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ORIGINAL ARTICLE

Maintenance of Y receptor dimers in epithelial cells depends on interaction with G-protein heterotrimers

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Abstract Treatment of CHO cells expressing human Y receptors (Y_1, Y_2 or Y4 subtype) with pertussis toxin results in a large decrease in functional receptors, with a preferential loss of heteropentameric assemblies of receptor dimers and G-protein trimers. This occurs in parallel to inactivation of the nucleotide site of Gi α subunits, with a half period of about 4 h. The loss could be mainly due to proteolysis at the level of recycling/perinuclear endosomes, and of receptor completion in the ER, since it is reduced by co-treatment with ammonium chloride, an inhibitor of particulate proteinases. Antagonists do not strongly decrease the heteropentameric fraction. These findings indicate that the upkeep of Y receptor dimers in epithelial cell lines depends on the association of receptor oligomers with functional Gi α subunits. This interaction could use the juxtamembrane helix 8 in the fourth intracellular domain, and could also be supported by the C-terminal helix of the third intracellular loop, as outlined in the companion review (Parker et al., Amino Acids, doi: 10.1007/s00726-010-0616-1, 2010).

Keywords G-protein coupling receptor · Neuropeptide Y · Receptor dimer · Receptor heteropentamer · Y1 receptor · Y2 receptor · Y4 receptor

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Abbreviations

BIIE0246	(*S*)-*N*(2)-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6 h)-oxodibenz[b, e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl] cyclopentyl] acetyl]-N-[2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2, 4-triazol-4-yl]ethyl]-argininеamide
GIR	Glucocorticoid-induced receptor (GPCR83)
GPCR	G-protein coupling receptor
H8	Helix 8
NPY	Human/rat neuropeptide Y
pPYY	Porcine/rat peptide YY
hPYY(3–36)	Human peptide Y(3–36)
hPP	Human pancreatic polypeptide
VD-11	$(IEPZYRLRY.NH_2)_2$ (Z = diaminopimelic acid)
PTX	Pertussis toxin (EC 2.4.2.-)
ic3, ic4	The third and fourth intracellular domain, tm1…tm7, the respective transmembrane domains 1–7 of GPCRs

Introduction

Coupling with transducing GTPases, especially with the α subunits of heterotrimeric G-proteins, is a salient

characteristic of most metazoan heptahelical receptors, called therefore the G-protein coupling receptors (GPCRs). The most represented and studied group of GPCRs, family A (Horn et al. 2003) or the rhodopsin family (Fredriksson et al. 2003) includes all metazoan visual opsins and more than 300 heptahelical receptor species with known specific agonists. Most of these canonical family A receptors (A-GPCRs) can activate the adenylyl cyclase-inhibitory α subunits (Gi or Go) of heterotrimeric G-proteins (see the listings in Alexander et al. 2007), although many prefer the α subunits of Gq and Gs classes. The visual opsins and the Taste-2 family GPCRs principally couple the Gt (transducin and gustducin, respectively) class α subunits, which are structurally very similar to Gi/o subunits, and the Frizzled family receptors prefer Go subunits.

The Gi, Go and Gt subunits are all inactivated by a bacterial enzyme, pertussis toxin (PTX; EC 2.4.2.-), which performs ADP ribosylation of cysteine near their C-terminus (see the supplementary Table S5 in the companion study), with very high preference for the α subunit linked to the $\beta\gamma$ dimer (the G-protein heterotrimer, G-trimer) over the free α subunit (Scheuring et al. 1998). In living cells, this inactivation is slow (see e.g. Parker et al. 2007b; Figs. 1–4 in this study), implying receptor-free G-trimers as targets, and effects at the level of receptor recycling and de novo synthesis.

Most membrane-residing proteins are known to form oligomers, which is of advantage in passing the ER quality control proteinase system (see e.g. Credle et al. 2005), as well as in retention in the membrane. Most A-GPCRs form homo- and heterodimers (for recent reviews see Milligan 2009; Parker et al. 2009). These dimers associate with G-trimers to produce heteropentamers (Baneres and Parello 2003; Nobles et al. 2005), which are stable in the absence of activation by agonists. All Y receptors characterized in terms of signal transduction [the Y_1, Y_2 and Y_5 receptors for neuropeptide Y (NPY) and Y_4 receptor for pancreatic polypeptide (PP)] prefer Gi-type α subunits, and form dimers (Berglund et al. 2003a; Dinger et al. 2003) which are detected as heteropentamers with G-trimers (Estes et al. 2008; Parker et al. 2007b, 2008c, and this work). The Y_1 and Y_2 heteropentamers are very much reduced by culture in the presence of PTX (Parker et al. 2007c, 2008c), which in the present study is also shown for the Y_4 receptor (and again found to tightly follow inactivation of Gi α subunits).

Materials and methods

Materials

Human Y receptor cDNAs packaged in Invitrogen pcDNA 3.1+ vector were donated by the University of Missouri at Rolla (MO, USA). The receptors were stably expressed in CHO-K1 cells (from the American Type Culture Collection, Baltimore, MD, USA).

Human/rat neuropeptide Y (NPY), porcine/rat peptide YY (pPYY), human peptide YY (3–36) (PYY (3–36)) and human pancreatic polypeptide (hPP) were obtained from the American Peptide Company (Sunnyvale, CA, USA), or from Bachem (King of Prussia, PA, USA). The Y_2 antagonist BIIE0246 was purchased from Tocris (Ellisville, MD, USA). The Y_1/Y_4 receptor antagonist VD-11 (see Parker et al. 2005) was synthesized by Dr. A. Balasubramaniam. Rabbit antibodies against human Gi_3 α subunit and Gq α subunits were from Upstate (Lake Placid, NY, USA).

