British Journal of Pharmacology (1997) 120, 481–487

Characterization of Y_3 receptor-mediated synaptic inhibition by chimeric neuropeptide Y-peptide YY peptides in the rat brainstem

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1 Neuropeptide Y (NPY) and peptide YY (PYY) act at receptors referred to as Y_1 and Y_2, while the Y_3 receptor is specific to NPY and does not recognize PYY. The effects of NPY, its related peptides and a series of newly constructed chimeric NPY-PYY peptides were examined on excitatory and inhibitory postsynaptic currents (e.p.s.cs and i.p.s.cs, respectively) in rat dorsomedial nucleus tractus solitarius (NTS) neurones recorded in coronal brainstem slices. Monosynaptic activity was evoked by electrical stimulation in the region of the tractus solitarius.

2 NPY (5–500 nM) inhibited e.p.s.cs and i.p.s.cs in a concentration-dependent manner. In contrast, PYY (500 nM) failed to affect either e.p.s.cs or i.p.s.cs. The N- and C-terminal parts of a series of chimeric NPY-PYY peptides were joined at positions where NPY and PYY sequences differ. In binding experiments the chimeric peptides were all about equipotent with NPY and PYY in displacing [^{125}I]-PYY from Y_1 and Y_2 binding sites on SK-N-MC cells and rat hippocampus respectively.

3 In the whole cell voltage clamp recordings of NTS neurones, NPY(1–23)-PYY(24–36) and NPY(1–14)-PYY(15–36) evoked a concentration-dependent inhibition of e.p.s.cs and i.p.s.cs, while NPY(1–7)-PYY(8–36) and NPY(1–3)-PYY(4–36) were inactive. The only differences in amino acid residues between NPY(1–14)-PYY(15–36) and NPY(1–7)-PYY(8–36) reside in positions 13 and 14.

4 Furthermore, [Pro^{34}]NPY (500 nM) was equivalent in potency to NPY itself at inhibiting monosynaptic transmission in NTS, while [Leu^{31},Pro^{34}]NPY and pancreatic polypeptide (both at 500 nM) failed to affect synaptic transmission.

5 The present study has shown that NPY acts at Y_3 receptors to suppress both excitatory and inhibitory currents in the NTS. The different efficacy of the chimeric NPY-PYY peptides suggests that positions 13 and 14 are of great importance for Y_3 receptor recognition. Finally, this receptor type readily recognizes [Pro^{34}]NPY, but not [Leu^{31},Pro^{34}]NPY.

Keywords: Neuropeptide Y; peptide YY; chimeric peptides; neuropeptide Y receptors; nucleus tractus solitarius; postsynaptic currents; electrophysiology; ligand binding; rat brainstem

Introduction

Neuropeptide Y (NPY) occurs in many neurones throughout the central and peripheral nervous systems (DeQuidt & Emson, 1986; Sundler *et al*., 1986). The peptide has been implicated in various physiological systems in the central nervous system, such as regulation of energy intake and autonomic functions (for a review see Grundemar & Håkanson, 1994). Other peptides in the NPY family include peptide YY (PYY) and pancreatic polypeptide (PP). PYY is mainly present in endocrine cells in the gut, although a sparse distribution of PYY mRNA has been shown in the central nervous system (Pieribone *et al*., 1992), while PP is confined to certain cells in the pancreatic islets (Larsson *et al*., 1975). PYY and PP display 70% and 50% sequence homology with NPY, respectively. NPY and PYY act as multiple receptors, of which human Y_1 and Y_2 types have recently been cloned and shown to belong to the superfamily of G-protein coupled receptors (Herzog *et al*., 1992; Larhammar *et al*., 1992; Gerald *et al*., 1995; Rose *et al*., 1995). Very recently, also a PP receptor has been cloned and it has been shown to recognize not only PP, but also PYY and NPY in the nanomolar range (Bard *et al*., 1995; Lundell *et al*., 1995). The most well characterized receptors are those of the Y_1 and Y_2 types, which are present in many parts of the brain and periphery. Both receptor types are equally activated by NPY and PYY (e.g. Wahlestedt *et al*., 1986; Grundemar *et al*., 1993b; Grundemar & Håkanson, 1994).

Moreover, a receptor that recognizes NPY but not PYY or PP, termed Y_3 has been described in the rat cardiac myocytes (Balasubramaniam *et al*., 1990) and nucleus tractus solitarii (NTS) (Grundemar *et al*., 1991b; 1993b). The NTS, the site of termination of primary afferent fibres of arterial baroreceptors, is richly innervated by neurones containing NPY (Hökfelt *et al*., 1984; Berger, 1990). Effects that are associated with activation of Y_3 receptors include hypotension bradycardia and inhibition of glutamate effects in response to unilateral injection of NPY into the NTS (Grundemar *et al*., 1991a,b). Receptors or binding sites corresponding to the Y_3 type have also been suggested in various peripheral tissues (e.g. Grundemar & Håkanson, 1994). The aim of this study was to examine effects of NPY-related peptides, including a series of newly constructed chimeric NPY-PYY peptides on whole cell voltage clamp recordings of excitatory postsynaptic currents (e.p.s.cs) and inhibitory postsynaptic currents (i.p.s.cs) in slices of the rat NTS.

