CHO cells rise strongly with concentration of the agonist in sub-nanomolar range [32], and the component with half-decrease at 2.5 nM hPYY(3-36) represents nearly 20% of hY2-CHO depletion (Table 2). These sites could be primed for internalization by phosphorylation, as indicated for C5a receptor [36], vasopressin and oxytocin receptors [3, 15] and opioid receptors [18]. Physical association with protein kinases and arrestins could in turn help mobilization of masked receptors.

In conclusion, this study indicates that the overall traffic of the Y2-CHO receptor is governed importantly by masking adhesive interactions. A dynamic masked pool comprises

the majority of surface Y2-CHO receptors. Similar Y2 pools could be present in other adherent cell lines and are also evident with brain receptors. Based on similarity in bulk composition at the N-termini [29], similar surface pools could be formed by other angiogenic GPCRs, as well as by compartmentalizing transporters [45]. The substantial hidden surface pools produced by adhesive masking of the Y2 receptor could be present in many cellular and tissue settings. Depending on the expression levels of the Y2 receptor, adhesive interactions could be of importance in regulation of angiogenesis and tubulogenesis, and deserve further careful examination and comparisons especially with chemokine and chemotactic A-GPCRs. In terms of adhesion-linked peptide receptor compartmentalization, the Y2 receptor could be a suitable reporter with high affinity, stability and selectivity of agonist binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was partly supported by the U.S. National Institutes of Health grant R01-HD13703.

Abbreviations

AcLeu	human neuropeptide Y(24-36), acetyl-Leu(28, 31)
BIBP326	R-N 2-(diphenylacetyl)-N-(4-hydroxyphenyl)methyl-arginineamide, BIIE0246, N-[(1S)-4-[(aminoiminomethyl)amino]-1-[([2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino)carbonyl]butyl]-1-(2-[4-(6,11-dihydro-6-oxo-5H-dibenz[b,e]azepin-11-yl)-1-piperazinyl]-2-oxoethyl)cyclopentaneacetamide
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
GTPγS	guanosine 5′-O-(3-thiotriphosphate
MTSET	2-(Trimethylammonium)ethyl] methanethiosulfonate
NEM	N-Ethylmaleimide
hNPY	human / rat neuropeptide Y
pPYY	porcine / rat peptide YY
hPYY(3-36)	human peptide YY(3-36)
hPP	human pancreatic polypeptide
PAO	phenylarsine oxide
pdb	Protein Data Bank
GPCR	G-protein coupling opsin-like receptor
m1…m7	transmembrane domains 1-7
ec1…ec4	extracellular domains 1-4

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Highlights

- Surface Y2 receptor in CHO cells are largely masked to the agonist peptides.
- This is due to adhesion of extracelular domains, especially the N-terminal.
- Removal of masking by cell dispersion strongly increases receptor traffic.

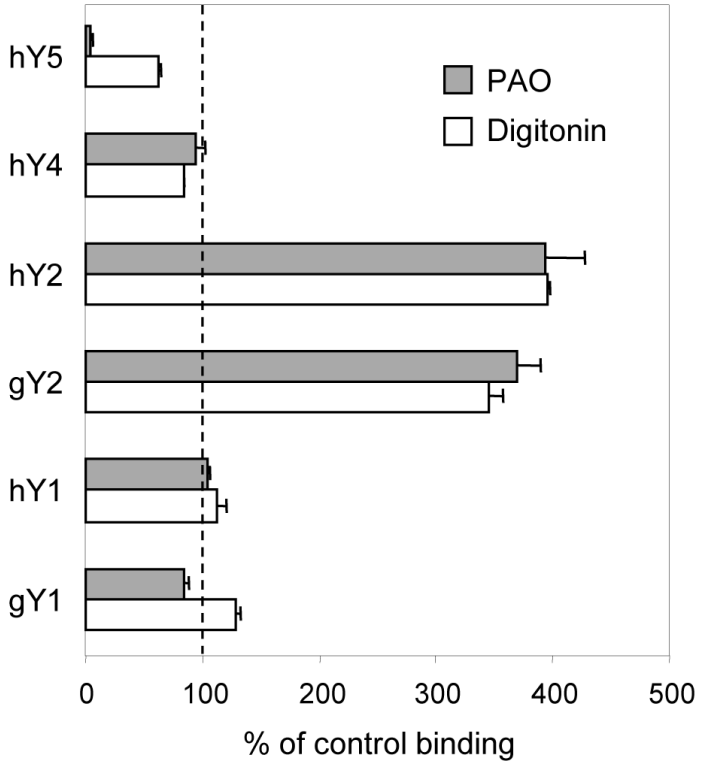

Fig. 1.