Monoiodinated HPLC-purified [^{125}I]human PYY(3–36) (hPYY(3–36)) was from Phoenix Pharmaceuticals (Shadyvale, CA, USA), while [^{125}I] porcine/rat peptide YY (pPYY) and human pancreatic polypeptide (hPP) were from PerkinElmer (Cambridge, MA, USA). Guanosine 5′-*O*-(γ-thiotriphosphate) (GTPγS) labeled by ^{35}S was from PerkinElmer. Digitonin (high purity) was from Calbiochem (La Jolla, CA, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

Cell culture and treatment with pertussis toxin

Confluent cells in culture wells were always washed three times with the medium (Ham/F12 1:1, 6% fetal calf serum) prior to exposure to pertussis toxin (PTX). The culture was at 5% CO_2. The toxin (from List Laboratories, Campbell, CA, USA) was reconstituted in 0.5 M NaCl–0.1 M Na phosphate (pH 7.0) and stored at 4°C up to 4 months without noticeable change in inhibitory activity. At up to 24 h of treatment, PTX at 1 ng/ml did not significantly change the cell numbers. Ammonium chloride, when used, was applied simultaneous with PTX.

Receptor and nucleotide site labeling and characterization in gradients

The receptors were labeled for 30 min at 25°C in respective particulates, employing 20 pM [^{125}I]-labeled agonist peptides (pPYY for the Y1, hPYY(3–36) for Y2, and hPP for Y4 subtype) in a buffer containing 0.2% proteinase-free bovine serum albumin, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM hepes.NaOH (pH 7.4), 1 mM diisopropylfluorophosphate, 0.04% bacitracin and 10 μg/ml each of proteinase inhibitors aprotinin, bestatin, chymostatin, leupeptin and pepstatin (Parker et al. 2008c). The nucleotide site labeling at 200 pM [^{35}S]GTPγS was for 30 min at 28°C after pre-activation for 30 min at 28°C at 3 μM GDP in the above buffer with 50 μM EDTA,

100 mM NaCl and 4 mM $MgCl_2$, and without $CaCl_2$ (Parker et al. 2008c). All subsequent operations were carried out at 5°C. The assays were terminated by sedimentation for 10 min at 30,000×g_{max}, followed by surface wash of the particulate pellets. After solubilization at 10 mM each of digitonin and sodium cholate, the samples were sedimented for 18 h at 218,000×g_{max} through linear 5–20% sucrose gradients (made in the receptor assay buffer). The gradients were divided into 23 fractions of 0.45 ml. The sedimentation positions were calibrated with [^{125}I]-labeled bovine γ-globulin (158 kDa), human iron-saturated transferrin (75 kDa) and ovalbumin (44 kDa) and with colorized myosin (211 kDa), producing a linear relation of migration to molecular weight ($R^2 > 0.99$). Most of the detergent that could get attached to receptor/G-protein complexes during solubilization at 5°C should be removed by dilution-dialysis over 18 h of passage through detergent-free gradients (see Wildenauer and Khorana 1977).

Immunodetection of G-protein α subunits coupled to receptors

For immunodetection of G-protein α subunits and associated receptors, aliquots of [^{125}I] agonist-labeled fractions from sucrose gradients (100 µl) were incubated with antibodies to Gi_3 or Gq α subunits (at 1:250 final dilution) for 12 h at 5°C. Protein A/protein G agarose (Santa Cruz, CA, USA) was then added (50 µl/250 µl final volume), the mixtures were rotated for 6 h at 4°C, loaded onto spin columns (Pierce, Rockford, IL, USA) and spin-washed with 2 × 1 ml of the cold receptor assay buffer prior to counting of the gels in a scintillation counter.

Adenylyl cyclase assay

Following overnight treatment with pertussis toxin or other agents, cultures of Y receptor-expressing CHO cells in 48 well plates were washed and brought to 1.2 µM forskolin and the desired concentration of Y receptor agonists or antagonists, at 100 µM isobutylmethylxanthine, all at 5°C and in the D-MEM/F-12 medium without antibiotics and fetal serum, but containing 0.2% BSA. The plates were then incubated for 20 min at 37°C, the medium removed by suction in ice and the cells extracted by 0.50 ml/well of 0.100 N HCl for 20 min at 21–23°C. The extracts were collected into a mixture of NaOH and CH_3COOH assuring adjustment to pH in the range of 6–7, and kept at −75°C until assayed for cAMP, using kits supplied by PerkinElmer (Cambridge, MA, USA). NIHRIA program by Dr. David Rodbard was used to estimate cAMP concentration.

Results

Detection of transductional complexes of Y receptors by labeled agonists

Of the six identified subtypes of the Y receptor (Bromee et al. 2006), four were shown to form dimers (the Y_1, Y_2 and Y_5 Dinger et al. 2003) and Y_4 (Berglund et al. 2003a receptor). All four subtypes transduce via Gi subunits and possibly also use Gq subunits (Grouzmann et al. 2001; Parker et al. 2008c). The Y_1, Y_2 and Y_4 subtypes show subnanomolar affinity for the respective primary agonists (which are 36-peptides for the Y_1 and Y_4 and a 34-peptide for Y_2 receptor), and attachment of these agonists to the receptors is stable at 5–25°C in the absence of signal transduction (Dautzenberg and Neysari 2005; Parker et al. 2001a; see Parker et al. 2007c for the dimers). At 5°C, the Y agonist attachment is stable to solubilization of particulates with digitonin and cholate (while there is a significant dissociation by a zwitterionic detergent (Parker et al. 2008a). The attachment of GTPγS to nucleotide sites of Gi α subunits has similar stability (Berglund et al. 2003a; Parker et al. 2008a). This allows a highly sensitive detection of transductional complexes and stages. The Y_5 subtype shows lower stability of agonist binding, and for that reason was examined only in terms of domain statistics, as were the Y2-like glucocorticoid-induced receptor (GIR or GPCR83, Sah et al. 2007) and prolactin-releasing peptide receptor (GPR10; Lagerstrom et al. 2005).

