Characterization of a Selective Antagonist of Neuropeptide Y at the Y2 Receptor

SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF A Y2 ANTAGONIST

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Neuropeptide Y (NPY) is a potent inhibitor of neurotransmitter release through the Y2 receptor subtype. Specific antagonists for the Y2 receptors have not yet been described. Based on the concept of template-assembled synthetic proteins we have used a cyclic template molecule containing two β-turn mimetics for covalent attachment of four COOH-terminal fragments RQRYNH2 (NPY 33–36), termed T4-[NPY(33–36)]. This structurally defined template-assembled synthetic protein has been tested for binding using SK-N-MC and LN319 cell lines that express the Y1 and Y2 receptor, respectively. T4-[NPY(33–36)] binds to the Y2 receptor with high affinity (IC50 = 67.2 nM) and has poor binding to the Y1 receptor. This peptidomimetic tested on LN319 cells at concentrations up to 10 μM shows no inhibitory effect on forskolin-stimulated cAMP levels (IC50 for NPY = 2.5 nM). Furthermore, we used confocal microscopy to examine the NPY-induced increase in intracellular calcium in single LN319 cells. Preincubation of the cells with T4-[NPY(33–36)] shifted to the right the dose-response curves for intracellular mobilization of calcium induced by NPY at concentrations ranging from 0.1 nM to 10 μM. Finally, we assessed the competitive antagonistic properties of T4-[NPY(33–36)] at presynaptic peptidergic Y2 receptors modulating noradrenaline release. the compound T4-[NPY(33–36)] caused a marked shift to the right of the concentration-response curve of NPY 13–36, a Y2-selective fragment, yielding a pA2 value of 8.48. Thus, to our best knowledge, T4-[NPY(33–36)] represents the first potent and selective Y2 antagonist.

Neuropeptide Y (NPY) is a 36-aminoc acid peptide amide distributed widely in the central and peripheral nervous system (1–3). NPY exerts many biological effects, especially on cardiovascular, metabolic, food intake, behavior, anxiety, and endocrine regulation (4). Several lines of evidence suggest potential roles for NPY in the pathophysiology of hypertension, obesity, diabetes, and psychiatric disorders (5). NPY acts through a number of G-protein-coupled receptors termed Y1, Y2, Y4/PP, Y3, and Y1-like receptors (4). Only Y1, Y2, and Y4/PP receptors have been cloned (6–10). Y1 receptors are present in the sympathetic nervous system mainly postsynaptically and mediate vasoconstriction. Those of the Y2 subtype are present prejunctionally and inhibit the release of catecholamines (11). Furthermore, it has been demonstrated that NPY Y2 receptors are located on noradrenergic nerve terminals within the hypothalamus and other brain regions, exerting an inhibitory action on [3H]norepinephrine release evoked by appropriate concentrations of potassium ions (12). Y2 receptors are also involved in endocrine control; NPY has been reported to inhibit through the Y2 receptor potassium-stimulated glutamate release (13), α-melanocyte-stimulating hormone release (14), release of luteinizing hormone in a steroid-free environment (15), prolactin release (16), and to potentiate the secretion of vasopressin from the neurointermediate lobe of the rat pituitary gland (17).

NPY belongs to the pancreatic polypeptide family characterized by a common helical structure termed PP fold (18). NPY consists of a polyproline type II helix for residues 1–8 followed by a β-turn through positions 9–14, an amphipathic α-helix at 15–32, and a flexible COOH terminus at 33–36 (18). The complete sequence of NPY is needed for binding to the Y1 receptor, whereas COOH-terminal fragments are selective for the Y2 receptor (19). Alanine scan studies performed on the NPY molecule have shown that the COOH-terminal part of NPY is essential for its biological activity on the Y1 and the Y2 receptors; the COOH-terminal pentapeptide amide is important for both receptors and probably represents the binding site (20). However, Arg-33 and Arg-35 may not be exchanged by t-alanine in the Y1 system, whereas Arg-35 and Tyr-36 are the most critical residues for the Y2 receptor. Based on these observations, specific peptidic and non-peptidic compounds have been designed providing potent Y1-selective antagonists (21–23). Earlier, we generated a cyclic truncated analog of NPY, [Ahx5–24,γ-Glu2–β-Lys36] NPY, which acts as a specific agonist for the Y2 receptor (24); however, no antagonists specific for the Y2 receptor have been described yet. NPY fragments shorter than NPY 27–36 are no longer able to bind to the Y2 receptor.

