Cloning, characterization and expression of the D2 dopamine receptor from the tilapia pituitary

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Abstract

A full-length cDNA encoding a dopamine receptor (DA-R) was obtained from the pituitary of tilapia (ta). This cDNA encodes a protein of 469 amino acids that exhibits the typical arrangement of GPCR. The taDA-R shows high similarity to the DA-Rs of mullet and fugu, and over 70% similarity to *Xenopus*, mouse and turkey D2 DA-Rs. Northern blot analysis revealed transcript for a single transcript in the pituitary, of approximately 3 kb. In a Southern analysis, the tilapia probe recognized specific bands in the genomic DNA of both mullet and catfish, suggesting high similarity between the corresponding genes. Phylogenetic analysis clearly aligned the taDA-D2-R with all vertebrate D2-like receptor sequences cloned to date, and it was therefore designated taDA-D2-R. taDA-D2-R was transiently expressed in COS-7 cells together with the reporter construct CRE-luciferase. Addition of the specific D2 dopamine agonists quinpirole or bromocriptine, in the presence of forskolin, led to a dose-dependent decrease in forskolin-induced cAMP levels. Both agonists yielded the same maximal inhibition (around 40%). However, the potency of taDA-D2-R for bromocriptine was higher than for quinpirole. As established for mammalian D2-like receptors, stimulation of the taDA-D2-R with quinpirole triggers pertussis-toxin-sensitive Gi/o-mediated, but not Gs-mediated signaling. In contrast to mammals, PCR analysis gave no evidence of alternative splicing in taDA-D2-R. Pharmacological and genetic manipulation of the taDA-D2-R should enable us to better define its physiological role and to further explore the usefulness of fish as a model system for understanding dopaminergic function in higher organisms.

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I. Introduction

Dopamine is a ubiquitous neurotransmitter found in both the central and peripheral nervous systems in many species. It is involved in a wide variety of cerebral and peripheral functions, such as control of movement, learning, behavior and regulation of the hypothalamo-pituitary axis. Disorders such as Parkinson’s disease, schizophrenia, Tourette’s syndrome and pituitary tumors are related to dopaminergic pathways. The transduction of dopaminergic signals is mediated by several subtypes of G-protein-coupled receptors (GPCRs). These receptors are divided into two major subclasses: the D1-like (D1 and D5) and D2-like (D2, D3, and D4) receptors, and are further characterized by their ability to stimulate or inhibit adenylate cyclase (AC) activity, respectively (Crivelli et al., 1991).

In mammals, dopamine released from the hypothalamic tuberoinfundibular neurons serves as a physiological inhibitor of prolactin secretion, mediated through D2 dopamine receptors (DA-Rs) residing on the pituitary lactotroph membranes (Ben-Jonathan, 1985). In fish, dopamine also inhibits prolactin release through D2-like receptors (James and Wigham, 1984) but stimulates growth hormone (GH) release through D1-like receptors (Melamed et al., 1995; Wong et al., 1993). Dopamine also inhibits the release of α-melanocyte-stimulating hormone (α-MSH) (Oliveira et al., 1987) through D2-like receptors in goldfish and tilapia (Lamers et al., 1991; Omeljaniuk et al., 1989) but has been...
found to stimulate α-MSH release as well, by means of D2-like receptors in tilapia exposed to low pH (Lamers et al., 1997). In many teleost species, dopamine inhibits both basal and gonadotropin-releasing hormone (GnRH)-stimulated luteinizing hormone (LH) secretion. This action is well documented in goldfish (Chang et al., 1984; Peter et al., 1986; Stedey et al., 1991), as well as in African catfish (De Leeuw et al., 1988; Van Asselt et al., 1990), coho salmon (Van der Kraak et al., 1986), rainbow trout (Linard et al., 1995; Vacher et al., 2002) eel (Vidal et al., 2004), mullet (Glubokov et al., 1994; Aizen et al., 2005) and tilapia (Gissis et al., 1991; Levavi-Sivan et al., 1995). In vitro experiments have also demonstrated the involvement of D2-like, but not D1-like receptors in the dopaminergic inhibition of gonadotropin secretion directly at the pituitary level (Chang et al., 1990; Vacher et al., 2000).

Dopaminergic innervation of the proximal pars distalis of the teleost pituitary emerges from the anterior part of the ventral preoptic region (Anglade et al., 1993; Kah et al., 1987, 1993; Linard et al., 1996b). In addition to direct actions at the level of the gonadotrophs, dopamine has also been shown, in goldfish, to exert inhibitory effects on GnRH neurons, blocking the synthesis of the peptide or inhibiting its release from the pituitary nerve terminals (De Leeuw et al., 1989).

