Receptors on phaeochromocytoma cells for two members of the PP-fold family – NPY and PP

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Pancreatic polypeptide (PP) and neuropeptide Y (NPY) belong to a family of regulatory peptides which hold a distinct tertiary structure, the PP-fold, even in dilute aqueous solution. High-affinity receptors, specific for both PP and NPY, are described on the rat phaeochromocytoma cell line, PC-12. The binding of [^{125}I-Tyr^{36}]PP to PC-12 cells was inhibited by concentrations of unlabeled PP which correspond to physiological concentrations of the hormone, 10^{-11}–10^{-9} mol/l. The affinity of the receptor for the neuropeptide, NPY, was 10^2-times lower than that of the PP receptor. C-terminal fragments of both PP (PP$^{24-36}$) and NPY (NPY$^{13-36}$) were between 10^2- and 10^3-times less potent in displacing the radiolabeled 36-amino-acid peptides from their respective receptors. It is concluded that PC-12 cells are suited for structure-function studies of the PP-fold peptides and studies on the cellular events following cellular binding of PP-fold peptides.

Neuropeptide Y; Pancreatic polypeptide; 3-Dimensional peptide structure; (PC-12 cell)

1. INTRODUCTION

The pancreatic polypeptide fold (PP-fold) family is a group of regulatory peptides containing 36 amino acids, with a common distinct tertiary structure [1]. The PP-fold consists of a long N-terminal polyproline helix followed by a type II β-bend and a long amphiphilic α-helix [1–4]. The two helices are held together by tightly packed, interdigitating hydrophobic side chains [1–4]. The C-terminal hexapeptide is relatively free-moving and the C-terminal amide group, at least, is of crucial importance for the biological function [5,6]. Three members of the family are known: pancreatic polypeptide (PP), a pancreatic hormone [7]; neuropeptide Y (NPY), an important neuropeptide in both the central and peripheral nervous systems [8–10]; and peptide YY (PYY), a hormone from the lower intestine [11]. The peptides are only between 45 and 70% homologous, when the whole primary structure is considered [1]. However, the key residues of importance for the stability of the PP-fold are either identical in all the peptides or very conservatively substituted, e.g. Phe for Tyr [1]. The structure of avian PP was determined by X-ray crystallography to a resolution of less than 1 Å [2–4]. By analogy to the avian structure, a computographic image of the PP-fold structure has been composed for the other members of the family, including the mammalian PPs [1,12]. The PP-fold is stable, even in dilute aqueous solution as indicated by circular dichroism studies [1,3,13]. The detailed knowledge of the PP-fold structure and its apparent stability under physiological conditions makes this peptide family particularly suited for structure-function studies.

Receptors for NPY have been demonstrated in synaptosomal preparations from brain tissues [14–16] and for PYY on enterocytes [17]. Avian PP binds specifically to receptors in bird

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cerebellum [18]. However, no true receptors for mammalian PP have been demonstrated, although the peptide and its physiological functions in the regulation of exocrine pancreatic secretions and biliary motility have been known for more than a decade [7]. Only high-capacity, low-affinity receptors of doubtful physiological relevance have been detected [19]. The action of PP is demonstrable in vivo only, not in in vitro preparations of pancreatic or biliary tissues [20,21]. Thus, it was suggested that PP must act through nerves [7]. Based on the occurrence of receptors for other peptides on neuroendocrine cell lines we looked for NPY and PP receptors on different phaechromocytoma and neuroblastoma cell lines, and here present evidence that PC-12 cells have high-affinity receptors for both PP and NPY.

