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Y_1 and Y_2 receptors for neuropeptide Y

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By using monoiodinated radioligands of both intact neuropeptide Y (NPY) and of a long C-terminal fragment, NPY^{13-36}, two subtypes of binding sites, which differ in affinity and specificity, have been characterized. The Y_1 type of binding site, characterized on a human neuroblastoma cell line, MC-IXC, and a rat pheochromocytoma cell line, PC-12, binds NPY with a dissociation constant (K_d) of a few nanomolar but does not bind NPY^{13-36}. The Y_2 type of binding site, characterized on porcine hippocampal membranes and on another human neuroblastoma cell line, SMS-MSN, is of higher affinity and binds both NPY and NPY^{13-36}. None of the binding sites distinguish between NPY and the homologous peptide YY (PYY). It is concluded that NPY/PYY-binding sites occur in two subtypes which may represent two types of physiological receptors.

Neuropeptide Y; Hippocampus; Brain receptor; (Neuroblastoma cell line, PC-12 cell)

1. INTRODUCTION

Neuropeptide Y (NPY) is an important regulatory peptide in both the central and peripheral nervous system [1,2]. Centrally, NPY is thought to be involved in regulation of food intake, memory processing and circadian rhythm [3–6]. In the periphery, NPY seems to function as a transmitter in sympathetic nerves where it interacts with norepinephrine mainly in the regulation of vascular tone [7–9]. NPY is also found in non-adrenergic neurones e.g. in the gastrointestinal tract [10,11].

The primary structure of the 36 amino acid NPY has been well conserved during evolution [12]. The peptide belongs to the pancreatic polypeptide-fold (PP-fold) family of peptides, which are characterized by a common tertiary structure, the so-called PP-fold [13]. This structural feature consists of two anti-parallel helices, an N-terminal polyproline helix and a long amphipathic α-helix, connected by a β-turn [14]. The PP-fold is stable, even in dilute aqueous solution, as indicated by circular dichroism studies [13–15]. In addition to NPY, pancreatic polypeptide (PP), a pancreatic hormone [16] and peptide YY (PYY), a hormone from the lower intestine [17] belong to the family.

Specific-binding sites for NPY have been described in membrane preparations from brain tissue [18–21], and in membranes from blood vessels [22] as well as on rat pheochromocytoma cells [23]. It has been suggested that in the sympathetic nervous system, two distinct subtypes of NPY receptors are responsible for the pre-synaptic and post-synaptic effects of the peptide [24]. In the test systems initially used, the post-junctional effect, vasoconstriction, could only be obtained with the intact NPY molecule, whereas a long C-terminal fragment, NPY^{13-36}, was sufficient to elicit pre-junctional inhibition of stimulated norepinephrine release [24]. However, these subtypes of NPY receptors were not generally accepted, mainly because the long C-terminal fragment in other biological test systems was capable of producing vasoconstriction [21,25]. In

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the present paper, we have used the long C-terminal fragment of NPY both in the labeled and unlabeled form to characterize two types of NPY-binding sites occurring on brain membranes and cell lines.

2. MATERIALS AND METHODS

2.1. *Peptides*

Porcine NPY^{1-36} and porcine PYY^{1-36} were purchased from Peninsula (St. Helens, England). Porcine NPY^{13-36} and porcine PYY^{13-36} were custom synthesized by Ferring, Sweden. The peptides were characterized and quantitated by several peptide chemical means, among which a radioimmunoassay (using rabbit antiserum no.8999) which is specific for the common C-terminal, amidated hexapeptide of NPY and PYY.