The assembly of bioactive peptides on topological template molecules according to the template-assembled synthetic proteins (TASP) concept has been shown to induce or stabilize specific conformations of various peptides and consequently, to modify their biological and pharmacokinetic properties (25). We have, for example, synthesized a TASP molecule able to bind to and to stimulate selectively the angiotensin II AT2 receptor (26). Here, we design a molecule composed of four truncated NPY peptide fragments (NPY 33–36) attached to a cyclic car-
T4 contains four lysine residues acting as attachment sites, and the cyclic template (I). A new methodology based on the analysis of Ca\(^{2+}\) ions, using an increase in intracellular calcium induced by NPY using a tetrakis aldehyde T4 was dissolved in 1 M sodium acetate, and the pH was adjusted to 5 with acetic acid. A 1.2-fold excess of the tetrapeptide was added, and the mixture was stirred at room temperature for 15 h. Bound radioactivity was determined by γ-counting. Half-maximal inhibition of the binding, obtained with 125I-NPY, is given as IC\(_{50}\). Each point represents the mean ± S.D. of at least four experiments.

**Materials and Methods**

Porcine NPY, NPY 13–36, and Leu-31-Pro-34 NPY were purchased from Novabiochem (Läufelfingen, Switzerland). Fluoro-3/AM and pluronic acid were obtained from Molecular Probes (Eugene, OR). Fluo-3/AM and pluronic acid were obtained from Molecular Probes (Eugene, OR), the Ca\(^{2+}\) ionophore 4-bromo A-23187 was from Sigma. Tween 20 was from Pierce.

**Synthesis of the TASP molecule T4-[NPY(33–36)]\(_4\)**

For the effective synthesis of the TASP (27) T4-[NPY(33–36)]\(_4\) (III in Fig. 1) chemoselective ligation methods were applied. Oxime bond formation (28) was used to attach the functionally modified NH\(_2\)-terminal (aminoxy group) peptide fragments to the cyclic peptide template, T4 (I). T4 contains four lysine residues acting as attachment sites and the \(\beta\)-turn mimic 8-aminomethyl-2-naphthoic acid (29). The \(\varepsilon\)-amino groups of the Lys residues were transformed to aldehydic functions by reaction with glyoxylic acid 1,1-diethylacetal and subsequent hydrolysis (30) to yield the unbound fraction by centrifugation.

**Cell Cultures**

SK-N-MC cells were derived from a human neuroblastoma and were cultured according to the American Type Cell Culture recommendations.
to be tested in varying dilutions. Cells were washed once in 100 mM sodium phosphate buffer (pH 7.5) and lysed with 0.75 ml of 0.1 M HCl. After centrifugation, the supernatant was recovered and lyophilized. cAMP concentration was measured by a radioimmunoassay using a commercially available kit (Amersham).

Antagonistic Properties of T₄-NPY(33–36)₄ on the Free Cytosolic Calcium Response to NPY