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Methods

Slice preparation and recording

The slice preparation and recording methods employed in the present study have been described in detail elsewhere (Brooks *et al*., 1992; Glaum & Miller, 1992; 1995). Briefly, transverse medullary slices (175–225 μm) at the level of the area postrema containing the NTS were prepared from 19–30 day old Sprague Dawley rats ($n=35$) and continuously superfused with artificial cerebrospinal fluid (ACSF) at 33°C, bubbled with 95% O_2, 5% CO_2, at a rate of 4 ml min^{-1}. The ACSF contained in (mM): NaCl 126, $NaHCO_3$ 26.2, NaH_2PO_4 1.0, KCl 3.0, $MgSO_4$ 1.5, $CaCl_2$ 2.5 and glucose 10. Visually-identified dorsomedial NTS neurones ($n=135$) were recorded in whole-cell mode (Glaum & Miller, 1992) by patch pipettes (1–3 M Ω tip resistance). Monosynaptic e.p.s.cs were evoked at 0.1 Hz by electrical stimulation (3–18 V, 300 μs) in the region of the ipsilateral tractus solitarius at $V_{hold}=-50$ to -80 mV in the presence of bicuculline (10 μM) and D-amino-5-phosphonopentanoic acid (AP5, 25 μM), recorded with electrodes containing (in mM): K^+ gluconate 145, $MgCl_2$ 2.0, HEPES 5.0, EGTA 1.1, $CaCl_2$ 0.1, K_2ATP 5, pH 7.2 (with KOH). The range of the e.p.s.cs was 40.5–212.0 pA, mean $=128\pm3$ pA. Monosynaptic i.p.s.cs were similarly evoked in ACSF supplemented with the excitatory amino acid receptor antagonists 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM) and AP5 (25 μM). I.p.s.c. recordings were made with electrodes containing (in mM): CsCl 145, $MgCl_2$ 2.0, HEPES 5.0, EGTA 1.1, $CaCl_2$ 0.1 and Mg_2ATP 5.0; pH 7.2 (with CsOH) at $V_{hold}=-60$ to -80 mV. The range of the i.p.s.cs was 79.7–305.0 pA, mean 159.9 ± 2.9 pA. All recordings were made in the discontinuous single electrode voltage clamp mode of an Axoclamp-2A amplifier, with switching frequencies (8–12 kHz) that allowed for complete settling of the headstage signal. Data were filtered at 3 Hz and stored both digitally and on analogue tape (Gould 6500) for subsequent analysis (WCP, Strathclyde electrophysiology software).

Whole cell voltage clamp experiments

Peptides were diluted in ACSF to a known concentration immediately before use. Baseline evoked synaptic currents were recorded for >5 min before bath application of a peptide for 90 s, which allowed for complete exchange of solution in the recording chamber. Peak inhibitory effects of the peptides were typically observed within 2 min of the initiation of drug application. A single peptide was applied per slice, except where otherwise noted in the text.

Analysis

The peak amplitudes of evoked e.p.s.cs or i.p.s.cs were determined from the individual synaptic records (WCP, Strathclyde electrophysiology software). Significant peptide inhibition of evoked transmission in a given experiment was determined from the running 1 min mean of p.s.c. amplitude by analysis of variance for repeated measures and Dunnett's test to compare control (predrug) amplitude with those following peptide application. Peak inhibition is presented as a percentage of control current and results were pooled for recordings receiving identical treatments. In recordings where no significant inhibition was noted for a peptide, the value presented reflects the minimum mean amplitude observed within 5 min of peptide application, a latency equivalent to 125% of the maximum latency observed for NPY itself in these experiments. Values are the mean values $\pm$ s.e.mean of the pooled results. Data points illustrate the running 1 min means obtained in an individual recording. Current traces are the average of 3–6 consecutive sweeps. Pooled values were compared by one way analysis of variance for repeated measures and Student's Newman Keuls test for multiple comparisons. Statistical significance was set at the $P<0.05$ level.

Binding experiments

Human neuroblastoma SK-N-MC cells, obtained from American Type Culture Collection were grown in Dulbecco's modified Eagle medium (Gibco) containing 10% foetal bovine serum and penicillin (100 μ ml^{-1}) + streptomycin (100 μg ml^{-1}) (Gibco). When cells were confluent, growth medium was replaced with fresh medium and cells were allowed to continue growing for an additional 24 h. Cells were harvested in a buffer consisting of: Tris 25 mM, $MgCl_2$ 6 mM, bacitracin 250 μg ml^{-1}, aprotinin 250 μg ml^{-1}, Peflabloc 250 μg ml^{-1} (Pentapharm AG, Germany, pH 7.4. Growth medium was removed and cells were lifted from the flasks with D-PBS containing EDTA 0.02%.