2.2. *Radioactive ligands*

NPY^{1-36} and NPY^{13-36} were iodinated by $Na^{125}I$ (Amersham) using the oxidative agent 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Scrva) as described in detail previously for NPY and PP [23,26]. The radioligands were purified by reverse-phase high-performance liquid chromatography (HPLC) using a Nucleosil 300-5 C_{18} column, 0.4 × 25 cm, with a flow rate of 1 ml/min at 50°C. For NPY^{1-36}, the column was eluted isocratically with 35% acetonitrile in 0.1% trifluoroacetic acid/water, and for NPY^{13-36} with 31% acetonitrile. Through this procedure, it was possible to prepare radioligands which were monoiodinated in specific positions. The radioligands were characterized by HPLC mapping of tryptic fragments on a Nucleosil C_{18} column (as above) eluted with a gradient of acetonitrile: 0–15% in 7 min, 15–20% from 7 to 22 min, and 20–50% from 22 to 30 min. The column was calibrated with tryptic fragments of 'cold'-iodinated NPY, and the radiolabeled fragments were further characterized with respect to mono- or di-iodination by HPLC of the radiolabeled amino acids after pronase digestion [26]. [^{125}I-Tyr^{36}]MonoiodoNPY$^{1-36}$, with a specific radioactivity of 1900 Ci/mmol, and [^{125}I-Tyr^{36}]monoiodoNPY$^{13-36}$, with a specific activity of 1550 Ci/mmol, were used for binding experiments in the present study.

2.3. *Cell culture*

The human neuroblastoma cell lines MC-IXC, a subclone of SK-N-MC, and SMS-MSN [27], were kindly provided by Dr June Biedler (Sloan Kettering Memorial Institute, NY, USA) and PC12 cells [28], subclone II-250, by Dr Hans Thoenen (Max-Planck-Institut für Psychiatrie, Martinsried, FRG). MC-IXC and SMS-MSN cells were grown at 5% CO_2 in a 1:1 mixture of Ham's F-10 medium and Dulbecco's modified Eagle's medium 1885 with 15% fetal calf serum and 1% non-essential amino acids, 2 mM glutamine and penicillin/streptomycin (100 IU and 100 g/ml, respectively). PC12 cells were grown at 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 5% fetal calf serum. All media and materials for tissue culture were from Gibco (Uxbridge, England).

2.4. *Binding experiments*

2.4.1. Cells

As described in detail previously for PC12 cells [23], binding studies with cells were performed with 25 pM of radioligand at 37°C using triplicates of 1.2×10^6 cells which had been preincubated for 2 days in petri wells, 6-well culture plates (Costar, Cambridge, MA, USA) coated with poly-Lys-Ala. The incubation time was standardized to 60 min, although experiments with PC-12 cells were performed with 30 min incubations.

2.4.2. Brain membranes

Synaptic membranes were prepared from porcine hippocampal tissue according to the method of Gray and Wittaker [29], in which the P_2 fraction was subfractionated on a discontinuous sucrose gradient and membranes from the interface between concentrations of 0.8 and 1.2 M sucrose were used. Binding was performed with 25 pM of radioligand at 37°C in 1 ml of a 25 mM Hepes buffer, pH 7.4, containing 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 g/l bovine serum albumin and 0.5 g/l bacitracin with a final concentration of 200 mg of membrane protein/l. After 60 min, triplicates of 0.25 ml were transferred to polypropylene tubes, 0.4 × 4.5 cm, and the membranes were centrifuged through 0.2 ml of ice-cold binding buffer. The pellet was washed gently and the tip of the tube was cut off and counted.

2.4.3. *Rebinding experiments*

In order to study the degradation of the radioligands during incubations, the tracers were incubated with either cells or hippocampal membranes for various time periods whereafter the binding buffer with unbound peptide was removed and subjected to rebinding with hippocampal membranes.

3. RESULTS

Specific steady-state binding to hippocampal membranes and cells was obtained with radiolabeled NPY^{1-36} within 45 min. The hippocampal membranes bound 27 ± 4% of the radioligand in the absence of cold peptide (mean ± SE); and this was reduced to 5 ± 1% (non-specific binding) in the presence of excess amounts of cold peptide, 10^{-6} M NPY^{1-36}. Specific and non-specific binding of radiolabeled NPY^{1-36} to the different cell lines were: SMS-MSN, 4.1 and 1.6%; PC-12, 4.8 and 1.4%, and MC-IXC, 3.8 and 1.6%.

