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Identification of a receptor protein for neuropeptide Y in rabbit kidney

G-Protein association and inhibition of adenylate cyclase

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We previously identified the receptor for neuropeptide Y (NPY) on rabbit kidney membranes as a 100 kDa protein [(1990) J. Biol. Chem. 265, 18142–18143]. It is demonstrated in this study that the stable guanine nucleotide analogue, guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS), is capable of decreasing the labeling of the 100 kDa protein in a concentration-dependent manner, with an IC_{50} value of 50 μM. This suggests that the 100 kDa protein represents a G-protein coupled receptor type. In isolated proximal tubules from rabbit kidney NPY decreases the parathyroid hormone stimulated cAMP production in a dose-dependent fashion. This indicates that the NPY receptor in rabbit kidney is negatively coupled to adenylate cyclase by a G_i-like protein.

Neuropeptide Y; Peptide receptor; Crosslinking; cAMP

1. INTRODUCTION

Neuropeptide Y (NPY) is a 36 amino acid peptide of the pancreatic polypeptide family, a group of structurally related brain-gut peptides including peptide YY (PYY) and pancreatic polypeptide (PP). NPY is found in the central and sympathetic nervous system of all vertebrates where it may act as a neurotransmitter/neuromodulator.

Receptors for NPY have been demonstrated in numerous tissues and cell lines [2–8], among which the proximal tubules of the rabbit kidney represent a surprisingly rich source of NPY binding sites. Here, we extend our previous studies on the characterization of NPY receptors in rabbit kidney [1] to crosslinking experiments in which a 100 kDa protein was identified that binds ^{125}I-NPY in a guanine nucleotide-dependent manner. Additionally, it is demonstrated that NPY can influence stimulated cAMP accumulation in isolated proximal tubules.

2. MATERIALS AND METHODS

2.1. *Chemicals*

NPY and parathyroid hormone were purchased from Bachem (Heidelberg, Germany). ^{125}I-NPY and the cAMP radioimmunoassay kit were purchased from Amersham (Buckinghamshire, UK). The other chemicals were obtained from the following sources: disuccinimidylsuberate (DSS) and disuccinimidyltartrate (DST), Pierce Chemical Co.; 3-isobutyl-1-methylxanthine (IBMX), *N*-hydroxysuccinimidyl-4-azidobenzoate (NHSAB), GTPγS, collagenase (type I), phenylmethylsulfonyl fluoride (PMSF), leupeptin, bacitracin and the 'Molecular Weight Markers' for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Sigma (Deisenhofen, Germany); Percoll, Pharmacia (Uppsala, Sweden); bovine serum albumin (BSA) and all other chemicals, except stated otherwise, were purchased from Serva (Heidelberg, Germany).

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2.2. *Preparation of rabbit kidney membranes*

Kidney membranes were prepared from New Zealand rabbits as described [1]. Membranes were stored at −20°C.

2.3. *Preparation of isolated proximal tubule cells*

The kidneys of exsanguinated New Zealand rabbits were removed, decapsulated and placed in a Hepes-buffered Krebs-Henseleit saline (KHS), pH 7.4, containing 0.1% BSA. Superficial cortical slices were incubated in KHS containing collagenase (1 mg/ml) for 45 min at 37°C in a shaking water bath. The digestate was pressed through a stainless steel mesh and gently centrifuged at 60 × *g* for 30 s. The pellet was washed twice in KHS at 4°C and was then resuspended in a 50% Percoll solution as described by Vinay et al. [10]. The F_4 layer consisting of proximal tubule segments was removed, resuspended and washed 3 times in oxygenated KHS at 4°C.

2.4. *Affinity labeling of neuropeptide Y binding sites*

Membrane fragments (100 μg/ml) were incubated with 100 pM ^{125}I-neuropeptide Y (2000 Ci/mmol) for 4 h at 0°C in binding buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.1% BSA, 0.1% bacitracin, 0.1 mg/ml aprotinin, 0.1 mg/ml soybean trypsin inhibitor, and 10 μg/ml leupeptin. The membranes were washed twice with ice-cold 50 mM Hepes, 5 mM $MgCl_2$, pH 7.4, and were finally resuspended in 300 μl of this buffer. The crosslinking reagents NHSAB, DSS, and DST were added to the membrane fragments at a final concentration of 1 mM. After 5 min in darkness the samples with the photoreactive NHSAB were exposed to UV-light. After 15 min at 0°C all the reactions were quenched by addition of 200 mM Tris-HCl, pH 6.8. The membranes were pelleted by centrifugation at 14 000 × *g* for 10 min at 4°C and analyzed by SDS-PAGE and autoradiography, as previously described [1].

