Molecular Characterization of the Human Neuropeptide Y Y_2-Receptor†

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ABSTRACT: Five neuropeptide Y receptors, the Y_1-, Y_2-, Y_4-, Y_5- and y_6-subtypes, have been cloned, which belong to the rhodopsin-like G-protein-coupled, 7-transmembrane helix-spanning receptors and bind the 36-mer neuromodulator NPY (neuropeptide Y) with nanomolar affinity. In this study, the Y_2-receptor subtype expressed in a human neuroblastoma cell line (SMS-KAN) and in transfected Chinese hamster ovary cells (CHO-hY2) was characterized on the protein level by using photoaffinity labeling and antireceptor antibodies. Two photoactivatable analogues of NPY were synthesized, in which a Tyr residue was substituted by the photoreactive amino acid 4-(3-trifluoromethyl)-3*H*-diazirin-3-ylphenylalanine ((Tmd)Phe), [N_α-biotinyl-Ahx_2,(Tmd)Phe^{36}]NPY (Tmd36), and the Y_2-receptor subtype selective [N_α-biotinyl-Ahx_2,Ahx^{5-24},(Tmd)Phe^{27}]NPY (Tmd27). Both analogues were labeled with [^{3}H]succinimidyl-propionate at Lys^4 and bind to the Y_2-receptor with affinity similar to that of the native ligand. A synthetic fragment of the second (E2) extracellular loop was used to generate subtype selective antireceptor antibodies against the Y_2-receptor. Photoaffinity labeling of the receptor followed by SDS−PAGE and detection of bound radioactivity and SDS−PAGE of solubilized receptors and subsequent Western blotting revealed the same molecular masses. Two proteins correspondingly have been detected for each cell line with molecular masses of 58 ± 4 and 50 ± 4 kDa, respectively.

Neuropeptide Y (NPY)[1] is a 36-amino acid peptide that is widely distributed both peripherally as well as centrally. Similar to many other neurotransmitters NPY elicits diverse physiological effects, e.g., induction of food intake and potent vasoconstriction (*1*−*4*). Accordingly, NPY is a neuronal and endocrine messenger that exerts its effects via several receptor subtypes of which the so-called Y_1- (*5*−*7*), Y_2- (*8*−*10*), Y_4/PP_1- (*11*, *12*), Y_5- (*13*), and y_6-receptors (*14*) have been cloned (*15*). Sequence comparisons show that the receptors Y_1, Y_4, and y_6 are more closely related to each other than to the receptors Y_2 and Y_5. The different receptor subtypes are localized in various tissues, in the central nervous system as well as in the periphery. Tissues with high density of NPY receptors are blood vessels, kidney, adrenal glands, colon, heart, pancreas, intestine, nerve endings, and brain. While their distribution appears to be species specific, all subtypes belong to the large superfamily of G-protein-coupled, heptahelical receptors (*16*). The Y_2-receptor is the predominant NPY receptor subtype in the brain and particularly abundant in the hippocampus (*17*). The cloned Y_2-receptor consists of 381 amino acids (*10*) and has the typical heptahelix receptor features including a potential glycosylation site in the amino-terminal part, two extracellular cysteines that may form a disulfide loop, and a single cysteine in the cytoplasmatic tail that probably serves as an attachment site for palmitate. Characterization of the receptor subtypes has been restricted so far to pharmacological experiments and investigations at the mRNA level. Because of possible posttranslational modifications such as glycosylation and palmitoylation, characterization on the protein level is necessary. For example, a receptor subtype identified on the mRNA level may or may not be functionally expressed. Moreover, since rapid axonal transport has been suggested for the Y_1-receptor (*18*), mRNA localization must not necessarily fit with the localization of the mature protein. Characterization and localization of the protein can be achieved by photoaffinity labeling and immunodetection. The first method, photoaffinity labeling, has been widely used for the identification of binding sites in different receptor systems (*19*−*21*). The use of this method to determine molecular mass depends on the specific labeling of the receptor. The problem of unspecific labeling may be overcome by constructing a ligand with a built-in photoreactive amino acid that generates a highly reactive, short-lived species, for example, a carbene (*21*, *22*). To increase the chance of productive coupling reactions, the photoactivatable amino acid should be placed near or within the

