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Pertussis toxin induces parallel loss of neuropeptide Y Y_1 receptor dimers and G_i α subunit function in CHO cells

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Abstract

Treatment with pertussis toxin in addition to a stable inhibition of $G_i\alpha$ subunits of G-proteins also strongly reduced human neuropeptide Y Y_1 receptors expressed in Chinese hamster ovary (CHO) cells. This was reflected in abolition of the inhibition by Y_1 agonists of forskolin-stimulated adenylyl cyclase in intact cells, and of Y_1 agonist stimulation of GTPγS binding to particulates from disrupted cells. The loss of both receptor and $G_i\alpha$ subunit function was attenuated by ammonium chloride, an inhibitor of acid proteinases, pointing to a chaperoning co-protection of active pertussis toxin-sensitive Gα subunits and Y_1 receptors. The surface complement of the Y_1 receptor was changed a little in conditions of ~85% decrease of the Y_1 population, but the rate of the Y_1 receptor-linked internalization of agonist peptides was reduced about 70%. The preserved receptor fraction consisted of monomers significantly coupled to $G_q\alpha$ subunits. The persistent pertussis toxin-insensitive internalization of agonists with the Y_1 receptor may reflect a rescue or alternative switching that could be important for cell functioning in neuropeptide Y-rich environments. The results are compatible with a loss, due to $G_i\alpha$ subunit inactivation by the toxin, of a large Y_1 receptor reserve constituted of oligomers associating with heterotrimeric G-proteins.

Keywords: Adenylyl cyclase; G-protein coupled receptor; G_i subunit; G_q subunit; Receptor organization; Receptor internalization; Receptor monomer; Receptor oligomer

1. Introduction

The Y receptors (i.e., the receptors for neuropeptide Y, peptide YY and the related pancreatic polypeptide) all interact with pertussis toxin-sensitive α subunits of heterotrimeric G-proteins (see Herzog et al. (1992) for the Y_1 receptor, Freitag et al. (1995) for the Y_2 receptor, Parker et al. (1998) for the Y_4 receptor, and Bischoff et al. (2001) for the Y_5 receptor). The CHO cell expressed Y_1 receptor is constitutively recycled, as evidenced by its loss to pertussis toxin treatment (Sah et al., 2005), but also rapidly cycles in response to agonists (Parker et al., 2001b; Gicquiaux et al., 2002). Similar is found for many other G-protein coupling receptors, e.g. the β2-adrenergic receptor (Moore et al., 1995), the cannabinoid CB1 receptor (Mukhopadhyay et al., 2000) and the thromboxane A2 receptor (Theriault et al., 2004). Cell membrane pools containing these receptors could be rich in pertussis toxin-sensitive G-proteins (Mukhopadhyay et al., 2000) and could also overlap with the dynamic pool of rafts and recycling endosomes that harbors the transferrin receptor (Apodaca et al., 1994). Inactivation of $G_i\alpha$ subunits could be expected to reduce the Y_1 receptor activity, traffic, and even levels. With cloned human Y_1 and Y_2 receptors expressed in CHO cells there indeed is a consistent and large reduction in levels of receptors by treatment with pertussis toxin, along with a decrease of natively expressed $G_i\alpha$ subunits

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and loss of Y agonist activity vs. stimulation of adenylyl cyclase by forskolin (Sah et al., 2005; Parker et al., 2007). With the Y_2 receptor, these changes are attenuated by co-treatment with ammonium chloride (Parker et al., 2007), an inhibitor of acid proteinases (Green et al., 1994), pointing to a possible interactive co-protection of the receptor and of $G_i\alpha$ subunits. Similar relations could be expected with the Y_1 receptor, with an activity highly sensitive to pertussis toxin (Sah et al., 2005). The large and fast cycling of the Y_1 receptor (Parker et al., 2001b; Gicquiaux et al., 2002) is also influenced by inactivation by the toxin of receptor's principal signal transduction partners.

2. Materials and methods

2.1. Materials

Human/rat neuropeptide Y, porcine/rat peptide YY and human (Leu^{31},Pro^{34})peptide YY were obtained from the American Peptide company (Sunnyvale, CA, USA), or from Bachem (King of Prussia, PA, USA). The Y_1 antagonist BIBP3226 ((*R*)-N2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]D-arginine amide) was purchased from Tocris (Ellisville, MD, USA). G_q-selective G-protein antagonist-2A (G-2A; RPKPQQdWFdWdWM.NH_2; dW=D-Trp) was from Bachem, King of Prussia, PA, USA). Rabbit antibodies against human G_{i1} and G_{i3} G-protein α subunits (not recognizing other G_i or pertussis toxin-insensitive α-subunits) and G_q α-subunits (recognizing neither the pertussis toxin-sensitive subunits, nor the G_s α-subunit) were obtained from Upstate (Lake Placid, NY, USA).

Monoiodinated HPLC-purified [^{125}I]human neuropeptide Y was supplied by Phoenix Pharmaceuticals (Shadyvale, CA, USA), while [^{125}I](Leu^{31},Pro^{34})human peptide YY and [^{125}I] porcine peptide YY were from PerkinElmer (Cambridge, MA, USA). All [^{125}I]-labeled Y peptides had specific activities above 1700 Ci/mmol. Guanosine 5′-*O*-(γ-thiotriphosphate) (GTPγS) labeled by ^{35}S (specific activity 1250 Ci/mmol) and *myo*-[^{3}H] inositol (specific activity 15–20 Ci/mmol) were purchased from PerkinElmer.

Pertussis toxin and cholera toxin were purchased, respectively, from List Laboratories (Campbell, CA) and from Sigma (St. Louis, MO, USA), 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. No preactivation of toxins was necessary for effects in intact cells, as ascertained by testing after activation by dithiothreitol pretreatment (Kaslow et al., 1987). At up to 24 h of treatment, neither the dithiothreitol-activated nor the nonactivated toxins at 1 μg/ml produced a significant change of cell numbers. However, prolonged treatment with pertussis toxin at 100 ng/ml (6–7 days) induced up to 40% cell loss, similar to previous results with adipocytes (Denis-Henriot et al., 1996).

All other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Cell cultures and labeling

The cDNAs for human Y_1 and Y_2 receptors packaged in Invitrogen pcDNA 3.1+ vector were donated by the University of Missouri at Rolla (MO, USA). The cDNA for guinea pig Y_1 receptor packaged in PTEJ-8 plasmid (Johansen et al., 1990) was a gift from Dr. Magnus Berglund (Department of Neuroscience, University of Uppsala, Uppsala, Sweden). The cDNA for mouse Y_4 receptor was a gift from Dr. Herbert Herzog (Garvan Institute for Medical Research, Sydney, Australia). All cDNAs were stably expressed in CHO-K1 cells (American Type Culture Collection, Baltimore, MD, USA) using lipofectamine-2000 cationic lipid (Invitrogen, Carlsbad, CA, USA). The cells were cultured at 400 μg/ml geneticin in D-MEM/F12 medium (Gibco, Long Island, NY, USA) containing 5% (v/v) of fetal calf serum. The culture medium was always replaced prior to treatment with pertussis toxin.

Short-term experimental incubations prior to or after any pretreatments were preceded by four washes with D-MEM/F12 medium without antibiotics or toxins, and with 0.2% proteinase-free BSA instead of fetal calf serum. The incubations were done in 48-well (0.8 cm^2 per well) plates, in a volume of 0.25 ml of the above D-MEM/F12 medium. The labeling with ^{125}I-tagged Y peptides was done at 100 pM ^{125}I-labeled peptide tyrosine, using 1 μM neuropeptide Y or 100 μM BIBP3226 to define the nonspecific binding. The incubations were terminated by the removal of the medium by suction, two washes with ice-cold D-MEM/F12 medium, and extraction for 10 min at 0–4 °C with ice-cold 0.2 M CH_3COOH–0.5 M NaCl (pH 2.7), which quantitatively dissociated the cell-surface attached Y peptides, without significant extraction of internalized peptides (Parker et al., 2002b). The binding of Y peptides to particulates from Y receptor-expressing CHO cells was, on the other hand, completely extracted by cold acid saline, as expected from the known importance of arginine residues in Y peptides for their binding to the cognate receptors (Beck-Sickinger et al., 1994).