Agonist binding to surface guinea-pig (g) and human (h) Y receptors in cell monolayers after exposure to digitonin or phenylarsine oxide (PAO). The hY5 receptor was expressed in HEK293 cells and other receptors in CHO cells. After pretreatment of 20 min at 37 °C with 30 μM digitonin or PAO, the cells were labeled with 50 pM [^{125}I] agonists (hNPY (Y1 and Y5), hPYY(3-36) (Y2), or hPP (Y4)) for 20 min at 37 °C. The results are averages of at least three experiments, ± S.E.M. . The surface receptor-bound agonist was extracted by cold acid saline. Note that the attachment of hNPY to hY5-HEK293 cells is inhibited by PAO (see the text). For other details see the Methods section.

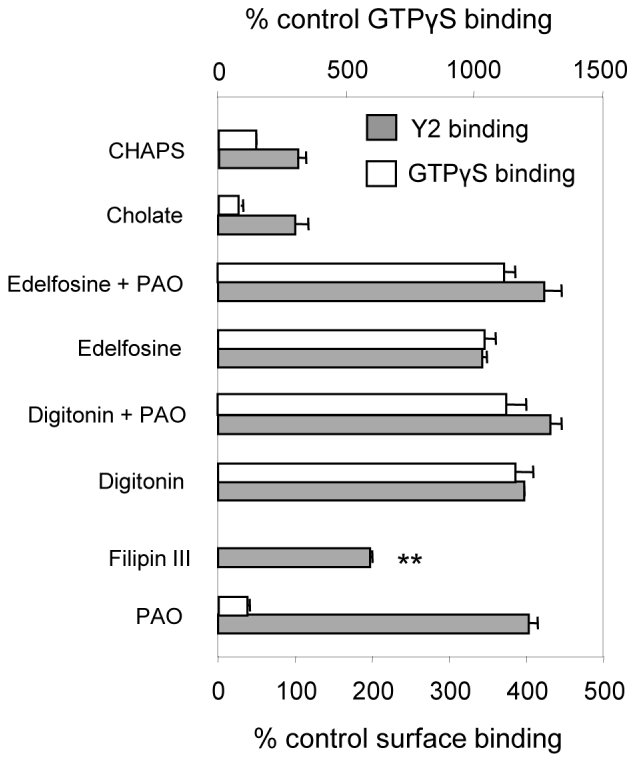

Fig. 2.

Compared unmasking of human Y2 receptors in adherent CHO cells by surfactants, filipin III and PAO. After pretreatment for 20 min at 37 °C with digitonin at 20 μM, edelfosine at 60 μM, CHAPS and cholate at 100 μM, filipin III at 3 μM and PAO at 30 μM (alone or with above molarities of digitonin or edelfosine), the binding of the Y2 agonist [^{125}I] hPYY(3-36) (50 pM) and of G-protein α subunit blocking agonist [^{35}S] GTPγS (200 pM) was for 20 min at 37 °C in the incubation medium, followed by extraction of surface receptor-bound agonist with cold acid saline. Data are averages of at least two experiments. ** difference with digitonin or PAO significant above 99% confidence in Scheffé *t* test. For other details see Methods. Note the different scales for the two agonists.

