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Cloning and characterization of a *Drosophila* tyramine receptor

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Receptors for biogenic amines such as dopamine, serotonin and epinephrine belong to the family of receptors that interact with G proteins and share a putative seven transmembrane domain structure. Using a strategy based on nucleotide sequence homology between the corresponding genes, we have isolated *Drosophila* cDNA clones encoding a new member of the G protein-coupled receptor family. This protein exhibits highest homology to the human α_2 adrenergic receptors, the human 5HT1A receptor and a recently cloned *Drosophila* serotonin receptor. The corresponding mRNA is found predominantly in adult *Drosophila* heads. Membranes from mammalian cells expressing this receptor displayed high affinity binding sites for [^{3}H]yohimbine, an α_2 adrenergic receptor antagonist (K_d = 4.45 × 10^{-9} M). Tyramine was the most efficient of the putative *Drosophila* neurotransmitters at displacing [^{3}H]yohimbine binding (EC_{50} = 1.25 × 10^{-6} M). Furthermore tyramine induced an inhibition of adenylate cyclase activity in NIH 3T3 cells expressing this receptor. The *Drosophila* tyramine receptor that we have isolated might therefore be an invertebrate equivalent of the mammalian α_2 adrenergic receptors.

Key words: adenylate cyclase/α_2-adrenergic receptors/G protein-coupled receptors/octopamine/yohimbine

Introduction

Biogenic amines such as dopamine, serotonin, octopamine and tyramine, have been suggested to play a role as neurotransmitters, hormones and neuromodulators in invertebrates (for a review see Kerkut and Gilbert, 1983). While dopamine and serotonin are present in both vertebrates and invertebrates, octopamine is most abundant in invertebrates and it has been proposed that octopamine is the invertebrate counterpart of norepinephrine (Robertson and Juorio, 1976). In the case of tyramine its role as a neuromediator has not yet been clearly established. Tyramine has been detected in rat brain and is abundant in the ganglion of crustaceans, insects and molluscs (Robertson and Juorio, 1976). It is synthesized from tyrosine by the enzyme tyrosine decarboxylase and is stored by a reserpine sensitive mechanism suggesting a localization in synaptic vesicles. There is no direct evidence, however, for intraneuronal localization or for release after nerve stimulation. In rat striatum [^{3}H]tyramine binding sites have been identified and it has been suggested that these sites are associated with dopamine storage vesicles (Vaccari, 1986). In invertebrates little is known about tyramine receptors and their physiological effects.

Most characterized receptors for biogenic amines (adrenaline, dopamine and serotonin) belong to the family of receptors that interact with guanine nucleotide binding proteins (G proteins) (O'Dowd *et al.*, 1989). These receptors share a predicted seven transmembrane domain structure with highly conserved amino acid sequences especially within certain transmembrane regions. In a previous study, we took advantage of this sequence homology to clone a *Drosophila* serotonin receptor (Witz *et al.*, 1990). Using a similar strategy we isolated a *Drosophila* cDNA clone which encodes a new member of the G protein-coupled receptor family.

This protein exhibits highest homology to the human 5HT1A receptor (Fargin *et al.*, 1988), to the human α_2A and α_2B adrenergic receptors (Kobilka *et al.*, 1987; Regan *et al.*, 1988) and to the *Drosophila* serotonin receptor (Witz *et al.*, 1990). Mammalian cells in which this receptor has been introduced display high affinity binding sites for [^{3}H]yohimbine, an α_2-adrenergic receptor antagonist. Among the putative *Drosophila* neurotransmitters, tyramine was the most effective at displacing [^{3}H]yohimbine binding. Furthermore, tyramine mediated an inhibition of adenylate cyclase activity in cells expressing this receptor. These data indicate that we have isolated a receptor for the putative neurotransmitter tyramine. We therefore named this protein Tyr-dro receptor.

Results

Isolation of Drosophila genomic and cDNA clones encoding a protein which exhibits homology to G protein-coupled receptors

Sequence comparisons of receptors for biogenic monoamines (adrenaline, dopamine and serotonin) reveal that these receptors constitute a subfamily among the larger family of G protein-coupled receptors. The highest sequence homology is found in the putative transmembrane domains VI and VII (O'Dowd *et al.*, 1989). We therefore constructed two series of degenerate oligonucleotides corresponding to consensus sequences found in these two domains. A *Drosophila* genomic library was probed with both series of oligonucleotides at low stringency. We obtained one genomic clone which hybridized strongly with both oligonucleotides. Southern blot analysis indicated that this genomic clone contained a 367 bp *Bgl*II fragment, which hybridized with both oligonucleotides. This fragment was therefore subcloned and sequenced.