Inactivation of Gi α subunit nucleotide site and of Y receptor binding sites by pertussis toxin

While the functional sensitivity to pertussis toxin (PTX) due to association with PTX-modifiable G-proteins was shown for the Y_1 receptor as early as 1990 (Aakerlund et al. 1990), and for the Y_2 receptor in 1996 (Nakamura et al. 1996), comparisons of inhibition by the toxin of the receptor and the Gi subunit type activity for Y_1, Y_2 and Y_4 receptors separately expressed in the same cell line were not presented thus far. Figure 1 shows the reduction in agonist peptide binding and of nucleotide site binding to CHO cell particulates after 24 h of culture at 0.01–100 ng/ml of PTX. The agonists used were porcine/rat peptide YY (pPYY) for the Y_1, human peptide YY(3–36) (PYY(3–36)) for the Y_2, and human pancreatic polypeptide (hPP) for the Y_4 expression. At up to 200 pM of tracer agonists, 15–20% of Y_1 sites ([^{125}I]pPYY binding) and Y_2 sites ([^{125}I]hPYY(3–36) binding) and 30–35% Y_4 sites ([^{125}I]hPP binding), but <5% of Y agonist-activated [^{35}S]GTPγS sites, are detected even after exposure to high concentrations of the toxin. These results confirm and expand our previous reports for the Y_1 (Parker et al. 2008c)

and the Y_2 expression (Parker et al. 2007c), and to our knowledge are the first direct characterization of the response of Y4 sites to the toxin.

The basal and the agonist-stimulated activity of the nucleotide site were followed at 200 pM of [^{35}S]GTPγS. This concentration of the nucleotide reveals (due to the low expression of Go α subunits in CHO cells, Raymond et al. 1993) only the status of Gi-type α subunits. Activation of Gs and Gq α subunits in vitro requires much higher concentrations of nucleotide site agonists (see e.g. Graziano et al. 1989 for the Gs, and Hepler et al. 1993 for the Gq subunit). This should be related to supply of effector enzymes and other proteinaceous co-factors, acting as accelerators (Ross and Wilkie 2000).

As shown in Fig. 1, up to 20% of Y_1 sites ([^{125}I]pPYY binding) and Y_2 sites ([^{125}I]hPYY(3–36) binding) and up to 35% Y_4 sites ([^{125}I]hPP binding) are detectable even after saturating exposure to the toxin. After subtraction of this asymptotic component (which should be due to receptor sites associating with PTX-resistant transducers, e.g. Gq-type α subunits, Grouzmann et al. 2001; Parker et al. 2008c), the toxin-sensitive agonist binding pooled for all receptors shows low standard errors, and sensitivity to the toxin reflects that found for the GTPγS binding (also pooled across receptor expressions). The ic_{50} values (best represented by single-component fits) in nanogram toxin per milliliter were 0.042 ± 0.005 for the agonist binding pool, and 0.023 ± 0.004 for the GTPγS binding pool.

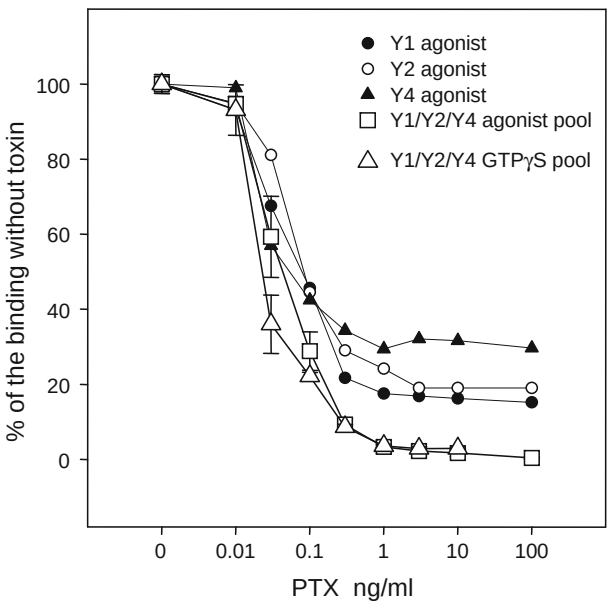

Fig. 1 Concentration dependence for inactivation by pertussis toxin of peptide agonist binding sites and G protein nucleotide sites in CHO cells expressing human Y receptors. The results represent at least four independent experiments for each subtype. Exponential ic_{50} values for inhibition of the Y_1 and Y_2 binding by PTX were 0.11 and 0.13 ng/ml, respectively. After subtraction of asymptotic binding, PTX-sensitive agonist binding pooled for all receptors (*open squares*) shows low standard errors, and sensitivity to the toxin is similar to that for GTPγS binding (also pooled across receptor expressions). The ic_{50} values (single-component fits) were (in ng toxin/ml) 0.042 ± 0.005 for the agonist binding pool, and 0.023 ± 0.004 for the GTPγS binding pool

The decrease in Y_2 receptor and Gi_3 subunit agonist binding is accompanied by the loss of immunoreactive mass of up to 70 and 46%, respectively, after 48 h of exposure to the toxin (Parker et al. 2007b). This was not examined with respect to transcription rates, which however are not affected for other receptors functionally responding to the toxin (e.g. Jajoo et al. 2006).