For comparisons of the various treatments affecting the Y_1 receptor in adherent cells, the confluent cells were incubated in wells for 30 min at 37 °C with D-MEM/F12 with the various additives, as indicated under the respective experiments. After 30 min at 23–24 °C, medium with the agents was removed, and the cells washed. Aliquots of resuspended cells, or cells harvested from appropriate wells, were assayed for the total particulate receptor binding. Other aliquots or wells were labeled for 20 min at 37 °C with 100 pM [^{125}I](Leu^{31},Pro^{34}) human peptide YY, washed in the cold, and the agonist attached to surface Y_1 sites was extracted at 0–4 °C with 0.2 M CH_3COOH–0.5 M NaCl.

2.3. Receptor characterization

Receptor assays were done essentially as described (Parker et al., 2002b), in a buffer containing 20 mM hepes.NaOH (pH 7.4), 3 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2% proteinase-free bovine serum albumin, 1 mM diisopropylfluorophosphate, 0.04% bacitracin, and 10 μg/ml each of proteinase inhibitors aprotinin, bestatin, chymostatin, leupeptin and pepstatin. The particulates were stored at −80 °C. Assay concentration of particulate protein was 100–125 μg/ml, the assay volume was 0.2 ml, and the incubation time was 40 min at 23–24 °C, with the appropriate competitors or inhibitors. The assay was terminated by

centrifugation for 12 min at 30,000 $\times g_{max}$ and 4 °C in a Sorvall (Newtown, CT, USA) T 21 centrifuge, the supernatants were aspirated, and the pellets surface-washed by cold assay buffer prior to counting in a gamma-scintillation counter. The binding properties of cell-surface receptors were characterized on monolayer cultures in D-MEM/F12 medium containing no antibiotics, and 0.2% BSA instead of fetal bovine serum. For competition assays, the iodinated Y peptides were used at 100 pM, and the competing peptides were input at 8–10 different concentrations ranging from 3×10^{-12} to 1×10^{-6} M.

2.4. The binding of ^{35}S-labeled GTPγS to particulates

The buffer used for homogenization and binding contained 100 mM NaCl, 4 mM $MgCl_2$, 20 mM hepes. NaOH (final pH 7.4), 50 μM EDTA, 1 mM diisopropylfluorophosphate, 0.05% bacitracin and 0.2% proteinase-free bovine serum albumin. The cells were homogenized in 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 particulates, which were stored at −80 °C prior to use in assays. The assay was done in a volume of 0.2 ml, with particulate protein input of 5–10 μg. A preincubation of 60 min at 28 °C without or with 100 nM of peptidic agonists was followed by labeling over 30 min at 28 °C by 0.2 nM of [^{35}S] GTPγS. The nonspecific binding was defined at 300 μM suramin. The assay was terminated by rapid filtration through Whatman GF/F filters, followed by three washes by 2 ml cold assay buffer (without GDP). The filters were soaked for 4 h at 37 °C in 2% sodium dodecyl sulfate — 0.02 M Tris. HCl pH 8.8, and the liquid scintillation solvent was added prior to counting in Beckman (Palo Alto, CA) LS 3801 liquid scintillation counter.

2.5. The assay of adenylyl cyclase

Confluent cultures of Y_1-CHO cells in 48-well plates were washed and brought to 1 μM forskolin and the desired concentration of Y_1 agonists or antagonists, at 100 μM isobutylmethylxanthine, all at 4 °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 rapid 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.

2.6. The assay for inositol phosphates

To measure the production of inositol phosphates, the procedure of Rosenkilde et al. (2004) was used with minor modifications. CHO cells stably expressing the human Y_2 receptor were cultured in 12-well plates for 24 h with *myo*-[^{3}H] inositol (PerkinElmer, Cambridge, MA, USA; 15 Ci/mmol), at 20 μCi in 2 ml per well of inositol-free Dulbecco's minimal Eagle medium (containing 2.5% (v/v) fetal bovine serum), with or without 1 ng/ml of pertussis toxin. The washed cells were then incubated for 3 h at 37 °C and 10 mM LiCl as described in Rosenkilde et al. (2004), without or with 100 nM human/rat neuropeptide Y. The selective Y1 receptor antagonist BIBP-3226 was used at 100 μM, and was added 5 min before 100 nM neuropeptide Y. The cells were washed with cold assay buffer and then extracted with 0.01 M HCOOH (1 ml/well, 30′/0 °C). The extracts were applied to 1-ml columns of Dowex-1, $HCOO^-$, and the columns washed with 0.02 M NH_4HCOO. Inositol monophosphate was then recovered by 0.2 M NH_4-HCOO + 0.1 M HCOOH, and the combined inositol di- and triphosphates were eluted by 1 M NH_4HCOO + 0.2 M HCOOH (after Berridge et al. (1983)). The eluates were then subjected to liquid scintillation counting.

2.7. Immunodetection of G-protein α-subunits

For immunoadsorption of G-protein α subunits and associated receptors, aliquots of [^{125}I] peptide YY-labeled fractions from sucrose gradients (0.1 ml) were incubated with antibodies to G_{i1}, G_{i3} or $G_q\alpha$ subunits (each at 1:250 final dilution) for 12 h at 4 °C. Protein A/protein G agarose 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 γ-scintillation counter.

2.8. Percoll gradient assays

After pretreatment with pertussis toxin and/or NH_4Cl or KCl for 20 h, the cells were incubated for 12 min at 37 °C with appropriate drugs and radioactive peptides, then washed three times in ice with cold incubation medium, and once with 0.25 M sucrose–10 mM HEPES · NaOH (pH 7.4). The cells were further processed as described before (Parker et al., 2002a). Briefly, the 500 ×g supernatants of individual homogenates were applied over pro-gradients consisting of layers of 1 ml 60% sucrose, 5 ml 18% (v/v) Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and 4 ml 10% (v/v) Percoll (both Percoll solutions in 0.25 M sucrose–10 mM HEPES · NaOH, pH 7.4), precooled to 5 °C in 14-ml UltraClear® tubes for the SW41 Ti rotor. The tubes were centrifuged for 55 min at 5 °C in a Beckman M-80 ultracentrifuge using SW41 Ti rotor at 20,000 rpm. (68,000 $\times g_{max}$). The tube contents were divided into 20 fractions prior to determination of radioactivity (in polyethyleneglycol-precipitated aliquots) and enzyme activity. Density at the end of centrifugation was determined from distribution of Percoll density marker beads (Amersham Pharmacia, Piscataway, NJ, USA).

2.9. Sucrose gradient assays

The particulates labeled by [^{125}I]porcine peptide YY as in Section 2.3, or by [^{35}S]GTPγS as in Section 2.4, were surface-washed in ice, and dispersed in cold receptor assay buffer at

≤0.25 mg particulate protein/ml. A mixture of sodium cholate and digitonin was added to 10 mM final, the mixtures gently passed eight times through a 25-gauge needle, and sedimented for 5 min at 10,000 $\times g_{max}$. The supernatants were then loaded on linear sucrose gradients (total volume 9.2 ml, made in the receptor assay buffer) and sedimented for 24 h (when using 10–30% sucrose, w/v) or 18 h (with 5–20% sucrose, w/v) at 35,000 rpm (218,000 $\times g_{max}$) and 5 °C in SW41 Ti rotor of Beckman-Spinco M8-80 ultracentrifuge. 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 the covalently colorized myosin (211 kDa), producing a linear relation of distance traveled in the gradient vs. molecular weight ($R^2 > 0.99$). The gradients were divided in 0.42-ml fractions, and the polyethyleneglycol-precipitated radioactivity determined. Aliquots of the gradient fractions were brought to 12% polyethyleneglycol-8000, 10 mM KI and 2 mg/ml bovine γ-globulin, allowed to precipitate for 10 min at 0–4 °C, and then sedimented for 10 min at 10,000 $\times g_{max}$, and the radioactivity counted in the pellets. This procedure recovers essentially only the Y_1 agonist associated with the receptor, or [^{35}S]GTPγS attached to G-proteins (Parker et al., 2002b).