The nucleotide sequence exhibited a striking homology

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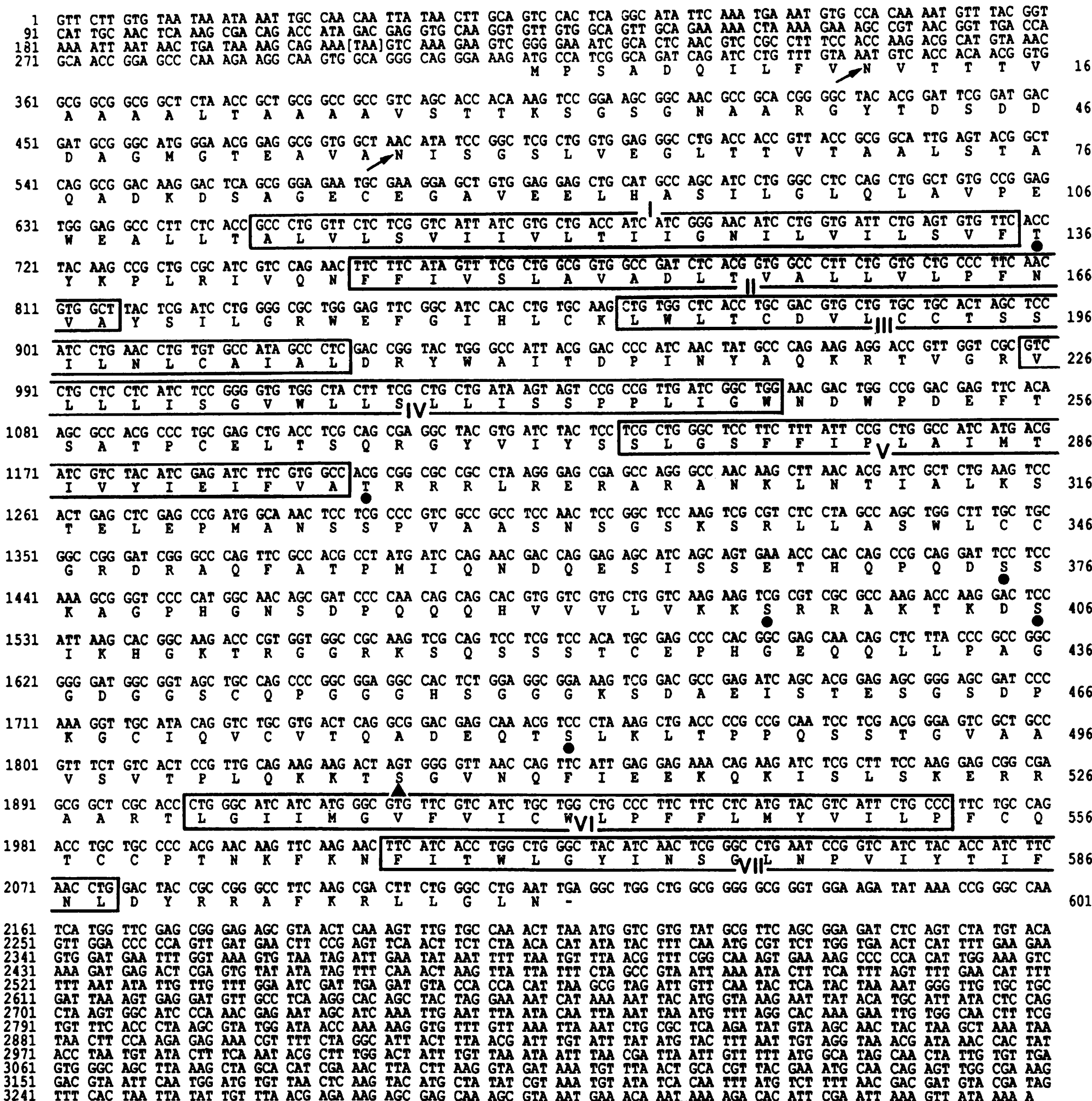

Fig. 1. Nucleotide sequence of the Tyr-dro receptor cDNA and predicted amino acid sequence. The nucleotide sequence of the 3329 bp long *Eco*RI fragment corresponding to the longest cDNA is represented (except the *Eco*RI sites). AATAAA is a putative polyadenylation sequence. The only differences between this cDNA sequence and the one recently published (Arakawa *et al.*, 1990) is the presence of a guanine instead of an adenine at position 413 (which transforms the serine 34 in an asparagine) and the presence of a short putative poly(A) tail in our clone. The seven putative transmembrane domains found in all G protein-coupled receptors are boxed and numbered (I–VII). Arrows indicate sites of potential N-linked glycosylation. Circles and triangles correspond to consensus sites for protein kinase C and protein kinase A respectively.

to transmembrane domains VI and VII of adrenergic receptors. 5′ of domain VI the open reading frame was interrupted suggesting the existence of an intron between transmembrane domains V and VI. In order to obtain the entire coding sequence we isolated corresponding cDNAs. A random primed *Drosophila* head cDNA library was probed with the *Bgl*II fragment. Several overlapping clones were obtained and sequenced. The sequence of the longest cDNA (Figure 1) contained a single open reading frame, 601 amino acids in length. Hydropathy profiles revealed seven hydrophobic domains which exhibited homology with the seven putative transmembrane domains of G protein-coupled receptors. The amino-terminal end of this protein contained an additional hydrophobic domain which might serve as a cleavable signal sequence, a feature observed in many membrane proteins but absent from most G protein-coupled receptors. Interestingly, the *Drosophila* muscarinic receptor (Onai *et al.*, 1989), the recently cloned *Drosophila* serotonin receptor (Witz *et al.*, 1990), and the receptors for glycoprotein hormones (LH, TSH and FSH) (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989; Parmentier *et al.*, 1989; Sprengel *et al.*, 1990) contain also a putative signal sequence. Since all these receptors have a long predicted extracellular tail (Figure 2) it is possible that the presence of a signal peptide is a characteristic of such receptors.

