Acidic Residues in Extracellular Loops of the Human Y1 Neuropeptide Y Receptor Are Essential for Ligand Binding*

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To investigate whether negatively charged residues of the human Y1 neuropeptide Y (NPY) receptor are required for ligand binding, a series of mutants were constructed in which aspartic acid and glutamic acid residues present in putative extracellular domains of the Y1 receptor were systematically replaced by alanines. The mutant cDNAs were transiently expressed in HeLa cells using a vaccinia virus-derived expression system, and their ability to bind NPY was evaluated. The level of expression of mutants unable to bind NPY was also tested immunologically. In addition, the ability of the mutant proteins to be recruited to the cell surface was assessed by confocal microscopy. Substitution of aspartic acids and glutamic acids of the N-terminal first extracellular domain had no effect on binding. On the other hand, substitution of acidic residues present in the second, third, and fourth extracellular loops resulted in proteins unable to bind [125I]NPY. These results demonstrate that the extracellular loops of the human Y1 NPY receptor are essential portions of its ligand binding domain.

Neuropeptide Y (NPY) is widely distributed throughout the central and peripheral nervous system (1). By activating specific G protein-coupled receptors, NPY is a mediator of diverse physiological responses (2). In the cardiovascular system, NPY exerts a number of important regulatory actions. First, NPY acts as a potent vasoconstrictor on certain blood vessels (3). Second, administered at nonpressor doses, NPY can strikingly potentiate the action of a number of vasopressive substances such as norepinephrine or angiotensin II (4). Third, in situations where the renin secretion has been stimulated by various agents (e.g. isoproterenol- or angiotensin-converting enzyme inhibitors) or treatments (e.g. sodium depletion or renal artery stenosis), NPY can drastically reduce plasma renin activity (5). Besides its effects in the cardiovascular system, NPY also inhibits glucose-induced insulin secretion in the mouse (6), and, when administered centrally, it causes hyperphagia and obesity (7). Therefore, NPY represents a target for the development of novel therapeutic agents. Three classes of NPY receptors have been described. Y1 receptors bind the entire peptide (NPY 1–36) as well as a Y1-specific agonist in which the glumatic acid residue at position 34 has been replaced by a proline (8). An NPY C-terminal fragment (NPY 13–36) interacts efficiently with the Y2 receptor type, whereas Y1 receptors bind this peptide fragment with a much lower affinity (9). A third class of NPY binding sites expressed in cardiac ventricular membranes has also been described (10). This Y3 subtype, unlike the Y1 and Y2 receptors does not bind the PYY peptide, a closely related peptide of gastrointestinal origin.

Most cardiovascular effects of NPY can be attributed to the stimulation of Y1 receptors. The cloning of the human and rat NPY Y1 receptors (11–13) demonstrated that this receptor is a member of the family of G protein-coupled receptors. This family of membrane receptors displays seven stretches of hydrophobic residues that probably constitute transmembrane domains. The N terminus is supposed to project in the extracellular space, whereas the C terminus is most probably located inside the cytoplasm. The potential transmembrane hydrophobic domains are formed by a helices probably packed in a sequential anticlockwise manner when observed from the extracellular side (14).

Among this family of receptors, the agonist binding site of catecholaminergic receptors is fairly well characterized (15, 16). The catecholamine binding site is buried at about 15 Å from the extracellular surface (14). Amino acid residues essential for catecholamine binding have been identified within transmembrane helices 3, 5, and 6 (16, 17). By contrast, very little is known about the ligand binding domain(s) of peptidergic G protein-coupled receptors. It has recently been demonstrated that epitopes at the top of transmembrane segments 5 and 6 are critical for the binding of non-peptide antagonist of the substance P (NK1) receptor (18). In addition, a valine residue present in the sixth transmembrane segment of the brain cholecystokinin-B/gastrin receptor has been implicated in the binding of a non-peptide antagonist (19). Nevertheless, little is known regarding the binding epitopes for the natural agonists of peptidergic G protein-coupled receptors.