2.10. Data evaluation

The receptor binding parameters were calculated in the LIGAND program (Munson and Rodbard, 1980). The ED_{50} and IC_{50} values were estimated from exponential or logistic curve fits with SigmaPlot software (version 8.02). Multiple comparisons in Scheffé *t* test following a positive ANOVA, and correlations of data series were done using ProStat software (Poly Software, Pearl River, NY, USA). Areas under the curve for radioactivity in gradient peaks were estimated in the ImageJ program (available at the U.S. National Institutes of Health website), employing .TIFF format graphics of the respective gradient profiles. SPSS 15 (Chicago, IL, USA) program was used for comparisons of trapezoid integrals.

3. Results

3.1. Exposure to pertussis toxin reduces agonist activity with Y_1 receptors, $G_i\alpha$ subunits and adenylyl cyclase of CHO cells

As we already reported (Sah et al., 2005), pertussis toxin induced a steep decrease in agonist binding to the Y_1 receptor. Over 24 h of treatment, the decrease was significant even below 0.1 ng toxin/ml, and saturated at 1 ng/ml (Fig. 1A). As there are no pertussis toxin-sensitive sites in the molecule of the Y_1 receptor, the decrease should be connected to pertussis toxin-sensitive receptor partners in the signal transduction system. Indeed, as seen in Fig. 1B, both the basal and the agonist-stimulated binding of [^{35}S]GTPγS to guinea pig Y_1 particulates were reduced by more than two thirds after 24 h at 1 ng pertussis toxin/ml. The basal production of cAMP in response to forskolin was not consistently affected by the toxin (not shown). As expected (Sah et al., 2005), the pertussis toxin-induced removal of the inhibition of forskolin-stimulated cAMP production by Y_1 agonist peptide YY (Fig. 1C) largely paralleled the inactivation of the respective ligand binding to the Y_1 receptor and to Giα subunits, and the corresponding regressions were significantly correlated (the legend of Fig. 1) (In contrast, pertussis toxin at 1 ng/ml did not modify the large stimulation of cAMP production by cholera toxin, an irreversible activator of $G_s\alpha$-subunit (e.g. Levis and Bourne, 1992), which saturably stimulated the production of cAMP by intact cells to a maximum between 1–10 ng/ml of that toxin; these results are not shown.)

3.2. Decrease in activity of the Y_1 receptor and $G_i\alpha$ subunits in response to pertussis toxin is parallel in time

At 10 ng pertussis toxin/ml, the decrease of agonist binding to both Y_1 receptor and $G_i\alpha$ subunits was fairly fast (Fig. 2A and B, respectively) and quite correlated in time (Fig. 2C and the legend of Fig. 2). It is of interest that there also was a significant (up to 50%) loss of the basal binding of [^{35}S]GTPγS

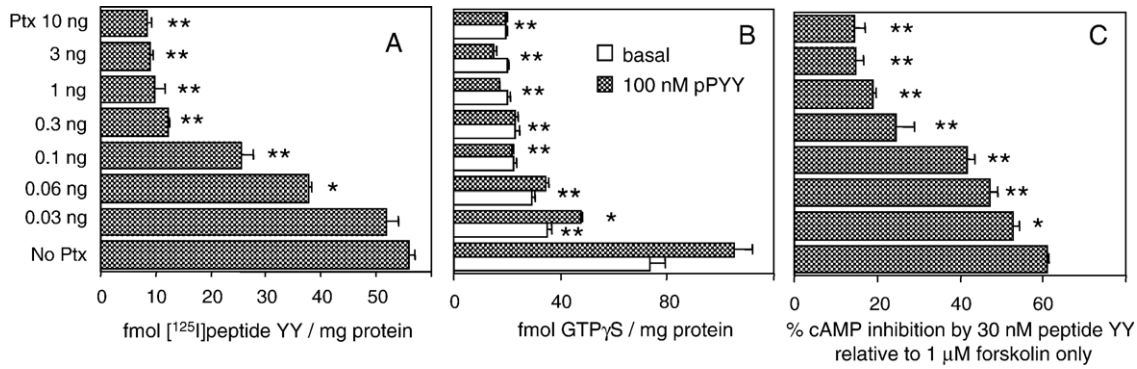

Fig. 1. Parallelism in the extent of pertussis toxin-induced decrease of the Y_1 receptor and G-protein agonist activity in CHO cells. The guinea pig Y_1 receptor expression in CHO cells was used. Asterisks indicate differences to the respective control significant at the level of 95% (*) and 99% (**) confidence in *post hoc* Scheffé *t* tests. All results are averages (±1 S.E.M.) of assays with particulates from six wells in two independent experiments, with pertussis toxin in the cell culture medium for 24 h at the indicated doses. A. The binding of [^{125}I] porcine peptide YY to particulate Y_1 receptors. B. The basal and Y_1 agonist-stimulated binding of [^{35}S]GTPγS to particulate G-protein nucleotide sites. C. Inhibition of forskolin-stimulated cAMP production in intact cells by 100 nM porcine peptide YY. Regressions of all three parameters were significantly correlated in Pearson and Spearman tests at >95% confidence.

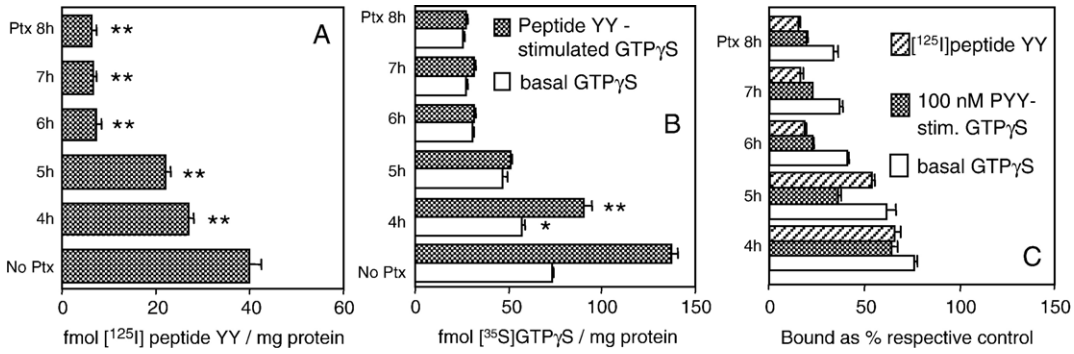

Fig. 2. Parallelism in the kinetics of pertussis toxin-induced decrease of the Y_1 receptor and G-protein agonist activity in CHO cells. The loss of function of Y_1 receptors and G α subunits was followed at 10 ng pertussis toxin/ml culture medium in CHO cells expressing the human Y_1 receptor. A. The binding to particulates of the Y_1 agonist [^{125}I] porcine peptide YY. B. The binding to particulates of the G-protein nucleotide site ligand [^{35}S]GTPγS. At ≥5 h of pertussis toxin treatment, decrease of both of the above parameters was highly significant for all points in Scheffé *t* tests. For clarity, this is not shown. C. The binding data from graphs A and B plotted as percentages of the respective controls indicate a high correlation of the inhibitions (confidence>99% in Pearson and Spearman correlation tests on the respective linear regressions).

(see also Fig. 5), part of which undoubtedly is due to receptor-$G_i\alpha$ precoupling, since the basal binding in untransfected CHO-K1 cells was less than 20% reduced by an overnight treatment with pertussis toxin at 10 ng/ml. The reduction observed with untransformed CHO-K1 cells could be related to G-protein precoupled natively expressed muscarinic receptors (Wang et al., 1995). For all treatments in Figs. 1 and 2, very similar results were obtained with human Y_1 receptor expressed in CHO cells, and with human Y_1 receptor natively expressed in SKN-MC cells.