LN319 cells were plated on glass coverslips 48 h before intracellular free calcium measurements. Intracellular free calcium concentration [Ca²⁺]ᵢ was determined using the fluorescent probe fluo-3/AM. The dye was loaded into the cells by adding the acetoxymethyl ester fluo-3/AM (2.5 μM) from a 1 mM stock in dry dimethyl sulfoxide to the culture medium (Dulbecco’s modified Eagle’s medium without serum or complements) and incubating the cells for 30 min at room temperature in the dark. Pluronic acid (2 μl/ml) in 25% dimethyl sulfoxide was added to fluo-3/AM to disperse the dye. After loading, the cells were washed three times with medium and placed in a chamber with 0.5 ml of physiological saline solution containing 140 mM NaCl, 2 mM CaCl₂, 4.6 mM KCl, 1.0 mM MgCl₂, 10 mM glucose, and 10 mM Hepes (pH 7.4). Tween 20 (Pierce) 0.0008% was present in the medium to prevent NPY from sticking to the walls of the exposed surfaces. Fluorescence images of the intracellular calcium localization were obtained with a laser-scanned confocal microscope (MRC 500 confocal imaging system, Bio-Rad) equipped with an argon ion laser and a fluorescein (488 nm) or rhodamine (514 nm) filter cartridge. The scanner and detectors were attached to an inverted microscope (Diaphot, Nikon).

The confocal microscopy technique with video recording provides serial readings, at 5-s intervals, of the individual fluorescence for a set of cells. The changes in Ca²⁺ were evaluated in single cells on whole images containing 5–15 cells using the NIH image analyzer program (29–385 cells were used to test each concentration of peptides). In each experiment it uses a fixed delineation of the cell borders, entered with a pointer device on the image screen. For each cell, five intensity readings were recorded. The base-line fluorescence was determined by averaging two consecutive images (Fbaseline); the signal induced by adding the T₄-NPY(33–36)₄ solution (Fantag); the peak response induced by adding the NPY solution (FNPY); the maximal response observed after adding the nonfluorescent Ca²⁺ ionophore A-23187 (10 μM) to saturate

FIG. 3. Representative concentration-response curves of the displacement of 125I-NPY by selective peptides for the Y1 (panel A) and Y2 (panel B) receptors in SK-N-MC cells (Y1) and LN319 cells (Y2). Four experiments were performed with each analog. The percentage inhibition of 125I-NPY binding to the receptor, which is caused by the increasing concentrations of competitors, is shown on the y axis. High affinity binding to the Y1 receptor on SK-N-MC cells was found for NPY (○) and Leu-31, Pro-34 NPY (◇). On LN319 cells NPY and NPY 13–36 (▲) exhibited high affinity binding, and T₄-NPY(33–36)₄ (●) slightly reduced affinity. NPY 33–36 (◇) and the template (♦) did not bind to either the Y1 or Y2 receptor.
the intracellular dye with calcium and thereby obtain maximal fluorescence ($F_{\text{max}}$); and the minimal fluorescence ($F_{\text{min}}$) was measured after addition of an excess of EDTA (5 mM). The $F_{\text{antag}}$ values were not used for further analysis, after it had been demonstrated that T$_4$-[NPY(33–36)]$_4$ did not induce any significant response.

The intracytoplasmic calcium concentrations at the peak of the NPY effect were derived from fluorescence readings by using the formula (32) in nmol/liter: $\text{Ca}_{\text{NPY}} = \frac{320}{(F_{\text{max}} - F_{\text{min}})(F_{\text{max}} - F_{\text{NPY}})}$.

Attempts were made to correct $\text{Ca}_{\text{NPY}}$ value for the base-line level; however, as the peak response was poorly correlated with the base-line value ($r^2 = 0.13, r_{\log/log} = 0.24$), this only added noise without modifying the results; so $\text{Ca}_{\text{NPY}}$ was retained as the response variable. It was transformed to logarithmic functions to normalize its distribution. Means and standard deviations of log$_{10}$($\text{Ca}_{\text{NPY}}$) were computed from the individual cell responses for each level of NPY and T$_4$-[NPY(33–36)]$_4$ (their antilog reflecting, respectively, the geometric means and coeffi-

FIG. 4. Frequency distribution of the logarithm of the peak intracellular calcium responses of single cells to NPY challenges at different concentrations, after pretreatment by vehicle only or T$_4$-[NPY(33–36)]$_4$ at three concentrations. See Table I for the corresponding number of cells and geometric means for each NPY and T$_4$-[NPY(33–36)]$_4$ level.