The binding sites for NPY appeared to fall into two categories which differed with respect to affinity and specificity. In the hippocampal membranes and the SMS-MSN cells the radiolabeled NPY^{1-36} was displaced by unlabeled NPY^{1-36} with an apparent 50% inhibitory concentration (IC_{50}) of 0.5 and 0.1 nM, respectively, and by NPY^{13-36} with an apparent IC_{50} of 2 nM in both systems (fig.1). In contrast, the apparent IC_{50} for NPY^{1-36} was 5 and 6 nM in MC-IXC and PC-12 cells,

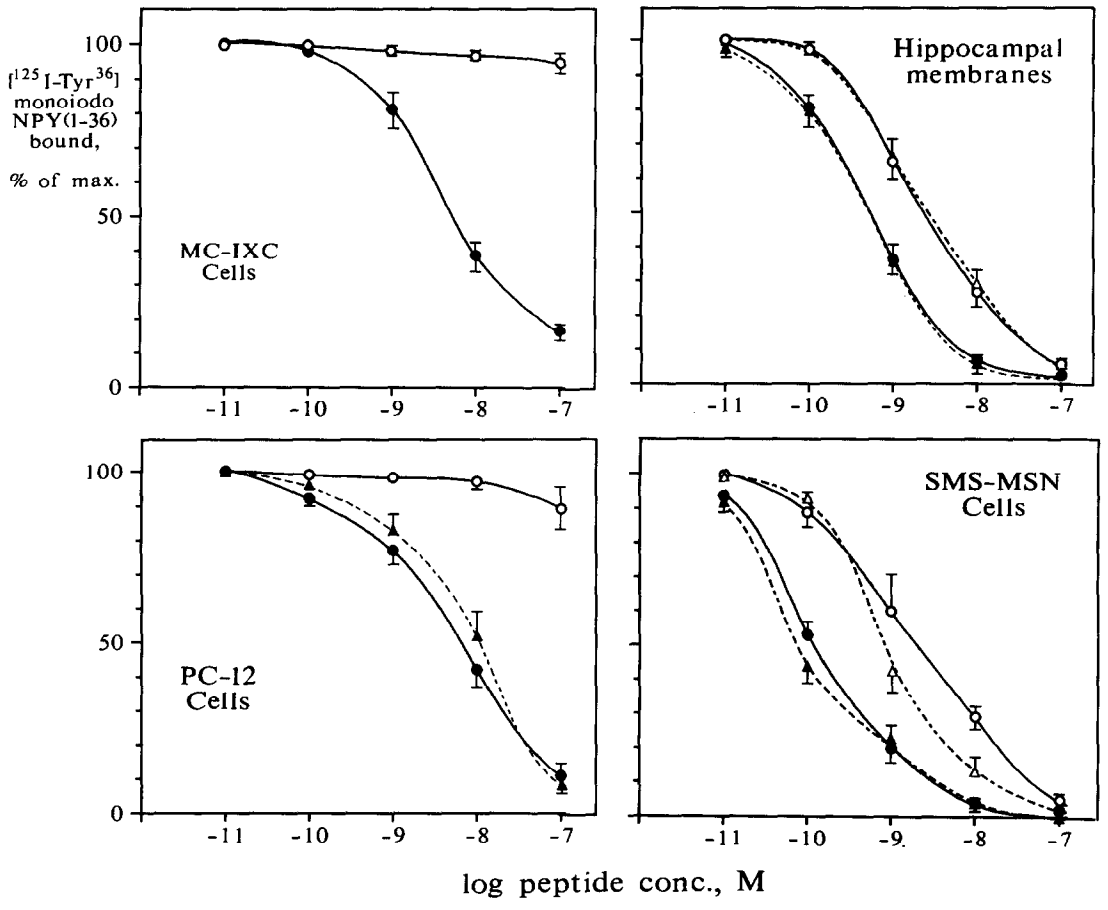

Fig.1. Inhibition of [^{125}I-Tyr^{36}]monoiodoNPY$^{1-36}$ binding by NPY, PYY and fragment 13–36 of NPY and PYY. NPY$^{1-36}$ (●), NPY$^{13-36}$ (○), PYY$^{1-36}$ (▲), and PYY$^{13-36}$ (△) were used for displacement. Experiments were performed in PC-12 cells (N = 12, NPY$^{1-36}$; N = 6 for both PYY$^{1-36}$ and NPY$^{13-36}$), in MC-IXC cells (N = 13 for NPY$^{1-36}$; N = 5 for NPY$^{13-36}$), in SMS-MSN cells (N = 5 for all peptides), and on hippocampal membranes (N = 6 for all peptides).

