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Neuropeptide Y as a partial agonist of the Y1 receptor

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Abstract

In absence of receptor cycling, human/rat neuropeptide Y was found to persistently occupy the guinea pig neuropeptide Y Y1 receptors expressed on the surface of Chinese hamster ovary (CHO) cells (IC_{50} ~8 nM); a lasting occupancy was also evident with active receptor cycling. A similar blockade was obtained with the human neuropeptide Y Y1 receptor (in CHO or SK-N-MC cells). Peptidic antagonists GR238118 (1229U91) and VD-11 blocked the Y1 receptor in the same molarity range. A neuropeptide Y-related Y1 agonist, ($Leu^{31}Pro^{34}$) human neuropeptide Y, also strongly adhered to the Y1 site. Similar blockade-like occupancy by neuropeptide Y was found with particulates from Y1-expressing CHO cells, and with native neuropeptide Y Y1 receptors of rat synaptosomes. Peptide YY and a related Y1-selective agonist, ($Leu^{31}Pro^{34}$) human peptide YY, showed a much less stable binding to the neuropeptide Y Y1 receptor with either the intact cells or particulates. The Y1 binding of neuropeptide Y was also less sensitive to chaotropic agents and guanine nucleotides than the binding of peptide YY, indicating a larger stability for association of neuropeptide Y with the receptor. Inhibition of forskolin-stimulated adenylyl cyclase showed a distinctly attenuating agonism for neuropeptide Y, with an activity similar to peptide YY below 1 nM, but considerably lower above 3 nM of the peptides. This activity was largely exerted via pertussis toxin-sensitive G-proteins of Y1-CHO cells. Our findings indicate that signaling by neuropeptide Y via its Y1 receptor could be self-restricting at higher levels of the peptide, in relation to a strong association of the agonist with the Y1 binding site.

Keywords: Receptor signaling; Adenylyl cyclase; G-protein; Pertussis toxin; Ligand attachment; Ligand dissociation

1. Introduction

Neuropeptide Y is abundant in the forebrain (Allen et al., 1983), and its synaptic concentration could reach high levels. The released neuropeptide Y acts on both presynaptic (Colmers et al., 1991) and postsynaptic (Kopp et al., 2002) specific receptors, including the Y1, Y2 and Y5 subtypes. Natively expressed pancreatic polypeptide receptors (Y4-like receptors) also bind neuropeptide Y at a high affinity (Parker et al., 1999). Receptors for neuropeptide Y are also found in glia (e.g. Glass et al., 2002), what may support a role for neuropeptide Y in modulation of the neuronal tone. While the activity of Y1 and Y5 receptors is in many respects similar, low levels of neuropeptide Y are likely to preferentially activate the Y1 sites, since the affinity of neuropeptide Y at the Y5 receptor is relatively less with either clonal or native expressions of the receptor (Parker et al., 2001a; Dumont et al., 2003).

As with many other neuropeptide receptors, numbers of active neuropeptide Y Y1 receptors are largely regulated by recycling endocytosis (Gicquiaux et al., 2002; Parker et al., 2002b), at rates that could depend on the availability of depletable vehicles such as Rab G-proteins (Croizet-Berger et al., 2002) and phosphoinositides (Aikawa and Martin, 2003), and also of large structures such as rafts (Nabi and Le, 2003). If maintained in nanomolar range, extracellular neuropeptide Y could overload the metabolic signaling through its Y1 receptors,

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which typically display high affinities for neuropeptide Y (Beck-Sickinger et al., 1994; Daniels et al., 1995a). Regulatory restraints could be in place to prevent oversignaling due to a continuing neuropeptide Y stimulus. In an examination of the concentration-related activity of neuropeptide Y at cell surface Y1 receptors, we found a persistent receptor occupancy not shared by peptide YY and the related Y1 agonist (Leu^{31},Pro^{34}) peptide YY, and reflected in a lower inhibition by neuropeptide Y of the activity of forskolin-stimulated adenylyl cyclase. The above findings show that the activity of the neuropeptide Y Y1 receptor in signal transduction as regulated by neuropeptide Y involves a concentration-dependent attenuation, which could be of importance at high levels of extracellular neuropeptide Y.

2. Experimental procedures

2.1. Chemicals

Dimeric nonapeptides VD-11 (compound 7 of (Balasubramaniam et al., 2001); bis(29/31′,29/31′ {{[Glu^{29},Pro^{30},Dpa^{31},Tyr^{32},Leu^{34},(Tyr-O-CH_3)^{36}]neuropeptide Y(28–36)}}) and GR231118 (also known as 1229U91) (Daniels et al., 1995a) (bis(29/31′,29/31′ {{[Glu^{29},Pro^{30},Dpa^{31},Tyr^{32},Leu^{34},(Tyr-NH_2)^{36}] neuropeptide Y(28–36)}}; Dpa = diaminopropionic acid) were synthesized by A.B. Human neuropeptide Y, porcine peptide YY (Leu^{31}, Pro^{34}) human peptide YY and (Leu^{31},Pro^{34}) human neuropeptide Y were purchased from the American Peptide Company (Los Angeles, CA). Porcine peptide YY and [^{125}I](Leu^{31},Pro^{34}) human peptide YY, and [^{35}S]-labeled guanosine-5′-*O*-(γ-thiotriphosphate) (GTP-γ-S) were obtained from PerkinElmer (Cambridge, MA, USA). Human/rat [^{125}I]neuropeptide Y was from Phoenix Pharmaceutical (Belmont, CA, USA). Non-labeled nucleotides were obtained from Calbiochem (Los Angeles, CA, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Cells, animals and particulates

Chinese hamster ovary (CHO) cells (CHO-K1 sub-line from the American Type Culture Collection, Baltimore, MD, USA) stably expressing the cloned guinea pig neuropeptide Y Y1 receptor (Berglund et al., 1999) were produced using cDNAs donated by Dr. M. Berglund. The CHO-K1 cells were also used to express the human neuropeptide Y Y1 receptor cDNA obtained from the cDNA Resource Center of the University of Missouri at Rolla (Rolla, MO, USA). SK-N-MC cells natively expressing the human neuropeptide Y Y1 receptor were purchased from the American Type Culture Collection (Rockville, MD, USA). No substantial differences were noted in sensitivity of expressions in CHO cells of the guinea pig and the human neuropeptide Y Y1 receptor to neuropeptide Y-related peptides or to pertussis toxin. Most experiments reported in this study were done with the guinea pig expression, and verified at least once with the human CHO expression.