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Antagonist Properties of T4-[NPY(33–36)]4 at Presynaptic Peptidergic Y2 Receptors Modulating Norepinephrine Release

Preparation of Synaptosomes—Nerve endings were prepared from the hypothalamus of adult Wistar rats (200–250 g) according to the method of Gray and Whittaker with minor modifications (34). Briefly, the rat hypothalamus was homogenized using a Teflon-glass tissue grinder (clearance 0.25 mm) in 40 volumes of 0.32 M sucrose buffered at pH 7.40 with phosphate. The homogenate was centrifuged (5 min, 3,000 g) to remove nuclei and debris, and the supernatant was again centrifuged (20 min, 10,000 g) to remove nuclei and debris, and the supernatant was again centrifuged (20 min, 10,000 g) to isolate crude synaptosomes. The synaptosomal pellet (at a protein concentration of 0.6–0.8 mg/ml) was then resuspended in a physiological medium with the following composition (in mM): 125 NaCl, 3 KCl, 1.2 CaCl2, 1.2 MgSO4, 1 NaH2PO4, 22 NaHCO3, 10 glucose, aerated with 95% O2 and 5% CO2 at 37 °C (pH 7.40). Protein was measured by the method of Lowry et al. (35).

Norepinephrine Release Experiments—Synaptosomes were incubated with [3H]norepinephrine (Amersham Radiochemical Center, Buckinghamshire, UK; specific activity, 39 Ci/mmol; 0.05 μM final concentration) for 15 min at 37 °C. The labeled particles were then distributed in several parallel superfusion chambers and superfused at 37 °C with continuously oxygenated medium, at a rate of 0.6 ml/min (36). After 10 min, to equilibrate the system and to reach a constant spontaneous efflux, T4-[NPY(33–36)]4 was added and perfusion continued for a further 15 min. The synaptosomes were depolarized with 15 mM KCl for 90 s (substituting for an equimolar concentration of NaCl). The NPY fragment, NPY 13–36 (Peninsula Laboratories, Merseyside, U.K.) was added concomitantly with K+. Fractions were collected every min, and the radioactivity (present as [3H]norepinephrine) in each fraction and in the filters was determined after separation on Biorex 70 columns (37).

Evaluation of Results—The [3H]norepinephrine found in each fraction collected was calculated as a percentage of the total [3H]norepinephrine recovered (fractions plus filters). The concentration-dependent effects of NPY 13–36 were calculated as follows. The area of baseline efflux curve was subtracted from the area of the total release curve obtained in the absence and in the presence of the compound tested. The areas under the release curves of the time course were recorded for each experiment according to the Newton-Cotes integration formula. Data obtained according to this method were used to calculate the percentage inhibition of the K+-evoked release of [3H]norepinephrine in the presence of NPY 13–36 or in the presence of NPY 13–36 plus T4-[NPY(33–36)]4. The apparent pA2 value for the antagonist was calculated by means of the Schild regression analysis according to the following formula: pA2 = logE/Ei – 1) – logB, where E′ and E are those concentrations of the agonist which caused half-maximum effects in the presence and in the absence of the antagonist, respectively. B is the concentration of the antagonist.