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[1] Abbreviations: Ahx, 6-aminohexanoic acid; DATD, *N*,*N*′-diallyltartardiamide; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; [^{3}H]Tmd27, [N_α-biotinyl-Ahx_2,[^{3}H]propionyl-Lys^4,Ahx^{5-24},(Tmd)Phe^{27}]NPY; [^{3}H]Tmd36, [N_α-biotinyl-Ahx_2,[^{3}H]propionyl-Lys^4,(Tmd)Phe^{36}]NPY; KLH, keyhole limpet hemocyanin; NPY, neuropeptide Y; (Tmd)Phe, 4-(3-trifluoromethyl)-3*H*-diazirin-3-ylphenylalanine; OSu, *N*-hydroxysuccinimide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; Tmd27, [N_α-biotinyl-Ahx_2,Ahx^{5-24},(Tmd)Phe^{27}]NPY; Tmd36, [N_α-biotinyl-Ahx_2,(Tmd)Phe^{36}]NPY; Tris, tris(hydroxymethyl)-aminomethane; Tween 20, polyoxyethylene−sorbitan monolaurate.

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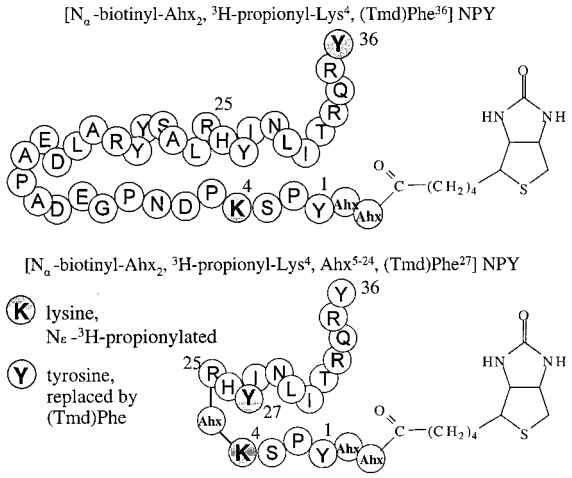

FIGURE 1: Sequence and modified positions of the photoactivatable NPY analogues.

binding site. To reach maximal specificity, we compared the results of photoaffinity labeling using two different [^{3}H]-propionylated analogues of NPY (Figure 1) with built-in photoreactive amino acids: [N_α-biotinyl-Ahx$_2$,[^{3}H]propionyl-Lys^4,(Tmd)Phe^{36}]NPY ([^{3}H]Tmd36) and [N_α-biotinyl-Ahx$_2$, [^{3}H]propionyl-Lys^4,Ahx$^{5-24}$,(Tmd)Phe^{27}]NPY ([^{3}H]Tmd27). Centrally truncated NPY analogues such as [Ahx$^{5-24}$]NPY and Tmd27 additionally have been shown to be selective for the Y_2-receptor subtype (*23*). Second, various studies have shown that antibodies produced against hormone receptors are valuable tools (*24*−*27*). Antireceptor antisera can be raised by immunization with purified, enriched receptors (*24*) or by immunization with receptor fragments (*25*−*27*). Molecular mass determination by SDS−PAGE and subsequent Western blotting, receptor purification by affinity chromatography on antibody columns (*26*), and investigations of the receptor localization (*28*) are only a few applications of antireceptor antibodies (*29*). Antibodies specifically recognizing the NPY receptor subtypes could be used to determine the localization pattern and the quantity of the receptor protein expression as well as to determine the topology and function of a receptor subtype. In this study, we compared the molecular masses of human neuropeptide Y Y_2-receptors expressed endogenously in a human neuroblastoma cell line (SMS-KAN) and in Chinese hamster ovary cells (CHO-hY2) that have been transfected with human Y_2-receptor cDNA (*10*). To increase the reliability of the results, molecular masses were determined by two independent methods: photoaffinity labeling and immunodetection.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. All peptides were prepared by solid-phase synthesis using the Fmoc strategy (Fmoc, 9-fluorenylmethoxycarbonyl) on a robot system (Syro, MultiSynTech, Bochum) (*30*). To obtain peptide amides, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin was used for anchoring. The polymer matrix was polystyrene−1% divinylbenzene (30 mg, 15 μmol). L-H-(Tmd)Phe-OH was prepared as described previously (*31*) and N-terminally protected with Fmoc-OSu (OSu, *N*-hydroxysuccinimide) (*32*). Cleavage of the peptide amides from the resin was achieved with trifluoroacetic acid/thioanisole/thiocresol within 2 h. Cleaved peptides were collected by centrifugation and lyophilized from water. All proceedings including (Tmd)-Phe have been performed in the absence of light. Characterization was achieved by reversed-phase HPLC (column C-18, 3 × 125 mm, 5 μm, flow 0.6 mL/min, gradient 25% acetonitrile to 75% acetonitrile in water/trifluoroacetic acid (100:0.1) within 30 min, Tmd27 5% acetonitrile to 50% acetonitrile in water/trifluoroacetic acid (100:0.1) within 30 min) and electrospray mass spectrometry (SSQ710, Finnigan, San Jose, CA). To test the photochemical properties, photoactivatable peptides were irradiated for 10 min with light of 366 nm and reinvestigated by HPLC using the same gradient.