The CHO cells were maintained at 400 μg/ml geneticin in D-MEM/F-12 medium (Gibco, Long Island, NY, USA) as described previously (Parker et al., 2002b). The SK-N-MC cells were cultured in Eagle's minimal essential medium with Earle's salts, 18 mM $NaHCO_3$, 1 mM sodium pyruvate, 2 mM Gluta-Max 1 (Gibco) and 10% fetal bovine serum. Experimental treatments of intact cells were done on 48- or 96-well plates, using Opti-Mem® medium (Gibco) without antibiotics and fetal serum, and with added 0.2% protease-free bovine serum albumin. The labeling was done at 50 pM [^{125}I]-peptides, using 100 μM BIBP-3226 ((*R*)-*N*2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]D-arginine amide) to define the nonspecific binding. After incubation and four cold washes, the wells were extracted for 6 min at 0–4 °C with 0.2 M CH_3COOH–0.5 M NaCl (pH 2.6) to dissociate agonist peptides attached to the cell surface (see Parker et al., 2002b). In internalization studies, the residues from the above extraction (containing the internalized peptides) were solubilized in 0.1 M NaOH prior to radioactivity counting. Radioactivity was measured in a gamma-scintillation counter for [^{125}I]-labeled, and in a liquid scintillation spectrometer for [^{35}S]-labeled samples.

All animal protocols followed the guidelines of the University of Tennessee Animal Health Care Committee. Sprague–Dawley male rats (2–2.5 months old; body weight 240–260 g) were obtained from Holtzman (Madison, WI, USA). The animals were sacrificed under isoflurane anesthesia. Synaptosomes from rat brain cortex were isolated as described by Nagy and Delgado-Escueta (1984). The Percoll vehicle was removed as a firm, transparent pellet underlying sedimented synaptosomes upon 1:3 dilution of Percoll gradient fractions with neuropeptide Y receptor assay buffer and sedimentation for 20 min at 30,000×g_{max}.

2.3. Receptor assays and assays employing the binding of [^{35}S] GTP-γ-S

Receptor binding assays employing up to 12 different concentrations of competitors at 50 pM of [^{125}I]-labeled agonist peptides were done as described (Parker et al., 2002b), but with 140 mM NaCl in the assay buffer. A Peltier-effect micro-incubator (MJ Research, Watertown, MA, USA) was used for temperature control in the range of 5–37 °C. Assays were terminated by sedimentation for 10 min at 4 °C and 30,000×g_{max} in a Sorvall (Newtown, CT, USA) T 21 centrifuge. The binding of [^{35}S]-GTP-γ-S was followed in a volume of 0.20 ml at 0.2 nM labeled triphosphate, 1 or 3 μM GDP and 100 nM of neuropeptide Y receptor agonists for 30 min at 28 °C after preincubation of particulates for 60 min at 28 °C with GDP and with or without Y peptides.

2.4. Assay of adenylyl cyclase

As expected from our previous study employing human neuropeptide Y Y1 receptor expressed in CHO-K1 cells (Fig. 2 in Parker et al., 1998), forskolin-stimulated adenylyl cyclase activity in CHO-K1 cells expressing the Y1 receptor could be up to 75% reduced by neuropeptide Y or peptide YY or by their Y1-selective derivatives, in a concentration-dependent fashion (see Fig. 5 in this study). In most experiments, confluent monolayers of Y1-CHO cells in 48-well plates were washed

and brought to 1 μM forskolin and the desired concentration of Y1 agonists or antagonists, at 100 μM isobutylmethylxanthine, all at 4 °C and in the Opti-Mem medium. The plates were then incubated for up to 60 min at 37 °C, the medium was removed by rapid suction in an ice bath, and the cells were extracted by 0.25 ml/well of 0.10 N HCl for 20 min at 21–23 °C. The extracts were placed into a mixture of NaOH and CH_3COOH assuring pH adjustment to the range of 6–7, and kept at −80 °C until assayed for cAMP using kits supplied by PerkinElmer (Cambridge, MA, USA).

2.5. Data evaluation

The receptor binding parameter calculations were done 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 (SPSS, Chicago, IL; version 8.0). Multiple comparisons following a positive analysis of variance were done in Tukey's *t*-test.

3. Results

3.1. Activity of Y peptides via neuropeptide Y Y1-CHO receptor in GTP-γ-S binding

As expected from a previous study using SK-N-MC cells natively expressing the human neuropeptide Y Y1 receptor (Gordon et al., 1990), treatment of guinea pig Y1-CHO cells in the growth medium with 1–100 ng/ml of pertussis toxin over 24 h produced a large (84±2.2% at 1 ng/ml, and 97±2.2% at 100 ng/ml; $n=6$) reduction of the basal binding of [^{35}S]GTP-γ-S (Fig. 1A). Stimulation of [^{35}S]GTP-γ-S binding by 100 nM neuropeptide Y at 3 μM GDP decreased from 61% to less than 3% above the basal binding after treatment by the toxin at 100 ng/ml, indicating an essentially complete dependence of this stimulation on the activity of pertussis toxin-sensitive G-protein α-subunits linked to the neuropeptide Y Y1 receptor (Fig. 1A). Treatment with pertussis toxin resulted in a lowering of adenylyl cyclase activity induced by 1 μM forskolin, but this decrease was not consistently significant (Fig. 1B). However, the inhibition of forskolin-stimulated cyclase activity by peptide YY was uniformly very much lower with any dosage of the toxin (Fig. 1B). This reduction of G-protein activity was accompanied by a loss of sensitivity of the Y1 binding of [^{125}I] peptide YY to G-protein nucleotide site agonist GTP-γ-S, and by a large reduction in the total specific binding (Fig. 1C).