2. MATERIALS AND METHODS

2.1. Radioactive ligands

Synthetic porcine NPY^{1-36} was purchased from Peninsula (St. Helens, England). Natural purified bovine PP^{1-36} was a generous gift from Ron Chance (Eli Lilly, Indianapolis, IN). Both peptides were iodinated with carrier-free $Na^{125}I$ (Amersham, Little Chalfont, England) using the oxidative reagent, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Serva) [22]. The chloroglycouril (3.7 mg) was dissolved in 2.0 ml dichloromethane (Merck) and an Eppendorf test tube (Sarstedt, Nümbrecht, FRG) was coated with 20 μl of this solution by gently turning the tube while the solvent evaporated. The peptide (5 nmol) was dissolved in 40 μl phosphate buffer, pH 7.38 (precision buffer solution, Radiometer, Copenhagen) and transferred to the coated tube. $Na^{125}I$ (1 mCi) was added and the tube was kept on ice. After 5 min, 50 μl of the high-performance liquid chromatography (HPLC) solvent was added before purification on a Nucleosil 300-5 C_{18} column (0.4 × 25 cm) eluted at 50°C at a flow rate of 1.0 ml/min. Fractions of 0.5 ml were collected into tubes containing 0.1 ml of 3 mol/l acetic acid containing BSA (100 mg/l). In the purification of the NPY tracer, the column was equilibrated and eluted isocratically with 35% acetonitrile (Merck) in 0.1% trifluoroacetic acid (TFA)/H_2O. The two peaks of labeled peptide eluting early after the free

iodine peak were used in the binding experiments. These were $[^{125}I\text{-}Tyr^1]NPY$ with a specific activity of 500 Ci/mmol and $[^{125}I\text{-}Tyr^{36}]NPY$ of 1900 Ci/mmol as determined in self-displacement experiments (details of characterization of ligands to be published elsewhere). In the purification of the PP tracer, the radiolabeled peptides were eluted with a gradient of acetonitrile from 28 to 50% over 60 min. The major, late peak of radioactivity (fig.1) which, by HPLC characterization of tryptic fragments, was shown to be $[^{125}I\text{-}Tyr^{36}]PP$ (not shown) had a specific activity of 1900 Ci/mmol and was used for binding.

2.2. Cell culture

PC-12 cells derived from a rat phaeochromocytoma cell line were of subclone II-250, kindly provided by Hans Thoenen (Max-Planck-Institut für Psychiatrie, Martinsried). These cells were originally subcloned from tumors induced by PC-12 cells in Diaconess rats. Cells were grown in 10% CO_2 in Dulbecco's essential
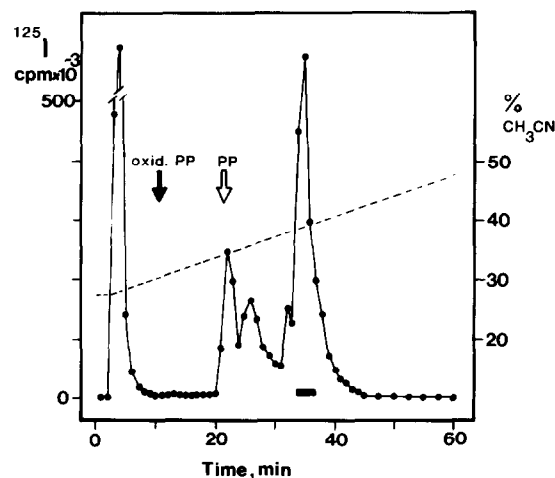

Fig.1. Purification of ^{125}I-labeled pancreatic polypeptide for receptor binding. HPLC of PP iodinated using chloroglycouril as described in detail in the text was performed on a Nucleosil C_{18} column eluted with TFA/water and a gradient of acetonitrile (– – –). The radiolabeled peptide (▬▬) was used for binding experiments; this peptide was shown by HPLC characterization of tryptic fragments to be mainly monoiodinated PP labeled in position 36. The elution positions of unlabeled PP and oxidized unlabeled PP are indicated by arrows.

medium (041-1885, Gibco, Uxbridge, England) supplemented with 10% horse serum, 5% fetal calf serum (both from Gibco), 1% glutamine, and 0.1% gentamycin.