Rescue of G-protein and Y receptor activity from pertussis toxin by co-treatment with ammonium chloride

Inhibition by PTX of receptor and Gi nucleotide site activity in cells expressing any of the Y receptor subtypes could be considerably attenuated (and at up to 0.1 ng/ml of the toxin even prevented) by ammonium chloride (an inhibitor of particulate proteinases (Green et al. 1994), in a concentration-dependent manner (Fig. 2). This was previously reported for the Y_1 (Parker et al. 2008c) and the Y_2 (Parker et al. 2007c), but not for the Y_4 expression. As in the case of inhibition itself, rescues of both Gi nucleotide site and of Y receptor binding (upon removal of the asymptotic component; see Fig. 1) show a consistent parallelism. At 30 mM NH_4Cl, the half-inhibition by PTX was right-shifted fourfold for the Y sites, and more than 20-fold for the Gi nucleotide site. With the Y_1 receptor, this effect was shown to be linked to receptor conservation at the level of endosomes/dense granules (Parker et al. 2008c), and similar was observed for the Y_2 and Y_4 subtype (data not shown).

Inhibition of adenylyl cyclase activity through Y receptors is prevented by pertussis toxin with parameters similar to those for the receptor and nucleotide site binding

Y receptors are known to inhibit adenylyl cyclase through PTX-sensitive activation of G-protein α subunits (Aakerlund et al. 1990; Parker et al. 2007b). Inhibition of forskolin-stimulated adenylyl cyclase by Y_1, Y_2 and Y_4 receptors activated by respective agonists is lost to cell treatment with pertussis toxin, in a concentration-related way (Fig. 3). For Y_1 and Y_2 expressions, this effect of PTX is best fit to a single specific component, with ic_{50} values of about 0.15 ng/ml toxin, similar to those for total receptor binding (see the legend of Fig. 1). For the Y_4 receptor, the best fit is to two about equal components, one of which is close to that for the receptor binding after subtraction of the

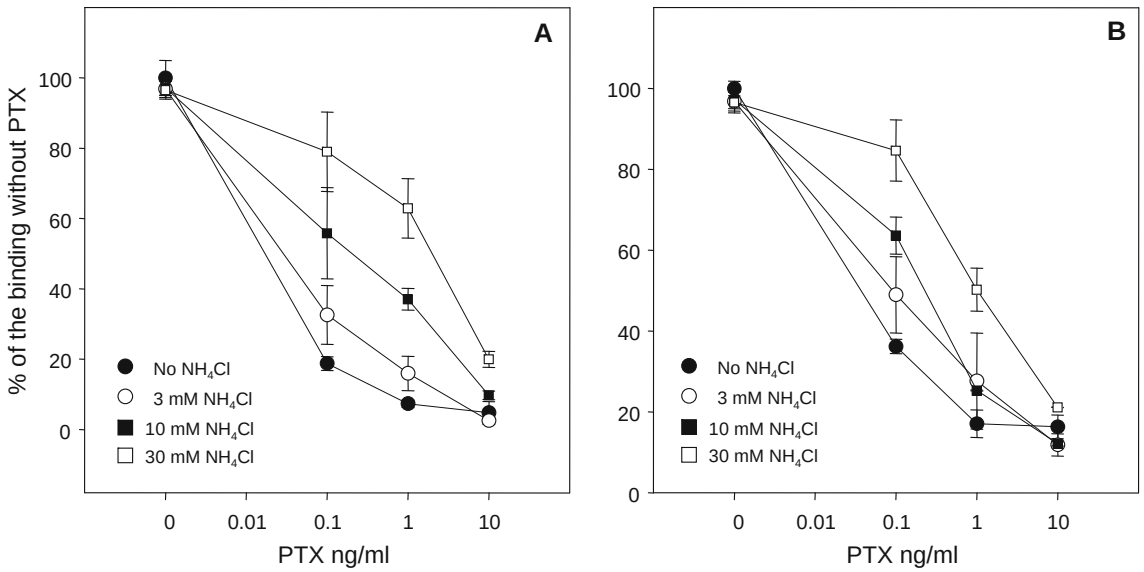

Fig. 2 Rescue of G protein and Y receptor agonist binding sites from pertussis toxin by ammonium chloride. CHO cells expressing Y_1, Y_2 or Y_4 receptors were cultured for 24 h with PTX at 0, 0.1, 1 or 10 ng/ml and NH_4Cl at 0, 3, 10 or 30 mM. Particulates from these cells were then assayed for the binding of [^{35}S]GTPγS and of Y receptor agonists (see "Methods"). The results are averages of profiles for the three receptor subtypes ± 1 SEM, and correspond to at least three independent experiments for each subtype. **a** Rescue of the nucleotide site binding. The labeling was with 200 pM [^{35}]GTPγS. The ic_{50} values (ng toxin/ml culture medium) at 0, 3, 10 and 30 mM NH_4Cl were, respectively, 0.041, 0.063, 0.15 and 0.96. **b** Rescue of agonist binding sites. The sites were labeled with 20 pM of [^{125}I]pPYY (Y_1 subtype), [^{125}I]hPYY(3–36) (Y_2 subtype) and [^{125}I]hPP (Y_4 subtype). The logistic asymptote was subtracted from each profile (see the legend of Fig. 1). The ic_{50} values (ng/ml of the toxin) at 0, 3, 10 and 30 mM NH_4Cl were, respectively, 0.046, 0.07, 0.1 and 0.17

asymptotic binding (see Fig. 1). The loss of inhibition due to PTX is attenuated by co-treatment of cells with NH_4Cl, in a concentration-dependent fashion (data not shown).