Comparison of the predicted amino acid sequence of the Tyr-dro receptor with the protein data banks revealed highest homology with the human 5HT1A receptor (47%), with the human α_2A and α_2B adrenergic receptors (46%) and with the *Drosophila* serotonin receptor (5HT-dro) (45%). Homologies were found in each putative transmembrane

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                              I                                    II
Tyr-dro   (111)TALVLSVIIVLTIIGNILVILSVFTYKPLRIVQNFFIVSLAVADLTVALLVLPFNVAYSI
5HT-dro   (165)VSIVLLIVILGTVVGNVLVCIAVCMVRKLRRPCNYLLVSLALSDLCVALLVMPMALLYEV
5HT1A-hu   (38)TSLLLGTLIFCAVLGNACVVAAIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQV
α2-hu      (35)LVCLAGLLMLLTVFGNVLVIIAVFTSRALKAPQNLFLVSLASADILVATLVIPFSLANEV
α1-ham     (47)VGLVLGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAIADLLLSFTVLPFSATIEV
D2-rat     (36)YAMLLTLLIFIIVFGNVLVCMAVSREKALQTTTNYLIVSLAVADLLVATLVMPWVVYLEV
dromu     (111)MGFVAAILSTVTVAGNVMVMISFKIDKQLQTISNYFLFSLAIADFAIGAISMPLFAVTTI

                                   III
Tyr-dro        LGRWEFGIHLCKLWLTCDVLCCTSSILNLCAIALDRYWAITDPINYAQ-KRTVGRVLLLI
5HT-dro        LEKWNFGPLLCDIWVSFDVLCCTASILNLCAISVDRYLAITKPLEYGV-KRTPRRMMLCV
5HT1A-hu       LNKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDRYWAITDPIDYVN-KRTPRPRAL-I
α2-hu          MGYWYFGKTWCEIYLALDVLFCTSSIVHLCAISLDRYWSITQAIEYNL-KRTPRRIKAII
α1-ham         LGYWVLGRIFCDIWAAVDVLCCTASILSLCAISIDRYIGVRYSLQYPT-LVTRRKAILAL
D2-rat         VGEWKFSRIHCDIFVTLDVMMCTASILNLCAISIDRYTAVAMPMLYNTRYSSKRRVTVMI
dromu          LGYWPLGPIVCDTWLALDYLASNASVLNLLIISFDRYFSVTRPLTYRA-KRTTNRAAVMI

                 IV                                                  V
Tyr-dro        SGVWLLSLLISSPPLIGWNDWPDEFTSATP----CELTSQRGYVIYSSLGSFFIPLAIMT
5HT-dro        GIVWLAAACISLPPLLILGNEHEDEEGQPI----CTVCQNFAYQIYATLGSFYIPLSVML
5HT1A-hu       SLTWLIGFLISIPPMLGWRTPEDRSDPDA-----CTISKDHGYTIYSTFGAFYIPLLLML
α2-hu          ITCWVISAVISFPPLISIEKKGGGGGPQPAEPR-CEINDQKWYVISSCIGSFFAPCLIMI
α1-ham         LSVWVLSTVISIGPLLGWKEPAPNDDKE------CGVTEEPFYALFSSLGSSYIPLAVIL
D2-rat         AIVWVLSFTISCPLLFGLNNTDQNE---------CIIA-NPAFVVYSSIVSFYVPFIVTL
dromu          GAAWGISLLLWPPWIYSWPYIEGKRTVPKDECYIQFIETNQYITFGTALAAFYFPVTIMC

Tyr-dro        IVYIEIFVATRRRLRERARANKL-(200)-NQFIEEKQKISLSKERRAARTLGIIMGVFV
5HT-dro        FVYYQIFRAARRIVLEEKRAQTH-( 72)-GSPHHKKLRFQLAKEKKASTTLGIIMSAFT
5HT1A-hu       VLYGRIFRAARFRIRKTVKKVEK-( 90)-ERNAEAKRKMALARERKTVKTLGIIMGTFI
α2-hu          LVYVRIYQIAKRRTRVPPSRRGP-(118)-GLPRRRAGAGGQNLEKRFTFVLAVVIGVFV
α1-ham         VMYCRVYIVAKRTTKNLEAGVMK-( 31)-PRSSIAVKLFKFSREKKAAKTLGIVVGMFI
D2-rat         LVYIKIYIVLRKRRKRVNTKRSS-( 95)-SLKTMSRRKLSQQKEKKATQMLAIVLGVFI
dromu          FLYWRIWRETKKRQKDLPNLQAG-(372)-NAAKKKKKSQEKRQESKAAKTLSAILLSFI

                  VI                          VII
Tyr-dro        ICWLPFFLMYVILPFCQT-CCPTNKFKNFITWLGYINSGLNPVIYTIFNLDYRRAFKRLL(  3)
5HT-dro        VCWLPFFILALIRPFET--MHVPASLSSLFLWLGYANSLLNPIIYATLNRDFRKPFQEIL( 39)
5HT1A-hu       LCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKII(  7)
α2-hu          VCWFPFFFTYTLTAVGG--CSVPRTLFKFFFWFGYCNSSLNPVIYTIFNHDFRRAFKKIL(  9)
α1-ham         LCWLPFFIALPIGSLFST-LKPPDAVFKVVFWLGYFNSCLNPIIYPCSSKEFKRAFMRIL(152)
D2-rat         ICWLPFFITHILNIHCD--CNIPPVLYSAFTWLGYVNSAVNPIIYTTFNIEFRKAFMKIL(  2)
dromu          ITWTPYNILVLIKPLTTCSDCIPTELWDFFYALCYINSTINPMCYALCNATFRRTYVRIL( 18)
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Fig. 2. Amino acid similarity between the Tyr-dro receptor and other G protein-coupled receptors. 5HT-dro, 5HT1A-hu, α_2-hu, α_1-ham, D_2-rat and dromu correspond respectively to the *Drosophila* serotonin receptor (Witz *et al.*, 1990), the human 5HT1A receptor (Fargin *et al.*, 1988), the human α_2A adrenergic receptor (Kobilka *et al.*, 1987), the hamster α_1 adrenergic receptor (Cotecchia *et al.*, 1988), the rat D_2 dopamine receptor (Bunzow *et al.*, 1988) and the *Drosophila* muscarinic receptor (Onai *et al.*, 1989; Shapiro *et al.*, 1989). Putative transmembrane domains are indicated by brackets (I and VII). Numbers in parentheses correspond to the number of amino acids that are not represented. The percentage of homology between these receptors was calculated over the domains that are represented in this figure.

domain (Figure 2) but not in the amino and carboxy terminal tails nor in the long third cytoplasmic loop.