3.2. Blockade of CHO cell surface neuropeptide Y Y1 receptors by Y1 agonists and antagonists

Our initial experiments indicated (Sallee et al., 2004) that neuropeptide Y, but not peptide YY, can substantially block the CHO cell surface Y1 sites. With receptor cycling inhibited by phenylarsine oxide and monensin, the occupancy by neuropeptide Y persisted 30 min of incubation at 37 °C following removal of the non-bound peptide (Fig. 2A). The observed profiles were quite similar for neuropeptide Y and for peptidic Y1 antagonists GR231118 and VD-11 in both the range of IC_{50} values (4–8 nM) and the extent of inhibition (>90%; Table 1). The dimeric nonapeptides examined, GR231118 (Matthews et al., 1997; originally coded as 1229U91) and VD-11 (compound 7 of Balasubramaniam et al., 2001), both also qualify as Y1 antagonists by not inhibiting the forskolin-stimulated adenylyl cyclase activity (Parker et al., 1998; Balasubramaniam et al., 2001; see also Fig. 5 in this paper). The IC_{50} values for this blockade with neuropeptide Y and the peptide antagonists were 30–100 times higher than K_I values in direct competition of the binding of peptide YY to particulates (Table 1). The small Y1 antagonist *N*2-diphenylacetyl-*N*-[(4-hydroxyphenyl)methyl]D-arginine amide (BIBP-3226) showed a blocking IC_{50} as high as 3.8 μM, exceeding by three orders of magnitude its K_I in direct competition with the binding of [^{125}I]peptide YY to particulate Y1 receptors (Table 1; see also Holliday and Cox, 2003). This indicated a fast dissociation of BIBP-3226 from cell surface Y1 sites. An Y1-selective agonist derived from neuropeptide Y, (Leu^{31},Pro^{34}) neuropeptide Y, also achieved a

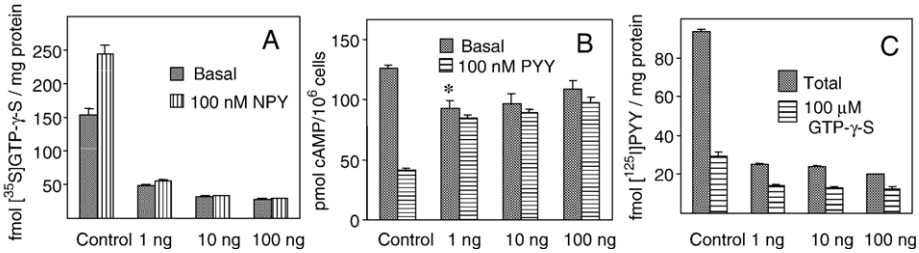

Fig. 1. Sensitivity to pertussis toxin of G-protein nucleotide site, Y1 receptor-linked adenylyl cyclase, and Y1 receptor binding of guinea pig Y1-CHO cells. All results are averages of at least six samples, shown with 1 S.E.M. Abbreviations used in graphs: NPY, neuropeptide Y; PYY, peptide YY. (A) Culturing Y1-CHO cells for 24 h at 1–100 ng/ml of pertussis toxin results in a large reduction of the basal binding of [^{35}S]GTP-γ-S, and in virtual elimination of the stimulation of GTP-γ-S binding (at 3 μM GDP) by Y1 agonists. All differences to controls were significant above 99% confidence in post hoc Tukey *t*-tests. (B) Treatment by pertussis toxin at 1–100 ng/ml medium consistently reduces the inhibition of forskolin (1 μM)-stimulated adenylyl cyclase in intact Y1-CHO cells by peptide YY (100 nM) to levels not consistently different from the basal. The basal activity differing from control without pertussis toxin at 95% confidence is indicated by an asterisk (*). The adenylyl cyclase activity at 100 nM peptide YY for any pertussis toxin concentration was significantly lower at the level of 99% confidence from control without the toxin. (C) Decrease in the labeling by [^{125}I]peptide YY of Y1 receptors and of GTP-γ-S-sensitive Y1 sites resulting from treatments by pertussis toxin described in (A). The particulate receptors were labeled by 200 pM [^{125}I]peptide YY for 60 min at 23 °C, defining the nonspecific binding at 100 μM (BIBP-3226). GTP-γ-S was used at 100 μM. All differences to controls for either the total or the nucleotide-resistant specific binding were significant above 99% confidence in post hoc Tukey *t*-tests.