When the positions of charged amino acid residues are labeled on a model of the NPY molecule (Fig. 2A) (20), a clear topological segregation appears between the positively (Arg, Lys, His) and the negatively (Asp, Glu) charged residues. While negatively charged amino acids are localized in the β turn, the positively charged residues are close to the joint N- and C-terminal ends of the peptide. Substitutions and deletions of various NPY residues have clearly demonstrated the importance of the N and C terminal regions of the peptide for receptor binding (21, 22). It is thus conceivable that ionic interactions between the positively charged amino acids of NPY and negatively charged residues of the Y1 receptor are involved in ligand-receptor interaction.

In this paper, we provide evidence that some acidic residues located in hydrophilic extracellular domain of the human neuropeptide Y (Y1) receptor are important to bind its natural ligand NPY.

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‡ The abbreviation used is: NPY, Neuropeptide Y.
**RESIDUES OF THE Y1 NPY RECEPTOR IMPORTANT FOR LIGAND BINDING**

**EXPERIMENTAL PROCEDURES**

**Materials**

125I-Neuropeptide Y and [3H]dATP were from Amersham. Restriction enzymes and T4 DNA ligase were from Boehringer Mannheim or Clonetech. Cell culture supplies were from GIBCO except Dulbecco's modified Eagle's medium and fetal calf serum from Seromed.

**Construction of Expression Vectors and Site-directed Mutagenesis**

The construction of pHGS-NPYr has been described previously (23). The addition of a histidine tag to generate pNHS-NPYr-His was performed by synthetic linkers addition. The FLAG epitope was introduced by site directed mutagenesis according to Deng and Nickoloff (24). The mutations were introduced into pHGS-NPYr-His by site-directed mutagenesis (24). In our nomenclature, the central number indicates the position of the mutated amino acid, the letter to the right indicates the wild type residue and the letter on the right indicates the mutant residue. All the mutated clones were analyzed by DNA sequencing. We systematically sequenced more than 200 base pairs around the mutation enzymes and T4 DNA ligase were from Boehringer Mannheim or Clonetech. Cell culture supplies were from GIBCO except Dulbecco's modified Eagle's medium and fetal calf serum from Seromed.

**Binding Assays on Whole Cells**

Binding assays were performed as described previously (23). To estimate the Kd, Ks, and IC50, the results of competition binding experiments were expressed in terms of the various constructs for DNA using V[1-(2,3-dioleoyloxypropyl)-N,N,N-trimethylammonium methylsulfate (Boehringer Mannheim) under conditions described by the supplier. The cells were incubated 16-18 h at 37 °C before analysis of expression.

**Immunodetection of Y1 Receptors Bearing the FLAG Epitope**

Indirect detection of the FLAG epitope by immunalkaline phosphatase assay or by immunofluorescence was performed using the M2 anti-FLAG antibody (IBI) and secondary antibodies from DAKOPATTS (Copenhagen, Denmark) using the supplier's recommendations. Cells transfected with a construct bearing the FLAG epitope at the C terminus were fixed with 4% paraformaldehyde and then permeabilized with 0.1% saponin. For cells transfected with N-terminal FLAG constructs, the permeabilization step was omitted.

**Computer-aided Protein Modeling**

**Sequence Alignments**—All sequence alignments were performed with the programs ALIGN/LALIGN (25), LFASTA (26), and the Wisconsin Genetic Computer Group Sequence Analysis Software Package (27). We used the computer graphics program Turbo-FRODO throughout this study. All force field calculations were performed with CHARMM (28) using the PARAM20 parameter set and a cut-off distance for nonbonded interactions of 8 Å.

**Model Structure of NPY**—A model for the neuropeptide Y was constructed based on the known three-dimensional structure of the avian pancreatic polypeptide (29) (Brokhaven Protein Data Bank entry IFFT), using the Protein Modeling tool ProMod (Peitsch and Joneung, 30). Briefly, the modeling procedure includes: (i) the construction of a framework for the NPY sequence based on the IFFT structure and (ii) the reconstruction of the missing and incomplete side chains using a library of allowed side chain rotamers (31). Optimization of the bond geometries and relief of unfavorable nonbonded contacts were performed by 10 steps of steepest descent followed by 500 steps of conjugate gradient energy minimization. This procedure is similar to those followed by other investigators (8, 32).