Tmd27: retention, 20.85 min; retention after irradiation, 18.11 min; mass^{calc}, 2763.5 amu; mass^{exp}, 2763.8 amu.

Tmd36: retention, 12.40 min; retention after irradiation, 11.29 min; mass^{calc}, 4795.37 amu; mass^{exp}, 4795.80 amu.

Synthetic fragment of second extracellular loop (sequence IFREYSLIEIIPDFEIVAF): retention, 17.3 min; mass^{calc}, 2313.7 amu; mass^{exp}, 2314.0 amu.

Peptide Modification. Tmd27 and Tmd36 were labeled with [^{3}H]succinimidyl-propionate (3.59 TBq/mmol; Amersham, Switzerland) at Lys^4. For each analogue 1 mL of [^{3}H]-succinimidyl-propionate solution (37 MBq) in toluene was dried in a nitrogen stream, and the analogue was added equimolar in 10 μL DMF. After addition of 0.1% DIPEA in DMF, the mixture was incubated for 20 h at room temperature in the dark. The complete reaction mixture was diluted in 1 mL 10% acetonitrile/0.1% trifluoroacetic acid and loaded onto a handpacked C-18-column (0.5 cm^3 bed volume), followed by washing with 5 mL 10% acetonitrile/0.1% trifluoroacetic acid. The peptides were eluted with 1 mL 60% acetonitrile/0.1% trifluoroacetic acid. Specific activity of [^{3}H]-Tmd27 and [^{3}H]Tmd36 was 3.0 and 2.8 TBq/mmol, respectively.

Membrane Preparation and Receptor Binding. Expression cloning of the human Y_2-receptor was performed as described previously (*10*); cultivation of SMS-KAN cells and membrane preparation were performed according to ref *33*. Displacement of [^{3}H]propionyl-NPY (3.18 TBq/mmol; Amersham, Switzerland) by NPY resulted in K_i = 0.67 nM for both cell lines (*33*). Binding of the tritiated photoactivatable analogues was assayed as follows. The membrane preparation was diluted in incubation buffer (MEM/25 mM Hepes, 1% bovine serum albumin, 50 μM Pefabloc SC, 0.1% bacitracin, 3.75 mM $CaCl_2$); 200 μL of the suspension containing 20 μg protein was incubated with 25 μL 8.3 nM solution of the analogues and 25 μL of solutions of NPY in increasing concentrations to give a total volume of 250 μL. After 1.5 h at room temperature, the incubation was terminated by centrifugation of the samples for 10 min at 3000*g* and 4 °C. The pellets were washed with PBS, resuspended in PBS, and mixed with scintillation cocktail, and radioactivity was determined. Nonspecific binding was defined in the presence of 10 μM NPY. K_i^{NPY} and IC_{50} were used to determine $K_D^{Analogue}$ (Figure 2) (*34*). The $K_D^{Analogue}$ values obtained were similar to K_D values of [^{3}H]propionyl-NPY at SMS-KAN cells (0.018 ± 0.008 nM).

[N_α-biotinyl-Ahx$_2$,[^{3}H]propionyl-Lys^4,Ahx$^{5-24}$,(Tmd)Phe^{27}]-NPY: K_D = 0.02 ± 0.015 nM.

[N_α-biotinyl-Ahx$_2$,[^{3}H]propionyl-Lys^4,(Tmd)Phe^{36}]NPY: K_D = 0.02 ± 0.008 nM.