SK-N-MC cells or hippocampus from male Sprague Dawley rats were homogenized with a polytron and broken membranes were pelleted by centrifugation at 18,000 r.p.m. for 10 min at 4°C. The pelleted membranes were resuspended in the buffer and frozen at −70°C until used. D-PBS (Gibco) pH 7.4, containing bacitracin 0.5 mg ml^{-1} and BSA 1 mg ml^{-1} was used in the preparation of drug and radiolabel. Each tube consisted of: 100 μl of unlabelled peptide or buffer, 100 μl of [^{125}I]-PYY (30 pM) and 50 μl of membrane preparation (50 μg/tube of SK-N-MC cell membrane protein or 150 μg/tube hippocampal membrane protein), resulting in a final volume of 250 μl. Non-specific binding was defined by the addition of 300 nM NPY. After addition of all agents, tubes were shaken in a water bath while incubating for 60 min at room temperature. The assay was terminated by filtering through a Whatman GF/C glass-fibre filter previously saturated with 0.1% (w/v) polyethylenimine in 10 mM Tris, pH 7.5 containing 0.1% BSA. Filters were punched from the filter mat, placed in tubes and retained radioactivity was counted for 1 min by a gamma counter. The experiments were done in triplicate.

Synthesis of chimeric peptides

Porcine NPY, porcine PYY and the chimeric peptides NPY(1–23)-PYY(24–36), NPY(1–14)-PYY(15–36), NPY(1–7)-PYY(8–36) and NPY(1–3)-PYY(4–36) were synthesized by solid-phase methodology from Fmoc-protected amino acids (Applied Biosystems, Foster City, CA, U.S.A.) by use of Applied Biosystems 430A automated peptide synthesizer (Applied Biosystems). Solvents and reagents were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.), respectively, and were used without further purification. Amino acid chains were not protected except for the following: Asp, Glu, Tyr, Ser, Thr, (tert-butyl); Lys (tert-butyloxycarbonyl; Asn, Gln, His (trityl); Arg (pentamethylchromansulphonyl). Peptides were assembled on Rink amide polystyrene resin (Bachem Bioscience, Philadelphia, PA, U.S.A.) by double coupling of 1-hydroxybenzotriazolyl esters of the protected amino acids which were prepared *in situ* with di-isopropylcarbodiimide and 1-hydroxybenzotriazole. Peptides were cleaved from the resin with 95% trifluoroacetic acid containing 2.5% ethane-1,2 dithiol and thioanisole as cation scavengers, then precipitated with ether to yield the crude peptide. Purification was achieved by preparative high performance liquid chromatography (h.p.l.c.) with a gradient of acetonitrile/water (0.1% trifluoroacetic acid) with a Waters 600 solvent delivery system and 490E programmable u.v. detector (Milford, MA, U.S.A.) and Vydac C18 preparative h.p.l.c. column (5 × 30 cm; Bodman Chromatography, Aston, PA, U.S.A.). Peptides were lysophilised and the actual peptide content determined by nitrogen determination. Purity was assessed by analytical h.p.l.c., amino acid analysis and high-resolution mass spectrometry.

Compounds

DNQX and AP5 were obtained from Research Biochemicals, Inc. (Natick, MA, U.S.A.). Other compounds employed were obtained from Sigma (St. Louis, MO) or standard commercial sources. [Pro^{34}]NPY was a kind gift from T.W. Schwartz.

Results

Electrical stimulation in the region of the tractus solitarius evokes monosynaptic AMPA receptor-mediated e.p.s.cs in dorsomedial NTS neurones (Glaum & Miller, 1992). Substantial evidence indicates that glutamate released from such tractus solitarius afferents conveys cardiorespiratory autonomic information from the periphery (Andresen & Kunze, 1994). As the site of the first central synapse, the NTS provides a central target for the actions of neuromodulators regulating cardiorespiratory function. In the present study, NPY, but not the homologous peptide PYY evoked a reversible inhibition of excitatory transmission in slices of the rat dorsomedial NTS (Figure 1).

Stimulation in the region of the tractus solitarius also evokes mixed mono- and polysynaptically-mediated i.p.s.cs in the dorsomedial NTS (Brooks *et al*., 1992). The monosynaptic component can be isolated following the addition of glutamate receptor antagonists (Glaum & Miller, 1992). NPY also suppressed monosynaptic inhibitory transmission evoked from γ-aminobutyric acid (GABA)ergic interneurones located in and around the tractus solitarius (Glaum & Brooks, 1996), while PYY was inactive (Figure 2). There were no differences in the overall latency or duration of effects of NPY on e.p.s.cs and i.p.s.cs other than those attributable to variability between recordings. Neither NPY nor PYY produced effects upon holding current or cell input resistance, assessed by 500 ms voltage steps to -90 mV, in NTS neurones recorded with either K^+- or Cs-containing electrodes.