**Model Structure of the Human Y1 NPY Receptor**—The building of an approximate model for the human neuropeptide Y1 receptor was based on the following data and assumptions: (i) the receptor is a seven-transmembrane helix protein similar to the visual pigment rhodopsin (14-17, 33, 34), and (ii) the seven antiparallel helices are arranged antiparallelwise as viewed from the outside the cell membrane (35). The model building procedures used are similar to those described by Dahl et al. (36) for dopamine receptor D2. The seven transmembrane helices of the Y1 receptor were located by sequence comparisons with other members of the superfamily of G protein-coupled receptors. This analysis confirmed the findings reported by J. Baldwin (35). The seven transmembrane helices were built from the sequence of the human neuropeptide Y1 receptor (SwissProt: Y1R HUMAN) using generic coordinates for all helices. The side chains were added in their most frequent rotamer (31) using ProMod. The seven helices were assembled by computer graphics. Their relative positions and orientations were derived from the study of J. Baldwin (35). Solvent-accessible surfaces (37) and electrostatic potentials (38) were also used to guide the helix assembly. The seven helices, without connecting loops, were then refined by energy minimization. Using the LOOP routine of ProMod, the connecting loops were reconstructed from their "stems" by structural homology searches through the Brookhaven Protein Data Bank, as described by Greer (39). Since no reasonable structural information is available for the 40 N-terminal residues and the C-terminal stretch, these were omitted in this model. The receptor model was refined by 2000 cycles of conjugate gradient energy minimization and 50-ps constrained molecular dynamics simulations using CHARMM. Due to its length, many very distinct structures are possible for the extracellular domain 3. In order to choose a possible conformation, secondary structure analysis was performed on the residues 175-205. The GOR algorithm predicts an helical conformation for the residues 186-195. This conformation was used for the final construction of the extracellular domain 3.

**RESULTS**

**Development of a Transient Expression System for the Human Y1 NPY Receptor**—To identify residues of the human Y1 neuropeptide Y receptor critical for ligand binding, we first developed a vaccinia virus-dependent expression system leading to the transient production of high levels of Y1 receptors. This strategy involved the infection of HeLa cells with wild type vaccinia viruses. One hour postinfection, the cells were transfected with expression vectors in which the vaccinia 11K late promoter was linked to a cDNA fragment encoding the human Y1 NPY receptor. The wild type vaccinia virus provides the transcription factors necessary for the transactivation of the 11K promoter used to drive the expression of the linked cDNAs. After overnight incubation at 37 °C, the cells were tested for expression of 125I-NPY binding sites. This system resulted in high level of transient expression of NPY receptors at the surface of the cells. Fig. 1 illustrates the design and the result of a typical transient expression experiment. About 3 x 106 binding sites are expressed per cells assuming that about 10% of the cells are efficiently expressing the transfected cDNA (see Table I). No specific binding of 125I-NPY was detected when a control plasmid lacking the Y1 coding region was transfected. The equilibrium binding of 125I-NPY measured in the presence of increasing amount of unlabeled NPY competitor revealed that the IC50 of NPY was 3.1 nM.

**Negatively Charged Residues Present in Extracellular Domains of the Human Y1 Neuropeptide Y Receptor Are Required for Ligand Binding**—To test the hypothesis that negatively charged residues present in extracellular domains of the human Y1 NPY receptor could be involved in ligand binding, we substituted negatively charged amino acid residues of the Y1 receptor with alamines. Within the human Y1 NPY receptor, the 30 negatively charged amino acids are distributed as follows: 14 are in extracellular domains (see Fig. 2, B and C), 1 is in transmembrane domain 2, 5 are in cytoplasmic loops, and 10 are located in the C-terminal intracytoplasmic tail of the receptor. A deletion of the 51 residues in the C-terminal portion of the receptor (∆333-384) resulted in the loss of 9 negatively charged residues but had no significant effect on the affinity of the receptor for NPY (Table I). Furthermore, it seems very unlikely that residues located in intracytoplasmic loops could directly participate in ligand recognition. Therefore we started...
by replacing the Asp and Glu residues present in the extracellular domains of the human Y1 receptor with the neutral amino acid alanine. As a wild type template for the generation of mutants we initially used pHGS-NPYr-His, a derivative of pHGS-NPYr (23) which encodes 6 adjacent histidine residues at the C terminus of the Y1 receptor. The addition of these histidine residues has no effect on the affinity of the receptor.