The Tyr-dro sequence exhibited also a series of features which are present in most members of this gene family: two putative N-linked glycosylation sites in the amino terminal tail and a putative phosphorylation site by the cAMP dependent kinase in the third putative intracellular loop (arrows and triangle in Figure 1).

Cos-7 cells expressing the Tyr-dro receptor display high affinity [^{3}H]yohimbine binding

To determine whether the Tyr-dro cDNA encoded a functional receptor this cDNA was cloned into the expression vector pSG5 (Green *et al.*, 1988) that contains an SV40 promoter and origin of replication and β-globin splice sequences. The resulting recombinant pTyr was introduced into Cos-7 cells by calcium phosphate transfection, after

which membranes from transfected and non transfected cells were analysed for their ability to bind a variety of radioligands. [^{3}H]Yohimbine displayed a high affinity for the membranes of transfected Cos-7 cells, while no binding was observed to membranes of untransfected cells. The calculated equilibrium dissociation constant (K_d) of [^{3}H]yohimbine was 4.45 nM and its B_{max} was 1.3 pmol of receptor per mg of membrane protein (Figure 3). The Tyr-dro cDNA therefore encodes a protein which has a high affinity for the α_2-adrenergic receptor antagonist [^{3}H]yohimbine. In order to determine which natural ligand might interact with the Tyr-dro receptor we tested the ability of a number of putative *Drosophila* neurotransmitters to displace [^{3}H]yohimbine binding (Table I). Tyramine displayed the highest affinity ($EC_{50} = 1.25 \times 10^{-6}$ M) for the Tyr-dro receptor. Octopamine, dopamine and epinephrine ($EC_{50} > 10^{-5}$ M) were less efficient than tyramine at displacing [^{3}H]yohimbine binding while serotonin, norepinephrine and histamine had a very low affinity for the Tyr-dro receptor ($EC_{50} > 10^{-4}$ M). We tested the affinity of various drugs for the Tyr-dro receptor in order to determine its pharmacological profile. These compounds displayed the following rank order of potency: yohimbine ($EC_{50} = 5.5 \times 10^{-9}$ M) > chlorpromazine ($EC_{50} = 2.5 \times 10^{-8}$ M) > phentolamine ($EC_{50} = 8.5 \times 10^{-8}$ M) > mianserine ($EC_{50} = 10^{-7}$ M) > cyproheptadine ($EC_{50} = 1.75 \times 10^{-7}$ M). The pharmacological profile of this receptor does not correspond with any of the receptors described in *Drosophila* or in other insects. In particular, octopamine receptors have been characterized in various insect preparations (Evans, 1981; Dudai, 1982; Guillen *et al.*, 1989) but none of them displays a high affinity for yohimbine. In addition, tyramine has less affinity than octopamine for these octopamine receptors. Our results therefore suggest that we have isolated a new *Drosophila* receptor, the pharmacology of which has not been reported previously.

Tyramine mediates a reduction of cAMP levels in cells expressing the Tyr-dro receptor

In order to determine to which second messenger machinery the Tyr-dro receptor might be coupled, we analysed the effects of various neurotransmitters on the levels of cAMP in transfected cells. We were unable to detect any reproducible change in cAMP levels in Cos-7 cells transfected with the pTyr recombinant. In control experiments where we transfected Cos-7 cells with recombinants expressing either the *Drosophila* serotonin receptor which activates adenylate cyclase or the mouse D_2 dopamine receptor which inhibits adenylate cyclase we could detect only a small change in cAMP levels (data not shown). However, when these two receptors were stably introduced and expressed in mouse NIH 3T3 cells, the effects of serotonin and dopamine on cAMP levels were stronger and more reproducible (not shown). We decided therefore to produce stable cell lines expressing the Tyr-dro receptor. Mouse NIH 3T3 cells were cotransfected with the pTyr recombinant and the pRSVneo recombinant encoding resistance to G418. G418 resistant colonies were isolated, amplified and analysed for expression of mRNA encoding the Tyr-dro receptor. We selected two cell lines expressing high levels of Tyr-dro mRNA, F4 and F5. To ensure that F4 and F5 cells were expressing the Tyr-dro receptor at their surface we analysed the binding of [^{3}H]yohimbine to these cells. [^{3}H]Yohimbine displayed a high affinity to membranes of F4 and F5 cells while it did not bind to membranes of control NIH 3T3 cells. Furthermore tyramine and octopamine were able to displace [^{3}H]yohimbine binding with the same respective affinities

Table I. Affinities of various compounds for the Tyr-dro receptor expressed in Cos-7 cells

	EC_{50} (μM)	
Neuromediators		
Tyramine	1.2	(4)
(±) Octopamine	40	(4)
Dopamine	50	(2)
(−) Epinephrine	70	(2)
(−) Norepinephrine	150	(2)
Serotonin	175	(2)
Histamine	200	(2)
Other ligands		
Yohimbine	0.0055	(2)
Chlorpromazine	0.025	(2)
Phentolamine	0.085	(2)
Mianserine	0.1	(2)
Cyproheptadine	0.175	(2)
Dihydroergotamine	0.2	(2)
Clonidine	15	(2)
(±) Synephrine	20	(2)

Competition by various compounds for the binding of [^{3}H]yohimbine to membranes of Cos-7 cells transfected with the pTyr recombinant. Membranes were incubated with 3 nM [^{3}H]yohimbine in the presence of increasing concentrations of the indicated drugs (100 pM to 1 mM). EC_{50} represents the average concentration required to displace 50% of [^{3}H]yohimbine specific binding. Numbers in parentheses represent the number of independent experiments with each point performed in duplicate.