We next compared the efficacy of a series of chimeric NPY-PYY peptides to modulate evoked transmission in the NTS. The strategy for the construction of the chimeric NPY-PYY peptides was to join an N-terminal part of NPY with a C-terminal end of PYY at positions where the amino acid residue sequences differ. The primary structures of the chimeric NPY-PYY peptides are shown in Table 1. The chimeric peptides NPY(1–23)-PYY(24–36) and NPY(1–14)-PYY(15–36) evoked significant inhibition of e.p.s.cs and i.p.s.cs, although they appeared to be somewhat less efficacious than NPY itself. In contrast, chimeric peptides with a shorter N-terminal NPY content, i.e. NPY(1–7)-PYY(8–36) and NPY(1–3)-PYY(4-36) were inactive (Figure 3). The concentration-dependent effects of NPY, NPY(1–23)-PYY(24–36) and NPY(1–14)-PYY(15–36) on e.p.s.cs and i.p.s.cs are illustrated in Figure 4. We observed a tendency for i.p.s.cs to be more strongly inhibited than e.p.s.cs at the highest concentrations of the peptides employed. However, the effects of these peptides were more variable upon i.p.s.cs than upon e.p.s.cs, suggesting a possible heterogeneity in the distribution of NPY receptors amongst the local inhibitory interneurone population in the NTS. In support of this contention, we observed that [Pro^{34}]NPY inhibited both i.p.s.cs and e.p.s.cs in the dorsomedial NTS, with significant effects noted in 6 of 6 e.p.s.c. recordings, while the peptide was active in only 3 of 8 i.p.s.c. recordings. Overall, the inhibitory effects of [Pro^{34}]NPY (500 nM) were $37.6 \pm 4.3\%$ on e.p.s.cs, $P < 0.05$ ($n = 6$) and $79.8 \pm 6.2\%$ on i.p.s.cs, $P < 0.05$ ($n = 3$). The efficacy of NPY to inhibit i.p.s.cs in the recordings showing no response to [Pro^{34}]NPY was not examined. In contrast, [Leu^{31},Pro^{34}]NPY (500 nM) failed to affect either e.p.s.cs ($106.2 \pm 6.0\%$ of control

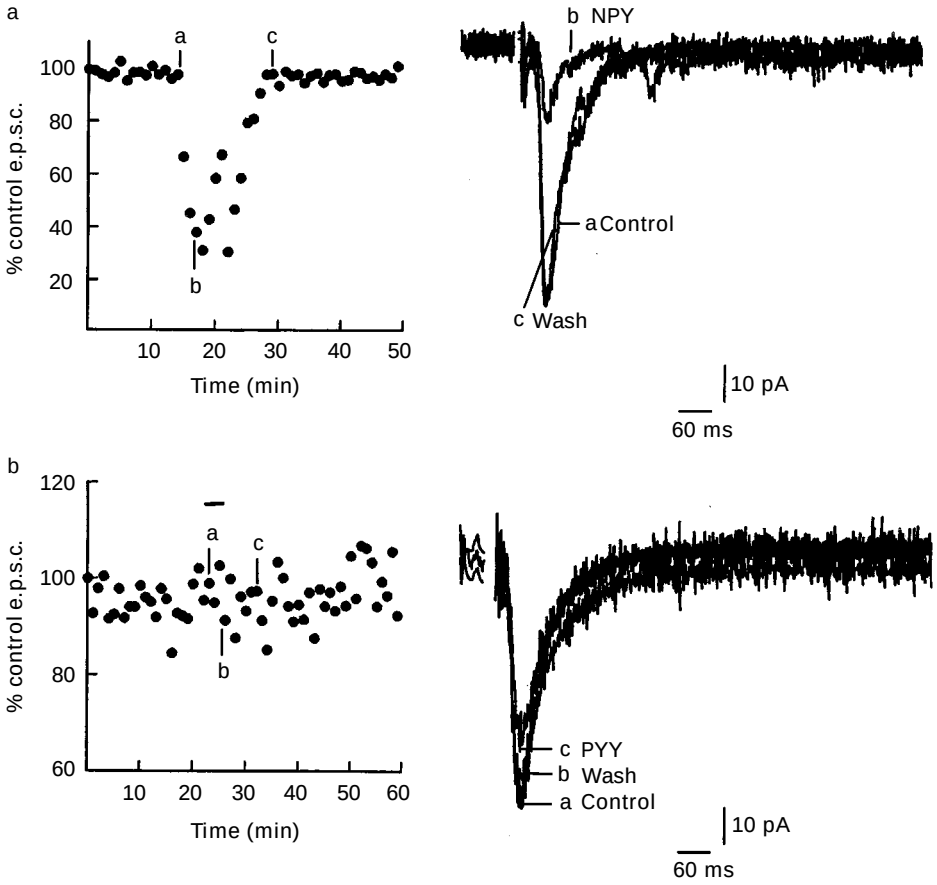

Figure 1 (A) Plot and trace showing suppression by neuropeptide Y (NPY, 500 nM) and recovery of electrically stimulated excitatory postsynaptic currents (e.p.s.cs) in rat dorsomedial nucleus tractus solitarius (NTS). (B) Plot and trace showing lack of effect of PYY (500 nM) of electrically stimulated e.p.s.cs in rat dorsomedial NTS. $V_{hold} = -60$ mV.