TABLE I

| Plasmid   | Location of the mutation | IC50 | Bmax | Expression |
|-----------|--------------------------|------|------|------------|
| Wild type |                          | 3.1  | 2.9 x 10^6 | 9.9 |
| Δ333–384 | C-terminal               | 4.2  | 0.5 x 10^6 | 9.9 |
| E10A      | ECD1                     | 2.5  | 9.0 x 10^6 | 9.9 |
| E20A      | ECD1                     | 3.0  | 3.4 x 10^6 | 9.9 |
| E289A     | ECD1                     | 1.5  | 2.1 x 10^6 | 9.9 |
| D21A, D32A| ECD1                     | 2.1  | 2.1 x 10^6 | 9.9 |
| D20A      | ECD1                     | 3.0  | 2.0 x 10^6 | 9.9 |
| D104A     | ECD2 No binding detected |      |        | 9.2      |
| E110A     | ECD2                     | 2.1  | 1.6 x 10^6 | 9.9 |
| D181A     | ECD3                     | 7.7  | 0.5 x 10^6 | 9.9 |
| E182A     | ECD3                     | 2.7  | 0.7 x 10^6 | 9.9 |
| D190A     | ECD3                     | 2.0  | 0.9 x 10^6 | 9.9 |
| D194A     | ECD3 No binding detected |      |        | 9.6      |
| D200A     | ECD3 No binding detected |      |        | 9.9      |
| D205A     | ECD3                     | 16.6 | 1.8 x 10^6 | 8.8 |
| D287A     | ECD4 No binding detected |      |        | 9.2      |

* Assuming that 10% of the cells are expressing the protein.

The IC50 of NPY and Bmax values obtained for the wild type (WT) and mutant receptors are summarized in Table I. None of the mutations introduced in the N-terminal extracellular domain affected NPY binding. The IC50 of these mutants was between 1.5 and 3.0 nM. Within extracellular domain 2, substitution of Asp104 resulted in a complete loss of NPY binding (D104A), whereas Glu110 could be substituted without loss of ligand binding activity (E110A). In extracellular domain 3, mutations D181A, E182A, and D190A had no significant effect on affinity for NPY. On the other hand, mutations D194A and D200A resulted in complete loss of NPY binding at 50 pM ligand concentration. The IC50 of mutant D105A was increased about 5-fold as compared with the wild type receptor. In the extracellular domain 4, mutation D287A resulted in a protein without affinity for NPY.

**Immunological Detection of Mutant Receptors Expressed in Transfected Cells**—It was essential to assess whether the mutant receptors leading to a complete loss of NPY binding activity were nevertheless expressed in transfected cells. To address this problem, we first constructed a new Y1 expression vector by adding a foreign epitope between the end of the Y1 receptor coding part and the 6 adjacent histidine residues encoded by the plasmid pHGS-NPYr-His. As an epitope we used the FLAG™ epitope from IBI (see “Experimental Procedures”). This new construct was called pNPYr-FLAG. The addition of this epitope at the C-terminal end of the Y1 receptor had no effect on its affinity for NPY. Using pNPYr-FLAG as a template for site-directed mutagenesis, we reintroduced the mutations that completely abolished NPY binding (D104A, D194A, D200A, D287A). All these constructs were transfected into HeLa cells, and the level of expression of the corresponding proteins was assessed by immunological techniques.
tion of the FLAG™ epitope. The percentage of labeled cells was measured for each mutants (Table I). Using this assay, we observed that about 10% of HeLa cells transfected with the vector pNPYr-FLAG expressed the Y1 receptor. No alkaline phosphatase activity was observed in cells transfected with pHGS-NPYr-His, a vector lacking the FLAG™ epitope. Mutants D104A, D194A, D200A, and D287A expressed the same level of FLAG™-labeled protein as the wild type receptor. Thus the lack of NPY binding to these mutants was not due to a lack of protein expression.