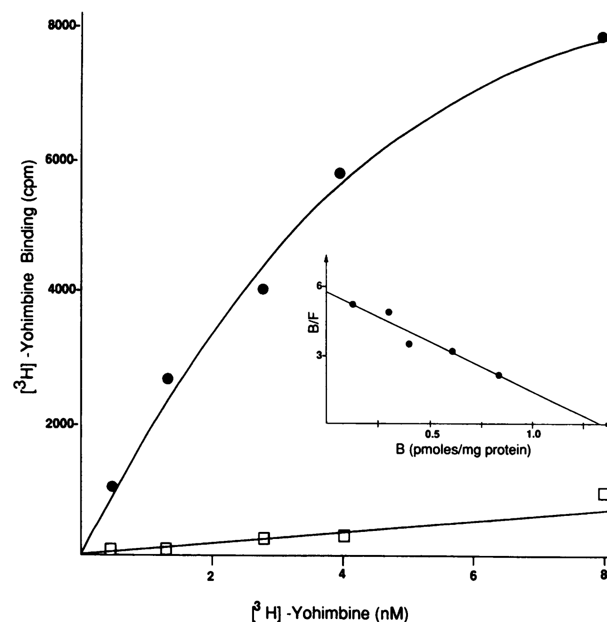

Fig. 3. Expression of the Tyr-dro receptor in Cos-7 cells. Saturation isotherm of [^{3}H]yohimbine binding to membranes of Cos-7 cells transfected with the pTyr recombinant. Membranes were incubated with concentrations of [^{3}H]yohimbine ranging from 0.5 nM to 8 nM plus (□) or minus (●) 10 μM phentolamine. Total yohimbine binding is indicated by closed circles (●) and non-specific binding by open squares (□). Data are representative of two independent experiments with each point performed in duplicate. Inset; Scatchard analysis of [^{3}H]yohimbine binding K_d = 4.45 nM; B_{max} = 1.3 pmol receptor/mg membrane protein.

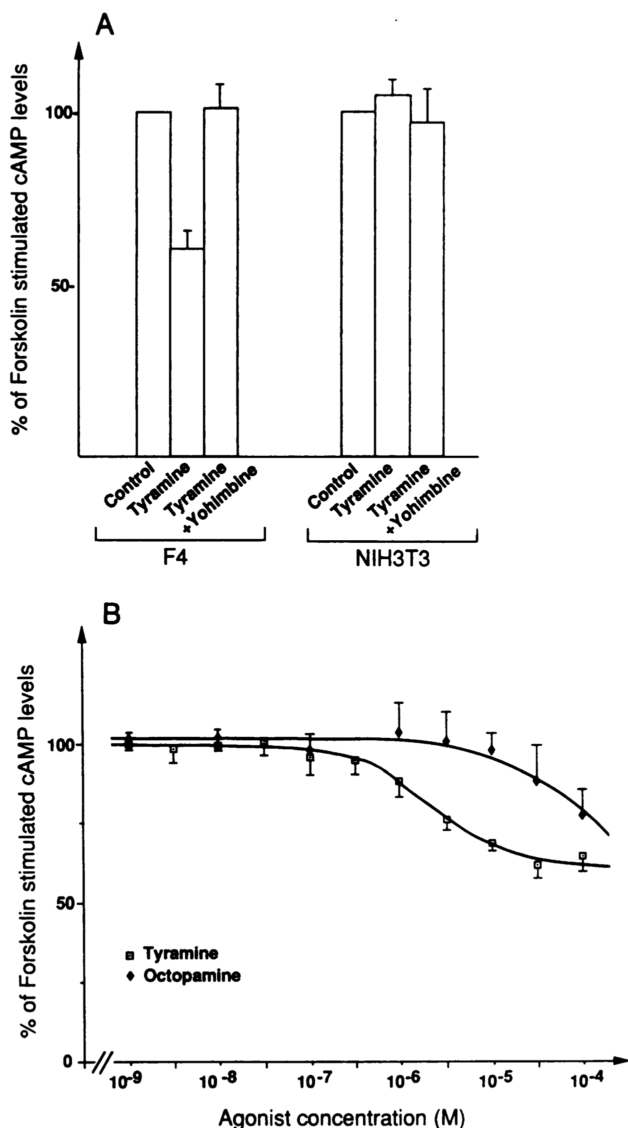

Fig. 4. Tyramine induced decrease in cAMP levels in cells expressing the Tyr-dro receptor. cAMP levels were determined as described in Materials and methods and were expressed as a percentage of the value obtained with 3 μM of forskolin (100%). cAMP levels presented are the mean of at least four independent experiments performed in duplicate. **Panel A.** Effect of 10 μM tyramine and 1 μM yohimbine on forskolin stimulated cAMP levels in NIH 3T3 cells expressing the Tyr-dro receptor (F4) or in control cells. **Panel B.** Effect of various concentrations of either tyramine or (±) octopamine on forskolin stimulated cAMP levels in F4 cells.

as in transfected Cos-7 cells (data not shown). The Tyr-dro receptor stably expressed in F4 and F5 cells appears, therefore, to have the same characteristics as in transfected Cos-7 cells.