2.5. *cAMP measurements*

The isolated proximal tubule preparations were divided into tubes and washed twice in 37°C warm KHS. The tubules were preincubated with the phosphodiesterase inhibitor IBMX at 0.4 mM for 5 min. Then the peptides were added as indicated. After 10 min the reaction was terminated by addition of ice-cold 0.65 M perchloric acid. The cells were homogenized and the protein pelleted down by centrifugation. The supernatant was neutralized and diluted 1:50 with 0.1 M sodium acetate buffer, pH 6.0. The samples were acetylated and cAMP was determined by radioimmunoassay.

2.6. *Protein determination*

Cell or membrane protein was measured according to a modified Lowry method [9], with BSA as a standard.

3. RESULTS AND DISCUSSION

In a previous paper we reported on the identification of a receptor for NPY in rabbit kidney as a 100 kDa protein by affinity labeling with the heterobifunctional photoreactive NHSAB [1]. We have now extended our crosslinking studies to the aminoactive homobifunctional reagents, DSS and DST, recently employed by other authors for the identification of NPY/PYY binding sites. As shown in Fig. 1, the labeling pattern generated with both crosslinkers was virtually identical. Besides the already mentioned 100 kDa complex a second protein with a molecular mass of approximately 120 kDa appeared. Competition studies performed for both crosslinkers showed diminuation of specific labeling with increasing concentrations of unlabeled NPY (Fig. 1b). Generally, crosslinking was markedly less efficient with DSS and DST than with the heterobifunctional NHSAB, which preferentially labeled the 100 kDa polypeptide (Fig. 2a). The reactivity of the homobifunctional agents is generally restricted to two readily accessible amino groups both in the receptor and in the ligand binding site coming into spatial proximity to each other. In view of the broader-ranged reactivity of the heterobifunctional reagent and the fact, that the Bolton-Hunter labeled NPY molecule contains only one free amino group in the N-terminal Tyr^1, the difference in crosslinking efficiency should not be very surprising. Since the 120 kDa protein was completely absent in crosslinking reactions with NHSAB and the monofunctional NPY derivative azidobenzoyl-NPY [1], we conclude that it may not represent a receptor protein. The 100 kDa complex could be detected with all the crosslinkers tested. Its identity with the covalently labeled NPY receptor therefore appears to be very likely.

Incubation of increasing concentrations of the stable guanine nucleotide analogue GTPγS, prior to crosslinking decreased the labeling of the 100 kDa band in a dose-dependent fashion (Fig. 2a). For quantification, the 100 kDa band was excised from the dried gels and radioactive incorporation was measured in a gamma-counter (Fig. 2b). Half-maximal inhibition of labeling occurred at about 50 μM GTPγS. This value agrees well