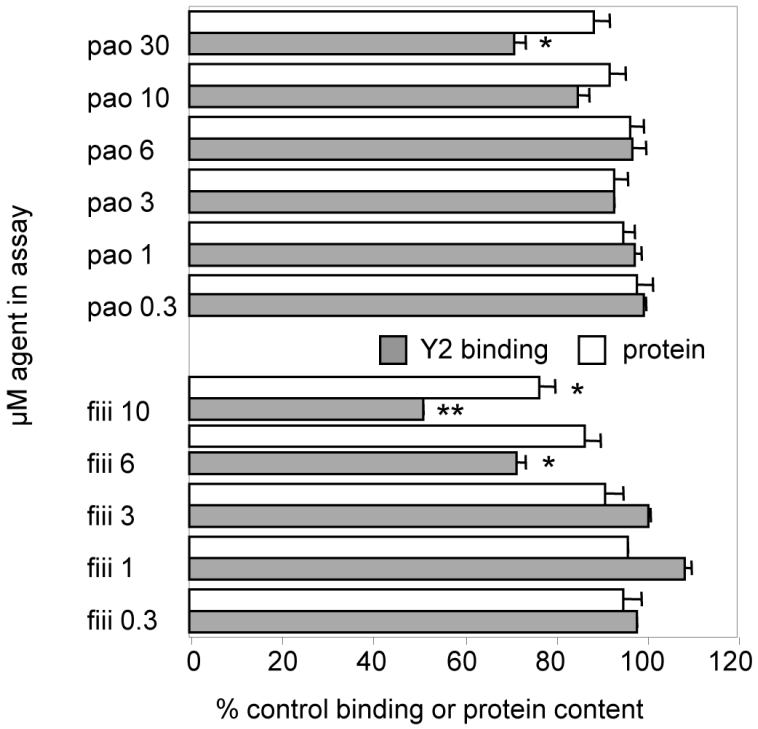

Fig. 3.

Agonist binding to surface hY2-CHO receptors of mechanically dispersed h Y2-CHO cells in the presence of filipin III (fiii; 0.3-10 μM) and phenylarsine oxide (pao; 0.3-30 μM). The labeling with 50 pM [^{125}I] hPYY(3-36) was for 20 min at 37 °C, followed by extraction of surface receptor-attached tracer with acid saline (see Methods). Cell protein as % control is shown in blank bars. The results represent three experiments. Scheffé *t* test differences with the respective control significant above 95% and 99% confidence are indicated by * and **, respectively.

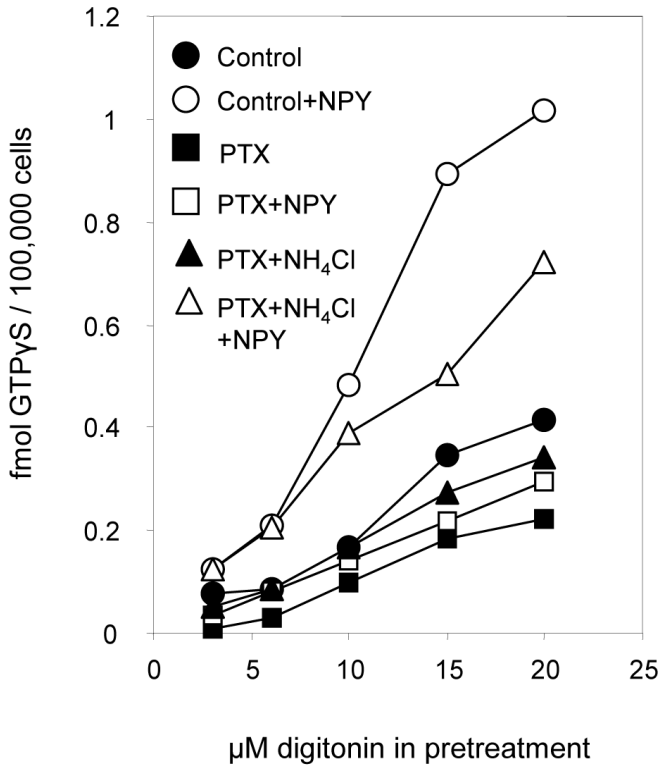

Fig. 4.

Compared binding of ^{35}S-GTPγS to digitonin-permeabilized Y2-CHO cells without (control) and with pretreatment with pertussis toxin (PTX), with or without NH_4Cl. The toxin was used at 1 ng/ml without or with 30 mM NH_4Cl for 21 h prior to unmasking with 3-20 μM digitonin and labeling for 20 min at 37 °C with 200 pM ^{35}S-GTPγS (see Methods), without or with 100 nM hNPY, followed by washing and extraction by cold acid saline. Data are representative of four experiments. The total particulate nucleotide sites were estimated at 32 ± 0.7 fmol/100,000 cells.