We analysed the effects of various concentrations of neurotransmitters on cAMP levels in transformed F4 cells and in control NIH 3T3 cells. Tyramine, octopamine, epinephrine, serotonin and dopamine did not elicit any increase in the basal level of cAMP indicating that the Tyr-dro receptor does not activate adenylate cyclase in these cells (data not shown). However, in the presence of forskolin, an activator of adenylate cyclase, tyramine reduced forskolin stimulated cAMP levels by 40% in F4 cells while it had no effect on control NIH 3T3 cells (Figure 4A). This effect was dose dependent and saturable. The half maximal reduction of cAMP levels (EC_{50}) was observed with 2.4×10^{-6} M of tyramine (Figure 4B). Octopamine was also able to reduce cAMP levels in F4 cells although less efficiently than tyramine ($EC_{50} = 3 \times 10^{-5}$ M) (Figure 4B). These values are in good correlation with those obtained in binding experiments (Table I). Epinephrine, dopamine and serotonin were unable to reduce forskolin stimulated cAMP levels in F4 cells. Among the putative *Drosophila* neurotransmitters, tyramine is therefore the most potent agonist of the Tyr-dro receptor at eliciting a decrease in cAMP levels. 10^{-6} M of yohimbine inhibited completely the effect of tyramine in F4 cells indicating that yohimbine is an antagonist of the Tyr-dro receptor (Figure 4A). Similar results were obtained with another cell line expressing the Tyr-dro receptor (F5) indicating that the results we have obtained with the F4 cells are not particular to that cell line (data not shown).

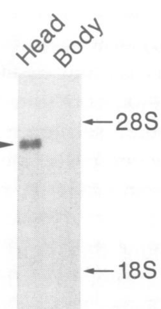

Fig. 5. Distribution of mRNA encoding the Tyr-dro receptor. Northern blot analysis of 10 μg of poly(A)$^+$ mRNA from adult *Drosophila* heads and bodies. Arrows denote positions in the gel of 28S and 18S mouse ribosomal RNAs. The arrowhead corresponds to the 4.3 kb mRNA detected in heads. The probe used was the ^{32}P-labelled *Eco*RI cDNA fragment. This experiment was performed twice with independent mRNA preparations.

The Tyr-dro receptor is expressed predominantly in Drosophila heads

In order to investigate the tissue distribution of Tyr-dro mRNA expression we performed a Northern blot analysis of poly(A)$^+$ mRNA from adult *Drosophila* heads and bodies. A single mRNA species 4.3 kb in length could be detected in heads but not in bodies (Figure 5). Interestingly, when the stringency of hybridization was reduced an additional band 3.5 kb in length was detected in head poly(A)$^+$ mRNA (not shown). This band might correspond to another closely related receptor. We are currently analysing expression of the Tyr-dro mRNA in the head by *in situ* hybridization.

Discussion

We have isolated a *Drosophila* cDNA encoding a member of the G protein-coupled receptor family. Sequence comparisons reveal that the members of that gene family which are the closest to the Tyr-dro receptor are the human 5HT1A serotonin receptor (47% homology), the human α_2A and α_2B adrenergic receptors (46%), the *Drosophila* serotonin receptor (45%), the hamster α_1 adrenergic receptor (43%) and the human β_1 adrenergic receptor (41%). The receptors that have the two highest scores (5HT1A and α_2) are activated by different ligands (serotonin and epinephrine) but they couple to the same second messenger machinery resulting in a decrease in

cAMP levels. The fact that the Tyr-dro receptor is also able to inhibit adenylate cyclase suggests therefore that its homology with the 5HT1A and α_2 adrenergic receptors is based more on a common coupling than on the nature of its ligand. In fact neither serotonin nor epinephrine can activate the Tyr-dro receptor. Reciprocally tyramine has a low affinity for the α_2 and 5HT1A receptors (Nathanson, 1985).

It is also worth noting that most receptors which are negatively coupled to adenylate cyclase have a very short carboxy-terminal tail (Figure 2), especially since this domain is involved in receptor−G protein interaction (for a review see Jackson, 1990).

While it has been clearly shown that octopamine acts as a neuromediator in various insect preparations there are only suggestions that tyramine might be a neuromediator in invertebrates (Robertson and Juorio, 1976). In particular, tyramine is abundant in insect brains and its distribution in different tissues does not parallel that of octopamine, suggesting that tyramine is not merely a precursor in the biosynthetic pathway of octopamine (Maxwell *et al.*, 1978; Juorio and Sloley, 1988). Furthermore some of the physiological responses elicited by tyramine such as the modulation of glycogenolysis are different from those generated by octopamine (Downer, 1979). It is therefore likely that tyramine interacts with specific receptors such as the one that we have isolated.

We should say at this point that we disagree in our conclusion with a recent article which reports the cloning of the same *Drosophila* cDNA and claims that it encodes an octopamine receptor (Arakawa *et al.*, 1990). Unfortunately these authors did not analyse the effects of tyramine on either [^{3}H]yohimbine binding or cAMP levels. Except for that, their results agree with ours: [^{3}H]yohimbine has a high affinity for their receptor and is displaced by a variety of drugs with affinities which are similar to the ones we observe. Furthermore, these authors report that octopamine elicits a decrease in forskolin-stimulated cAMP levels with an EC_{50} similar to ours. The reason why these investigators did not analyse the effects of tyramine is unclear to us. Tyramine might have cross-reacted with an endogenous receptor present in the cell line they used (CHO cells) and its effect might therefore have been obscured.

Our studies point toward tyramine rather than octopamine as the natural ligand of the Tyr-dro receptor for the following reasons.

(i) Tyramine is able to elicit a physiological response in cells expressing the Tyr-dro receptor and is the most efficient of the putative *Drosophila* neurotransmitters at doing so. The only other neurotransmitter which can activate the Tyr-dro receptor is octopamine but its EC_{50} is ~ 12 times higher than that of tyramine (Figure 4).