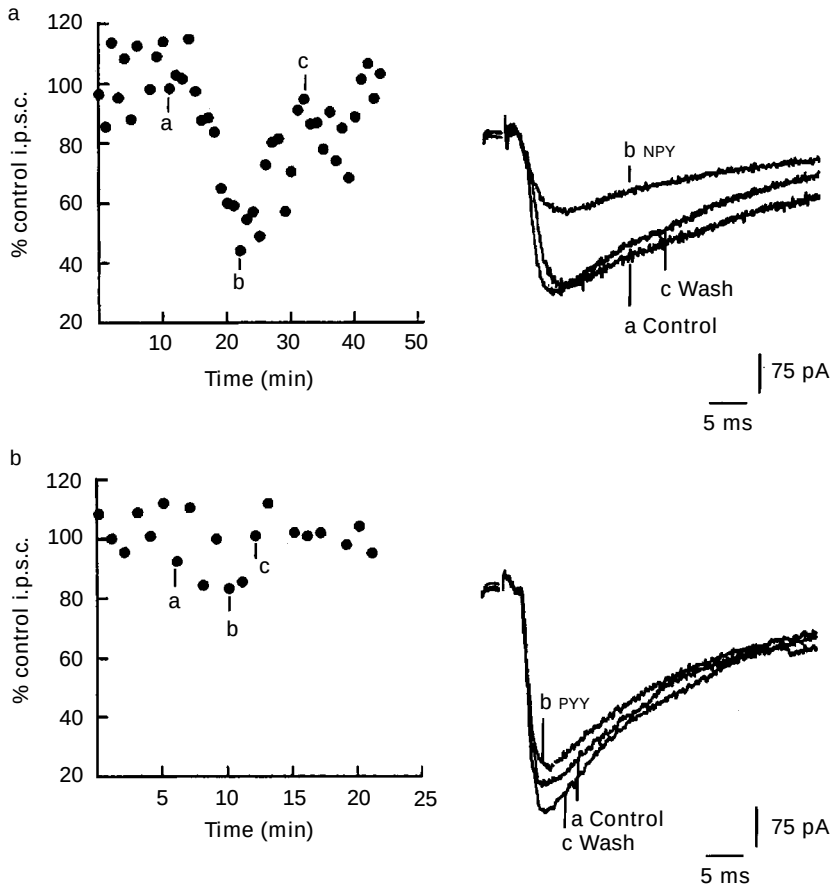

Figure 2 (a) Plot and trace showing suppression by neuropeptide Y (NPY, 500 nM) and recovery of electrically stimulated inhibitory postsynaptic currents (i.p.s.cs) in rat dorsomedial nucleus tractus solitarius (NTS). (b) Plot and trace showing lack of effect of PYY (500 nM) on electrically stimulated i.p.s.cs in rat dorsomedial NTS. $V_{hold} = -80$ mV.

Table 1 Amino acid alignment of NPY, PYY and chimeric NPY-PYY peptides

NPY (1–36) (porcine)
Y-P-S-K-P-D-N-P-G-E-D-A-P-A-E-D-L-A-R-Y-Y-S-A-L-R-H-Y-I-N-L-I-T-R-Q-R-Y amide
NPY (1–23)-PYY (24–36)
Y-P-S-K-P-D-N-P-G-E-D-A-P-A-E-D-L-A-R-Y-Y-S-A-L-R-H-Y-L-N-L-V-T-R-Q-R-Y amide
NPY (1–14)-PYY (15–36)
Y-P-S-K-P-D-N-P-G-E-D-A-P-A-E-E-L-S-R-Y-Y-A-S-L-R-H-Y-L-N-L-V-T-R-Q-R-Y amide
NPY (1–7)-PYY (8–36)
Y-P-S-K-P-D-N-P-G-E-D-A-S-P-E-E-L-S-R-Y-Y-A-S-L-R-H-Y-L-N-L-V-T-R-Q-R-Y amide
NPY (1–3)-PYY (8–36)
Y-P-S-K-P-E-A-P-G-E-D-A-S-P-E-E-L-S-R-Y-Y-A-S-L-R-H-Y-L-N-L-V-T-R-Q-R-Y amide
PYY (1–36)
Y-P-A-K-P-E-A-P-G-E-D-A-S-P-E-E-L-S-R-Y-Y-A-S-L-R-H-Y-L-N-L-V-T-R-Q-R-Y amide

Underlined amino acid residues are identical with NPY and amino acid residues in bold are those that differ between NPY and PYY.