respectively; whereas NPY$^{13-36}$ in neither of these cell lines, not even in concentrations of 100 nM, significantly displaced the radiolabeled NPY$^{1-36}$ (fig.1). By using the nomenclature proposed by Wahlested et al. [24], the binding sites on hippocampal membranes and SMS-MSN cells, for which NPY$^{13-36}$ is a relatively good ligand, could be classified as Y_2 type NPY receptors; whereas the binding sites on PC-12 cells and MC-IXC cells, for which NPY$^{13-36}$ is a poor ligand, are of the Y_1 type. NPY and the homologous PYY could not be distinguished by the Y_1 and the Y_2 types of NPY-binding sites either in the intact molecular form, as shown in PC-12 cells, SMS-MSN cells, and on hippocampal membranes, or as the respective 13–36 fragments as shown in the two Y_2 receptor systems, SMS-MSN cells and hippocampal membranes (fig.1).

In order to produce a specific Y_2 receptor radioligand, NPY$^{13-36}$ was radiolabeled. As shown in fig.2, it was possible by HPLC to isolate [^{125}I-Tyr^{36}]monoiodoNPY$^{13-36}$ from the ascending part of the composed peak of differently radiolabeled peptides. Specific steady-state binding of the radiolabeled NPY$^{13-36}$ was obtained within 45 min and maintained for 2 h in hippocampal membranes and SMS-MSN cells. The specific binding of monoiodinated NPY$^{13-36}$ to hippocampal membranes was saturable and of high affinity as shown in fig.3. The linearity of Scatchard plots of the binding experiments with [^{125}I-Tyr^{36}]monoiodo-NPY$^{13-36}$ indicates that NPY$^{13-36}$ binds to a

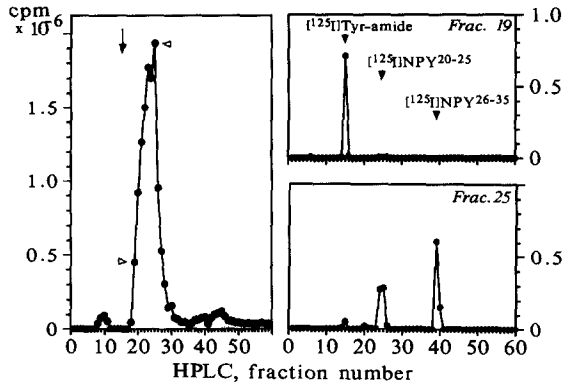

Fig.2. Purification and characterization of ^{125}I-labeled NPY$^{13-36}$ for receptor binding. (Left panel) The HPLC profile of NPY$^{13-36}$, iodinated as described in detail in the text, and purified on a Nucleosil C_{18} column eluted isocratically at 31% acetonitrile in 0.1% TFA/water; 10 l of each fraction were counted. The elution position of unlabeled NPY$^{13-36}$ is indicated by a vertical arrow. (Righthand panels) HPLC mappings of radiolabeled tryptic fragments of fractions 19 and 25 (arrowheads in the left panel); for chromatographic details see text. The elution positions of the radioiodinated fragments are indicated; [^{125}I]Tyr-amide corresponds to [^{125}I]Tyr^{36} of NPY.

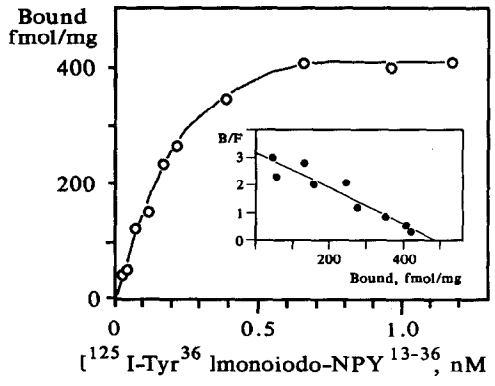

Fig.3. Saturation binding of [^{125}I-Tyr^{36}]monoiodoNPY$^{13-36}$ to porcine hippocampal membranes. The specific binding, i.e. total minus nonspecific binding in the presence of 10^{-6} M NPY$^{13-36}$, of increasing amounts of [^{125}I-Tyr^{36}]monoiodo-NPY$^{13-36}$ to porcine hippocampal membranes is indicated (○). (Inset) Scatchard transformation of the binding data; K_d was 0.15 nM and B_{max} was 485 fmol/mg membrane protein.

