Cloning, Functional Expression, and Developmental Regulation of a Neuropeptide Y Receptor from Drosophila melanogaster*

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Neuropeptide Y, peptide YY, and pancreatic polypeptide are homologous 36-amino acid peptides that differ from most other peptide transmitters by having a relatively rigid conformation in aqueous solutions, defined as the pancreatic polypeptide fold, and a critical C-terminal tyrosine amide. These peptides serve as gastrointestinal hormones and neurotransmitters. A cDNA encoding a novel G protein-coupled receptor activated by neuropeptide Y was cloned from Drosophila by use of degenerate oligonucleotide primers and polymerase chain reaction amplification of cDNA prepared from transcripts expressed early in embryogenesis. The cDNA encodes a protein of 449 amino acids with the characteristics of a G protein-coupled receptor and shares significant amino acid identity with mammalian tachykinin receptors. When expressed in Xenopus oocytes, the PR4 protein is activated by mammalian neuropeptides in the order: peptide YY > neuropeptide Y > pancreatic polypeptide. Northern analysis showed that PR4 receptor is expressed at equivalent levels in adult Drosophila head and body and that the expression of the PR4 receptor is regulated during development. The molecular characterization of this receptor should lead to a better understanding of the functional role of this important family of hormone receptors in adult organisms and during development.

Neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide are homologous peptides that function as gastrointestinal hormones and neurotransmitters (1-4). NPY is often co-localized in nerves with noradrenaline (5, 6) and markedly potentiates vasoconstriction by noradrenaline (5, 6). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M81490.

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The abbreviations used are: NPY, neuropeptide Y; PYY, peptide YY; PCR, polymerase chain reaction; TM, transmembrane domain; Aco, 8-amino octanoic acid.

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Fig. 1. Restriction map (A) and nucleotide sequence and derived amino acid sequence of PR4 (B). Nucleotide 1 is the first nucleotide of the ATG translation start codon. The termination codon is indicated by a dot. Six potential N-glycosylation sites are indicated by asterisks. The likely transmembrane domains (I-VII) of the deduced amino acid sequence are indicated. C, alignment of the amino acid sequence of PR4 with those of Drosophila tachykinin receptor (DTKR) (16) and the mammalian tachykinin receptors NK1 (15) (or substance P, SPR), NK2...
coupled receptors were used to amplify members of this receptor family in cDNA prepared from transcripts expressed in 2-6-h Drosophila embryos. Sequence analysis of one subcloned PCR product showed that it represented a novel G protein-coupled receptor. This fragment was then used to probe a λcDNA library made from adult Drosophila heads. Restriction mapping and partial sequence analysis indicated that the two clones identified were identical. One clone, APR4, was further characterized.

Fig. 1 shows the restriction map, nucleotide sequence, and derived amino acid sequence of the APR4 cDNA and protein. The longest open reading frame encodes a 449-amino acid protein with a relative molecular mass of 49.3 kDa. Hydrophathy analysis of this protein indicated seven stretches of hydrophobic amino acids forming potential transmembrane domains characteristic of G protein-coupled receptors (data not shown). Four potential asparagine-linked glycosylation sites (Fig. 1B) are found at the N terminus preceding TM I. Several serine and threonine residues are present in the third intracellular loop and in the C-terminal cytoplasmic tail that are potential phosphorylation sites (12). The APR4 protein also possesses a number of residues conserved in most G protein-coupled receptors (Fig. 1C), such as the Asp-Arg-Tyr sequence in the second intracellular loop, cysteine residues in the first and second extracellular loops that may participate in a structurally important disulfide bonds, and prolines in TM VI and VII. APR4 protein shares significant homology with the tachykinin receptors (Fig. 1C). From residue 79 to 390, APR4 is 32-34.5% identical to mammalian (13-15) and Drosophila (16) tachykinin receptors. Unlike tachykinin receptors, APR4 protein does not have a His residue in TM VI; instead, APR4 possesses two His residues in the TM VII. Moreover, unlike most G protein-coupled receptors, the APR4 protein does not have a cysteine in the C-terminal intracellular domain that could serve as a substrate for palmitoylation.