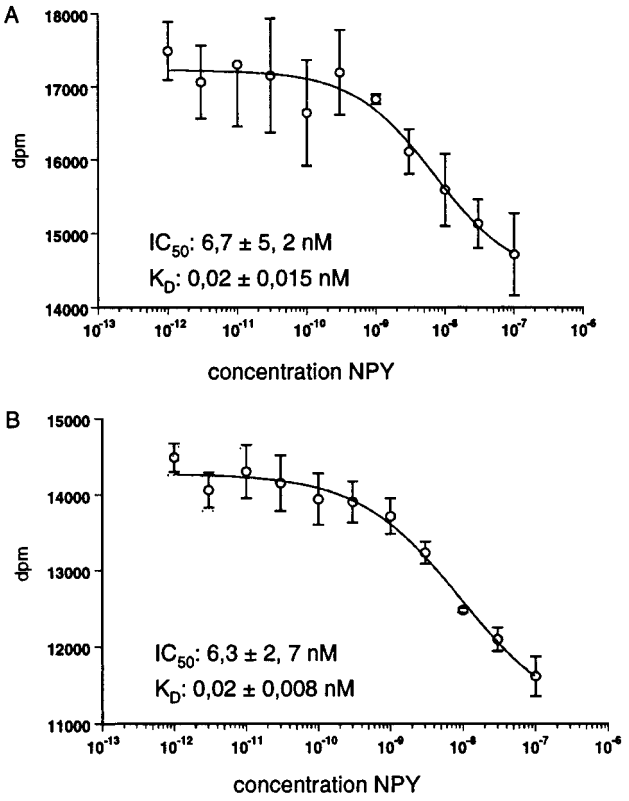

FIGURE 2: Competition assay of [N_α-biotinyl-Ahx$_2$,[^{3}H]propionyl-Lys^4,Ahx$^{5-24}$,(Tmd)Phe^{27}]NPY (A) and [N_α-biotinyl-Ahx$_2$,[^{3}H]-propionyl-Lys^4,(Tmd)Phe^{36}]NPY (B) against NPY at CHO-hY2 membranes. For details, see Experimental Procedures.

Photo-Cross-Linking of Y_2-Receptor Containing Membranes. Membranes were prepared as described above, and 500 μg protein from this suspension was incubated with 10 nM solution of the photoactivatable analogue for 90 min in 2 mL binding buffer (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM K_3PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1% bovine serum albumin, 50 μM Pefabloc SC, 0.1% bacitracin, pH 7.4). A control probe was handled in parallel, but with the addition of NPY in excess (1 μM). The incubation was stopped by centrifugation at 2000*g* for 5 min at 20 °C. The resulting pellets were suspended in 2 mL cold cross-linking buffer (25 mM Hepes, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 50 μM Pefabloc SC, 0.1% bacitracin, pH 7.4), centrifuged again for 5 min, resuspended in 2 mL ice-cold cross-linking buffer, and transferred to a 6-well cell culture plate on ice. The samples were irradiated for 20 min using a 180-W high-pressure mercury lamp (366 nm). The membranes were pelleted and washed three times with 1 mL cold binding buffer, dissolved in SDS sample buffer (10% glycerol, 2.3% SDS, 1.5% mercaptoethanol, 30 mM Tris (pH 6.8), 0.1% bromophenol blue, 8 M Urea), treated at 95 °C for 5 min, and subjected to SDS−PAGE (12% polyacrylamide gel) according to the procedure of Laemmli (*35*) with *N*,*N*′-diallyltartardiamide (DATD) as cross-linker (*36*). The gels were cut into 1.3-mm slices, incubated in 2% periodic acid for 60 min at 37 °C with shaking, and resuspended in liquid scintillation cocktail, and radioactivity was measured.

Specificity of photoaffinity labeling was shown by competition with different concentrations of NPY (Figure 3) as follows. Membranes (50 μg) were incubated with the photoactivatable analogue (3 nM) and increasing concentrations of NPY (1 pM to 10 μM). After washing the probes were irradiated. A control set of probes was handled in parallel, but without irradiation. After irradiation and washing, all probes were incubated with NPY (10 μM) to remove receptor-bound ligand which was not covalentely attached to the receptor. Bound radioactivity was determined after three washing steps. All determinations were performed in triplicate with mean values and errors given in Figure 3.