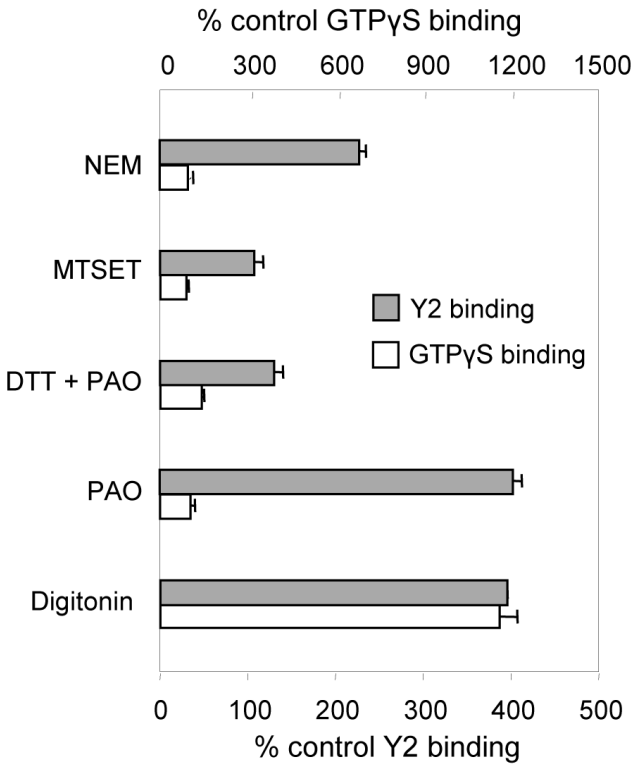

Fig. 5.

Compared unmasking of hY2-CHO receptors in adherent cells by cysteine bridging and cysteine alkylation. Pretreatment with PAO or NEM at 30 μM, MTSET (100 μM) and PAO (30 μM) + dithiothreitol (DTT; 300 μM) was for 20 min at 37 °C, followed by labeling with 50 pM [^{125}I] hPYY(3-36) or 200 pM [^{35}S] GTPγS (also for 20 min at 37 °C) , washing and extraction with cold acid saline. Labeling after pretreatment with 20 μM digitonin is included to illustrate availability of Y2 receptor and G-protein nucleotide sites upon permeabilization without cell detachment. Note the different scales for the two agonists. For other details see the Methods section.

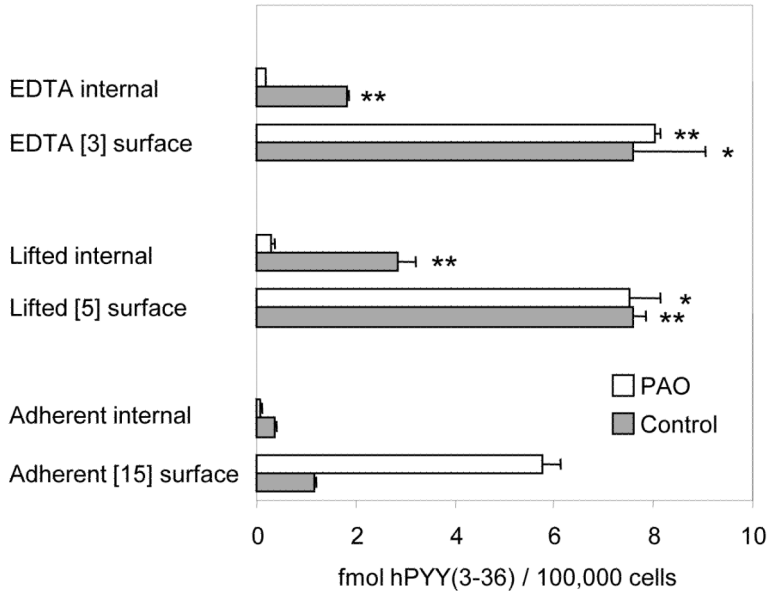

Fig. 6.

Effects of dispersion of the monolayer on accessibility of binding sites and internalization of human Y2 receptor in CHO cells. The EDTA label refers to cells dispersed at 1 mM EDTA, and the 'Lifted' label to cells dispersed mechanically, as described in Methods. The labeling at 50 pM [^{125}I] hPYY(3-36) was for 20 min at 37 °C, and phenylarsine oxide (PAO) was used at 20 μM over the labeling period. The number of experiments is shown in brackets after group labels. Scheffé *t* test differences with the respective adherent control significant above 95% and 99% confidence are indicated by * and **, respectively, For other details see Methods.