The binding of the Y_1 agonist [^{125}I]peptide YY to the total (particulate) receptors (graph A, Fig. 2) decreased more than 70% within 8 h at 10 ng/ml of the toxin. Both the basal and the Y_1 agonist-induced particulate binding of the G-protein nucleotide site agonist [^{35}S]GTPγS were significantly reduced already at 4 h. The basal binding decreased ~50%, and the agonist-stimulated binding ~80% within 8 h of culture at 10 ng/ml of the toxin, following a dynamics very similar to that for the binding of the Y_1 agonist to the receptor (graph A, Fig. 2). After 24 h at ≥10 ng/ml of the toxin, decrease in the binding of both agonists saturated above 80% (as observed in more than 30 experiments), without a decrease of cell numbers or protein within this interval. This very high level of inhibition was stable for at least 14 days (including two cell passages) following removal of the medium containing the toxin, indicating a substantial conservation of the cell-bound toxin. The correlation of percentages related to agonist activity in Fig. 2A and B is shown in Fig. 2C. This correlation was highly significant in both Pearson and Spearman tests. There also was a significant correlation of the decrease of agonist binding and the decrease of inhibition of adenylyl cyclase at these toxin concentrations (see graph C, Fig. 1).

It should be noted that at the low molarity used (0.2 nM), [^{35}S]GTPγS would not significantly label either the Gq (Hepler et al., 1993) or the Gs α-subunit (Higashijima et al., 1990). The above results therefore indicated a critical dependence on functional $G_i\alpha$ subunits in the conservation of Y_1 receptors expressed in CHO-K1 cells ($G_o\alpha$ subunits are not significantly expressed in CHO cells (Raymond et al., 1993)). However, while ~15% of Y_1 agonist binding persisted the 24-h pertussis toxin treatment at 10 ng/ml (Fig. 1A), the stimulation by peptide YY of the binding of [^{35}S]GTPγS to $G_i\alpha$ subunits was essentially eliminated after this length of exposure (Fig. 1B). This is consistent with the finding of a larger relative Y_1 receptor coupling to α subunits of the G_q group after pertussis toxin treatment (Fig. 3).

3.3. The Y_1 receptors persisting steady-state exposure to pertussis toxin show stronger coupling to Gq-type α subunits

Sensitivity to G_q-selective agonist G-2A of [^{125}I]peptide YY binding to particulates from CHO cells expressing the human Y_1 receptor was much larger after prolonged pertussis toxin treatment (Fig. 3A), with a large decrease in K_I (from 8.9 μM in the absence of pertussis toxin to 1.1 μM at 0.1 μg toxin/ml (the legend of Fig. 3)). This indicated a substantial increase in the fraction of the Y_1 receptor coupling to G_q-type α subunits. Sensitivity of the binding of [^{125}I] peptide YY to the general G-protein antagonist suramin decreased after toxin treatment, with a significant rightward shift in IC_{50} from 55 μM without pertussis toxin to 94 μM after exposure to 0.1 ng/ml of the toxin over 24 h (Fig. 3B). Profiles with particulates from pertussis toxin-pretreated cells correspond to the results in several studies of receptors that preferentially associate with the G_q-type α subunits (Hohenegger et al., 1998; Ancellin and Hla, 1999; Moore et al., 2002; Kemp et al., 2004). At 100 and 300 μM unlabeled GTPγS, there was a significant increase of [^{35}S]GTPγS binding with particulates from pertussis toxin-treated cells relative to particulates from cells not treated with pertussis toxin (Fig. 3C). This is in the expected range for the activation by GTPγS of the nucleotide site of $G_q\alpha$ subunit (Hepler et al., 1993).

The Y_1 receptors persisting long-term exposure to pertussis toxin were found to immunoadsorb with G_q/11 α antibody consistently more than the receptors from untreated cells. As seen in Fig. 3D, up to 10% of the 80–120 kDa gradient zone receptors from cells exposed for 24 h to 0.1 ng/ml of pertussis toxin was precipitated by the G_q/11 antibody, while with control

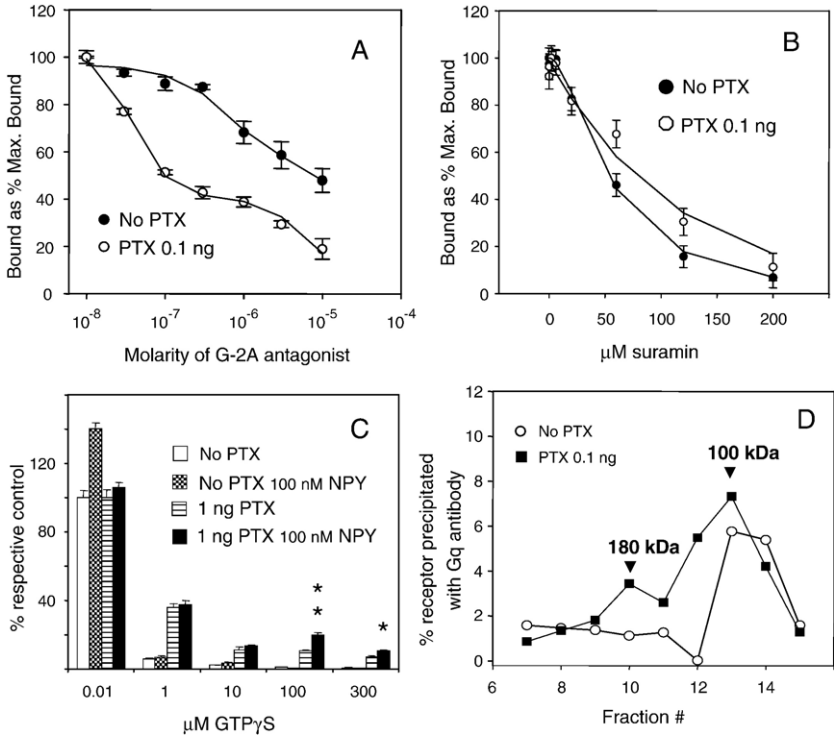

Fig. 3. Treatment by pertussis toxin results in loss of G_i and gain of G_q-related Y_1 receptor agonist and G-protein ligand binding to CHO cell particulates. Graphs A and B show competitions of the binding of Y_1-selective agonist [^{125}I](Leu^{31},Pro^{34})human peptide YY to particulates from cells treated with 0.1 ng/ml pertussis toxin for 24 h, and from the respective control cells. A. Competition by G_q-selective antagonist G 2A. The IC_{50} values were 8.9±3 µM with control, and 1.1±0.3 µM with particulates from toxin-treated cells (n=3 for both). B. Competition by G-protein antagonist suramin. The IC_{50} values (µM) were 55±4 (control) and 94±16 (pertussis toxin) (n=3 for both). C. The binding of [^{35}S]GTPγS (0.2 nM) competed by 1–300 µM unlabeled GTPγS with particulates from cells treated by 1 ng/ml of the toxin over 24 h, and with the corresponding control particulates. Asterisks indicate activation by 100 nM neuropeptide Y of [^{35}S]GTPγS binding significant at the level of 95% (*) and 99% (**) confidence in Student's t tests vs. the respective basal binding. D. A typical immunoadsorption with G_q antibody of human Y_1 receptor labeled by [^{125}I]porcine peptide YY. The particles were labeled for 20 min at 20 pM of the agonist, lysed by cholate/digitonin, and sedimented for 18 h in 5–20% sucrose gradients. For other details see Section 2.9 and Figs. 6 and 7.

cells this fraction represented at most about 8% of total 80–120 kDa [^{125}I]peptide YY-labeled receptor. Areas under the percentage curves were significantly different in the trapezoidal rule comparison (the legend of Fig. 3).

After loading for 24 h with 10 µCi/ml of *myo*-[^{3}H]inositol, the control cells as well as the cells exposed to pertussis toxin (1 ng/ml, 24 h) were found to significantly increase production of inositol phosphates in response to a high concentration of neuropeptide Y (Table 1). Both the basal and the neuropeptide Y-stimulated production of inositol phosphates was quite similar for the cells treated with pertussis toxin, and the cells not exposed to the toxin (Table 1). The increase induced by neuropeptide Y was more than 40% over the basal production in the case of the monophosphate, and more than 50% for the combined di- and triphosphates.