Kinetics of inactivation of Y receptors and Gi α subunit nucleotide site by pertussis toxin

From the results shown in Figs. 1, 2, 3, effects of pertussis toxin are linked to inactivation of Gi α subunits. This is further supported by inactivation kinetics of the Y_1 and Y_2 sites and of Gi nucleotide sites at a saturating concentration of PTX (Fig. 4). As seen, the overall sensitivity to the toxin is larger for the G-protein subunits. The half period of inactivation of the subunits is shorter from that for the Y_1 receptor by 0.9 h, and from that for the Y_2 receptor by 1.6 h. This indicates dependence of receptor inactivation upon that of the Gi subunits, and also is consistent with lower sequestration/internalization rates for the Y_2 receptor as compared to the Y_1 subtype (Berglund et al. 2003b; Parker et al. 2001b).

Y receptor dimers in cells are affected by pertussis toxin much more than by antagonists

Many GPCRs are principally detected as complexes of receptor dimers with G-protein heterotrimers, which can subserve receptor survival, expression and function. This includes the Y receptors (Berglund et al. 2003a; Parker et al. 2007c, 2008c). These complexes were found to disband in response to PTX treatment much faster than the total specific binding (Fig. 5), indicating a larger dependence on de novo receptor synthesis. Less than 15% dimers (as opposed to ~65% in untreated cells) were found at saturating (≥80%) inactivation of Y_2 sites by PTX, and this also paralleled the complete loss of surface compartmentalization of the receptor (Parker et al. 2007c). The Y_2 heteropentamer fraction, however, decreased not more than 40% after ~70% receptor knockout in cell culture by the blocking antagonist BIIE0246 (Fig. 5), and upon withdrawal of the antagonist recovered completely within 24 h, with a kinetics inverse to that observed for receptor loss to PTX, and parallel to recovery of the total receptor binding (the caption of Fig. 5). The treatment of cells expressing the Y_4 receptor with slowly reversible antagonist VD-11 (Parker et al. 2005, 2007a) resulted in <50% loss of total receptor binding in 2 h, and the dimeric fraction was not significantly changed by the antagonist (Fig. 5).

Loss of Y receptor heteropentamers to pertussis toxin treatment is coupled to Gi inactivation and both are attenuated by ammonium chloride

Pretreatment with pertussis toxin results in depletion of ~180 kDa heteropentamers (Fig. 6a for the Y1, d for Y2,

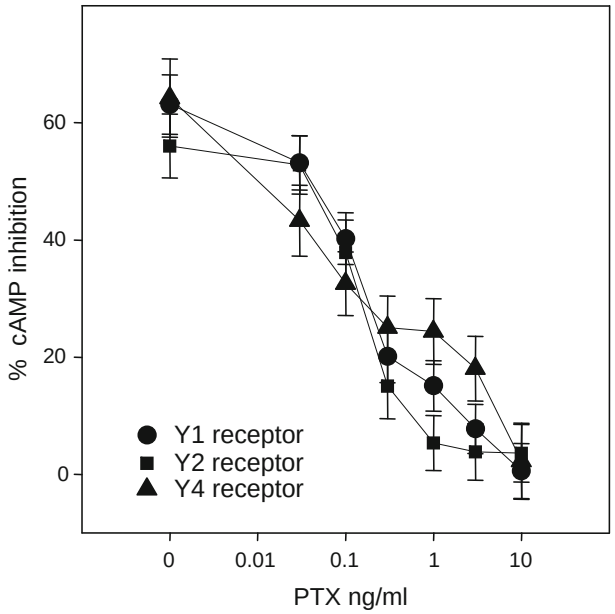

Fig. 3 Loss of inhibition of the forskolin-stimulated adenylyl cyclase activity by Y receptor agonists in relation to pretreatment of cells with pertussis toxin. CHO cells expressing human Y_1, Y_2 or Y_4 receptor were cultured for 24 h at 0, 0.1, 1 or 10 ng/ml of the toxin prior to adenylyl cyclase assay (see "Methods"). The production of cAMP stimulated by 1.2 μM forskolin was challenged by 100 nM pPYY (Y_1 expressing cells), hPYY(3–36) (Y_2 cells) and hPP (Y_4 cells). The best fit for the Y_4 receptor has two approximately equal components with ic_{50} values of, respectively, 0.024 ± 0.006 and 3.6 ± 0.8 ng/ml of the toxin. The Y_1 and Y_2 profiles are best represented by single-component fits with the respective ic_{50} values of 0.16 ± 0.05 and 0.14 ± 0.03 ng PTX/ml

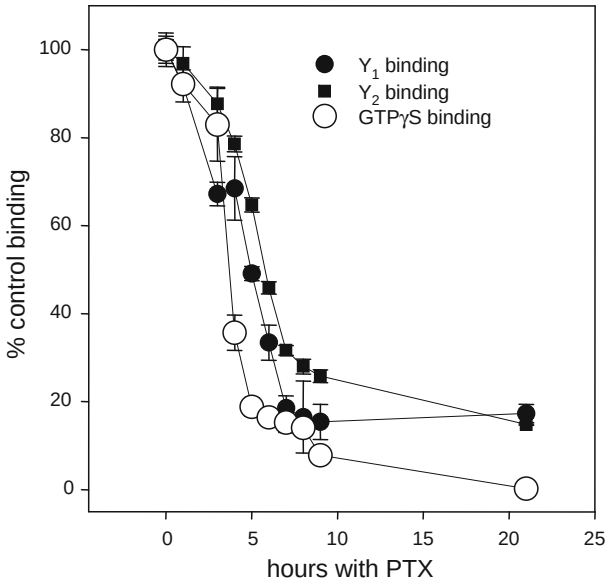

Fig. 4 Compared kinetics of inactivation of Y_1 and Y_2 receptors and Gi α subunits by pertussis toxin. Particulates from CHO cells cultured at 10 ng/ml toxin over the indicated time periods were labeled with 20 pM [^{125}I]pPYY (Y_1 receptor) or hPYY(3–36) (Y_2 receptor), or with 200 pM [^{35}S]GTPγS (see "Methods"). The half periods of inactivation (h) were 4.5 ± 0.4 for the Y_1 receptor, 5.2 ± 0.15 for the Y_2 receptor, and 3.6 ± 0.14 for nucleotide sites. The results are averages of two independent experiments