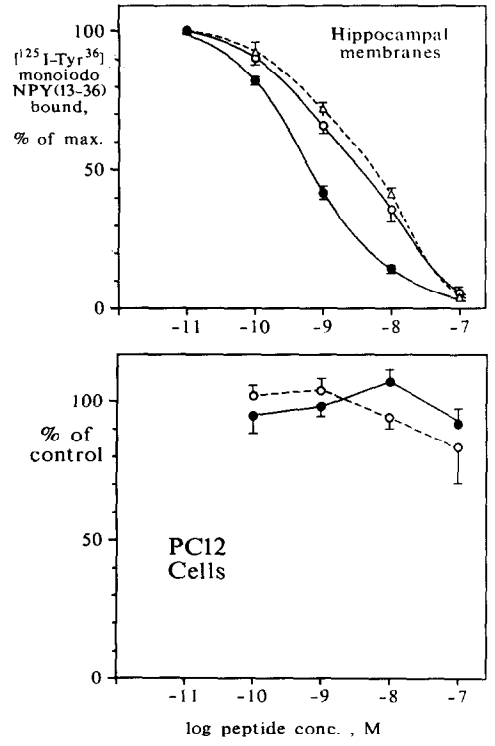

Fig.4. Binding of [^{125}I-Tyr^{36}]monoiodoNPY$^{13-36}$ to hippocampal membranes and PC-12 cells. The binding of [^{125}I-Tyr^{36}]monoiodoNPY$^{13-36}$ to porcine hippocampal membranes was inhibited by NPY$^{1-36}$ (●), $N = 7$; by NPY$^{13-36}$ (○), $N = 8$; and by PYY$^{13-36}$ (△), $N = 6$. Binding of [^{125}I-Tyr^{36}]monoiodo-NPY$^{13-36}$ to PC-12 cells was not significantly inhibited by NPY$^{1-36}$, $N = 4$, or by NPY$^{13-36}$, $N = 4$.

homogeneous population of binding sites (fig.3B). The dissociation constant was calculated to be 0.15 nM and the concentration of binding sites 485 fmol/mg membrane protein. The radiolabeled NPY$^{13-36}$ was displaced from the hippocampal membranes by NPY$^{1-36}$, NPY$^{13-36}$, and PYY$^{13-36}$ in a similar manner as the radiolabeled intact NPY molecule (fig.4). In contrast, radiolabeled NPY$^{13-36}$ bound to PC-12 cells (shown in fig.4) and to MC-IXC cells in a non-specific manner only, as it could not be displaced by unlabeled NPY$^{1-36}$ or NPY$^{13-36}$.

The different performance of the intact molecule and the long C-terminal fragment might reflect differences in the degradation of these molecules during the incubation. However, the intact molecule and the 13–36 fragment of NPY appeared to be equally stabile as judged by the ability of the radiolabeled species to bind to hippocampal membranes after being incubated with MC-IXC cells or SMS-MSN cells for various periods (fig.5). Similar results were obtained in PC-12 cells and with primary incubation with hippocampal membranes (data not shown). Thus, the inability of NPY$^{13-36}$ to bind to and its inability to displace