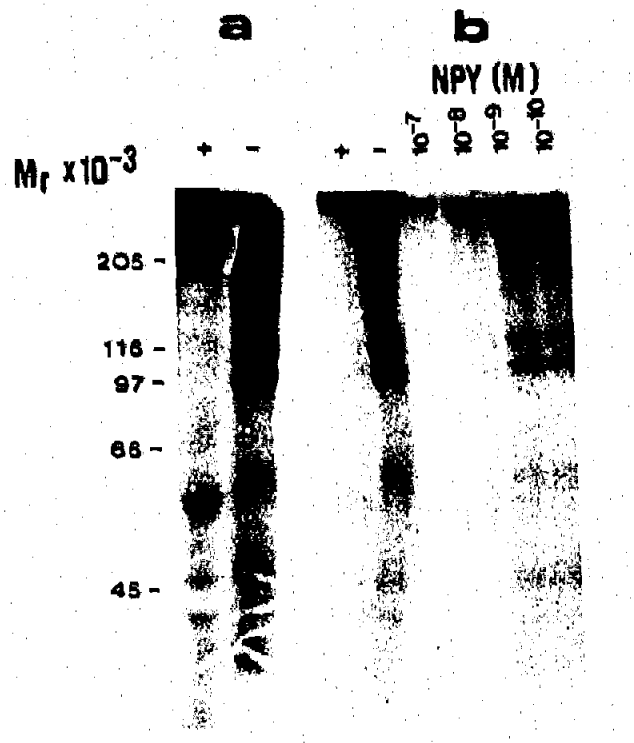

Fig. 1. Crosslinking of ^{125}I-NPY to rabbit kidney membranes (100 μg/ml) with the homobifunctional agents DSS (a) and DST (b) at a final concentration of 1 mM. Equilibrium binding was performed with 0.1 nM ^{125}I-NPY in the absence (−) or presence of unlabeled NPY at concentrations of 0.1 μM (+) or as indicated.

with the IC_{50} of 28 μM which characterizes the inhibitory effect of GTPγS on NPY binding to rabbit kidney membranes [1]. This provides further evidence that the 100 kDa binding protein described here may represent a G-protein coupled NPY receptor type.

Crosslinking experiments for NPY and PYY have been performed by several groups in a number of tissues and resulted in specific labeling of a variety of proteins: 62 and 39 kDa proteins in rat brain [11], 50 kDa proteins in membranes of rat hippocampus [12], porcine hippocampus [13], and rabbit kidney [12], 52–59 kDa and 42–44 kDa complexes in rat intestinal membranes [14], 70 kDa and 45 kDa polypeptides in a human neuroblastoma cell line [12], and a 65 kDa species that binds all three PP family peptides with similar affinities on intact rat cells [15]. The use of differentially labeled ligands may partly explain the discrepancy between these and our results. It appears rather unlikely that the 100 kDa protein described here represents an artefactual aggregate of the receptor with another protein since the same protein was labeled when the monofunctional NPY derivative azidobenzoyl-NPY was used [1].

Receptors linked to a guanine nucleotide binding protein may couple to ion channels, to adenylate cyclase, and/or to phospholipase C. A number of studies, in which the possibility of NPY stimulating