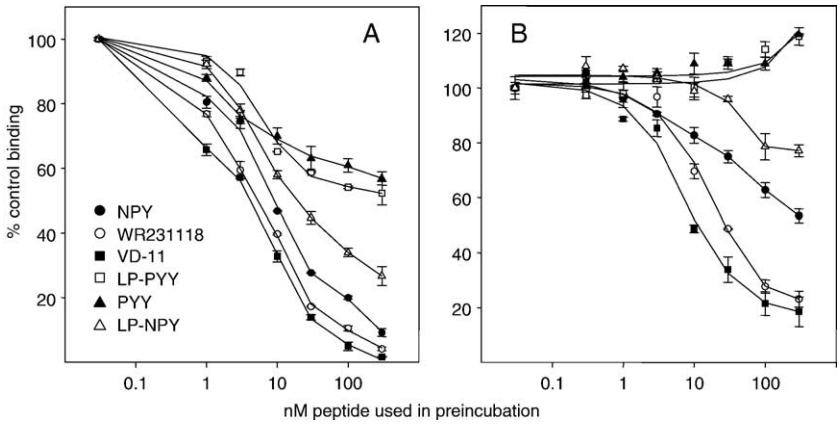

Fig. 2. Blockade of surface guinea pig Y1 receptors of CHO cells by agonists and antagonists. Abbreviations used in graphs: NPY, neuropeptide Y; PYY, peptide YY; LP-PYY, (Leu^{31},Pro^{34}) peptide YY; LP-NPY, (Leu^{31},Pro^{34}) neuropeptide Y; for full names of GR231118 and VD-11 see Section 2.1. (A) Recovery of guinea pig Y1 sites in the absence of receptor cycling after a pre-treatment with Y1 receptor-active peptides. The cells were preincubated with up to 300 nM Y1-active agents for 45 min at 37 °C (corresponding to at least four times the reported half-interval of Y1 receptor cycling at 37 °C; Parker et al., 2001b; Gicquiaux et al., 2002). After fourfold washing at 4 °C, endocytosis inhibitor phenylarsine oxide (30 μM; a concentration reducing Y1 endocytosis more than 90%; Parker et al., 2001b) and sodium transport/receptor recycling inhibitor monensin (60 μM) were added, and the bound agents were allowed to dissociate over 30 min at 37 °C, followed by medium removal, replacement with fresh medium containing 30 μM PAO and 60 μM monensin, and labeling with 50 pM [^{125}I]peptide YY for 20 min at 37 °C. (At the above concentrations, PAO and monensin competed less than 10% of the specific binding of [^{125}I]peptide YY to CHO-Y1 particulates.) (B) Recovery of guinea pig Y1 sites with active receptor cycling after a pre-treatment with Y1-active peptides. The peptides were input at concentrations indicated for (A). The unblocking incubation for 30 min at 37 °C, the washing, and the labeling incubation with 50 pM [^{125}I]peptide YY for 20 min at 37 °C were all done in Opti-Mem medium without inhibitors of internalization/recycling. The IC_{50} values in nM (followed by % persisting blockade) were 5.4±2.6 (49.5%) for neuropeptide Y, 16.5±2.3 for GR231118, and 7.5±1.5 (82%) for VD-11. (These IC_{50} values are estimates from logistic fits with asymptotes equaling the above % of persisting blockade.) After pre-treatment with 300 nM of the peptides, the internalization of 50 pM [^{125}I]peptide YY as percent of control value was 65.3±1.2% (neuropeptide Y), 82.8 ±0.3% (peptide YY), and 6.4±4.1 (GR231118) (n=6 for all).

significant blockade, but at concentrations about eight times higher than neuropeptide Y (Table 1 and Fig. 2). Peptide YY and the related (Leu^{31},Pro^{34}) peptide YY, however, blocked less than 50% Y1 sites in preincubation at 300 nM (Table 1 and Fig. 2).

At 30 μM of the internalization inhibitor phenylarsine oxide (PAO) and 60 μM of the recycling blocker monensin, internalization of 50 pM [^{125}I]peptide YY over 20 min at 37 °C was reduced to 7±0.5% of the corresponding control values (n=24). In cells labeled with [^{125}I]peptide YY or [^{125}I]neuropeptide Y before adding the above inhibitors, the loss of sequestered or internalized labeled peptide over 30 min at 37 °C was only 16±1.4% (n=6) of that found for control incubations (as determined by Bio-Gel P-4 chromatography). This indicated that cycling is of a large importance for re-activation of the neuropeptide Y-blocked Y1 receptor.

Without inhibitors of endocytosis, the surface Y1 sites showed a slow recovery after treatment with dimeric nonapeptide antagonists, and a faster recovery following exposure to neuropeptide Y and (Leu^{31},Pro^{34}) neuropeptide Y (Fig. 2B). However, recovery after treatment with peptide YY and (Leu^{31}, Pro^{34}) peptide YY was complete after 30 min of dissociation at 37 °C, and there even was an increase in active surface sites after preincubation at high concentrations of these peptides (Fig. 2B), possibly related to a slight decrease in internalization. The internalization of [^{125}I]peptide YY decreased less than

Table 1
A comparison of competition of particulate binding of [^{125}I]peptide YY by Y1 active agents and of blockade of [^{125}I]peptide YY binding to surface guinea pig Y1 sites of intact CHO cells by pretreatment with the same agents

Cold peptide	K_I (nM) in competition of the binding to particulates [a]	IC_{50} (nM) for the inhibition of cell surface PYY binding by pretreatment [a]	% inhibition of cell surface binding [b]
Peptide YY	0.172±0.021	~460	43.2
(Leu^{31},Pro^{34})peptide YY	0.428±0.025	~310	47.7
Neuropeptide Y	0.195±0.012	8±2.4	90.9
(Leu^{31},Pro^{34})neuropeptide Y	0.191±0.039	62±15	73.3
GR231118	0.193±0.036	5.1±0.78	95.9
VD-11	0.151±0.065	4.2±1.3	98.4
BIBP-3226	6.86±0.33	3790±860	91.7

All profiles were corrected for the nonspecific binding at 100 μM BIBP-3226, representing ≤8% of the total binding of 50 pM [^{125}I]peptide YY. Very similar profiles of inhibition of cell surface sites by neuropeptide Y and peptide YY were obtained with human Y1 expressions in CHO and SK-N-MC cells (IC_{50} 6.4±1.5 nM (CHO cells) and 8.1±1.1 nM (SK-N-MC cells) with NPY, and >300 nM with peptide YY for both cell types; n=2).

[a] The binding parameters for [^{125}I]-peptide YY (PYY), shown ±1 S.E.M., are averages of at least three independent measurements.

[b] At 300 nM peptides or 100 μM (*R*)-*N*2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]D-arginine amide (BIBP-3226).

35% after exposure to 300 nM of the agonists, but more than 90% following the same dosage of the antagonist GR231118 (see the legend of Fig. 2B).