3.4. The agonist binding affinity at the Y_1 receptor changes only at high degree of decrease induced by pertussis toxin

As seen in Fig. 4, the affinity of Y_1 binding (evaluated by the Scatchard linearization) did not change with pertussis toxin treatment at up to 0.05 ng/ml over 20 h of culture (reducing the apparent B_{max} ~ 39%). At least in this range of reduction, the decrease of Y_1 agonist binding at 50 pM of input should reflect the loss of receptor sites. At higher reduction of total Y_1 binding by pertussis toxin, there was a decrease of the apparent affinity. The Y_1 receptor binding is strongly dependent on functional G_i α subunits, and a large part of the binding is inhibited by exposure of particulates to GTPγS, a tightly-binding ligand of the G_iα nucleotide site (Table 2). As seen in Table 2, treatment with pertussis toxin at 10 ng/ml lowered the total binding of [^{125}I] peptide YY by almost 80%, in the same time increasing the fraction of the binding insensitive to GTPγS from 14% to 74%. In parallel, as we already reported (Parker et al., 2007), the immunoreactivity to G_{i3}α antibody was reduced significantly more than 40%, while there was a significant upregulation of G_q immunoreactivity by about 30%.

3.5. The pertussis toxin-induced loss of Y_1 receptor and Gi activity is reduced by ammonium chloride at the level of endosomal/lysosomal system

The concerted loss of Y_1 receptor and G_i function induced by pertussis toxin indicated involvement of endosomal proteinases in both receptor and G_i inactivation. This could be confirmed by exposing Y_1-CHO cells to the toxin in the presence of endosome alkalinizer/acid cathepsin inhibitor NH_4Cl. As seen in Fig. 5, NH_4Cl dose-dependently prevented the loss of both Y_1 (graph

Table 1
Increased production of inositol phosphates in response to neuropeptide Y is similar for the Y_1 receptor-expressing CHO cells exposed to pertussis toxin and for the untreated cells

Treatment	Inositol monophosphate, disintegrations/min/10^6 cells	Inositol di- and triphosphates, disintegrations/min/10^6 cells
None	165±12	99±6
Pertussis toxin only	161±7	93±10
100 nM neuropeptide Y without the pertussis toxin pretreatment	235±12*	158±6*
100 nM neuropeptide Y after the pertussis toxin pretreatment	238±6*	155±9*
100 μM BIBP-3226+100 nM neuropeptide Y after the toxin	164±6	112±6

The results represent averages ±1 S.E.M. for Dowex-1 eluates of the extracts obtained after the indicated treatment (3 h) and pretreatment/inositol loading of triplicate wells labeled with 10 μCi/ml of *myo*-[^{3}H] inositol for 24 h as detailed in Section 2.6. Pretreatment with the pertussis toxin was at 1 ng/ml over the inositol loading period. Asterisks indicate differences significant at the level of 95% confidence in Student's *t* test against either the untreated control or the pertussis toxin-pretreated cells.

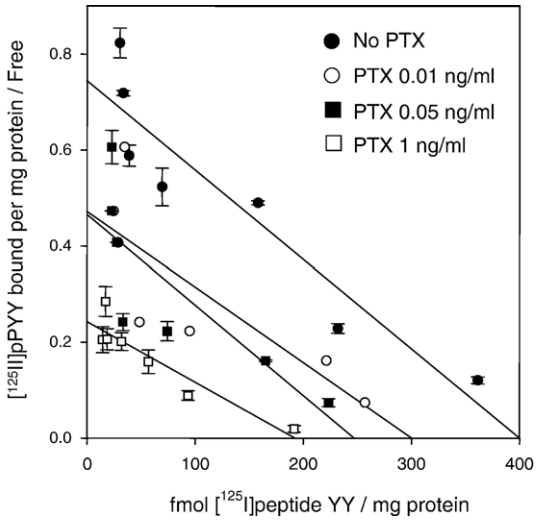

Fig. 4. The Y_1 receptor affinity is not changed significantly by pertussis toxin at up to 40% of the maximal decrease in the uncompeted binding of [^{125}I] porcine peptide YY. The cells were cultured in the presence of indicated concentrations of the toxin for 20 h. The Scatchard K_{diss} in pM, and B_{max} in fmol/mg protein (with% control in parenthesis for B_{max}) were: no pertussis toxin, 502±44 and 400±32; 0.01 ng pertussis toxin/ml, 537±38 and 302±32 (75% control); 0.05 ng pertussis toxin/ml, 496±55 and 247±22 (61% control); 1 ng pertussis toxin/ml 791±140 and 194±64 (48% control) (n=3 for all profiles).

A) and Gα subunit (graph B) agonist binding; this is strikingly similar to the rescue from pertussis toxin of the Y_2 receptor and G-protein binding by this agent (Parker et al., 2007). At 30 mM NH_4Cl, the loss of agonist binding to the receptor, or of GTPγS to G-protein, was completely prevented at 0.1, and significantly attenuated at 1 ng pertussis toxin/ml. Exposure to ammonium chloride alone at up to 30 mM did not significantly change the binding of either ligand. However, as seen in graph 5C, NH_4Cl caused a concentration-dependent accumulation of internalized (and largely Y_1 receptor-attached) [^{125}I]peptide YY. This was observed both at the level of secondary endosomes (density range 1.04–1.06 in Percoll/sucrose), and of lysosomes and heavy granules (density ≥ 1.10). In contrast, co-treatment with the NH_4^+-isomorphic K^+ ion at 30 mM did not change the effects of the toxin. After solubilization at 10 mM cholate and digitonin, up to 25% of the [^{125}I] agonist from gradient fractions could be recovered by immunoadsorption with $G_{i3}\alpha$ antibody, similar to yields in Fig. 7.

In agreement with the above findings, NH_4Cl also strongly and consistently attenuated the loss of Y_1 receptor dimers and of Gi association with Y_1 receptor monomers induced by pertussis toxin (see Fig. 7).

3.6. The Y_1 receptor dimers are disbanded in parallel to the loss of $G_{i\alpha}\alpha$ subunit function

All Y receptors have been identified as extensively dimerizing (Berglund et al., 2003; Dinger et al., 2003). Also, oligomerization of G-protein coupling receptors could be expected to change in response to stable large-scale inactivation of signal transduction partners, as induced by pertussis toxin. We therefore characterized Y receptor dimers by gradient centrifugation of detergent-solubilized particulates labeled by the respective primary agonists, as a procedure that does not require covalent modification of the receptors, provides estimates of molecular size for multiple interaction complexes, and also permits a direct cross-characterization of immunoreactivities of interacting receptors and G-proteins in the same preparation. This approach is made possible by the quasi-irreversible association of Y agonists with their cognate receptors (Parker et al., 2001a; Dautzenberg and Neysari, 2005; Sah et al., 2005). Indeed, solubilization of particulate Y_1 receptors labeled by [^{125}I]peptide YY could be achieved with less than 15% dissociation of the bound agonist by dispersion in a mixture of 10 mM each of cholate and digitonin at 0–4 °C. An even better stability was found for [^{125}I]peptide YY(3–36) binding to the Y_2 receptor, and for [^{125}I]human pancreatic polypeptide binding to the Y_4 receptor expressed in CHO cells (not shown). The principal agonist-labeled species in control Y_1 receptor-expressing cells sedimented in sucrose gradients at ~180 kDa (Fig. 6A). Average control labeling in the dimer peak, assessed as area under the curve in the ImageJ program, was 55±7% (n=12). This zone was poorly labeled by [^{35}S]

Table 2
The 1 μM GTPγS-resistant fraction of [^{125}I]peptide YY binding to the Y_1 receptor in CHO cell particulates is strongly increased by pertussis toxin

Group	Total specific binding and % of no toxin	Specific binding at 1 μM GTPγS and % of group's total
No toxin	33.8±2.1 (100)	4.83±0.56 (14.3)
PTX 8 h	4.07±0.23 (12.0)	1.33±0.08 (32.7)
PTX 24 h	2.97±0. 29 (8.8)	2.21±0.39 (74.4)

Pertussis toxin (PTX) was used at 10 ng/ml culture medium over 24 h. The results are averages of four wells per treatment, shown ±1 S.E.M. The specific binding represents fmol [^{125}I]peptide YY bound per mg particulate protein after correction for the binding at 100 μM Y_1 antagonist (*R*)-N2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]D-arginine amide (BIBP-3226).