and g for Y_4 receptor), in parallel to a large loss of ~100 kDa complexes of agonist-activated Y receptor monomers and Gi_3-α (Fig. 6c, f, i for the Y_1, Y_2 and Y_4 expression, respectively). This is accompanied by a similar loss of activated Y agonist-carrying monomer immunoreacting with Gi_3 α subunit antibody (Fig. 6b for the Y_1, e for Y_2, and h for Y_4 receptor). The losses are attenuated by ammonium chloride. This is shown in Fig. 6b for the Y_1 and e for Y_2 receptor; NH_4Cl also attenuates decrease in the Y_4 binding by PTX (Fig. 2), but that was not characterized by sedimentation.

The observed ~185 kDa size of heteropentameric complexes (Fig. 6) is close to the value expected with both protomers occupied by 4.1–4.3 kDa agonists; occupancy of both protomers by the respective agonists was established at the level of gradient-separated heteropentamers (Estes et al. 2008; Parker et al. 2008a). For the agonist–receptor–Gα complex, the expected value is about 90 kDa, while the observed peaks of both the agonist peptide and the GTPγS-labeled complex are at 100–120 kDa (Fig. 6). This could mainly be due to an admixture of receptor dimer–Gα complexes, since ~90 kDa complexes prevail at high Y agonist inputs (Estes et al. 2008). However, possible presence of regulators of G-protein signaling (e. g. Hooks et al. 2003) needs to be examined.

It is important to note that only small fractions of 40–50 kDa Gα-free agonist-labeled receptors are observed with any subtype in the absence of PTX (Fig. 6a, d, g), with the radioactivity recovery in the 60–300 kDa zone of gradients consistently above 90%. A somewhat broad 40–120 kDa profile with Y_4 preparations from PTX-treated cells (Fig. 6g) should include a fraction of Gα-free liganded receptors, since it is not observed for Gi_3-immunoreacting agonist-labeled Y_4 receptor (Fig. 6h). This could be the receptor that prior to solubilization interacts with effectors of transporter type (for cation and amiloride sensitivity of the Y_4 binding, see Parker et al. 2002), and is currently being investigated. However, most receptors of the three examined subtypes appear to be coupled to heterotrimeric G-proteins at the level of the dimer or the monomer.

Discussion

Both transducing and non-transducing membrane-resident proteins of many types form (frequently obligatory) oligomers at the level of ER/Golgi (for general reviews, see Christis et al. 2008; Trombetta and Parodi 2003). This allows an adequate processing following translation (which

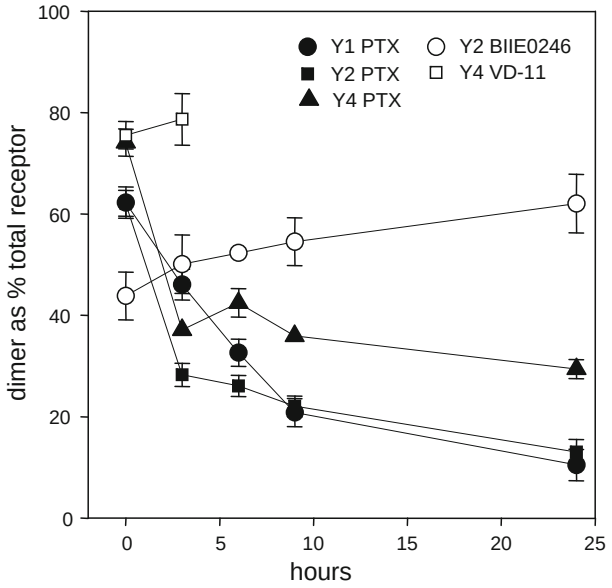

Fig. 5 Abundance of Y receptor dimers in relation to treatment with pertussis toxin or Y receptor antagonists. After treatments of cells with the respective agents (see below), particulates were labeled with 20 pM [^{125}I]pPYY (Y_1 receptor), hPYY(3–36) (Y_2 receptor), and hPP (Y_4 receptor), solubilized and sedimented in sucrose gradients (see "Methods"). To follow effects of PTX, CHO cells expressing the receptors were cultured at 1 ng/ml toxin over the indicated time periods (*filled symbols*). For effects of antagonists (*open symbols*), the Y_2 and Y_4 receptor-expressing cells were cultured for 60 min at, respectively, 300 nM BIIE0246 (an irreversible Y_2 antagonist) and VD-11 (a slowly reversible Y_4 antagonist, Parker et al. 2007a) and then without antagonists for the indicated periods prior to isolation of particulates. The results are averages of at least two independent experiments. In untreated control cells, percentages of the dimers (as ~180 kDa heteropentamers, see Fig. 6) were 62.3 ± 2.6 for Y_1, 62.1 ± 2.6 for Y_2, and 74.1 ± 2.7 for Y_4 receptor. After 24 h of PTX treatment, Y site labeling was reduced 74.4% (Y_1), 74.1% (Y_2) and 65.7% (Y_4). At the end of treatment with antagonists, the Y_2 and Y_4 sites were respectively reduced 70 and 49.5%. The half period of recovery for the total agonist binding was 3.9 ± 0.8 h ($n = 3$) for Y_2 receptor after BIIE0246, and ~1 h for Y4 receptor after VD-11