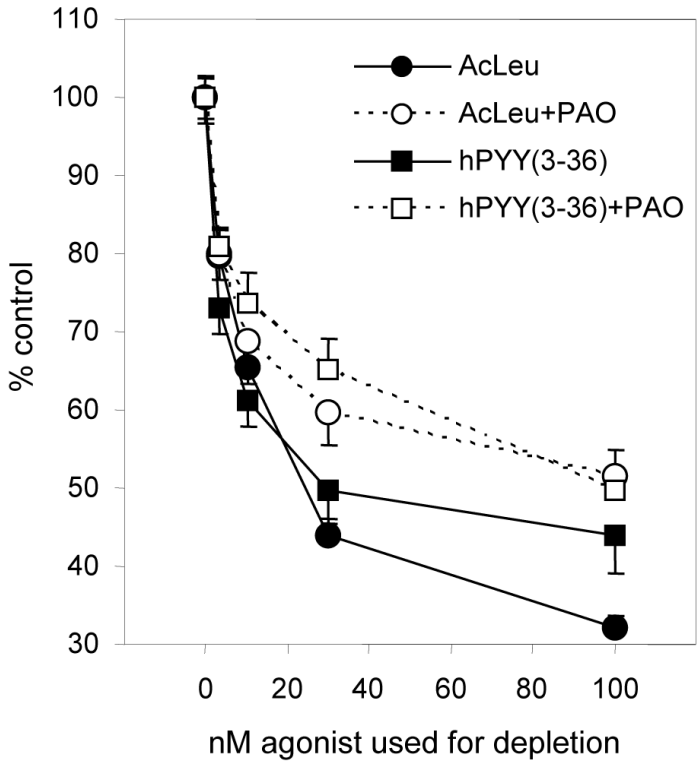

Fig. 7.

Depletion of hY2-CHO surface receptors by two agonists of highly different size and structure. hPYY(3-36) (34 residues, 44.1% helicity (pdb 2DF0) K_{diss} 0.13 nM [26]) and acetylLeu(28,31)-hNPY(24-36) (AcLeu) (13 residues, 84.6% helicity (pdb 1QFA), K_{diss} 0.5 nM [37]) were incubated with monolayers at 0, 3, 10, 30 and 100 nM for 30 min at 37 °C, followed by washing and incubation with [^{125}I] hPYY(3-36) at 50 pM with or without 30 μM PAO for 20 min at 37 °C and extraction of tracer bound to surface receptors. The results are averages of four experiments. For other details see Methods.

Table 1

Sequences and secondary structures of agonists used in comparisons of the surface Y2 receptor depletion

Peptide	Sequences	Access	
hNPY	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY	*up* P01303	
hNPY(24-36) acetyl-Leu(28, 31)	LRHY**L**NL**L**TRORY	*pdb* 1QFA	
pPYY	YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY	*up* P01305	
hPYY(3-36)	IKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY	*up* P10082	
C-terminal 13-residue alignment	****:**:*****		
	Structures		% helicity
hNPY	CCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHHC	*pdb* 1TZ4	61.1
hNPY(24-36) acetyl-Leu(28, 31)	CHHHHHHHHHHHC	*pdb* 1QFA	84.6
pPYY	CCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHHHHCCCCC	*pdb* 2RLK	50
hPYY(3-36)	CCCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHHCCCCC	*pdb* 2DF0	44.1

up indicates sequences from *uniprotein* base (*expasy* site); *pdb* denotes structures as rendered in the indicated Protein Data Bank files. Abbreviations for the type of structure: C = coiled, H = helical; no sheet-type structure is reported for the Y peptides. **Boldfaced** leucines in the AcLeu peptide are acetylated.

Table 2

Depletion of hY2-CHO receptors by two Y2-selective agonists differing in size, structure and affinity

Agonist and treatment	% depletion	K1, nM	% total depletion in component 1	K2, nM
AcLeu	67.8 ± 2.9 *	6.2 ± 1.6 *	32.8 ± 8.5 *	153 ± 17
+ PAO	49.5 ± 1.5	3.0 ± 0.75	15.6 ± 3.9	243 ± 15
hPYY(3-36)	56.1 ± 4.8 *	2.5 ± 0.75 *	19.6 ± 4.5 *	216 ± 22
+ PAO	52.5 ± 5.0	1.76 ± 0.42	9.32 ± 2.2	157 ± 7.1

The parameters are from a model assuming one exponential (K1) and one linear (K2) component (see Methods). The results are averages of four experiments. Pretreatment at 0, 3, 10, 30 and 100 nM unlabeled peptides was for 30 min at 37 °C, followed by washing, incubation for 3 min with or without 30 μM PAO, addition of [^{125}I] hPYY(3-36) to 50 pM, and labeling for 20 min at 37 °C. The non-specific binding was defined at 2 μM BIIE0246. The agonist attached to surface receptors was extracted with cold acid saline. The % depletion is the decrease after preincubation with 100 nM relative to control with no unlabeled agonist.

* indicates agonist (without PAO) differences between non-masked receptors significant above 95% confidence in Student's *t* test . For other details see Methods. The Scatchard affinity at hY2-CHO receptor is 0.13 nM for hPYY(3-36) [26]and 0.5 nM for acetyl-Leu(28,31)-hNPY(24-36) (AcLeu) [37].