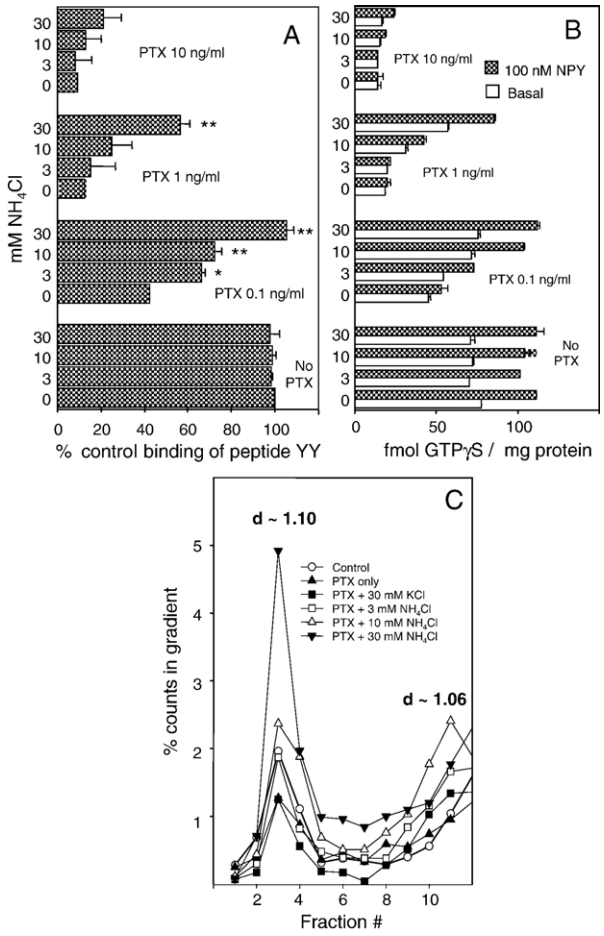

Fig. 5. Endosome alkalinizer/cathepsin inhibitor NH_4Cl reduces the pertussis toxin-induced loss of both human Y_1 receptor and G-protein in CHO cells via endosomal/lysosomal processing. The Y_1-CHO cells were cultured at 0.1, 1 or 10 ng/ml of pertussis toxin for 24 h without or with ammonium chloride (3, 10 or 30 mM). Suppression of either the receptor or the G-protein loss by 30 mM ammonium chloride was complete at 0.1 ng, highly significant at 1 ng/ml, and significant even at 10 ng/ml of the toxin. A much larger protection by NH_4Cl was observed at shorter intervals of exposure to the above dosages of the toxin (not shown). The results are averages of six wells in two independent experiments, shown with 1 S.E.M.. Significance in *post hoc* Scheffé *t* tests vs. toxin alone is indicated by asterisks (*, $p<0.05$; **, $p<0.01$). A. The binding of [^{125}I]porcine peptide YY binding to Y_1 receptors. B. The basal and neuropeptide Y (100 nM)-stimulated binding of [^{35}S]GTPγS to G-proteins. All significances of attenuation by NH_4Cl relative to pertussis toxin (PTX) alone were at least equal to those in graph A, and for clarity are not shown. C. Percoll gradient profiles of [^{125}I]peptide YY-labeled (12 min, 37 °C) secondary endosomes (density region 1.04–1.06) and lysosomes (density ≥ 1.10) after 20 h of culture without or with 0.1 ng/ml pertussis toxin and the indicated molarities of KCl and NH_4Cl. The toxin-treated area under the curve was 45% larger than the control, and significantly different in trapezoidal rule comparison. For other details see Section 2.8.

GTPγS (Fig. 6B), indicating an absence of free $G_i\alpha$ subunits, but [^{125}I]peptide YY bound to the 180 kDa complex was significantly precipitated by antibodies to $G_i\alpha$ subunits 1–3, or by antibody to $G_{i3}\alpha$ used alone (Fig. 6B–E and 7D). This material should represent a pentameric association of the receptor dimer with G-protein heterotrimers, already reported for leukotriene B4-BLT1 receptor (Baneres and Parello, 2003), the α2A-adrenergic receptor (Nobles et al., 2005), and the human Y_2 receptor (Parker et al., in press). A shoulder of Y_1 agonist radioactivity usually observed at about 50 kDa should correspond to G-protein-free Y_1 receptor monomer.

The bound [^{35}S] GTPγS sedimented in a somewhat broad zone with a peak at about 100 kDa (Fig. 6B–E). As seen in Fig. 6B, the labeling of this zone more than trebled (without change in the sedimentation rate) by incubation with 100 nM of the Y_1 agonist peptide YY, indicating a strong $G_i\alpha$ subunit activation by agonist-liganded monomeric Y_1 receptor. The large stimulation of [^{35}S] GTPγS binding by Y_1 agonists indicates that the receptor could be one of the principal partners of Gi α subunits in CHO cells

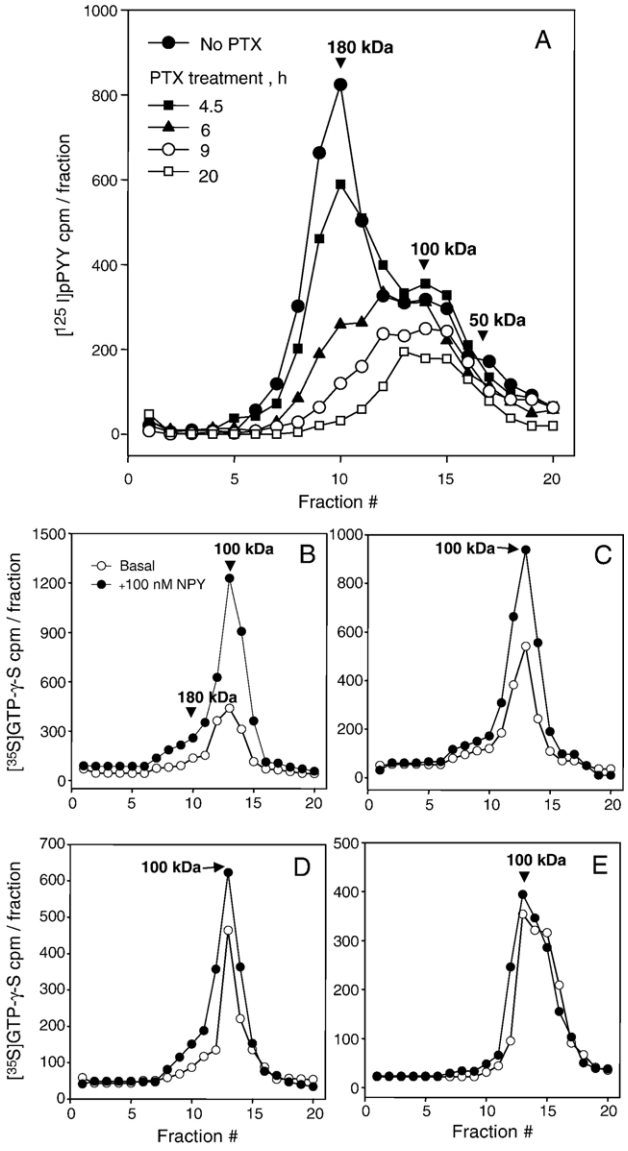

Fig. 6. Loss of Y_1 dimers and of G_i nucleotide site activation by Y_1 agonist vs. the length of exposure to pertussis toxin (PTX) in culture. The toxin was used at 10 ng/ml medium. The 5–20% sucrose gradients were centrifuged for 18 h at 218,000 $\times g_{max}$ and 5 °C. For other details see Section 2.9. A. Profiles of [^{125}I] porcine peptide YY-labeled solubilized Y_1 receptor at indicated periods of cell exposure to pertussis toxin. B, C, D, E. Profiles of sedimentation of [^{35}S]-labeled G-protein after 0 (B), 4.5 (C), 9 (D) and 20 h (E) of cell exposure to the toxin, without (basal) and with preincubation/coincubation at 100 nM human neuropeptide Y.

expressing the receptor. This should also be important in the constitutive activation of $G_i\alpha$ in the absence of Y_1 agonists. Indeed, after an exposure of 24 h to 4 ng/ml of pertussis toxin there was a stable reduction of basal GTPγS binding at the level of 55–63% over 7 days of further culturing.