RESULTS

Binding Assays—As described above, the SK-N-MC and LN319 cells express Y1 and Y2 receptor subtypes, respectively. For competitive binding studies, in addition to the native NPY, we used peptides with differential selectivity for Y1 and Y2 binding. The Leu-31- and Pro-34-substituted NPY has been shown to be a Y1 agonist (38), whereas the NPY 13–36 has been reported to bind preferentially to the NPY Y2 receptor subtype. Fig. 3 depicts the results of binding experiments obtained with the two cell lines. SK-N-MC cells (Fig. 3A) bind NPY and Leu-31, Pro-34 NPY equally well as shown by the similar competition displacement curves (Fig. 3A). In contrast, NPY 13–36 binding was 2,000-fold less as this cell line does not express Y2 receptors.
Neither the template nor NPY33–36 bound to SK-N-MC cells (IC_{50} > 10 μM), and T_{4}[NPY(33–36)]_{4} shows only a poor affinity for the Y1 receptor (IC_{50} = 6.6 μM). The LN319 cells (Fig. 3B) exhibited a comparable high affinity for NPY and NPY 13–36 with IC_{50} of 0.085 and 0.126 nM, respectively. In contrast, Leu-31, Pro-34 NPY bound poorly to the Y2 receptor. Similar to the observation for the Y1 receptor, neither the template nor NPY 33–36 exhibited affinity for LN319 cells (IC50 >10 μM), but good binding to the Y2 receptor was obtained with T_{4}[NPY(33–36)]_{4} (IC_{50} = 67.2 nM). T_{4}[NPY(33–36)]_{4} was also added to angiotensin II type 1 and type 2 and muscarinic receptor preparations at concentrations up to 10 μM, and there was no binding. A slight interaction was observed at a high concentration of the antagonist in binding experiments with the a1B-adrenergic receptor (42% inhibition at 20 μM).

**cAMP Measurements**—To assess whether this peptidergic had intrinsic agonist properties we tested its ability to inhibit cAMP accumulation in LN319 cells. Whereas NPY inhibits forskolin-stimulated cAMP accumulation in LN319 cells with an IC_{50} of 2.5 nM, T_{4}[NPY(33–36)]_{4} at concentrations up to 10 μM was devoid of effect.

**Calcium Measurements in LN319 Cells**—During the study, 3,674 individual cell responses were measured, with an average number of 131 responses (range 30–385) for each concentration of NPY and T_{4}[NPY(33–36)]_{4}. The distribution of individual peak cytosolic calcium responses for each concentration of NPY and T_{4}[NPY(33–36)]_{4} is shown on Fig. 4. Preincubation of the cells for 1 min with T_{4}[NPY(33–36)]_{4} had no agonistic effect. Logarithmic expression conferred a quite symmetrical distribution of the intracellular calcium level observed after adding various concentrations of NPY, in individual T_{4}[NPY(33–36)]_{4} pretreated by vehicle only (○), by T_{4}[NPY(33–36)]_{4} at 1,000 (△), 5,000 (●), and 10,000 nM (□). The lines represent the predicted response according to the pharmacokinetic model fitted to the individual data, for the same values of T_{4}[NPY(33–36)]_{4} (r² = 0.91). Vehicle, —; T_{4}[NPY(33–36)]_{4}; 1 μM (— —), 5 μM (— —), 10 μM (— —).
pended manner (IC$_{50}$ values in the presence of 1, 10, and 100 nM were 9, 22, and 109 nM, respectively). The concentration-response curve of the NPY 13–36 fragment was shifted to the right by the addition of 10 nM antagonist (Fig. 7). The antagonist appeared competitive for the Y2 receptor that modulated norepinephrine release because the effect of each antagonist concentration could be overcome by high doses of the agonist NPY 13–36. To assess competitive antagonism of T$_4$-[NPY(33–36)]$_4$, the apparent pA$_2$ value for this compound was determined using three different concentrations (1, 10, and 100 nM). The half-maximum inhibitory concentrations of the NPY 13–36 fragment on norepinephrine release obtained in the presence of various concentrations of the antagonist have been used in the Schild regression analysis. The compound T$_4$-[NPY(33–36)]$_4$ caused a marked shift to the right of the concentration-response curve of the NPY 13–36 fragment, yielding a pA$_2$ value of 8.48. The pA$_2$ value of the antagonist T$_4$-[NPY(33–36)]$_4$ was calculated from concentration-response curves corresponding to those shown in Fig. 7.

**DISCUSSION**

The present study shows that T$_4$-[NPY(33–36)]$_4$ is a potent and selective ligand for the NPY Y2 receptor and exhibits in vitro antagonistic properties in two models of Y2-mediated effects. Several NPY Y1 receptor antagonists have been characterized and demonstrated to inhibit the pharmacological vasopressor effect of NPY. This compound displays a high affinity for the Y2 receptor (IC$_{50}$ = 62 nM) but exhibits 2 orders of magnitude less interaction with the Y1 receptor. The antagonistic properties of the molecule have been confirmed by demonstrating inhibition of function.