often includes a long-lasting modification in the ER (e.g. Alarcon et al. 1988), and provides shielding from the quality control proteinase system (e.g. Hirsch et al. 2009) at the level of ER/Golgi (Hampton 2002; Trombetta and Parodi 2003) and of the recycling endosome (Colombo et al. 1994; Scarselli and Donaldson 2009). Dimerization could be important for cell surface retention of GPCRs (Decaillot et al. 2008; Salahpour et al. 2004), and appears to be a ubiquitous form of intracellular organization for these receptors.

Dimers of A-GPCRs mainly are non-covalent (see reviews Milligan 2007; Parker et al. 2009), and their formation could generally be helped by association with G-proteins, and may even depend on a stabilization and/or induction of a specific conformation, and on reduction of the random mobility by G-protein attachment to the first protomer. However, covalent dimerization via extracellular domains for Frizzled receptors (Carron et al. 2003) should also be helped by a PTX-sensitive anchoring of intracellular domains to Go-containing heterotrimers.

Dimers of many A-GPCRs are detected as stable pentameric complexes with G-protein heterotrimers (Baneres and Parello 2003; Jastrzebska et al. 2006; Nobles et al. 2005; Parker et al. 2007b, 2008c). However, GPCR monomers also stably associate with G-protein heterotrimers (Jastrzebska et al. 2009). Pre-assembly with G-proteins could assure a prompt response to agonist attachment (Baneres and Parello 2003; Cheng and Miller 2001; Estes et al. 2008), and possibly help in association of other partners and repetitive signaling (see e.g. Ross and Wilkie 2000). The association also should increase receptor survival at the level of completion in ER/Golgi (Decaillot et al. 2008; Salahpour et al. 2004; Ulloa-Aguirre et al. 2007), or during recycling, as indicated by the slow response to PTX (Parker et al. 2007b, 2008c and this work). These losses again point to an effect at the level of completion in the ER/Golgi compartment, and are sustained in tandem over long periods of cell culture (Parker et al. 2007c; Ramkumar and Stiles 1990).

Large dimeric fractions for Y receptors are found in many epithelial-type cell lines (Berglund et al. 2003a; Dinger et al. 2003; Parker et al. 2008c; Parker et al. 2007b) as well as in kidney epithelia (Estes et al. 2008). Most of these receptors in the absence of signal transduction appear to stably interact with Gi-containing heterotrimers, and their decrease after exposure of cells to pertussis toxin follows depletion of functional Gi subunits. This was shown for the Y_2 receptor (Parker et al. 2007b, c), the Y_1 receptor (Parker et al. 2008c), and in this work for the Y_4 receptor. Other Gi-associating GPCRs could respond to PTX similarly, e.g. the adenosine A_1 receptor (Hansen et al. 2003; Jajoo et al. 2006). The functional sensitivity should depend on relative affinities for α subunits of various classes, and other factors (including abundance of α subunits, effectors, and other protein partners (Krumins and Gilman 2006; Ross and Wilkie 2000).

Dimerization of Y receptors is importantly connected to functionality of Gi subunits, since the receptors detected at saturating exposure to the toxin are largely α subunit-associated monomers (Parker et al. 2007b, 2008c; Fig. 6 in this work). A lack of functional Gi subunits could impact survival of the receptors both at the completion of biosynthesis in the ER and in the recycling/perinuclear endosomes, as clearly is indicated by the attenuating effect of NH_4Cl, an inhibitor of particulate proteinases (Green et al. 1994). The effect of PTX has a long half period (Fig. 4; see also Parker et al. 2007c, 2008b), indicating dependence on de novo synthesis and recycling, which in the case of Y receptors involves the perinuclear endocytotic compartment