In many fish, the inhibitory impact of endogenous dopamine is so strong that it severely compromises the ability of externally applied GnRH to increase LH release (Yaron, 1995) or to induce spawning (Drött et al., 1994; Gissis et al., 1991). In these fish, the simultaneous removal of dopamine inhibition and an increase in GnRH release constitute one of the neuroendocrine events leading to the preovulatory LH surge and spawning. Accordingly, the widely adopted method of spawning induction in fish with strong dopaminergic inhibition relies on the simultaneous application of a potent DA-R antagonist and GnRH superactive analog (Peter et al., 1988; Yaron, 1995). Nevertheless, the importance of dopamine’s inhibitory regulation varies among teleosts. Dopaminergic inhibition is relatively weak in salmonids and some perches, and blocking it only slightly potentiates GnRH-induced LH secretion; fish belonging to these families can spawn after induction by GnRH analog alone (Mylonas and Zohar, 2001). Dopaminergic inhibition is totally absent in the Atlantic croaker (Copeland and Thomas, 1989). On the other hand, although belonging to the evolved order Perciformes, LH release in tilapia is under considerable dopamine inhibition and an increase in GnRH release constitute one of the neuroendocrine events leading to the preovulatory LH surge and spawning. Accordingly, the widely adopted method of spawning induction in fish with strong dopaminergic inhibition relies on the simultaneous application of a potent DA-R antagonist and GnRH superactive analog (Peter et al., 1988; Yaron, 1995). Nevertheless, the importance of dopamine’s inhibitory regulation varies among teleosts. Dopaminergic inhibition is relatively weak in salmonids and some perciforms, and blocking it only slightly potentiates GnRH-induced LH secretion; fish belonging to these families can spawn after induction by GnRH analog alone (Mylonas and Zohar, 2001). Dopaminergic inhibition is totally absent in the Atlantic croaker (Copeland and Thomas, 1989). On the other hand, although belonging to the evolved order Perciformes, LH release in tilapia is under considerable dopamine inhibition, both in vivo (Gissis et al., 1991) and in vitro (Levavi-Sivan et al., 1995). A direct inhibitory action of dopamine at the pituitary level has also been reported in rabbit, whereby dopamine inhibits GnRH-stimulated LH release in normal, as well as as-tall-sectioned animals (Dailey et al., 1978).

The present study describes the cloning and characterization of a newly discovered tilapia DA-R. We expressed and pharmacologically assessed this receptor, and identified its G-protein coupling.

2. Materials and methods

2.1. Fish

Approximately 600 14-day-old Oreochromis niloticus fry were obtained from the Institut für Tierzucht und Haustier- genetik, Georg-August Universität, Göttingen, Germany. The fish were originally from different families of a population originating from Lake Manzala (Egypt). The fish were kept and bred in the fish facility unit at the Hebrew University in 500-L tanks, at 26°C under natural photoperiod. They were fed every morning ad libitum with commercial pellets and flakes, containing 50% protein, 6% fat, 5.6% ash and 2.6% cellulose (Zencam Feed Mills, Zemach, Israel). Fish were anesthetized with 2-phenoxyethanol (Sigma, Ness Ziona, Israel) at a concentration of 1 ml/L before being weighed and injected i.p. with the indicated doses of estradiol (E2; Sigma). Controls were injected with fish saline. Fish received E2 at an injection volume of 1 μl/g body weight (BW). All experimental procedures were in compliance with the Animal Care and Use Guidelines at the Hebrew University and approved by the local Administrative Panel on Laboratory Animal Care Committee.

2.2. Molecular cloning of a tilapia dopamine receptor cDNA

Total RNA was extracted with Total RNA Isolation Reagent (Trizol, Gibco BRL, Paisley, UK) from the pituitary of four mature female tilapia (O. niloticus) according to the manufacturer’s protocol, and was treated with RNase-free-DNase I (Promega, Madison, WI) to remove contaminating genomic DNA. A total of 2 μg of the pooled RNA was used to synthesize the first cDNA strand using Superscript System II (Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol. A degenerate primer pair, DA-F1 and DA-R1 (Table 1; Sigma), was designed based on