3.3. Blockade of particulate neuropeptide Y Y1 receptors by Y1 receptor agonists and antagonists

The neuropeptide Y Y1 receptors on particulates from CHO cells were strongly blocked by pretreatment with neuropeptide Y, and less strongly but significantly by (Leu^{31},Pro^{34}) neuropeptide Y (Fig. 3A). However, peptide YY and (Leu^{31},Pro^{34}) peptide YY even at 300 nM did not produce a blockade of particulate Y1-CHO receptors that would persist dissociation over 30 min at 37 °C following removal of external peptides. The dissociation of the peptide antagonists was apparently aided by shearing of the particulates (resuspension and resedimentation), resulting in shallow profiles of apparent blockade. However, the K_I values against [^{125}I]peptide YY in direct competition at particulates without resuspension and dissociation were below 200 pM for either antagonist (Table 1), indicating a high potency of inhibition.

The synaptosomal neuropeptide Y Y1 receptors from rat cortex were also strongly blocked by neuropeptide Y (Fig. 3B). To avoid fast deterioration of rat cortical neuropeptide Y Y1 receptor-coupling G-proteins at 37 °C (more than 50% loss of [^{35}S]GTP-γ-S binding within 30 min at 37 °C) which also greatly reduces the Y1 binding, all procedures with synaptosomes were done at 21–23 °C. The Y1-selective receptor agonist [^{125}I](Leu^{31},Pro^{34}) peptide YY was used as the tracer instead of [^{125}I]peptide YY, to exclude labeling of the neuropeptide Y Y2 receptors. While the inhibition was less stable than found with CHO cell particulates, neuropeptide Y at 300 nM blocked more than 70% of synaptosomal Y1 sites, and (Leu^{31},Pro^{34}) neuropeptide Y more than 50% (Fig. 3B); peptide YY and (Leu^{31},Pro^{34}) peptide YY blocked, respectively, only 43% and 30% of the sites. Dissociation of the neuropeptide Y Y1 receptor antagonists was assisted by resuspension and resedimentation of synaptosomes even more than found with CHO cell particulates. However, the K_I values vs. the binding of [^{125}I] (Leu^{31}, Pro^{34}) peptide YY as measured without resuspension and dissociation were 28±6 pM with GR231118 and 14.8±0.24 pM with VD-11 (n=3 for each), indicating a very high potency of both receptor antagonists in the direct competition of this neuropeptide Y Y1-selective receptor agonist at synaptosomal Y1 receptors.

3.4. The Y1-CHO binding of neuropeptide Y and peptide YY shows different sensitivity to chaotropes and guanine nucleotides

Differential blockade of neuropeptide Y Y1 receptors by neuropeptide Y and peptide YY could mainly depend on a different tenacity of attachment of these peptides. To examine this, we first compared sensitivity of the guinea pig Y1 binding of neuropeptide Y and peptide YY to urea, a non-ionic chaotrope, and $NaClO_4$, an ionic chaotrope. The maximal molarities that could be applied without loss of particulate protein sedimentable at 30,000×g were 2.0 M for urea, and 0.3 M for $NaClO_4$. With particulate neuropeptide Y Y1 receptors of CHO cells the binding of [^{125}I] peptide YY was inhibited much more efficaciously than the binding of [^{125}I]neuropeptide Y by either urea (Fig. 4A) or $NaClO_4$ (Fig. 4B). With urea, the half-inhibition molarity for neuropeptide Y binding was 60% higher, and with perchlorate 45% higher than for the binding of peptide YY (the legend of Fig. 4A and B). The steady-state binding of either peptide was fairly resistant to 2 M urea within 30 min at 23 °C (Fig. 4A). However, the steady-state binding of peptide YY was about 76% dissociated by 0.3 M

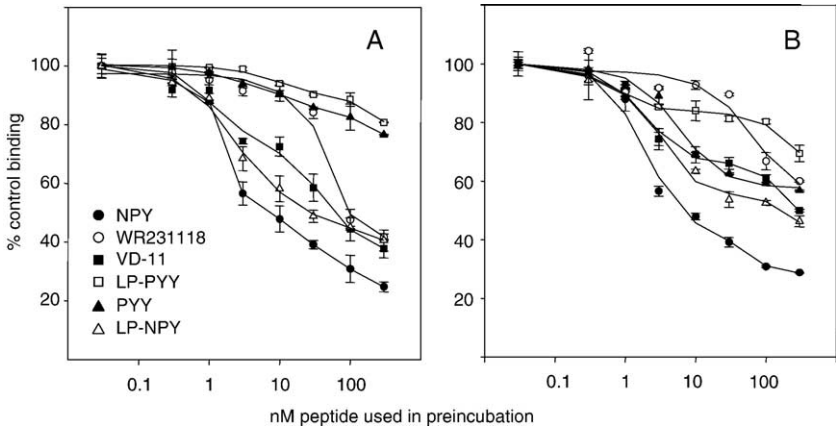

Fig. 3. Blockade of neuropeptide Y Y1 receptors by peptidic agonists and antagonists. (A) Guinea pig Y1 receptors on particulates from CHO cells are strongly blocked by neuropeptide Y. The indicated concentrations of peptides were applied for 45 min at 37 °C, followed by washing, resuspension, dissociation of 30 min at 37 °C, resedimentation, and labeling with [^{125}I]peptide YY. The IC_{50} values in nM (from logistic fits; followed by % inhibition) were: neuropeptide Y, 2.8±0.9 (75), (Leu^{31},Pro^{34}) neuropeptide Y, 3.2±0.7 (59), GR231118, 49±9 (58), and VD-11, 27±5 (62). (B) The native Y1 receptors on synaptosomes from rat cortex are also strongly blocked by neuropeptide Y. The indicated concentrations of peptides were applied for 30 min at 21 °C, followed by washing, dissociation of 30 min at 21 °C, and labeling with [^{125}I](Leu^{31},Pro^{34}) peptide YY for 30 min at 21 °C. The IC_{50} values in nM (from logistic fits; followed by % inhibition) were: neuropeptide Y, 2.5 ±0.7 (71), (Leu^{31},Pro^{34}) neuropeptide Y, 3.7±0.7 (54), and VD-11, 17±5 (50). Other peptides produced less than 50% inhibition of the binding of (Leu^{31},Pro^{34}) peptide YY in the above conditions. See the legend of Fig. 2 for abbreviations in the graphs.