(ii) The characteristics of the known octopamine receptors are different from those of the Tyr-dro receptor. Octopamine receptors in insects have been classified in different subtypes (Evans, 1981), some of which are positively coupled to adenylate cyclase (Garcia *et al.*, 1981; Orchard *et al.*, 1982; Nathanson, 1985; Guillen *et al.*, 1989). In *Drosophila* heads, [^{3}H]octopamine binding sites have been found (Dudai, 1982) as well as an octopamine sensitive cyclase (Uzzan and Dudai, 1982). However, none of the known octopamine receptors display a high affinity for [^{3}H]yohimbine as does the Tyr-dro receptor. In addition, tyramine has less affinity than octopamine, for octopamine receptors, while it is the opposite in the case of the Tyr-dro receptor (Table I). Furthermore, there is no report of octopamine inhibiting adenylate cyclase.

(iii) Two observations suggest that tyramine and octopamine might have opposite effects on adenylate cyclase. It has been shown that octopamine stimulates glycogenolysis in cockroach nerve cord and fat bodies possibly through activation of adenylate cyclase (Downer, 1979). In contrast tyramine caused a decrease in glycogenolysis in isolated fat bodies of cockroaches (Downer, 1979). In *Drosophila* head membranes it was shown that tyramine stimulates adenylate cyclase presumably by cross-reacting with octopamine receptors (Uzzan and Dudai, 1982). However, the maximal stimulation obtained with tyramine was only 1.6-fold while the maximal stimulation obtained with octopamine was 4-fold. In addition when octopamine and tyramine were mixed the stimulation of adenylate cyclase was reduced by 50% (Uzzan and Dudai, 1982). These two observations suggest that tyramine might activate receptors that are negatively coupled to adenylate cyclase in *Drosophila* head membranes and in cockroach fat bodies.

We have isolated a *Drosophila* receptor which is a member of the G protein-coupled receptor family. Its pharmacological characteristics are different from those of the receptors so far described in insect preparations. Our results suggest that tyramine is the best candidate for the natural ligand of the Tyr-dro receptor. However, more experiments are needed to demonstrate that tyramine is a neuromediator in insects. In particular we will try to localize more precisely tyramine and the Tyr-dro receptor using antibodies directed against these two molecules. In any case the availability of the Tyr-dro cDNA combined with the powerful genetic techniques available in *Drosophila* should enable us to generate mutant flies that do not express the Tyr-dro receptor or express altered versions of this receptor and should therefore allow us to study its function *in vivo*.

Materials and methods

Reagents

All drugs were obtained from Sigma. Tyramine = para-tyramine; (±) Octopamine = (±) para-octopamine.

Isolation and sequence of genomic and cDNA clones

A genomic library from the wild-type Oregon R strain of *Drosophila melanogaster* constructed in the lambda phage EMBL3 was probed with two degenerate oligonucleotides corresponding to consensus sequences found in transmembrane domains *VI* [5′-TT(C/T)(A/G)(C/T)(C/G)(C, A/G) TCTGCTGGCTGCCCTTCTTC-3′] and *VII* [5′-TGG(T,C/A)T(G/T) GGCTA(T/C)G(T/C)CAA(T/C)(A/T)(G/C)-3′]. Oligonucleotides were labelled at their 5′ end with polynucleotide kinase. Hybridizations (40°C, 5 × SSC, 5 × Denhardt's, 20 mM sodium phosphate buffer, pH 6.5, 1% SDS, 100 μg/ml tRNA) and washings (40°C, 2 × SSC, 1% SDS) were performed in non-stringent conditions.

The cDNA library (a gift from Lily Jan) was prepared from heads of *Drosophila* from the wild-type Oregon R strain. [The library contains both random primed and oligo(dT) primed cDNAs and was prepared from RNAs >3 kb.] This library was probed with a random primed *Bgl*II genomic fragment, extending from positions 1865 to 2232 (Figure 1) at high stringency (42°C, 50% formamide, 5 × SSC, 20 mM sodium phosphate buffer, pH 6.5, 1 × Denhardt's, 100 μg/ml tRNA). Sequence of the 3329 bp cDNA (Figure 1) was performed on both strands by the dideoxy technique using successive synthetic oligonucleotides.

Expression of the Tyr-dro receptor in mammalian cells

The 3329 bp *Eco*RI fragment corresponding to the longest cDNA was inserted into the *Eco*RI site of expression vector pSG5 (Green *et al.*, 1988).

The resulting recombinant pTyr was introduced into mouse NIH 3T3 cells by calcium phosphate mediated transfection, together with the recombinant pRSVneo which encodes resistance to G418 (20 μg of pTyr and 1 μg of pRSVneo per 10 cm dish). Transformed clones were selected in the presence of 0.5 mg/ml of G418. Isolated foci were amplified and total RNA was prepared and analysed for expression of Tyr-dro mRNA. Two cell lines were selected (F4 and F5) which expressed high levels of Tyr-dro mRNA as measured by Northern blot analysis.

For transient expression of the Tyr-dro receptor, Cos-7 cells were transfected by the calcium phosphate technique with the pTyr recombinant alone (20 μg per 10 cm dish) and analysed 48 h after transfection.

RNA analysis

Heads and bodies of adult *Drosophila* were separated by freeze fracture. Poly(A)$^+$ mRNA was prepared, fractionated on 1% agarose-formaldehyde gel and transferred to a nitrocellulose filter. DNA probes were ^{32}P-labelled by random priming and hybridized to filters at high stringency (42°C, 50% formamide, 5 × SSC, 1 × Denhardt's, 20 mM sodium phosphate buffer, pH 6.5, 0.1% SDS, 100 μg/ml tRNA). Washings were performed at high stringency (60°C, 0.1 × SSC, 0.1% SDS).

cAMP assays

Cells were seeded into six-well plates at a density of $\sim 3 \times 10^5$ cells/well, washed once with PBS and incubated for 15 min at 37°C with 100 μM of isobutylmethylxanthine (IBMX) and test agents in PBS. The reaction was stopped by aspiration of the media, followed by the addition of 1 ml of ice-cold ethanol. After 2 h at room temperature, the ethanol was collected and lyophilized. The pellet was reconstituted and cAMP was quantitated using a radioimmunoassay kit (NEN: NEK-033). The basal level of cAMP observed in the absence of drugs was about the same in all cell lines (~300 pmol/mg of protein). Forskolin (3 μM) typically yielded a 10-fold increase in cAMP levels.