These considerations suggested that the PR4 cDNA encodes a G protein-coupled receptor that is activated by a peptide. The cRNA was injected into Xenopus oocytes, and voltage clamp recordings were made 3-5 days later; of several peptides applied (Fig. 2), only NPY and PYY had any effect. These peptides evoked inward currents typical of those induced by other agonists that activate a calcium-dependent chloride current. No responses were obtained in the same oocytes with NPY free acid; this analog, which lacks the C-terminal amide of NPY, does not share the biological activity of NPY. PYY was more potent than NPY, and pancreatic polypeptide had almost no effect (Fig. 2); this order of potency is typical for the actions of these peptides on mammalian tissues (17). Peptides that had no effect on oocytes expressing PR4 protein included (n = 3 or 5) neurenomed K (4 μM), kassinin (10 μM), eledoisin (10 μM), substance P (10 μM), physalaemin (10 μM), bombesin (10 μM), arginine vasopressin (6 μM), bradykinin (10 μM), oxytocin (5 μM), cholecystokinin (4 μM), and vasoactive intestinal polypeptide (2 μM).

Two subtypes of receptors for NPY and PYY have been postulated from ligand binding and bioassay experiments (17). C2-NPY ([Cys5,Ac13,Cys17]NPY), a shortened, disulfide-stabilized analog of NPY (18), was as effective as NPY in activating the PR4 receptor (Fig. 2), and the analog NPY(2-36) (1 μM) also caused an inward current of 500 pA in three of four oocytes tested; these two analogs are somewhat Y2-selective (17). On the other hand, the substitution of Pro for Gln in NPY [Pro4]NPY reduces binding to Y2 receptors but has no effect on the binding of this compound to Y1 receptors; as shown in Fig. 2, [Pro4]NPY was a relatively poor agonist at the PR4 receptor. These data suggest that the PR4 receptor is more similar to the Y2 than the Y1 mammalian receptor, but it is possible that differences between Drosophila and mammalian receptor may confound the pharmacological classification. The inward current evoked by activation of the PR4 receptor in the oocyte is similar to that elicited by other transmitters known to stimulate phosphatidylinositol metabolism (e.g. Refs. 13-16). Activation of NPY receptors in mammalian cells has also been shown to increase phosphatidylinositol turnover (19, 20). However, it is not clear whether the Y1 or Y2 receptor type is normally associated with this transduction pathway.

Expression of the PR4 receptor was assessed by Northern analysis of poly(A)+ RNA prepared from adult head and body. A dominant 2.4-kilobase transcript appeared to be equally abundant in both head and body (Fig. 3A). Two weakly hybridizing bands (about 2.8 and 1.5 kilobases) were also present. These transcripts may represent alternative processing of a common RNA precursor for PR4 or transcripts from distinct yet highly related receptor genes. During development, the level of PR4 is subject to precise regulation. Northern analysis (Fig. 3B) and PCR analysis (not shown) indicate that PR4 is expressed at low levels during early embryonic stages (0-10 h). The expression of PR4 receptor increases later (10-14 h) and reaches the highest level during late stages of embryogenesis (14-18 h). This pattern of expression during embryogenesis corresponds to periods of rapid neuronal proliferation and differentiation. Subsequently, PR4 levels are reduced during larval stages and increased again during pupal

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(13) (or substance K, SKR), and NK3 (14) (or neurenomed K, NKR) receptors. Identical residues are boxed. Dashes indicate deletion of amino acid residues. Dots indicate the position of amino acid residues conserved in most G protein-coupled receptors. Likely transmembrane domains I-VII are indicated. bp, base pairs.
stages. The location of the gene encoding PR4 was identified by in situ hybridization to salivary gland chromosomes. Hybridization was present at position 97E1.2 on the right arm of the third chromosome (data not shown).

Most G protein-coupled receptors of *Drosophila* show quite high homology to their mammalian counterparts (see for example, Refs. 22–24); therefore, the PR4 cDNA should be useful for the isolation of mammalian receptors for NPY and related peptides. It has been very difficult to resolve subclasses of the family of receptors activated by NPY and PYY, and transfected cells expressing individual molecular species of NPY receptor will be particularly helpful in the development of selective ligands. Such ligands, particularly receptor antagonists, are needed to determine the functional role of this class of the peptides. Recently, the development and anatomic distribution of specific populations of neurons that contain NPY immunoreactive peptides has been described in *Drosophila*. Thus, the genetic approaches possible in *Drosophila* will also facilitate the study of the functional role of NPY-related peptides and their receptors in development.

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