2.3. Binding experiments

Cells (1.2 × 10⁶) were transferred to petri wells, 6-well culture plates (Costar, Cambridge, MA), which had been pretreated for 1 min with 1 ml per well of poly-Lys-Ala (P-1276, 0.2 g/l; Sigma, St. Louis, MO) and extensively washed in phosphate-buffered saline. After 2 days media were removed, and the cells were washed in binding buffer (pH 7.4) of the following composition (in mmol/l): NaCl, 150; KCl, 5; CaCl, 2.5; KH_2PO_4, 1.2; Mg_2SO_4, 1.2; $NaHCO_3$, 25; Hepes, 10, supplemented with 1% BSA. The cells were incubated with 0.9 ml binding buffer to which peptide was added in 0.05 ml binding buffer followed by 50000 cpm radiolabeled peptide, dissolved in 0.05 ml binding buffer. After incubation for 30 min in a CO_2 incubator at 37°C, the incubation buffer was removed and cells were washed twice in 1 ml ice-cold binding buffer before 1 + 0.5 ml of lysis buffer was added, 3 mol/l acetic acid with urea (8 mol/l) and Nonidet P40 (2%, v/v). The lysis mixture was transferred to test tubes before counting for 10 min. In all experiments, triplicate
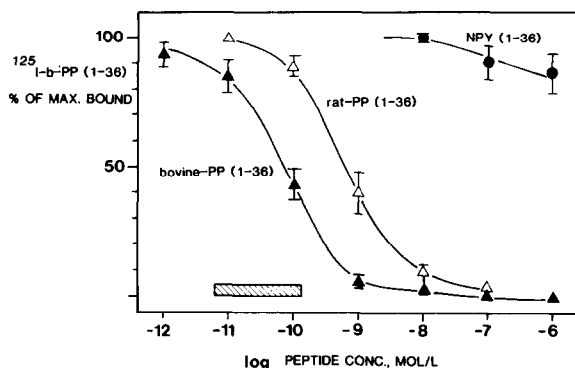
incubations were performed for each concentration of peptide.

2.4. Peptides used for displacement

Porcine NPY^{1-36} and bovine PP^{1-36} were from the same source as those used for radiolabeling. Porcine PYY^{1-36} and rat PP^{1-36} were both purchased from Peninsula. PP^{24-36} was a Staphylococcus protease fragment of natural bovine PP^{1-36} generously provided by Melvin Johnson and Ron Chance (Eli Lilly). Synthetic NPY^{13-36} was generously provided by Rolf Håkanson (University of Lund).

3. RESULTS

Specific steady-state binding to PC-12 cells was obtained with radiolabeled NPY within 10 min and with radiolabeled PP within 30 min at 37°C. The PC-12 cells bound 2.48 ± 0.61% (mean ± SE) of the PP label of which 0.44 ± 0.07% was unspecific binding, i.e. binding in the presence of 1 × 10⁻⁶ mol/l of unlabeled peptide. NPY binding was 4.78 ± 0.66% of which 1.37 ± 0.19% was

Fig.2. Inhibition of ^{125}I-PP binding to PC-12 cells by members of the PP-fold family. Binding of radioiodinated bovine PP^{1-36}, ^{125}I-b-PP (1–36), was inhibited by purified natural bovine PP^{1-36} (▲—▲, $N = 9$), synthetic rat PP^{1-36} (△—△, $N = 5$) and synthetic porcine NPY^{1-36} (●—●, $N = 5$). Means ± SE of the % of maximal binding of radiolabeled PP are indicated for each concentration of peptide.
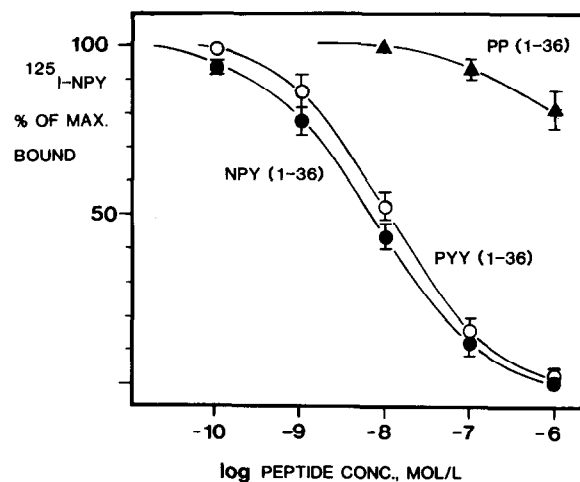
Fig.3. Inhibition of ^{125}I-NPY binding to PC-12 cells by members of the PP-fold family. Binding of ^{125}I-NPY was inhibited by synthetic porcine NPY^{1-36} (●—●, $N = 12$), synthetic porcine PYY^{1-36} (○—○, $N = 4$), and naturally purified bovine PP^{1-36} (▲—▲, $N = 4$). Means ± SE of the % of maximal binding of radiolabeled NPY are indicated for each concentration of peptide.