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>DA-F1</td>
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<td>DA-R1</td>
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<tr>
<td>3</td>
<td>(D2-R-645F)</td>
<td>5'-AAGCGCGCTGGCTGATGAGCTC-3'</td>
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<td>(D2-435R)</td>
<td>5'-CTGATGCTGGCTGATGAGCTC-3'</td>
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<td>(D2-Bos)</td>
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<td>5'-CTGATGCTGGCTGATGAGCTC-3'</td>
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<td>9</td>
<td>(D2-610F)</td>
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<td>(D2-910F)</td>
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<td>14</td>
<td>18S-897R</td>
<td>5'-CAGTATGCTGCTGCTGATGAGCTC-3'</td>
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</table>

Table 1: Primers used in present study.
analysis of a nucleotide sequence that is conserved among D2 DA-Rs of many genera. cDNA (1 µl) was used as a template for a hot-start PCR in the presence of 1.5 µM of each degenerate primer, 2 µl of 10× buffer, 1.5 mM MgCl₂, 1 µl of dNTPs (final 200 µM each), and 5 units of Taq DNA polymerase (Promega). PCR conditions were 94 °C for 5 min, and 30 cycles of 94 °C for 50 s, 50 °C for 2 min and 72 °C for 1 min. The PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide. The PCR product of the predicted size (approximately 436 bp) was obtained and cloned into a pGEM-T Easy Vector System (Promega). The nucleotide sequence of the cloned fragment was obtained with T7 and SP6 primers at the Weizmann Institute Sequencing Unit (Rehovot, Israel). First- and second-strand cDNA were synthesized with the Advantage 2 PCR System (Clontech, Palo Alto, CA). Adaptor primers were ligated to the cDNA synthesized from the pituitaries. Gene-specific primers D2-435R and D2-645F (Table 1) were used for 5′- and 3′-RACE, respectively, along with the primer 3′-RACE-PCR following the manufacturer’s instructions. The RNA was fixed by baking for 2 h at 80 °C. The membrane was prehybridized at 50 °C in 50% formamide, 5× SSC, 2× Denhardt’s reagent, 0.1% SDS and 250 µg/ml denatured calf thymus DNA (Sigma) for 4 h. Hybridization was carried out at 60 °C for 18 h in prehybridization buffer containing 50 ng/ml of digoxigenin (DIG)-labeled DNA probe. The probe used for both Northern and Southern blots was created by PCR using primers D2-27F and D2-463R (Table 1) corresponding to nt 27–50 and 440–463 for forward and reverse primers, respectively. The PCR product was used as a DNA probe and was labeled with the PCR DIG Probe Synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany). The membrane was washed twice with 2× SSC/0.1% SDS for 5 min at room temperature and then twice with 0.5× SSC/0.1% SDS for 15 min at 55 °C.

For Southern blot analysis, genomic DNA was isolated from the liver of mature tilapia, mullet or catfish using DNeasyTM Tissue kit (Qiagen; Alameda, CA), according to the manufacturer’s recommendations. Each genomic DNA (20 µg) was digested with the restriction endonucleases HindIII, BamHI or EcoRI (New England BioLabs, MA), and electrophoresed in a 0.8% agarose gel. The gels were processed as described by Sambrook et al. (1989), washed with 0.25 N HCl, treated with a denaturing solution (0.5 M NaOH and 1.5 M NaCl) and a neutralizing solution (0.5 M Tris–HCl, 1.5 M NaCl), and 1 mM EDTA, pH 7.2), and transferred by capillary action to a GeneScreen Plus nylon membrane. The PCR product used as a DNA probe was labeled with Random-Prime (Biological Industries, Beit Haemek, Israel) [α-32P]dCTP (Amersham Pharmacia Biotech, Buckinghamshire, UK). Hybridization was performed according to manufacturer instructions. The membranes were washed twice with 2× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and 0.1% SDS at room temperature for 5 min each, and then once with 0.1× SSC and 0.1% SDS at 65 °C for 30 min. The membranes were then exposed to X-ray films (X-Omat; Kodak, Rochester, NY, USA) at −70 °C.

For Northern blot analysis, total RNA was extracted from the pituitaries of 10 male fish [53.00 ± 0.34 g BW, and 0.23 ± 0.0078% gonado-somatic index (GSI; gonadal weight percentage of body weight)] by Trizol reagent (Gibco) according to the manufacturer’s protocol. RNA (40 µg) was run through a 1.5% agarose-formaldehyde gel and transferred to positively charged nylon membranes (GeneScreen Plus; DuPont, Wilmington, DE) according to the manufacturer’s instructions. The RNA was fixed by baking for 2 h at 80 °C. The membrane was prehybridized at 50 °C in 50% formamide, 5× SSC, 2× Denhardt’s reagent, 0.1% SDS and 250 µg/ml denatured calf thymus DNA (Sigma) for 4 h. Hybridization was carried out at 60 °C for 18 h in prehybridization buffer containing 50 ng/ml of digoxigenin (DIG)-labeled DNA probe. The probe used for both Northern and Southern blots was created by PCR using primers D2-27F and D2-463R (Table 1) corresponding to nt 27–50 and 440–463 for forward and reverse primers, respectively. The PCR product was used as a DNA probe and was labeled with the PCR DIG Probe Synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany). The membrane was washed twice with 2× SSC/0.1% SDS for 5 min at room temperature and then twice with 0.5× SSC/0.1% SDS for 15 min at 55 °C.