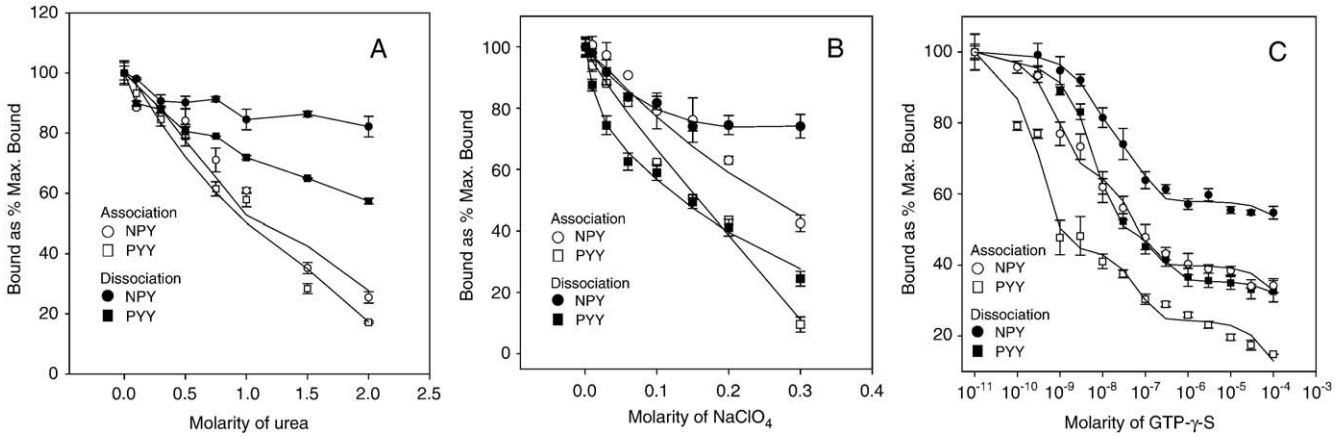

Fig. 4. Different sensitivity to chaotropic agents and GTP-γ-S in the binding of [^{125}I]neuropeptide Y and [^{125}I]peptide YY to guinea pig Y1-CHO particulate receptors. The labeling with either peptide was at 25 pM over 60 min at 21–23 °C. In the binding to a steady state, the chaotropes or GTP-γ-S were added before particulates. For dissociation from the steady state, the treatment agents were added after 60 min of labeling, and allowed to act for 30 min at 21–23 °C. All assays were terminated by centrifugation (see Section 2). See the legend of Fig. 2 for abbreviations in the graphs. (A) Inhibition of the association and dissociation of [^{125}]-labeled peptide YY and neuropeptide Y by urea (eight concentrations in the range of 0.1–2.0 M; three runs in either mode of labeling). The steady-state binding of neuropeptide Y was maximally inhibited by 86±7% (IC_{50} 0.96±0.08 M), and that of peptide YY by 89±16% (IC_{50} 0.6±0.11 M). At 2.0 M urea, the dissociation from steady state was 18±0.9% for neuropeptide Y, and 42±0.9% for peptide YY. (B) Inhibition of the association and dissociation of [^{125}]-labeled peptides by $NaClO_4$ (eight concentrations in the range of 0.01–0.3 M; six runs in either condition). The steady-state binding of neuropeptide Y was inhibited by 90±2% (IC_{50} 0.196±0.02 M), and that of peptide YY by 94±6% (IC_{50} 0.135±0.02 M). At 0.3 M $NaClO_4$, the dissociation from steady state was 25±1.3% for neuropeptide Y, and 76±7% for peptide YY. (C) Effects of GTP-γ-S on the association and dissociation of [^{125}]-peptides. The nucleotide was used at 12 concentrations in the range of 1×10^{-10} to 1×10^{-4} M. Five determinations were done in association, and seven in dissociation. The steady-state specific binding of neuropeptide Y and peptide YY was maximally inhibited, respectively, 66±2% and 86±1.4%. The neuropeptide Y profile fitted to components of inhibition with IC_{50} values of 1.4±0.45 nM and 28 ±1.4 nM, respectively, representing 29% and 71% of the inhibition. The corresponding IC_{50} values in the profile for peptide YY were 0.97±0.24 nM (41%) and 11 ±0.7 nM (59%). At 100 μM GTP-γ-S, dissociation of neuropeptide Y and peptide YY was, respectively, 46±1.8% and 67±3%.

perchlorate, and that of neuropeptide Y only 25% (Fig. 4B), indicating a much larger stability of neuropeptide Y binding to ionic chaotropes.

The primary association of [^{125}I]neuropeptide Y with the Y1-CHO receptor was much less sensitive to guanine nucleotides than that of [^{125}I]peptide YY (Fig. 4C). At 25 pM labeled peptide, GTP-γ-S in the range of 0.1–100 μM inhibited more than 85% of the specific binding of [^{125}I]peptide YY, showing components of 0.97±0.24 nM and 11±0.7 nM that represented, respectively, 41% and 59% of the