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unspecific binding. ^{125}I-Tyr^{36} and ^{125}I-Tyr^{1} bound equally well, however, the latter was generally used.

The specificity of binding was tested in competition experiments with different members of the PP-fold family (figs 2,3). The binding of ^{125}I-PP was inhibited by unlabeled PP at very low concentrations (10^{-12}–10^{-9} mol/l); 50% inhibition was obtained with 6×10^{-11} mol/l (fig.2). The number of binding sites for PP on PC-12 cells was calculated to be 750 per cell, and the dissociation constant $K_d = 4.5 \times 10^{-11}$ mol/l. The potency of NPY$^{1-36}$ in inhibiting PP binding was less than 10^{-5}-times that of PP. Surprisingly, synthetic rat PP$^{1-36}$ inhibited the binding of the bovine ^{125}I-PP$^{1-36}$ to rat PC-12 cells with a potency of only

Fig.4. Inhibition of ^{125}I-NPY and ^{125}I-PP binding to PC-12 cells by C-terminal fragments. (Upper panel) Inhibition of ^{125}I-NPY binding by synthetic porcine NPY$^{1-36}$ (●——●, $N = 12$) and synthetic NPY$^{13-36}$ (◇——◇, $N = 6$) is shown. (Lower panel) Inhibition of ^{125}I-PP binding by purified, natural bovine PP$^{1-36}$ (▲——▲, $N = 9$) and an enzymatic fragment of bovine PP, PP$^{24-36}$ (◆——◆, $N = 3$) is shown. Means ± SE of the % of maximal binding of radiolabeled tracer are indicated for each concentration of peptide.

around 10% of that of unlabeled bovine PP$^{1-36}$ (fig.2).

The binding of radiolabeled NPY was inhibited by unlabeled NPY$^{1-36}$ in a dose-dependent manner in the concentration range 10^{-10}–10^{-7} mol/l; 50% inhibition was obtained with 6.0 nmol/l of unlabeled NPY$^{1-36}$ (fig.3). Mathematical transformation of the binding data for NPY indicated either the presence of more than one type of receptor or different affinity states of a single receptor (not shown). The high-affinity receptor is characterized by a maximal binding capacity of 55 000 binding sites per cell, and a dissociation constant $K_d = 3$ nmol/l. PYY$^{1-36}$ competed with ^{125}I-NPY binding to PC-12 cells almost as well as NPY$^{1-36}$, whereas the potency of PP$^{1-36}$ was less than 0.1% of that of NPY$^{1-36}$ (fig.3).

A partial structure-binding characterization was performed with long C-terminal fragments of the peptides (fig.4). NPY$^{13-36}$ needed to be added at 200-times higher concentrations than NPY$^{1-36}$ to obtain a similar inhibition of ^{125}I-NPY binding. PP$^{24-36}$ was also less potent than the intact molecule, by a factor of 5×10^{3}, in inhibiting the binding of ^{125}I-PP to PC-12 cells (fig.4). Nevertheless, PP$^{24-36}$ was able to prevent totally ^{125}I-PP binding at a concentration of 3×10^{-6} mol/l. This finding is in agreement with the observation of a full agonistic effect but low potency of PP$^{31-36}$ on physiological functions [5].
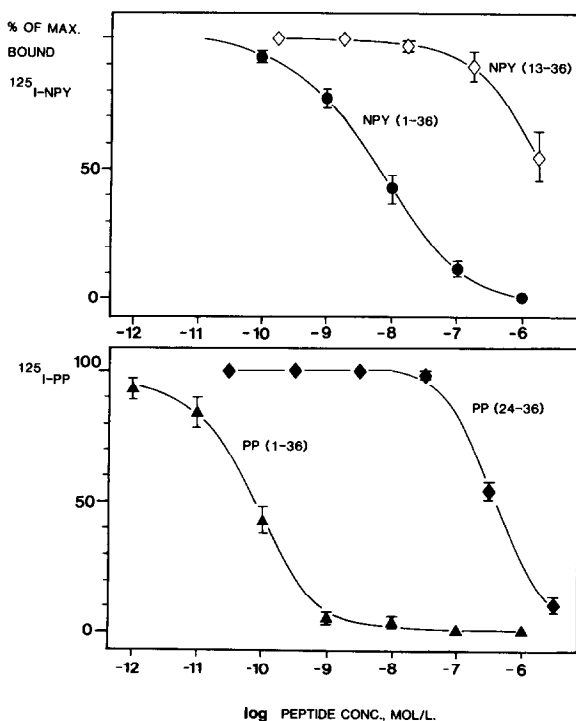
4. DISCUSSION