For these functional studies, we used an original experimental approach involving single-cell recordings of cytosolic calcium responses to a biochemical stimulus. Other investigators have reported a similar technique on a limited number of cells (39–41). The ability to perform hundreds or thousands of single-cell measurements required the development of appropriate statistical methods to cope with the large amount of data. The pharmacological characterization of an antagonist with this new methodology cannot rely on simple Shild plots drawn across average points. Thus, we used computer-based nonlinear regression methods, with special attention to the variability structure displayed by the data.

Preliminary explorations led us to use the intracytoplasmic calcium concentrations at the peak of the NPY effect as the response variable. Correction of this value for the base-line calcium level could be theoretically justified. However, this only added noise to the data, as the peak response was poorly correlated with the base-line value, and this correction was discarded. The response variable showed a strongly skewed distribution. A logarithmic transformation brought the results closer to a Gaussian distribution, allowing the application of a least square approach to estimate pharmacodynamic parameters. This observa-
tion indicates a log-normal behavior of calcium concentrations, consistent with its contamination by multiplicative randomness and warrants the description of results by geometric rather than arithmetic means. Biologically, the NPY stimulus is indeed linked to the intracyttoplasmic calcium response by a cascade of amplification steps. The high response variability must be emphasized: most coefficients of variation associated with the geometric means exceeded 100%. A pharmacodynamic study could have recorded the average response of a cluster of cells, a piece of tissue, or a whole organ. In that situation, however, only a global effect would have been measured, without consideration for the heterogeneity of individual cell responses. Moreover, averaging would have distorted the signal in many ways. Thus, the results shown here may not reflect what might be observed in multiple cell or tissue preparations. On the other hand, the individual cell behavior itself can be considered as the integration of many subcellular quantal responses (42, 43). Interestingly, when the same procedure of calculation was used for only the responding cells, we obtained an almost identical mathematical model. This finding may indicate that the free calcium response to NPY does not obey an all-or-none rule. Thus, the shape of the dose-effect relationship at the cellular level not only reflects the dose-effect relationship at the subcellular scale but also its variable nature (42).

Despite the high number and variability of the single-cell responses, a pharmacodynamic model was fitted satisfactorily to the whole data set by nonlinear regression. We have chosen a dual Hill model, able to account for the fading of the response at high doses, which is frequent in the field of peptide pharmacology. This model included a competitive antagonism, which adequately describes the pharmacodynamics of T₄[NPY(33–36)] in this experimental setting. However, all terms of the model must be interpreted with caution. As this was not a binding study, the EC₅₀, FC₅₀, and AC₅₀ cannot simply be equated with receptor affinities. Neither can they be considered equivalent to values measured in a living organism. This may account for the apparent differences observed between the pharmacodynamic parameters of the two functional assays we have used. Nevertheless, we cannot exclude the possibility that LN319 cells and hypothalamic synaptosomes exhibit different Y2 receptors (44). The slope coefficients γ and δ have also no straightforward interpretation; they rely strongly on the variance structure of the underlying phenomena (42).

Second, T₄[NPY(33–36)] also inhibited in a dose-dependent manner the action of a Y2 agonist on K⁺-induced [³H]noradrenaline release from perfused rat hypothalamic synaptosomes. This effect was prevented by a large dose of the Y2 agonist NPY 13–36, demonstrating that T₄[NPY(33–36)] acts as a fully competitive antagonist.

No intrinsic agonistic properties either on calcium or cAMP transduction systems were observed with T₄[NPY(33–36)] using LN319.

The NPY gene has recently been disrupted in mice (45). These NPY-deficient mice exhibit seizures, an effect that may be due to inhibition of glutamate release which is known to be mediated by Y2 receptors present on the endings of presynaptic excitatory neurons of NPY (13). Therefore, this compound may be a useful tool for the study of the role of NPY in various disorders such as seizures or abnormalities in the reproductive axis.

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