The loss of 180 kDa complex was already significant after 4.5 h of treatment with the toxin at 10 ng/ml (Fig. 6A), i.e. in conditions of less than 30% decrease of the total Y_1 receptor (see also Fig. 2A), and much more pronounced after 6 and 9 h of the treatment (Fig. 6A). This complex was largely lost in response to steady-state (20 h at ≥1 ng/ml) pertussis toxin treatment (Fig. 6A). This was observed in more than 30 experiments. The decrease was accompanied by a complete loss of immunoreactivity to $G_i\alpha$ antibodies in the 180 kDa zone (Figs. 6E, 7F). At 20 h, there was >80% decrease in the labeling of the 100 kDa material by [^{35}S]GTPγS, and an almost complete loss of stimulation of GTPγS binding by the Y_1 agonist (Fig. 6E).

As expected from results in Fig. 5, 30 mM ammonium chloride at 0.1 ng/ml pertussis toxin prevented both the loss of dimers (Fig. 7C) and the loss of Y binding immunoadsorbed with $G_{i3}\alpha$ antibody (Fig. 7D). There was also a significant attenuation by NH_4Cl of toxin's effect at 1 ng/ml (Fig. 7E, F). At this dose of the toxin, NH_4Cl appeared to help stabilization of an intermediate complex of about 120 kDa (Fig. 7E,F) which did not strongly react with the $G_{i3}\alpha$ antibody (Fig. 7F). At 0.1 ng/ml of the toxin, ammonium chloride at least restored the fraction of the agonist-labeled dimeric receptor precipitated by $G_{i3}\alpha$ antibody (Fig. 7D).

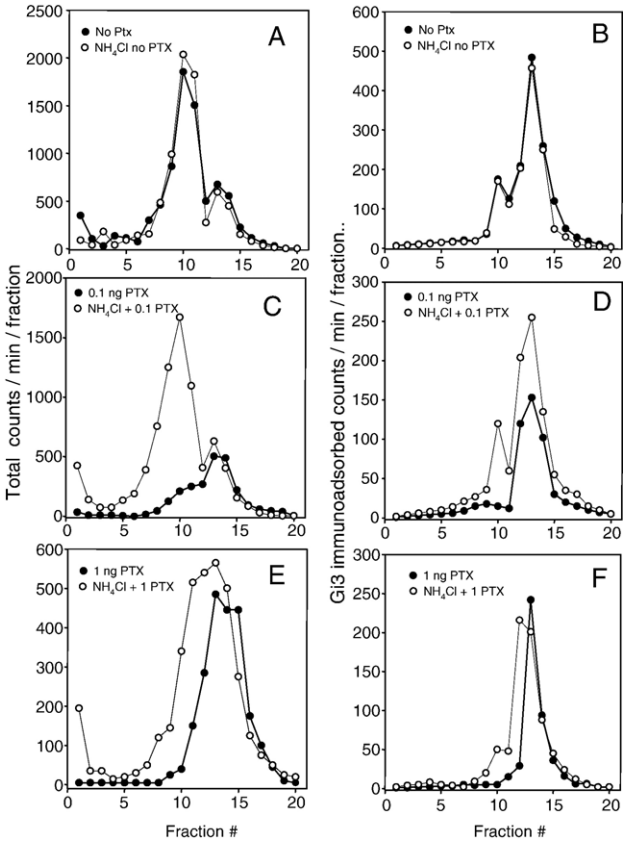

Fig. 7. Ammonium chloride attenuates the pertussis toxin-induced losses of Y_1 receptor dimers and of immunoadsorption of the labeled receptor with antibody to the $G_{i3}\alpha$ subunit. The cells were exposed for 20 h to 0, 0.1 and 1 ng/ml of pertussis toxin without or with 30 mM NH_4Cl in the medium. The particulates were solubilized with cholate plus digitonin, followed by sedimentation through 5–20% sucrose gradients for 18 h at 218,000 $\times g_{max}$ (see Section 2.8). Aliquots of fractions were precipitated with polyethyleneglycol (Section 2.9), or immunoreacted with antibody to $G_{i3}\alpha$ subunit (Section 2.7).

3.7. The numbers and cycling of surface Y_1 receptors are maintained in the presence of pertussis toxin

Agonist attachment to surface human Y_1 receptors (defined as the sites that attach peptide agonists dissociable with cold 0.2 M CH_3COOH–0.5 M NaCl from intact cell monolayers, and representing 10–12% of total Y_1 receptors in our expression in CHO cells) was somewhat slower after exposure to 10 ng/ml of pertussis toxin over 24 h (legend of Fig. 8), but the decrease relative to untreated cells was not significant past 10 min of labeling. At saturation (20–30 min at 37 °C), the number of labeled surface sites was about equal to the labeling found with untreated cells (Fig. 8A). Internalization of these receptors was also maintained, but at a rate that was almost twice slower after pretreatment with the toxin (the legend of Fig. 8). Very similar results were obtained with the guinea pig Y_1 receptor expression in CHO cells (results not shown). Thus the surface complement of Y_1 sites is little changed by pertussis toxin treatment, but the rate of cycling is strongly reduced. At full effect of the toxin, these sites represented more than a half of the total specific Y_1 binding (the legend of Fig. 8), and should be mainly monomeric (see Figs. 6 and 7).

4. Discussion

The Y_1 receptor is known to associate with pertussis toxin-sensitive G-protein α subunits in many cell types and lines expressing the native (Shigeri and Fujimoto, 1992; Michel, 1998) as well as the cloned (Herzog et al., 1992; Sah et al., 2005) receptor. A preferential transduction from the Y_1 receptor via the G_{i2} and $G_{i3}\alpha$ subunits was noted, respectively, in the smooth muscle cells (Misra et al., 2004) and in HEL cells (Michel, 1998). There also is direct evidence for association of the Y_1 receptor with G_q-transduced cascades (Heredia et al., 2005), and the present study also provides proofs of association of the Y_1 receptor and G_q-type α subunit(s) in CHO cells. The amounts of inositol phosphates produced via stimulation of Gq-type α subunits are typically small (e.g. ref. (Rosenkilde et al., 2004)), as would be expected from the well-known slow GTPase activity of the $G_q\alpha$ subunit (Higashijima et al., 1990; Hepler et al., 1993). However, our experiments show a clear increase of phosphoinositide turnover by neuropeptide Y in the Y_1 receptor-expressing CHO cells.

Both the Y_1 receptors and the G_i subunits could be preferentially localized in raft-like formations (e.g. Oh and Schnitzer, 2001), possibly overlapping with the dynamic endosome pool that also houses recyclable transferrin (Apodaca et al., 1994). The $G_{i3}\alpha$ subunit is also involved in inactivation