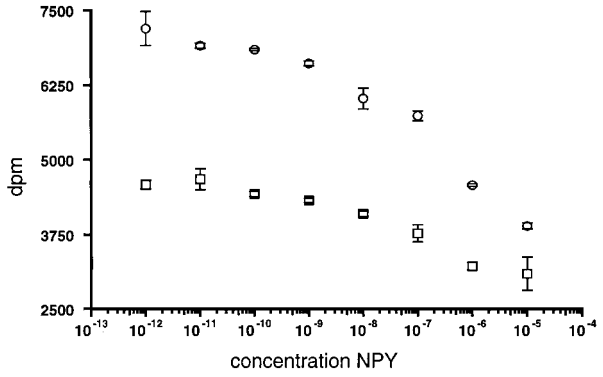

FIGURE 3: Dose-dependent inhibition of cross-linking at CHO-hY2 membranes with [N_α-biotinyl-Ahx$_2$,[^{3}H]propionyl-Lys^4,Ahx$^{5-24}$,(Tmd)Phe^{27}]NPY by NPY (circles). Control probes were handled in parallel without irradiation (squares). A second incubation with an excess of NPY after irradiation was used to remove receptor-bound but not covalentely attached ligand. All determinations were performed in triplicate with mean values and errors given.

Preparation of the Conjugates and Immunization. For immunization the peptide from the second extracellular loop (sequence IFREYSLIEIIPDFEIVAF) was coupled to keyhole limpet hemocyanin (KLH) (Biotrend, Cologne, Germany). Polyclonal antibodies against the KLH-coupled receptor fragment were obtained by immunization of rabbits (Biotrend, Cologne, Germany). Antibodies were tested for binding and specificity by ELISA and Western blotting (*37*) and were found to be Y_2-receptor subtype selective.

Immunoblot. Membranes from SMS-KAN and CHO-hY2 cells were prepared by incubation at 95 °C for 7 min in a urea sample buffer (8 M urea, 63 mM Tris/HCl (pH 6.8), 2% SDS, 5% mercaptoethanol, 0.01% bromophenol blue) and were separated on a 12% polyacrylamide gel according to Lämmli (*35*). After blotting for 60 min (350 mA/20 V) in a semidry electrophoretic transfer cell (Biorad) onto nitrocellulose membrane in a transfer buffer containing 25 mM Tris/HCl (pH 8.3), 150 mM glycine, 20% methanol, the blots were blocked overnight with TBS/Tween (0.5% Tween 20 in TBS/HCl, pH 7.4) containing 1% BSA. The blots were incubated with antibodies for 90 min with gentle shaking, washed three times with TBS/Tween, incubated for 60 min with alkaline phosphatase-bound secondary antibodies, and washed three times again. Immunoreactivity was detected with 5-bromo-4-chloroindolyl phosphate (Sigma, Buchs, Switzerland). Control was performed with membranes from SMS-KAN, CHO-hY2, and not transfected CHO cells using anti-hY2 serum preincubated with the peptide against which the antibodies were raised and 0-sera (Figure 5).

RESULTS

Synthesis and Analysis of Photoactivatable NPY Analogues. Since NPY analogues containing Phe but not Trp are still recognized at the hY_2-receptor (*38*), we decided to