Radioligand binding assays

Membranes were prepared as described in Amlaiky and Caron (1985). [^{3}H]Yohimbine binding assays and competition displacement experiments were performed with 10 μg protein per sample in a final volume of 500 μl in 50 mM Tris–HCl, pH 7.4, at 30°C for 20 min. Reactions were terminated by filtration over Whatman GF/B glassfibre filters.

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References

Amlaiky,N. and Caron,M.G. (1985) *J. Biol. Chem.*, **260**, 1983–1986.

Arakawa,S., Gocayne,J.D., McCombie,W.R., Urquhart,D.A., Hall,L.M., Fraser,C.M. and Venter,J.C. (1990) *Neuron*, **2**, 343–354.

Bunzow,J.R., Van Tol.H.H.M., Grandy,D.K., Albert,P., Salon,J., Christie,M., Machida,C.A., Neve,K.A. and Civelli,O. (1988) *Nature*, **336**, 783–787.

Cotecchia,S., Schwinn,D.A., Randall,R.R., Lefkowitz,R.J., Caron,M.G. and Kobilka,B.K. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7159–7163.

Downer,R.G.H. (1979) *Comp. Biochem. Physiol.*, **62C**, 31–34.

Dudai,Y. (1982) *Neurosci. Lett.*, **28**, 163–167.

Evans,P.D. (1981) *J. Physiol.*, **318**, 99–122.

Fargin,A., Raymond,J.R., Lohse,M.J., Kobilka,B.K., Caron,M.G. and Lefkowitz,R.J. (1988) *Nature*, **335**, 358–360.

Garcia,J.L., Haro,A. and Municio,A.M. (1981) *Comp. Biochem. Physiol.*, **70B**, 57–62.

Green,S., Issemann,I. and Sheer,E. (1988) *Nucleic Acids Res.*, **16**, 369–370.

Guillen,A., Haro,A. and Municio,A.M. (1989) *Life Sci.*, **45**, 655–662.

Jackson,T.R. (1990) *Cell Biol.*, **2**, 167–173.

Juorio,A.V. and Sloley,B.D. (1988) *Brain Res.*, **444**, 380–382.

Kerkut,G.A. and Gilbert,L.I. (1983) *Comprehensive Insect Physiology, Biochemistry and Pharmacology.* Pergamon Press, **11**, 477–489.

Kobilka,B.K., Matsui,H., Kobilka,T.S., Yang-Feng,T.L., Francke,U., Caron,M.G., Lefkowitz,R.J. and Regan,J.W. (1987) *Science*, **238**, 650–656.

Loosfelt,H., Misrahi,M., Atger,M., Salesse,R., Thi,M.T.V.H.-L., Jolivet,A., Guiochon-Mantel,A., Sar,S., Jallal,B., Garnier,J. and Milgrom,E. (1989) *Science*, **245**, 525–528.

Maxwell,G.D., Tait,J.F. and Hildebrand,J.G. (1978) *Comp. Biochem. Physiol.*, **61C**, 109–119.

McFarland,K.C., Sprengel,R., Philipps,H.S., Köhler,M., Rosemblit,N., Nikolics,K., Segaloff,D.L. and Seeburg,P.H. (1989) *Science*, **245**, 494–499.

Nathanson,J.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 599–603.

O'Dowd,B.F., Lefkowitz,R.J. and Caron,M.G. (1989) *Annu. Rev. Neurosci.*, **12**, 67–83.

Onai,T., FitzGerald,M.G., Arakawa,S., Gocayne,J.D., Urquhart,D.A., Hall,L.M., Fraser,C.M., McCombie,W.R. and Venter,J.C. (1989) *FEBS Lett.*, **255**, 219–225.

Orchard,I., Carlisle,J.A., Loughton,B.G., Gole,J.W.D. and Downer, R.G.H. (1982) *Gen. Comp. Endocrinol.*, **48**, 7–13.

Parmentier,M., Libert,F., Maenhaut,C., Lefort,A., Gerard,C., Perret,J., VanSande,J., Dumont,J.E. and Vassart,G. (1989) *Science*, **246**, 1620–1622.

Regan,J.W., Kobilka,T.S., Yang-Feng,T.L., Caron,M.G., Lefkowitz,R.J. and Kobilka,B.K. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6301–6305.

Robertson,H.. ι. and Juorio,A.V. (1976) *Int. Rev. Neurobiol.*, **19**, 173–224.

Shapiro,R.A., Wakimoto,B.T., Subers,E.M. and Nathanson,N.M. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9039–9043.

Sprengel,R., Braun,T., Nikolics,K., Segaloff,D.L. and Seeburg,P.H. (1990) *Mol. Endocrinol.*, **4**, 525–530.

Uzzan,A. and Dudai,Y. (1982) *J. Neurochem.*, **38**, 1542–1550.

Vaccari,A. (1986) *Br. J. Pharmacol.*, **89**, 15–25.

Witz,P., Amlaiky,N., Plassat,J.-L., Maroteaux,L., Borrelli,E. and Hen,R. (1990) *Proc. Natl. Acad. Sci. USA*, in press.

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