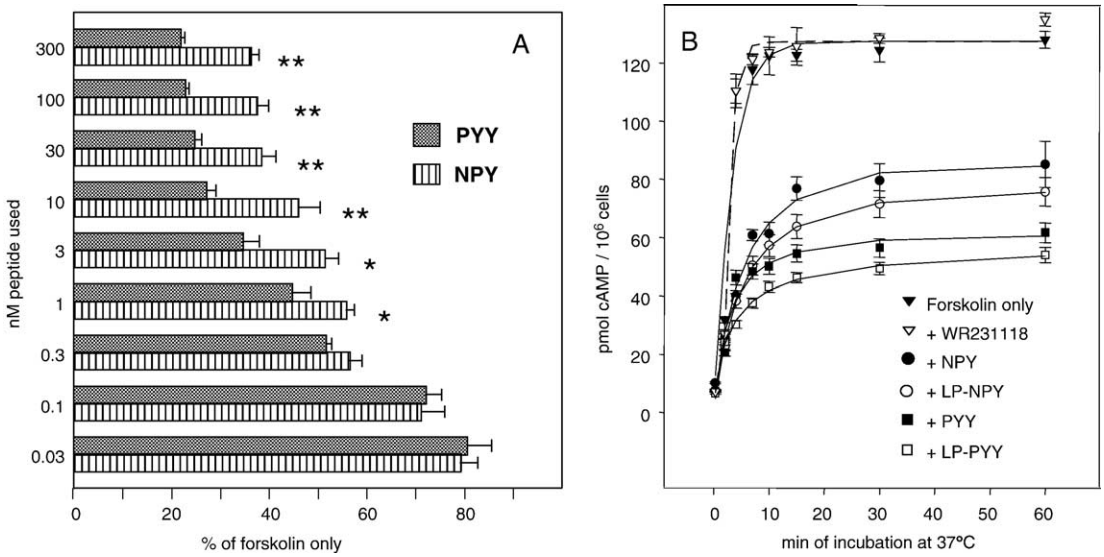

Fig. 5. Inhibition of forskolin-stimulated adenylyl cyclase activity by neuropeptide Y, peptide YY and their Y1-selective analogues in intact CHO cells expressing the guinea pig Y1 receptor. Forskolin was used at 1 μM in all experiments. (A) Inhibition of cyclase activity over 20 min at 37 °C by different inputs of neuropeptide Y and peptide YY. The results are averages of 12 measurements, shown with 1 S.E.M. Concentration of neuropeptide Y and peptide YY is shown on *Y*-axis. Asterisks indicate significance at 95% (*) or 99% (**) confidence in post hoc Tukey *t*-tests. Similar profiles were obtained with CHO cells expressing the human Y1 receptor (data not shown). (B) Kinetics of inhibition of forskolin-stimulated cyclase activity by Y1 receptor active peptides. All peptides were used at 100 nM. The results are averages of six measurements for each point, shown with 1 S.E.M. At 10 to 60 min of incubation, differences between neuropeptide Y and either (Leu^{31},Pro^{34}) peptide YY or peptide YY were significant in post hoc Tukey *t*-tests. With neuropeptide Y and peptide YY, similar differences were observed in SK-N-MC cells.

displacement. However, the association binding of 25 pM [^{125}I]neuropeptide Y was inhibited by GTP-γ-S to only 68% of the specific binding, with components of 1.4±0.45 nM and 28±1.4 nM (29% and 71% of the displacement, respectively). These components could correspond to prevailing affinity states (e.g. Kent et al., 1980) of the Y1 receptor. For either peptide, the binding inhibited by GTP-γ-S is largely dependent on the activity of pertussis toxin-sensitive G-proteins (see Fig. 1). The fraction of the binding not inhibited by GTP-γ-S could reflect presence of additional affinity states as well as association with toxin-insensitive G-proteins. The steady-state binding of peptide YY was also much more sensitive to GTP-γ-S than the binding of neuropeptide Y (Fig. 4C). However, for both peptides the sensitivity to GTP-γ-S in the steady-state binding was much reduced compared to the association binding, indicating a restructuring of the ligand–receptor–Gα complex following the primary agonist attachment. This is also indicated by the low sensitivity of the steady-state binding to urea (Fig. 4A).

3.5. Inhibition of forskolin-stimulated adenylyl cyclase in Y1-CHO cells by agonists of the neuropeptide Y Y1 receptor

As expected (Parker et al., 1998), neuropeptide Y, peptide YY, and their analogs active as Y1 receptor agonists, at 0.3–300 nM inhibited in a concentration-dependent manner the activity of adenylyl cyclase stimulated by 1 μM forskolin in intact monolayers of CHO cells expressing guinea pig or human Y1 receptors. A saturation of the inhibitory effect was reached at about 30 nM for all agonist peptides tested. Fig. 5A shows this for neuropeptide Y and peptide YY. After 20 min of in vivo exposure, the inhibition by peptide YY was highly significantly ($p<0.01$; $n=12$) above that of neuropeptide Y over the range of 10–300 nM. However, differences at 1 and 3 nM were less significant, and the activity of peptide YY and neuropeptide Y was quite similar at or below 0.3 nM. A kinetic comparison of four Y1 agonists at 100 nM vs. stimulation of adenylyl cyclase by 1 μM forskolin showed a fast and steady inhibition by (Leu^{31},Pro^{34}) peptide YY and peptide YY that persisted to 60 min of incubation (Fig. 3B). At 100 nM, the inhibition by neuropeptide Y was at longer incubation times much lower than observed with peptide YY, and especially with (Leu^{31},Pro^{34}) peptide YY (Fig. 5B). As expected (Parker et al., 1998; Balasubramaniam et al., 2001), antagonists GR231118 and VD-11 did not inhibit the forskolin-stimulated adenylyl cyclase activity; the results with 100 nM GR231118 are shown in Fig. 5B.

4. Discussion

Our results indicate that neuropeptide Y, unlike peptide YY, persistently engages both clonal and native Y1 receptors. A similar blockade-like occupancy is found at the Y4 receptor with its pancreatic polypeptide agonists (Parker et al., 2001a). Such stabilization of the receptor–ligand complex could significantly depend on a post-attachment restructuring, evident from the increase of resistance to chaotropes for the steady-state relative to primary binding (as shown in this study for the Y1 receptor, and previously for the Y4 receptor; Parker et al., 2001a). The increase in blocking with ligand concentration is likely connected to an occupancy-forced decrease of site availability, and of the apparent agonist affinity (on which see e.g. the review by Kenakin, 2002). The prolonged occupancy by neuropeptide Y could be verified with guinea pig and human Y1 clonal expressions in CHO cells, as well as with the native expression of the human Y1 receptor in SK-N-MC cells, and with the synaptosomal rat neuropeptide Y Y1 receptor. This uniformity across species should be expected from the high conservation of sequence of the neuropeptide Y Y1 receptor in the mammal (Larhammar et al., 2001).