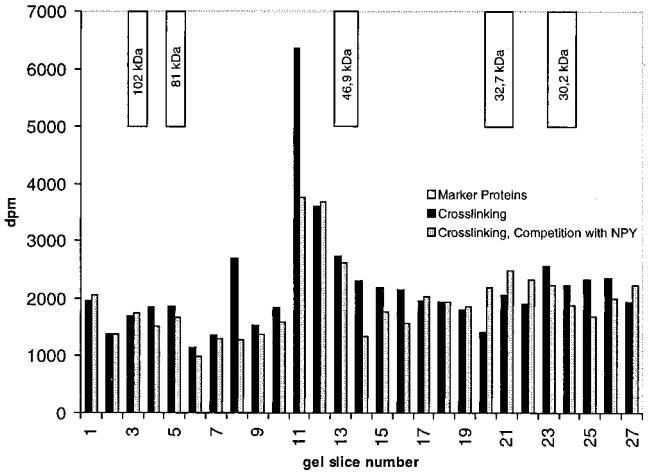

FIGURE 4: Cross-linking experiment of [N_α-biotinyl-Ahx$_2$,[^{3}H]-propionyl-Lys^4,Ahx$^{5-24}$,(Tmd)Phe^{27}]NPY at CHO-hY2 membranes. Control was performed with an excess of NPY. After SDS−PAGE the gel was cut into 1.3-mm slices, and radioactivity was measured. A set of marker proteins was run on the same gel. The numbers of the gel slices in which the marker proteins turned up are shown as bars and were used to establish a calibration curve. The curve was used to determine the molecular mass range for the proteins covered by each gel slice.

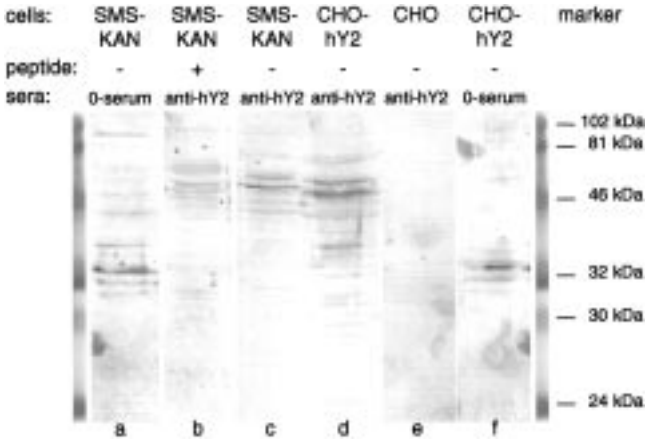

FIGURE 5: Western blot of solubilized SMS-KAN (lane c) and CHO-hY2 (lane d) membranes. Specificity of the Y_2-receptor recognition by the anti-Y_2-receptor antibodies is shown by staining with 0-sera (lanes a and f), anti-hY2 serum preincubated with the peptide against which the antibodies were raised (lane b), and not transfected CHO cells (lane e). Staining was achieved using alkaline phosphatase conjugated secondary antibodies.

use (Tmd)Phe instead of other photoactivatable amino acids of larger size as, for example, *p*-benzoylphenylalanine, which previously has been used for efficient photo-cross-linking of other peptides (*39*, *40*). Fmoc-(Tmd)Phe was coupled manually in 2-fold excess; all the subsequent cycles were performed using a peptide synthesizer. Preparation of Fmoc-(Tmd)Phe-OH as well as all synthesis, cleavage, and purification steps were carried out in the absence of light. The peptide was characterized by means of analytical HPLC and electrospray mass spectrometry. In addition, a small sample of each peptide was dissolved in water and illuminated, which caused a change in the retention time as observed by HPLC. This result confirmed that the side chain of (Tmd)Phe remained intact during the remaining cycles of peptide synthesis after coupling of (Tmd)Phe. Tmd27 and Tmd36 were labeled successfully with [^{3}H]succinimidyl-propionate. Both peptides contained a single reactive amino group (side chain of Lys^4) which facilitated the coupling of a single label per peptide. The labeled peptides, [^{3}H]Tmd27 and [^{3}H]Tmd36, showed a high specific activity of 3.0 and 2.8 TBq/mmol, respectively, which suggests a labeling efficiency of at least 90%. The binding of both NPY analogues to the Y_2-receptor was determined in a competition assay against NPY using the labeled analogues as radioactive tracer (Figure 2). Although both peptides were modified at three positions (N-terminal spacer and biotinylation, propionylation at Lys^4, and exchange of Tyr against (Tmd)Phe in a single position), their binding properties at the Y_2-receptor were only slightly reduced compared to those of [^{3}H]propionyl-NPY used in the binding assay ([^{3}H]propionyl-NPY, $K_D = 0.018 \pm 0.008$ nM; [^{3}H]Tmd27, $K_D = 0.02 \pm 0.015$ nM; [^{3}H]Tmd36, $K_D = 0.02 \pm 0.008$ nM).