($n = 3$)) or i.p.s.cs (101.0 ± 2.0% of control ($n = 3$)). Also rat PP at 500 nM failed to inhibit evoked transmission in the NTS (not shown). In recordings where peptide application had no effect upon the evoked synaptic activity (i.e. PYY, NPY(1–7)-PYY(8–36), NPY(1–3)-PYY(4–36), [Leu^{31},Pro^{34}]NPY and rat PP), NPY was applied after 30–40 min of washing to confirm the presence of functionally active NPY receptors. In all instances, potent inhibition of evoked activity by NPY was observed.

The chimeric NPY-PYY peptides were examined as ligands at [^{125}I]-PYY binding sites in tissues known to contain predominant populations of Y_1 receptors (SK-N-MC cells) (e.g. Schwartz *et al*., 1990) and Y_2 receptors (rat hippocampus) (e.g. Kahl *et al*., 1994). It was shown that NPY, PYY and the chimeric NPY-PYY peptides all displaced [^{125}I]-PYY with a high potency. For each peptide the respective IC_{50} values for Y_1 and Y_2 binding sites were: NPY 1.5 and 4.6 nM, NPY(1–23)-PYY(24–36) 2.3 and 2.3 nM, NPY(1–14)-PYY(15–36) 2.0 and 0.9 nM, NPY(1–7)-PYY(8–36) 1.5 and 2.6 nM, NPY(1–3)-PYY(4–36) 1.4 and 1.1 nM and PYY 1.4 and 1.0 nM.

Discussion

NPY is known to depress synaptic transmission in various parts of the brain, such as in the rat hippocampus (Colmers & Bleakman, 1994) and rat locus coeruleus (Illes & Regenhold, 1990). In agreement with these observations, the present study has shown that NPY evoked a concentration-dependent inhibition of the e.p.s.c. and i.p.s.c., events which reflect a reduction of transmitter release onto neurones of the rat dorsomedial NTS.

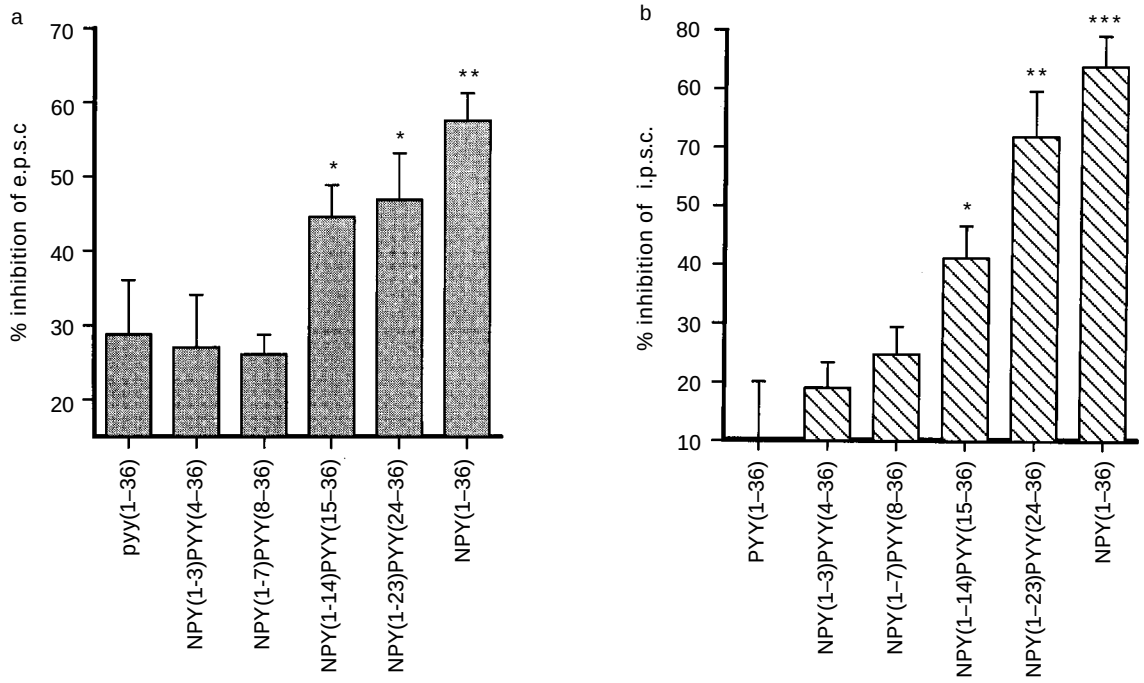

Figure 3 The effects of neuropeptide Y (NPY) and a series of chimeric NPY-PYY peptides, and lack of effects of PYY and chimeric peptides with long C-terminal PYY content on electrically stimulated (a) excitatory postsynaptic currents (e.p.s.cs) and (b) inhibitory postsynaptic currents (i.p.s.cs) in neurones of rat dorsomedial nucleus tracts solitarius (NTS). Significant differences from control recordings $^{*}P<0.05$, $^{**}P<0.01$; $n=4-7$ for each peptide. All peptides were applied at 500 nM concentration.