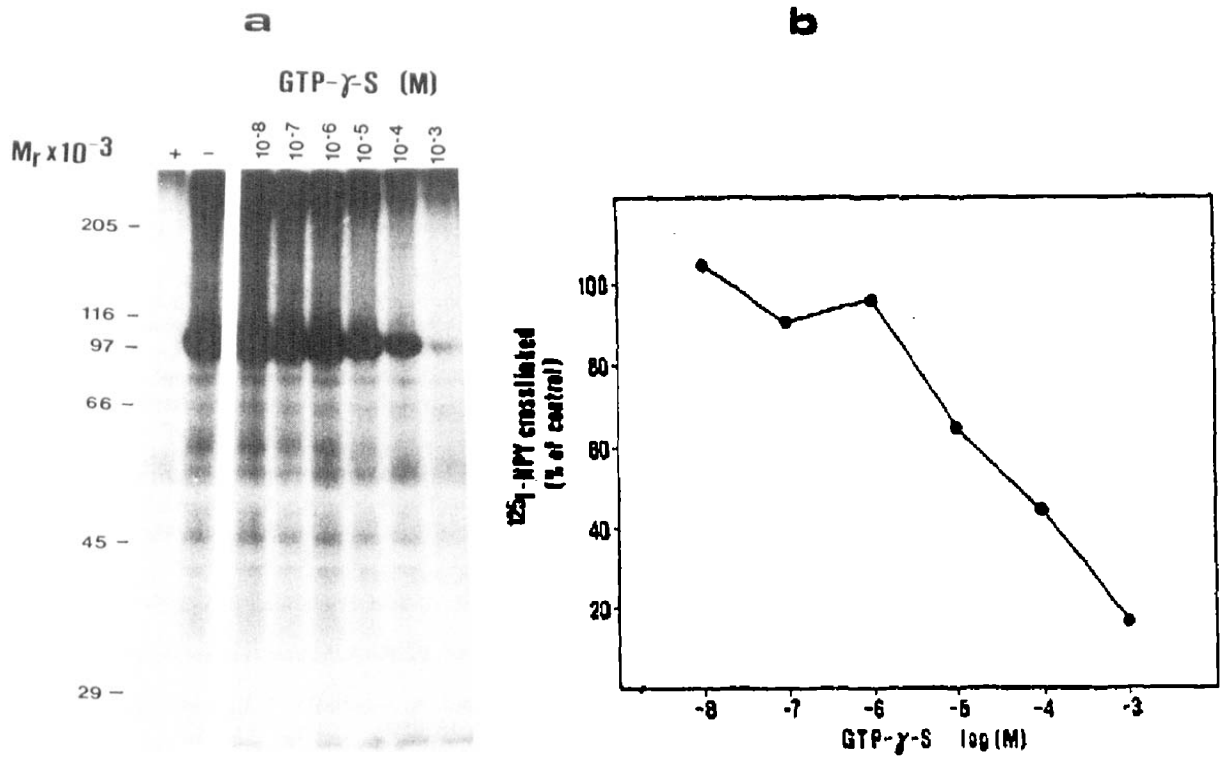

Fig. 2. Effect of GTPγS on the crosslinking of ^{125}I-NPY. Rabbit kidney membranes (100 μg/ml) were incubated with 0.1 nM ^{125}I-NPY and increasing concentrations of GTPγS. Crosslinking was performed by addition of NHSAB (1 mM, final concentration) and subsequent UV irradiation for 15 min. Then the samples were analyzed by SDS-PAGE and processed for autoradiography (a). The 100 kDa band was excised from the dried gel and its radioactivity was determined in a gamma-counter (b).

phosphoinositide hydrolysis was investigated, failed to detect any effects of the peptide [3,16,17]. On the other hand, inhibition of adenylate cyclase or stimulated cAMP generation has been reported for many tissues including brain, spleen, vascular smooth muscle cells and vas deferens [3,18–25]. We therefore tested whether NPY is capable of affecting adenylate cyclase activity in proximal tubule cells of rabbit kidney. As shown in Fig. 3, the basal levels of cAMP did not change in response to the peptide. However, NPY suppressed in a concentration-dependent manner the accumulation of cAMP induced by 0.1 μM parathyroid hormone (Fig. 3). Half-

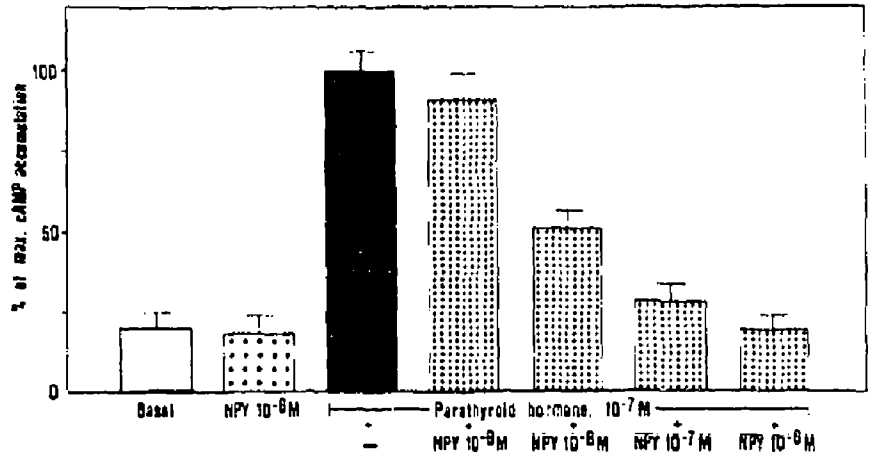

Fig. 3. Effects of NPY in suppressing parathyroid hormone-stimulated cAMP levels. Isolated proximal tubules were incubated with the indicated concentrations of peptides or no stimulatory agent (basal). Data are the mean ± SE of three experiments and are expressed as percentage of the parathyroid hormone-stimulated cAMP level, with the 100% value being equivalent to 45 ± 4 pmol/mg protein.

maximal inhibition was observed at about 10 nM NPY, which is markedly above the concentration required for half-maximal binding of NPY to kidney membranes (K_d = 0.09 nM) [1]. A similar discrepancy has also been found in pig spleen tissue [3]. So it may be possible that there are several populations of NPY/PYY receptors and that only a subpopulation with lower affinity is involved in the mediation of the NPY effects on cAMP generation. Since parathyroid hormone could not alter ^{125}I-NPY binding to rabbit kidney membranes (unpublished observations), interaction of both peptides on the receptor level is very unlikely.

These results confirm that negative coupling to adenylate cyclase is part of the signaling pathway initiated by binding of NPY to proximal tubule cells of rabbit kidney.

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