The possibility remained that the mutant receptor proteins might be impaired in their ability to be recruited to the cell surface. To examine this possibility, we transfected cells with the wild type or the mutants receptors, all bearing the FLAG™ at the C terminus, and compared the cellular localization of the mutant proteins with that of the wild type receptor by indirect immunofluorescence and confocal microscopy (see Fig. 3). We could detect no difference in the localization of the wild type protein (pNPYr-FLAG) and the mutant proteins (D104A, D194A, D200A, and D287A). The staining pattern indicates that the receptor proteins are present at the cell surface. To further demonstrate the presence of the mutant proteins at the cell surface, we placed the FLAG™ epitope at the extracellular N terminus of the receptor between residues 2 and 3. The addition of the FLAG™ epitope at this position abolished ligand binding activity. Nevertheless the presence of this N-terminal tagged protein was unambiguously localized at the cell surface, since 10% of the cells transfected with this new construct and fixed with paraformaldehyde were fluorescent after indirect immunofluorescence detection of the FLAG™ epitope (data not shown). Because paraformaldehyde fixation does not allow access of the antibody to the cell interior, only cell surface-associated epitopes are detected with this approach. We then introduced mutations D104A, D194, D200A, and D287A into the N-terminal tagged receptor and examined the expression of the resulting proteins at the cell surface. A clear labeling of the surface of about 10% of the cells was seen for all the mutants (data not shown), demonstrating that the presence of these mutations do not impair the recruitment of the mutant receptors to the cell surface.

Molecular Modeling of the Interactions between the Human Y1 Receptor and NPY—In order to get spatial insight into the relative positions of the mutations described above, we constructed a molecular model of both NPY and the Y1 receptor. Based on the above results, and previous NPY analogue binding studies (8) which show that the N- and C-terminal regions of NPY are important for receptor binding, we searched for possible peptide-receptor interactions. Using the positions of the mutated acidic residues as guide lines, we placed NPY in a manner that maximises the interaction between its basic residues and the acidic residues of the Y1 receptor that were found to be crucial for peptide binding (Fig. 4). Since the mutation of Glu10, Glu30, Glu39, Asp31, and Asp32 of the first extracellular domain did not affect ligand binding, its absence in the model would not affect these studies. The orientation that best fits the experimental results is shown in Fig. 4. The long axis of NPY is almost parallel to the transmembrane helices, and both N and C termini are placed close to the cell membrane. In this orientation, both NPY:Arg33 and NPY:Arg39, which are essential for ligand binding, form salt bridges with residues Y1:Asp287 and Y1:Asp290, respectively. The more distant Y1:Asp290 may also participate in electrostatic interactions with NPY:Arg33 and NPY:Arg39, explaining its partial implication in peptide binding (Table I). In our model Y1:Asp290 also interacts with NPY:His26. Y1:Asp194 forms a salt bridge with NPY:Arg19 and may form electrostatic interactions with Y1:Lys4. Y1:Asp194 is also essential for ligand binding and could be implicated in hydrogen bonding with NPY:Arg25. The acidic residues of the Y1 receptor, in which mutation did not affect ligand binding, are not implicated in electrostatic interaction with NPY. Furthermore, this orientation of NPY on the Y1 receptor allows the positioning of NPY:Tyr2 in a hydrophobic pocket formed by Y1:Phe105, Y1:Met112, Y1:Tyr176, and Y1:Tyr211. Similarly, NPY: Tyr36 is placed in another hydrophobic area rimmed by the helices I, II, and VII.

DISCUSSION

The elucidation of the precise physiological role of NPY awaits the development of specific NPY receptor antagonists. To provide tools for a more rational development of such com-
pounds, and to attempt an understanding of how a peptide ligand interacts with a G protein-coupled receptor, we investigated the NPY binding epitopes of the human Y1 neuropeptide Y receptor. Positively charged residues of NPY are located in regions of the peptide essential for binding to the Y1 receptor (22). Thus, we hypothesized that NPY binding to its receptor...
might involve ionic interactions between positively charged residues of the ligand and negatively charged residues of the receptor. In this paper we used a site-directed mutagenesis approach to identify the negatively charged residues present in extracellular domains of the human Y1 receptor involved in receptor-ligand binding.