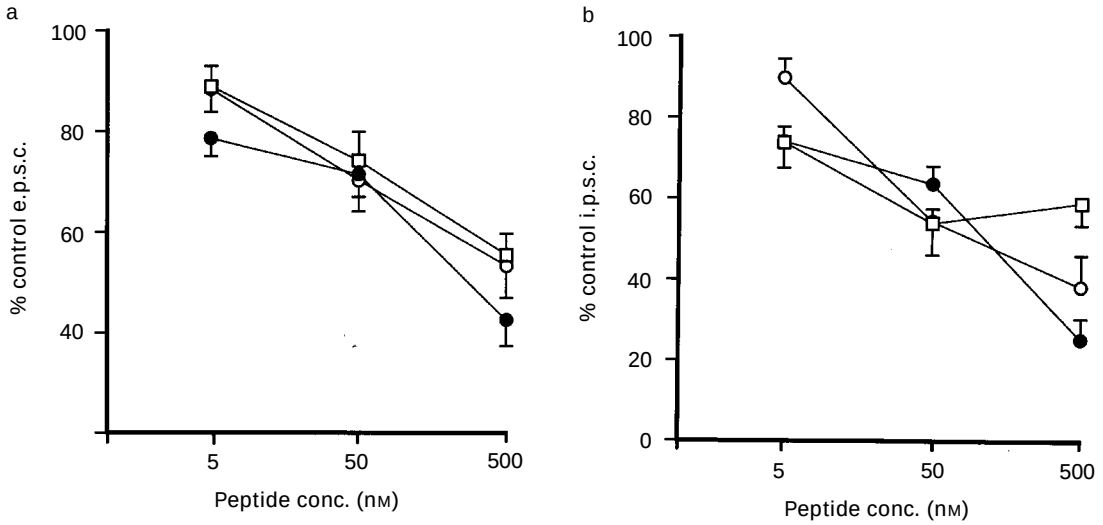

Figure 4 Concentration-response curves for neuropeptide Y (NPY) (●), NPY(1–23)-PYY(24–36) (○) and NPY(1–14)-PYY(15–36) (□) on (a) electrically stimulated excitatory postsynaptic currents (e.p.s.cs) and (b) inhibitory postsynaptic currents (i.p.s.cs) of rat dorsomedial nucleus tractus solitarius. $n=4-6$ for each concentration.

Central prejunctional NPY receptors share certain characteristics with prejunctional $GABA_B$ receptors. *In vitro*, both receptor types depress the e.p.s.c. and i.p.s.c. in the NTS and inhibit transmitter release in a Ca^{2+}-dependent manner (Colmers *et al.*, 1988; Brooks *et al.*, 1992; Colmers & Bleakman, 1994; Wall & Dale, 1994; Brooks & Glaum, 1995). However, there are also notable differences between NPY receptor and $GABA_B$ receptor activity in the NTS. While baclofen markedly hyperpolarizes NTS neurones via a G-protein-dependent activation of K^+ channels (Brooks *et al.*, 1992), NPY did not produce any discernable effects upon membrane properties of dorsomedial NTS neurones. Thus, while projections to the dorsomedial NTS from the region of tractus solitarius may have both presynaptic $GABA_B$ and NPY receptors, intrinsic dorsomedial NTS neurones appear to lack functional NPY receptors.

The type of NPY receptor that mediates the inhibitory effects on e.p.s.cs and i.p.s.cs in the NTS appears to be NPY-specific, because it did not recognize the other membranes of this peptide family, PYY and PP. Furthermore, the different effects obtained by the peptides cannot be attributed to an absence of functional NPY receptors on projections to dorsomedial NTS neurones, as inhibition by NPY itself was observed. The lack of effect of PYY contrasts with the ligand requirements for Y_1 and Y_2 receptors, both of which are equally well activated by NPY and PYY. In accordance with

the results from the present study, it has previously been shown that unilateral injections of NPY into the NTS evoke a dose-dependent fall in arterial blood pressure and heart rate in the anaesthetized rat, while PYY and PP are inactive (Grundemar *et al*., 1991a,b). This NPY-specific receptor has been referred to as a Y_3 type of receptor, and seems to attenuate also glutamate effects in the NTS and the baroreceptor reflex (Grundemar *et al*., 1991a,b; 1993b). Moreover, autoradiographic studies have shown that NPY, but not PYY (or PP) displace radiolabelled NPY in the rat brainstem, consistent with the presence of NPY-specific receptors in this region (Nakajima *et al*., 1986). It is likely that the similarities of the ligand requirements in the present study with those in the *in vivo* study of NTS (Grundemar *et al*., 1991b) reflect activation of a common NPY receptor type. Receptors or binding sites corresponding to Y_3 receptors have also been suggested in certain peripheral tissues, such as the rat superior cervical ganglia (Foucart *et al*., 1993), bovine adrenal medulla (Higuchi *et al*., 1988; Wahlestedt *et al*., 1992; Nörenberg *et al*., 1995), rat colon (Dumont *et al*., 1994) and rat cardiac myocytes (Balasubramaniam *et al*., 1990). However, the NPY-specific Y_3 receptor has remained poorly characterized. For instance, there is a lack of generally available sources of Y_3 receptor-containing membranes or cell lines and there are problems in binding studies with radiolabelled NPY, which gives a high degree of unspecific binding.