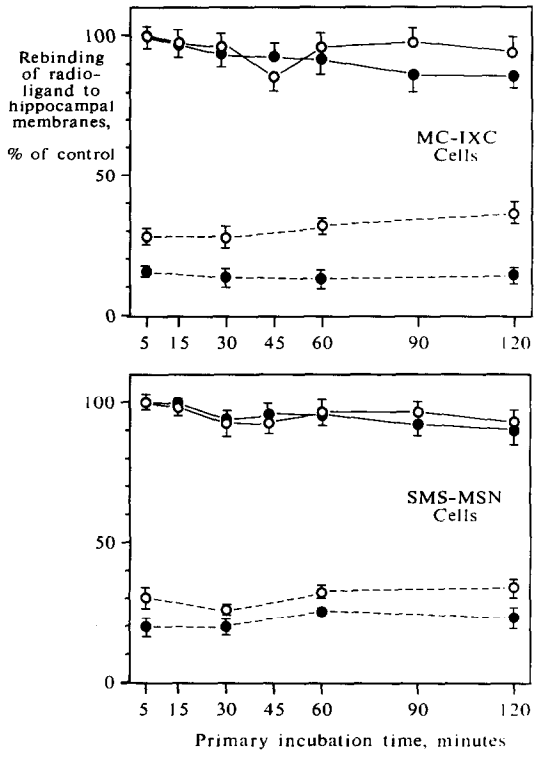

Fig.5. Stability of radioligands as indicated by rebinding of [^{125}I-Tyr^{36}]monoiodoNPY$^{1-36}$ and [^{125}I-Tyr^{36}]monoiodo-NPY$^{13-36}$ to hippocampal membranes. (Top pand) Rebinding of [^{125}I-Tyr^{36}]monoiodoNPY$^{1-36}$, total (●——●) and non-specific (●---●), and of [^{125}I-Tyr^{36}]monoiodoNPY$^{13-36}$, total (○——○) and non-specific binding (○---○) after exposure to MC-IXC cells for various periods. (Bottom panel) Rebinding data after primary exposure to SMS-MSN cells.

radiolabeled NPY$^{1-36}$ from cells with Y_1 receptors does not reflect rapid degradation of the C-terminal fragment.

4. DISCUSSION

In the present paper we have, by using monoiodinated radioligands of both the intact NPY molecule and of NPY$^{13-36}$, characterized two subtypes of NPY-binding sites, which differ in affinity as well as specificity. The Y_1 type of NPY receptors, which are found e.g. on the human neuroblastoma cell line, MC-IXC, and the rat pheochromocytoma cell line, PC-12, binds NPY with a dissociation constant (K_d) of a few nanomolar but does not bind the long C-terminal fragment. The Y_2 type of NPY receptors binds NPY with a dissociation constant in the subnanomolar range and more importantly, NPY$^{13-36}$ is a relatively good ligand for these receptors (fig.3). These two types of binding sites could represent the biochemical equivalents to the two types of biological receptors described by Wahlestedt et al. [24].

Y_2 receptors are found pre-junctionally on sympathetic nerves [24], whereas the post-junctional receptors are apparently of either the Y_1 or the Y_2 types, as described mainly on the basis of biological responses [9,13,14,18]. In the central nervous system, the Y_2 type of receptor seems to be predominating. The receptors originally described by Unden et al. [17] in the rat cortex have an affinity and specificity similar to those of the porcine hippocampal Y_2 receptors of the present study [26,30]. The NPY (Y_2) receptors in the hippocampus have been classified as being pre-junctional on the basis of electrophysiological data [31]. It is likely that Y_1 receptors are also found in certain areas of the central nervous system, because unlike intact NPY, the fragment, NPY$^{13-36}$, cannot evoke e.g. the centrally mediated lowering of blood pressure and the stimulation of feeding behaviour (Fuxe, K. and Kalra, S.P., personal communications).

Both Y_1 and Y_2 receptors accept NPY and PYY equally well (figs 1 and 3). Whether the hormone, PYY, the neuropeptide, NPY, or both act as physiological ligands for any particular receptor is dependent on the actual concentration of the peptide in a specific location in relation to the affinity of the receptor. Thus, Y_2 receptors in e.g. the hippocampus, where only NPY is found, are probably 'NPY receptors', whereas Y_2 receptors on tubular cells in the kidney, where no NPY innervation is found, may be 'PYY receptors', responding to circulating PYY.

From the data presented in the present paper it is not possible to establish whether the two types of NPY/PYY-binding sites in fact represent two different molecular species of receptors or just two different states of one receptor. However, as the genes for receptors for e.g. biogenic amines are being cloned, it appears that receptors are found in even more variant forms than those known from pharmacological experiments [32]. The presence of these NPY receptor subtypes on cell lines offers

special possibilities in respect of molecular characterization of the receptors and characterization of secondary messengers systems involved in their action.

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