The PC-12 cell line is a classical cell line for studies in neurobiology [23]. In the present paper we present evidence for the presence of two distinct types of receptors for members of the PP-fold family on PC-12 cells. This observation should permit further studies on, e.g. secondary messenger systems, and other cellular events following stimulation with NPY and PP. Also, the structure-function relationship of this family of peptides can now be studied in more detail, based on the knowledge of the distinct three-dimensional structure of the PP-fold. Finally, the presence of the receptors on a cell line offers unique possibilities for isolation and characterization of the receptor by, e.g. expression cloning based on mRNA from PC-12 cells.

The normal counterpart to PC-12 cells (original-

ly derived from a phaeochromocytoma) is the chromaffin cell of the adrenal medulla. NPY, a neurotransmitter predominantly located in the sympathetic nervous system [8,24,25], is also found in a subset of chromaffin cells [26,27]. In fact, PC-12 cells synthesize and release NPY [28]. NPY receptors on NPY-producing cells could be expected to be 'presynaptic'. However, with respect to specificity, the PC-12 cell receptor resembles the postsynaptic, Y_1, receptor. The biological function of NPY has been characterized as a combination of a postsynaptic action, mediated through a hypothetical Y_1 receptor and a presynaptic action, mediated through a Y_2 receptor [6]. The postsynaptic effects are only observed with the intact NPY or PYY molecule, whereas, e.g. PYY^{13-36} can stimulate the hypothetical presynaptic receptor equally well to the whole molecule [6]. Since the NPY receptor on PC-12 cells did not bind NPY^{13-36} as well as NPY^{1-36}, it is presumably of a postsynaptic type. The affinity of the NPY receptors on PC-12 cells is around 10-times less than that reported for brain synaptosomal NPY receptors [15,16]. We find that the NPY receptor on PC-12 cells does not discriminate well between NPY and PYY, which supports observations in biological assays for postsynaptic function where PYY is as potent as NPY [6].

High-affinity receptors for mammalian PPs have not been described previously. It has been postulated that PP may act through nerve cells [7], and since the effect of PP on its target organs, the exocrine pancreas and the biliary system, could be considered 'anticholinergic', it was expected that inhibitory PP receptors would be found on cholinergic ganglionic cells. In fact, we initially looked for PP receptors on cholinergic cell lines, e.g. the NS-20-Y cells, without success. PP per se has not previously been suspected as having effects on adrenal function and the present observation will thus lead to physiological studies in PC-12 cells and their normal counterpart, chromaffin cells. It should be noted that the normal range of plasma PP concentrations during e.g. food intake and hypoglycemia [29], covers the upper part of the dose-inhibition curve for bovine PP^{1-36} (fig.2). The notion that PC-12 cells do have two distinct receptors, one for NPY and another for PP, is illustrated by the lack of potency of PP in displacing ^{125}I-NPY and the lack of potency of NPY in

displacing ^{125}I-PP from binding to the cells (figs 2,3). Unexpectedly, it was found that synthetic rat PP^{1-36} was rather poor in competing with the binding of natural bovine PP^{1-36} to these rat cells (fig.4). The amino acid sequence of rat PP is extraordinarily poorly conserved in comparison to other mammalian species [30,31]. The difference in binding observed here could be due to some error in synthesis of the peptide or even in the sequence determination of rat PP. More likely, however, it could be a true physiological observation. A similar observation has been made in guinea pigs which have poorly conserved insulin. In this species, other mammalian insulins, e.g. porcine, react better with the insulin receptor than the endogenous guinea pig insulin itself [32,33].

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