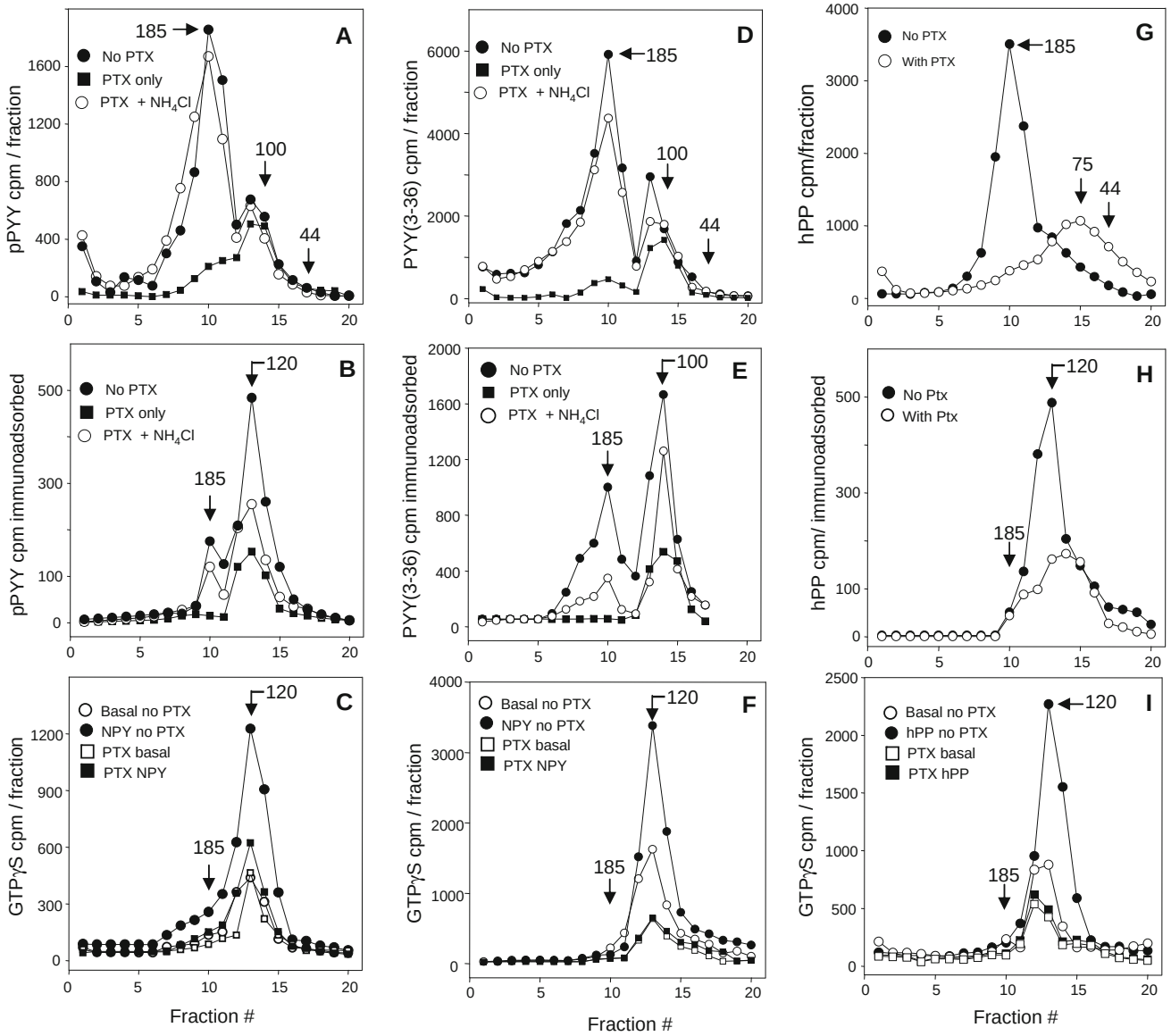

Fig. 6 Stoichiometric 1:1 heteropentamers (~180 kDa) of Y dimers and G protein heterotrimers are the most abundant form of organization of these receptors in CHO cell expressions. The receptors were solubilized by digitonin and cholate and sedimented through sucrose gradients (see "Methods"). The attachment of [^{125}I]-labeled agonist peptides (pPYY for the Y_1, hPYY(3–36) for Y_2, and hPP for Y_4 receptor) following solubilization and isolation by gradient centrifugation is stable for 48 h at 5°C for all three receptors, and for the Y_2 and Y_4 receptors even at 25°C (Dautzenberg and Neysari 2005; Parker et al. 2001a). The molecular weight estimates are based on sedimentation of [^{125}I]-labeled or colorized marker proteins (see "Methods"). The heteropentamers are largely depleted by 24 h of exposure in cell culture to 1 ng/ml of pertussis toxin (*first row* **a** Y_1, **d** Y_2, **g** Y_4). This is due to inactivation of Gi α subunits (**c**, **f**, **i**; also see Figs. 1, 2, 3) here evident from the large decrease in receptor reaction with antibody to Gi_3 α subunit (*second row* **b** Y_1, **e** Y_2, **h** Y_4 receptor). At 1 ng/ml toxin in cell culture, this decrease is strongly attenuated by co-treatment with 30 mM NH_4Cl (**b** Y_1 and **e** for Y_2 receptor; large attenuation is also achieved for Y_4 receptor (characterization not done in the gradient centrifugation format). Decrease at the level of activation of nucleotide sites of the Gi α subunits, as followed by attachment of the quasi-irreversible agonist GTPγS, is shown in the *last row* (**c** Y_1, **f** Y_2, **i** Y_4 receptor)

(Pheng et al. 2003). In any case, association with the Gq α subunit (which is expressed in CHO cells at levels similar to Gi subunits (e.g. Mullaney et al. 1993) appears not to provide sufficient support for oligomerization of Y receptors, probably due to a lower degree of Y receptor contact with the Gq relative to Gi subunits, with the consequently smaller oligomer stability and protection from proteinases.

As expected, heteropentamers of Y receptors are not preferentially lost to subtype-specific antagonists (Parker et al. 2007c, 2008c; and this work). With the irreversible

Y2 antagonist BIIE0246, recovery of the dimeric fraction is achieved with a kinetics inverse to that observed with PTX, reflecting renewal by de novo synthesis. Similar could apply to a cannabinoid CB1 receptor mutant (Anavi-Goffer et al. 2007).

Complexes of both GPCR monomers and dimers with G-protein trimers in the absence of nucleotide site activation are stable at 5–28°C either in particulates or in solution, withstanding elaborate processing and allowing immunodetection (Baneres and Parello 2003; Jastrzebska et al. 2006, 2009; Parker et al. 2007c). Mild nonionic and weak anionic detergents do not disband Y2 receptor heteropentamers, but zwitterionic detergents concentration dependently detach the G-protein component (Parker et al. 2008a). This indicates involvement of ionic motifs in Y2 receptor–transducer interaction.

Activation of G-proteins has been reported to use residues and motifs in all intracellular domains of GPCRs. However, the C-terminus of the ic3 domain (ic3-Ct) and helix 8 are found to interact with G-proteins much more frequently than other intracellular tracts. This is reviewed in detail in the companion study (Parker et al. 2010).

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