Table 1: Molecular Masses Identified for the NPY Y_2-Receptor Subtype

determination method of molecular mass	masses (kDa) of labeled proteins in SMS−KAN cells	masses (kDa) of labeled proteins in CHO-hY2 cells [kDa]
cross-linking with [^{3}H]Tmd27	57 ± 4/47 ± 4	62 ± 4/48 ± 4
subtracted by mass of cross-linker	54 ± 4/44 ± 4	59 ± 4/45 ± 4
cross-linking with [^{3}H]Tmd36	58 ± 4/49 ± 4	63 ± 4/51 ± 4
subtracted by mass of cross-linker	53 ± 4/44 ± 4	58 ± 4/46 ± 4
Western blotting	58 ± 4/54 ± 4	57 ± 4/51 ± 4

Photoaffinity Labeling. SMS-KAN and CHO-hY2 membranes were cross-linked using the labeled photoactivatable NPY analogues [^{3}H]Tmd27 and [^{3}H]Tmd36. For each cross-linking experiment a probe was handled in parallel with the addition of 1 μM NPY as control. SDS−PAGE was performed subsequently, followed by cutting of the gels in 1.3-mm slices and determination of the radioactivity of each gel slice. Four sets of experiments have been performed. SMS-KAN membranes were cross-linked using [^{3}H]Tmd27 and [^{3}H]Tmd36, respectively, and the same experiments were processed with CHO-hY2 membranes. Two major radioactive protein bands (Figure 4, Table 1) were found in all cases. In parallel, a set of marker proteins was always run on the same gel. The numbers of the gel slices in which the marker proteins turned up were used to establish a calibration curve for each gel. The curves were used to determine the molecular mass range for the proteins covered by each gel slice. Depending on the calibration method, the molecular masses obtained for the proteins varied up to 4 kDa. Molecular masses of the receptor proteins detected for SMS-KAN membranes were found to be 54 and 44 kDa using [^{3}H]Tmd27 for cross-linking and 53 and 44 kDa using [^{3}H]-Tmd36. Photoaffinity labeling on CHO-hY2 membranes led to the identification of two proteins as well with molecular masses of 59 and 46 kDa with [^{3}H]Tmd27 and 58 and 46 kDa with [^{3}H]Tmd36. In some cases minor bands in the range of 30 kDa were observed, which we presume to represent partially degraded receptor proteins. Both sequence positions (Tyr^{27} and Tyr^{36}) exchanged against (Tmd)Phe allowed insertion of the photoactivatable group into the receptor protein upon irradiation at 366 nm. Specificity of photoaffinity labeling was shown by competition with different concentrations of NPY (Figure 3). Using both analogues for cross-linking of SMS-KAN membranes, the molecular masses identified by SDS-PAGE (and subtracted by the mass of the photoactivatable NPY analogue used) correspond well

in both cell lines. A slight difference was found between the two cell lines for the upper mass (54 versus 58 kDa) which most likely is due to difficulties in exact weight determination by SDS−PAGE.

Immunoblot. Membranes from SMS-KAN and transfected CHO-hY2 cells were solubilized and proteins separated on a gel followed by Western blotting using the Y_2-receptor selective antibodies. Control was performed with membranes from SMS-KAN, CHO-hY2, and not transfected CHO cells using anti-hY2 serum preincubated with the peptide against which the antibodies were raised and 0-sera (Figure 5). Again, two major protein bands were detected, and the marker proteins were used to establish a calibration curve. The molecular masses observed for the Y_2-receptor expressed in SMS-KAN cells were approximately 58 and 54 kDa. For CHO-hY2 membranes slightly lower masses of 57 and 51 kDa were found (Figure 5, Table 1). Depending on the calibration curve, these masses varied up to 4 kDa. As in the photoaffinity labeling experiments, weaker bands in the range of 30−40 kDa were found too, which are believed to represent degradation products of the receptor protein and which are still recognized by the antibody.