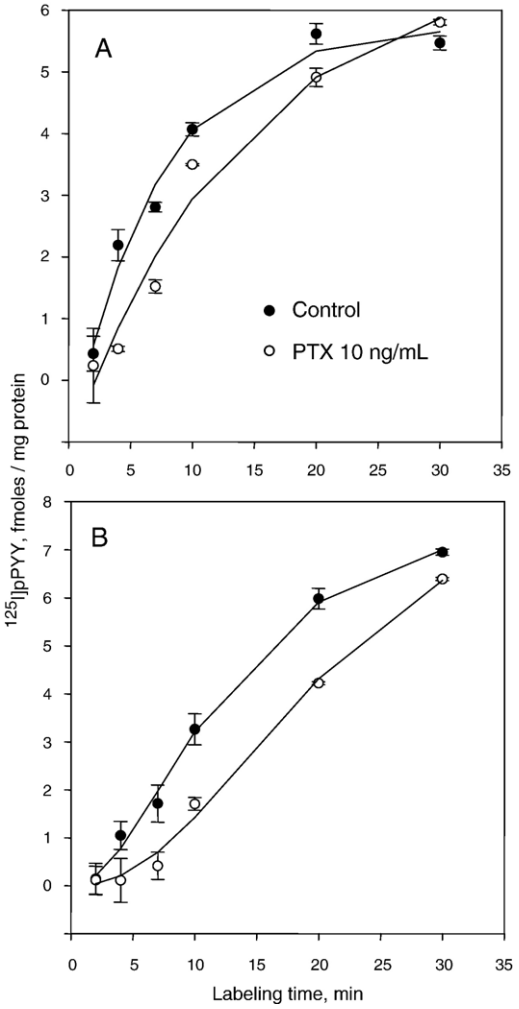

Fig. 8. The rate of internalization of agonists by the human Y_1 receptor in CHO cells is decreased by treatment with pertussis toxin. Shown is the kinetics of surface labeling and internalization of [^{125}I] porcine peptide YY in Y_1-CHO cells after exposure to 10 ng/ml of pertussis toxin over 24 h of culture. A. Surface labeling. B. Internalized tracer. The results are averages of six wells in two independent experiments. Very similar results were obtained with guinea pig Y_1 expression in CHO cells. In these experiments, surface Y_1 receptors represented 10.2±0.2% of total in untreated, and 77.5±4.4% of total in pertussis toxin-treated cells. At 30 min (i.e., near the steady state), the surface site number (in fmol per mg total particulate protein) was 5.47±0.13 in control, and 5.80± 0.33 in toxin-treated cells, and the respective amounts of internalized tracer were 6.9±0.07 and 6.40± 0.03 fmol/mg total particulate protein. The half-period for maximal surface labeling increased from 6.7±1.3 min in control cells to 9.2±0.8 min (37%) with toxin. However, internalization of the tracer half-saturated in 12.5±1.7 min in control cells, and in 21.4±4 min with toxin (an increase of 71%). The regression slopes were different at ≥99% confidence. At 7–30 min of labeling, all individual point differences were highly significant in Student's *t* test.

by pertussis toxin of phospholipase C/protein kinase C-linked dopamine D_3 receptor activity in the proximal tubuli of the rat kidney (Pedrosa et al., 2004). Our finding of a parallel loss of pertussis toxin-sensitive Gα activity and Y_1 receptor numbers points to a role of functional $G_i\alpha$ subunits in both organization and conservation of the receptor in CHO cells. This also is found, and to a similar degree, for the Y_2 receptor expressed in CHO cells (Parker et al., 2007), indicating that both receptors as expressed in epithelial cells strongly depend on association with pertussis toxin-sensitive G-protein α subunits for organization and maintenance. To some degree this should be a co-protection, as $G_{i3}\alpha$ subunit can be downregulated by ubiquitin ligase (Fischer et al., 2003), and G_t, another pertussis toxin-sensitive α subunit, was shown to be regulated by ubiquitins (Obin et al., 1994). Also, interaction with heat-shock chaperone proteins could help elimination of the $G_{i/o}$ α subunits through the proteasome pathway (Busconi et al., 2000).

Ammonium chloride (an endosome alkalinizer/acid proteinase inhibitor (Green et al., 1994; Ling et al., 1996) is known to inhibit proteolytic degradation of lysosome-sorted adhesion molecules (Green et al., 1994), and of internalized receptors in lysosomes as well as in endosomes (Brown et al., 1986; Neumann et al., 1993). Our results are entirely in agreement with the above findings, and point to a large reduction by NH_4Cl in the rate of catheptic disposal of the Y_1 receptor. Based on protection of the Y_1 receptor dimer levels and of G_i function by NH_4Cl, Y_1 receptors obviously need considerable contact with functional Gi subunits to avoid association with ubiquitins or endosomal cathepsins. From our immunoadsorption profiles, G-protein antagonist profiles, and inositol phosphate production results, the CHO-cell expressed Y_1 receptor obviously interacts with G_q-type α subunits. This could include other pertussis toxin-insensitive G-proteins, e.g. Rab-type catalysts of endocytosis (Croizet-Berger et al., 2002) (especially in view of the fast cycling of the Y_1 receptor (Parker et al., 2001b; Gicquiaux et al., 2002)), and would produce a degree of chaperoning protection from proteasomal or endosomal proteinases. There is a stable inhibition by the toxin of fast GTPases that associate with G-protein coupling receptors (in CHO cells apparently represented mainly by G_i subunits (Raymond et al., 1993)). Since the interactivity of $G_q\alpha$ is much slower than that of $G_i\alpha$ (Higashijima et al., 1990; Hepler et al., 1993), there will be a large loss of receptors, as the supply of the incoming *de novo* synthesized receptor might not keep with the degradation by endosomal and lysosomal proteinases (Pillay et al., 2002), or by proteasomes. A physiological protection by kinase(s) phosphorylating the G-protein coupling receptors, which may not attach to the receptor in the absence of appropriate Gα subunit (Penela et al., 1998; Theilade et al., 2005), should also be lacking.

Profiles of the loss of Y_1 receptor dimer to pertussis toxin indicate association with heterotrimeric G-proteins (e.g. (Evanko et al., 2005)) or even with aggregates of $G_{i3}\alpha$ subunits (as inferred from $G_i\alpha$ modeling (Mixon et al., 1995; Tesmer et al., 2005)). The parallel reduction of G_i activity and Y_1 dimers by pertussis toxin found in the present work, as well as in our recent study with the Y_2 receptor (Parker et al., in press), does support the presence of such complexes. A concatenation of $G_i\alpha$ subunits (see Mixon et al., 1995) may, through a reduction of mobility, support ADP-ribosylation even in the absence of βγ dimers; the ADP-ribosylation by pertussis toxin could be obtained with high concentrations of free α subunits (see Scheuring et al. 1998). However, there also is evidence for a degree of association of α and βγ subunits throughout the signaling cycle (Ganpat et al., 2000; Evanko et al., 2005).

We did not observe affinity change in the loss of up to 40% of the Y_1 agonist, and this corresponds with previous findings on two other pertussis toxin-sensitive receptors, the *N*-formyl peptide receptor (Hoffman et al., 1996) and the neuropeptide Y Y_2 receptor (Parker et al., 2007). Thus, there is a net loss of receptors, which also ties with a relatively low sensitivity of receptor internalization to the toxin. The severe decreases of the Y_1 binding, immunoreactivity and agonist activity at steady-state exposure to pertussis toxin indicate a very large loss of receptor numbers. Absence of functional pertussis toxin-sensitive α subunits physically associated with G-protein coupling receptors (e.g. Damaj et al., 1996; Martin Shreeve, 2002) is likely to result in an over-exposure of the Y_1 receptor to ubiquitins and other chaperones, including proteinases, with the receptor being either escorted to lysosomes (for a review see Hicke, 1999), or degraded at the site of capture by endosomal cathepsins or by proteasomes. Our findings of suppression by NH_4Cl of the loss of both the Y_1 receptor and the functional Giα subunits, and of accumulation of internalized receptor with NH_4Cl treatment, strongly support the role of degradative pathways in the downregulation. Upregulation of G_q-type α subunits, on the other hand, could be related to an increased availability (possibly linked to decrease in $G_i\alpha$ subunit), of membrane carriers and processors of G-proteins, such as GDP/GTP exchange factors (GEFs) and GTPase-activating proteins (GAPs).

We observed an about 70% reduction of the rate of Y_1 receptor-linked internalization of agonist peptide YY in consequence to prolonged exposure to pertussis toxin. This is another indication of a partnership switch of the Y_1 receptor to G-proteins that get activated slowly. The half-period of cycling for the Y_1 receptor in CHO cells from our previous (Parker et al., 2001b) and present work is about 12 min, and similar was found for the HEK 293 expression of the Y_1 receptor (Gicquiaux et al., 2002). A nearly two-fold increase in this parameter at steady-state exposure to pertussis toxin would relate to multiple factors, including the rate of activation of the involved endocytotic vehicles, profiles of their interaction with the Y_1 receptor, and also the availability of these vehicles. The $G_i\alpha$ subunits should be among the principal physiologic vehicles of Y_1 receptor internalization in CHO cells, correspondent with previous findings on the m2 muscarinic receptor (Roseberry et al., 2001), and the calcium-sensing receptor (Holstein et al., 2004).

Acknowledgments

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