A series of chimeric NPY-PYY peptides have been synthesized where an N-terminal part of NPY has been linked with a C-terminal end of PYY. The N- and C-terminal parts of the peptide were joined at positions where NPY and PYY sequences differ. In the binding study the chimeric peptides behaved as expected, since they were all equipotent with NPY and PYY on Y_1 and Y_2 binding sites, respectively. These peptides were subsequently used to identify which unique amino acid residues in the NPY molecule are important for activation of Y_3 receptors. NPY(1–23)-PYY(24–36) and NPY(1–14)-PYY(15–36) evoked a concentration-dependent inhibition of e.p.s.cs and i.p.s.cs, while NPY(1–7)-PYY(8–36) and NPY(1–3)-PYY(4–36)-PYY were inactive. It seems that an important part of the biological activity resides in positions 13 and 14, which are the only positions that differ between the active peptide NPY(1–14)-PYY(15–36) and the inactive peptide NPY(1–7)-PYY(8–36). The amino acid residues are Pro13 and Ala14 in the NPY molecule and Ser13 and Pro14 in the PYY molecule. In the case of Y_1 and Y_2 receptors, the central part of the NPY/PYY molecules does not serve as a signal epitope, but seems to keep the N- and C-terminal ends in a proximity for receptor recognition (Schwartz *et al*., 1990; Grundemar *et al*., 1993a,b). It is possible that Pro (an amino acid residue known to alter the helical structure) in position 14 creates a conformation of the PYY molecule, which is not recognized by the Y_3 receptor. Certain fragments of NPY, such as NPY 13–36 and NPY 16–36 (Grundemar *et al*., 1991b; Nörenberg *et al*., 1995) have been shown to be about equipotent with the native peptide on Y_3 receptors, indicating that the C-terminus contains a signal epitope for Y_3 receptor recognition.

Both [Leu^{31},Pro^{34}]NPY and [Pro^{34}]NPY have been claimed to be Y_1 receptor-specific agonists and been much used for identification and characterization of Y_1 receptors in the brain and periphery (e.g. Fuhlendorff *et al*., 1990; Schwartz *et al*., 1990; Grundemar *et al*., 1992). Interestingly, the present study has shown that [Pro^{34}]NPY is equipotent with NPY on inhibitory synaptic transmission, and this ligand was also equipotent with NPY on cardiovascular function when injected into the rat NTS *in vivo* (Grundemar *et al*., 1991b). Furthermore, [Pro^{34}]NPY was shown to be only slightly less potent than NPY on Y_3 binding sites in the bovine adrenal medulla (Wahlestedt *et al*., 1992). Hence, it seems that [Pro^{34}]NPY cannot be regarded as an Y_1 receptor specific agonist, since it is also readily recognized by Y_3 receptors. In contrast, [Leu^{31},-Pro^{34}]NPY was inactive in the present study and this peptide was also shown to be much less potent than NPY on Y_3 receptors in the bovine adrenal medulla (Nörenberg *et al*., 1995). Thus, it seems that Leu in position 31, instead of the naturally occurring Ile disrupts Y_3 (but not Y_1) receptor recognition. However, this seems only to apply to the situation where Pro is at position 34, since Val, which is present at position 31 in all functionally active NPY-PYY chimeric peptides did not impair Y_3 receptor recognition.

The ligand requirements of the NPY receptors mediating the inhibitory effects on synapatic transmission were quite distinct from those of Y_1, Y_2 or PP receptors, and although the Y_3 receptor has not yet been cloned, the present results support the existence of the Y_3 receptor as a separate entity. The rank order of potency of various NPY analogues at the Y_3 receptor was shown to be: NPY = [Pro^{34}]NPY = NPY(1–23)-PYY(24–36) > NPY(1–14) - PYY(15–36) > > > NPY(1–7) - PYY(8–36),PYY, PP, [Leu^{31},Pro^{34}]NPY.

Taken together, the present results have shown that NPY acts at Y_3 receptors to suppress both excitatory and inhibitiory currents in the rat dorsomedial NTS. The different efficacy of the chimeric NPY-PYY peptides suggest that positions 13 and 14 are of great importance for Y_3 receptor recognition. In addition, this receptor type readily recognizes [Pro^{34}]NPY, but not [Leu^{31},Pro^{34}]NPY, which makes the former peptide less useful as a specific Y_1 receptor agonist.

This study was supported by NINDS 25PONS 21442-0981 and by PHS grants DA2575, DA02121, MH40165 and Crafoord foundation.

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(Received June 17, 1996
Revised September 23, 1996
Accepted October 1, 1996)