DISCUSSION

We successfully synthesized photoactivatable, tritium-labeled analogues of NPY with high receptor affinity ([^{3}H]-Tmd27 and [^{3}H]Tmd36). Using these ligands, photoaffinity labeling of the NPY Y_2-receptor subtype expressed in SMS-KAN and transfected CHO-hY2 cells identified two labeled proteins with molecular weights of 54/44 and 59/46 kDa, respectively. Specificity of photoaffinity labeling was shown by competition with different concentrations of NPY (Figure 3). Y_2-Receptor subtype selective antibodies were raised by immunization of rabbits with a synthetic fragment of the second extracellular loop of the Y_2-receptor, and subtype selectivity was shown by ELISA and Western blotting. These antibodies were used for Western blotting in order to confirm the molecular mass determination by photoaffinity labeling. Again, two proteins were identified with molecular weights of 58/54 kDa for SMS-KAN and 57/51 kDa for transfected CHO-hY2 cells. The molecular weight determination is based on a mass calibration curve established for 5−6 marker proteins. The ratio of the migration of the proteins in the gel and their molecular masses is not linear. Thus a curve has to be fitted to the marker weights, which might cause differences in the molecular weights of the proteins detected, depending on the mathematical model used. In addition to the inherent inaccuracy of SDS−PAGE, the masses determined for the Y_2-receptor might vary up to 4 kDa. The masses identified by photoaffinity labeling with two different photoactivatable analogues are in good agreement for both cell lines. When compared to the mass determination via Western blotting, the upper masses identified are in good agreement for both cell lines as well, whereas the masses of the lower protein bands seem to differ slightly (Table 1). This might be explained by the error in mass determination or the different experimental efforts for photoaffinity labeling and Western blotting and the different detection methods that have been applied. Thus, the results of the molecular mass determination of the Y_2-receptor suggest the same protein masses of 58 and 50 kDa in two different cell lines. SMS-KAN cells are human neuroblastoma cells which are endogenously expressing the Y_2-receptor subtype, whereas CHO-hY2 cells have been transfected with human Y_2-receptor cDNA. The corresponding molecular masses suggest similar posttranslational modifications in both cell lines. When compared to the mass of the Y_2-receptor calculated from the sequence (42 kDa), a difference of up to 16 kDa caused by posttranslational processing was observed. Glycosylation alters the migration behavior of proteins in SDS−PAGE in a nonlinear way (*41*). This means that the exact increase in the mass of the Y_2-receptor caused by glycosylation cannot be determined exactly by SDS−PAGE, but our results suggest a remarkable glycosylation of the Y_2-receptor at the consensus sequence of the N-terminus. Preliminary deglycosylation experiments showed a decrease of the molecular masses after application of endoglycosidase F and peptide-*N*-glycosidase F. Therefore, the two different masses of the proteins identified in each cell line are most likely due to different glycosylation of the Y_2-receptor.

Previous studies using chemical cross-linking with PYY and NPY analogues in different cells or tissues revealed huge differences in the molecular masses for the Y_2-receptor. Accordingly, in rat hippocampus and rabbit kidney membranes, the Y_2-receptor was identified as a glycoprotein of 50 kDa (*42*), which was found also for bovine (*43*), human (*44*), and porcine (*45*) hippocampal membranes. The same molecular weight was identified in a renal proximal tubule cell line of mice (*46*), whereas molecular masses from 39 up to 70 kDa were found in other tissues and species (*47*−*50*). These results suggest that NPY Y_2-receptor subtypes in hippocampal membranes from different species are conserved in their size and different from those in other tissues (*43*). The existence of at least two different Y_2-receptor subtypes, located centrally and peripherally, is suggested by Northern hybridization (*51*). Our approach for molecular mass determination of the Y_2-receptor subtype identified two glycoproteins of 58 and 50 kDa in SMS-KAN cells as well as in transfected CHO-hY2 cells, which are believed to represent the same protein with two different amounts of glycosylation. This approach included two different methods for molecular mass determination, specific cross-linking by photoaffinity labeling and Western blotting using Y_2-receptor selective antibodies, which provides a high reliability of the masses determined.

Both photoactivatable analogues have been shown to allow the formation of a covalent ligand−receptor complex. In the putative receptor binding region the ligands were only slightly modified, which suggests, in combination with the observed high receptor affinity, a receptor binding mode similar to that of the native ligand. The biotin label of the photoactivatable analogues, as well as the subtype selective antireceptor antibodies, will facilitate the purification of the covalent ligand−receptor complex. This will be used to identify the cross-linked positions of the receptor after enzymatic cleavage. Thus, the photoactivatable NPY analogues and antireceptor antibodies described are valuable tools for identification of the receptor regions involved in ligand binding. Because of the Y_2-receptor subtype selectivity of [^{3}H]Tmd27 and the used antibody, this will be possible even in tissues in which other Y-receptor subtypes are present.

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