The extended occupancy of the binding site could be due to activity of residues or sequences of neuropeptide Y not directly attaching to the Y1 site, and residing in the extensive non-helical portions of neuropeptide Y molecule (Lerch et al., 2004) different from the short Y1-binding epitopes (Beck-Sickinger et al., 1994) (which should be identical for human/rat neuropeptide Y and porcine/rat peptide YY). Peptide YY has a larger degree of helical self-structuring than neuropeptide Y(Lerch et al., 2004) and, from our results, is less involved in secondary interactions that result in a blockade. The extent of such interactions is probably also reflected in non-saturable binding of different neuropeptide Y-related peptides to intact cells or particulates, which is typically much higher for neuropeptide Y than for peptide YY, and very low for pancreatic polypeptide (see e.g. Table 1 in Parker et al., 2002b). The high lipid affinity of neuropeptide Y (McLean et al., 1990) should facilitate intramembrane presentation of the peptide to neuropeptide Y receptors (Lerch et al., 2004). This could be especially important for the Y1 binding, which uses short sequences at both termini of the neuropeptide Y molecule (Beck-Sickinger et al., 1994; Daniels et al., 1995b) that may not productively associate with the binding site via random collisions from the extracellular fluid. Different chaotrope sensitivities of neuropeptide Y and peptide YY also indicate larger secondary binding interactions for neuropeptide Y.

As possibly related to the above, peptide YY and pancreatic polypeptide do not readily cross the hematoencephalic barrier (Whitcomb et al., 1990; Hernandez et al., 1994), while neuropeptide Y is non-saturably shuttled in both directions (Kastin and Akerstrom, 1999). The persistent Y1 receptor occupancy by neuropeptide Y below 10 nM could be of physiological consequence in tissues enriched in this receptor, including forebrain and kidney (see e.g. Parker et al., 1999). The relatively low blockade by (Leu^{31}Pro^{34}) neuropeptide Y points to importance of Ile^{31} of neuropeptide Y in stabilization of the attachment to the Y1 site. Residue 31 is leucine in both peptide YY, which poorly blocks the Y1 receptor, and in pancreatic polypeptides (which do not attach to the Y1 site, and share the C-terminal hexapeptide with (Leu^{31},Pro^{34}) neuropeptide Y; see e.g. Parker et al., 2002a).

Neuropeptide Y qualifies as a partial agonist based on a progressive decrease of the inhibition of adenylyl cyclase in

intact cells relative to peptide YY or (Leu^{31},Pro^{34}) peptide YY. We did not detect either desensitization or superactivation of adenylyl cyclase (Wang et al., 2003) by long exposure to neuropeptide Y or the closely related Y1 agonist (Leu^{31},Pro^{34}) neuropeptide Y. The larger potentiation of GTP-γ-S binding to particulates via the Y1 receptor by neuropeptide Y relative to peptide YY (Holliday and Cox, 2003) also points to a larger stability in association of the neuropeptide Y-Y1 complex with Gα. This is further supported by a much lower sensitivity to inhibition by GTP-γ-S of neuropeptide Y association with the neuropeptide Y Y1 receptor. Mobility of the agonist-engaged Y1 receptor should be reduced compared to the non-liganded receptor, analogous with e.g. the cholecystokinin receptor engaged by a partial agonist (Roettger et al., 1999). Under physiological conditions, strong secondary attachment(s) of neuropeptide Y at the Y1 site might reduce the G-protein cycling and interaction with effectors, resulting in the observed lower inhibition of adenylyl cyclase relative to peptide YY. The affected molecular switches need not be located exclusively on G-protein α subunits (e.g. Kisselev and Downs, 2003). The binding of GTP-γ-S to the Gα nucleotide site is enhanced by neuropeptide Y in preference to peptide YY (Holliday and Cox, 2003), with an ED_{50} value close to the IC_{50} for the blockade of the Y1 binding by neuropeptide Y. The concentration-related contribution of a motif (and possibly even a single residue) of neuropeptide Y might become important already at low nanomolar levels of the peptide. This can be related to differences in the solution structure of neuropeptide Y and peptide YY (Lerch et al., 2004).

The significant removal of two peptidic antagonists of the neuropeptide Y Y1 receptor by shearing involved in resuspension and resedimentation of particulates could be related to tight intramolecular associations forced by covalent crosslinking within these dimeric peptides, resulting in a decreased ability for stabilizing interactions in the vicinity of the Y1 binding site. However, blockade of intact cell Y1 receptors by these antagonists will probably not be affected by the low physiological levels of shear.

Protracted occupation of the Y1 site by neuropeptide Y and not by peptide YY, two peptides of similar Y1 affinity (see also Holliday and Cox, 2003), could indicate an important regulatory difference. The more stable Y1 receptor attachment of neuropeptide Y might boost signaling at low concentrations, and at higher concentrations limit the transduction via decreased mobility and increased rigidity of the complex with the receptor. Prolonged receptor occupancy is known to be connected to surface immobilization in the case of peptidic antagonists (for an early study, see Loumaye et al., 1984). However, the blockade-like occupancy by neuropeptide Y is largely overcome by receptor cycling, which is not the case with peptidic antagonists. Agonist blockade relieved by endocytotic processing is also found with endothelin-B receptor (Grantcharova et al., 2002) and with Y4 receptors (Parker et al., 2002a), and possibly also with corticotropin releasing factor receptors (Roseboom et al., 2001). Extended occupancy of receptors of an abundant peptide, such as neuropeptide Y, could represent an evolutionary solution contributing to a balancing of metabolic signaling, peptide clearance and receptor conservation.

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