The transfection system we describe in this study results in the expression of binding sites displaying an IC₅₀ for NPY of about 3 nm. This value is slightly reduced when compared with endogenous binding sites expressed in SK-N-MC cells or in rat brain membrane preparations where the IC₅₀ values are close to 0.5 nm (40). On the other hand, the IC₅₀ value we measured using this transient expression system is similar to what is observed for the human Y1 cDNA clone expressed in CHO cells (10 nm) (11). A high density of Y1 binding sites is generated following this transient transfection protocol (about 3 × 10⁶ binding sites per cell) thus allowing sensitive detection of the effect of mutations on ligand binding. NPY receptors are coupled to the adenyl cyclase through G proteins. The signaling pathway most frequently associated with the stimulation of Y1 NPY receptors is the inhibition of cAMP accumulation (41). Using the transient expression system described in this study, we could not detect a faithful coupling of the receptor to this signaling pathway (not shown). We think that this apparent lack of coupling is due to the fact that only about 10% of the transfected cells are expressing the Y1 receptor (Table I and Fig. 3). Even an efficient coupling of receptors present in such a low proportion of the cell population would not result in a dramatic decrease of cAMP accumulation measured in the whole cell population.

Systematic mutations of the different acidic residues located in extracellular domains of the human Y1 neuropeptide Y receptor suggests that the N-terminal part of the human Y1 receptor is not involved in NPY binding through ionic interaction. On the other hand, we could identify acidic residues located in the three putative extracellular loops of the human Y1 receptor that, when mutated to alanine, yielded proteins unable to bind NPY. This absence of NPY binding was not due to a lack of expression nor to an impaired cell surface recruitment of the mutants, since immunocytochemical detection of the proteins showed identical levels of expression and similar cell staining pattern for the wild type receptor and the mutants unable to bind NPY. These mutations might result in the destruction of salt bridges between the ligand and the receptor. Alternatively, the three-dimensional conformation of the extracellular loops may be modified in such a way that no ligand-receptor interaction could take place. Site-directed mutagenesis does not allow differentiation between these possibilities. Being aware of these considerations, it is nevertheless interesting to note that most mutations affecting NPY binding (D194A, D200A, D205A, and D287A) are clustered at the top of transmembrane helices 5 and 6. Thus, whether the effects of mutations on ligand binding are direct or indirect, the clustering of the mutations affecting the receptor’s affinity for NPY suggests that the extracellular domains, at the top of transmembrane helices 5 and 6, are involved in ligand binding. These helices are connected by intracellular loop C₃H₂, which has been implicated in G protein coupling in receptors of the same family (42). Thus, binding of the ligand above these transmembrane segments might induce conformational changes that could affect the three-dimensional structure of loop C₃H₂, thereby promoting the coupling to the transducing G protein. Furthermore, it has recently been demonstrated that residues in two epitopes at the top of transmembrane domains 5 and 6 of the NK1 Substance P receptor were essential for the binding of a non-peptide Substance P antagonist (18).

In an attempt to explain the experimental results obtained, we have built three-dimensional models for both NPY and the Y1 receptor. Our modeling procedures being in agreement with those used by other investigators, our model for NPY must be very similar to the previously reported model structures (8, 20, 32). For the same reasons, the proposed model for the Y1 receptor should not differ markedly from the reported dopamine D2 receptor model (36), except for the connecting loops which are of different lengths. These models, however, are solely meant for providing structural insight into our experimental results. The interaction between NPY and the Y1 receptor seems to be largely dictated by electrostatic interactions, and a reasonable fit of the NPY and the Y1 receptor models was found based on the relative charge distributions on both molecules. Since the extracellular domains 2–4 of the Y1 receptor bear mainly negative charges, we oriented the NPY model in a way that maximizes the contacts between the acidic residues of the Y1 receptor and the basic amino acids of NPY. In doing so, we found that both essential N- and C-terminal domains are automatically located in the binding pocket. We are now using these models to assist us in designing new mutagenesis experiments, which should provide a more detailed understanding of the NPY-Y1 receptor interaction.

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