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2.5. Expression of taDA-D2- R in extrapituitary tissues

Tissue samples were collected from sexually mature post-vitellogenic female and milt-producing male tilapia. Brain, gill, heart, liver, muscle, ovary, pituitary, spleen and testis samples were flash-frozen and then stored at −80 °C. Total RNA was isolated as already described. A total 5 µg of...
RNA was digested with RQ 1 DNase (Roche) for 15 min following the manufacturer’s protocol. After heat inactivation of RQ 1 DNAse at 70 °C for 10 min, 0.5 μg RNA was reverse-transcribed with the MMLV reverse transcriptase and oligo d(T) (Promega). In an attempt to characterize the tissue-specific expression pattern of the taDA-R, semi-quantitative RT-PCR analysis was performed according to Levavi-Sivan et al. (1998). 18S ribosomal RNA was used for standardization, using primers 18S-660F and 18S-897R (Table 1). The PCR amplification was performed in a DNA thermal cycler (MJ Research Inc., Line Village, NV), using primers tD2-27F and tD2-463R (Table 1) in a 20-μl PCR, using 1.5 units of Taq polymerase, 10 mM buffer (Promega), 3 mM MgCl₂, dNTPs (2.5 mM final concentration of each nucleotide), 1.5 μM of each primer, and 4 μl of tilapia-tissue cDNA. Cycling parameters were: 5 min at 94 °C, then 20–30 cycles of 30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C. Final elongation was at 72 °C for 8 min. Twenty cycles were carried out for 18S and 30 cycles for the receptor. The amount of RNA taken for transcription before the PCR and the linear range of amplification for each primer pair were established in independent preliminary studies (data not shown). Following amplification, the samples were run on a 1.5% agarose gel, stained with ethidium bromide and visualized using a Gel Imaging Workstation. As a negative control, PCR was conducted in the absence of cDNA template.

2.6. Identification of alternatively spliced transcripts

A forward primer from the end of transmembrane (TM) V (tD2-610F; Table 1), which encodes nucleotides 1191–1211, with 18S mRNA, the latter was normalized to the amount of an endogenous RNA and its expression is considered stable. The amplification was performed according to Levavi-Sivan et al. (1998). The CMV-β-gal plasmid was used to monitor transfection efficiency. Twenty-four hours prior to stimulation, the cells were starved of serum: the standard medium was replaced with serum-free DMEM (with 0.5% bovine serum albumin). Cells were treated with vehicle or various concentrations of quinpirole (Sigma) or bromocriptine (Sigma) for 4 h, and then harvested and analyzed for luciferase and β-gal activity. When toxins were used, the cells were cultured in the presence or absence of pertussis toxin (PTX, 1.3 ng/ml; Sigma) or chola toxin (CTX, 20 μg/ml; Sigma), and after an additional 24 h, the cells were assayed for AC as described further on.

2.8. Luciferase and β-galactosidase assays

Fifty-eight hours post-transfection, cells were exposed to ligands for 4 h in serum-free medium, and then the cells from each well were lysed in 0.5 ml lysis buffer (Promega) and luciferase activity was determined using a Biocounter M apparatus (Labdata, Copenhagen, Denmark) at 595 nm. The results were expressed as fold induction, and the ratio of stimulated to non-stimulated cells, after luciferase activity, was normalized by correcting for β-gal activity, taking into account the transfection efficiency.

2.9. Adenylyl cyclase (AC) activity

In general, AC activity was determined according to Obadiah et al. (1999). Briefly, COS-7 cells were incubated for 2 h with medium containing 5 μCi/ml [3H]adenosine (New England Biolabs, Boston, MA). AC activity was measured in the presence of the phosphodiesterase inhibitors 1-methyl-3-isobuthylxanthine (IBMX; 0.5 mM; Sigma), RO-20-1724 (0.5 mM; Calbiochem, La Jolla, CA) and forskolin (20 μM; Sigma) in the presence or absence of quinpirole for 20 min at 37 °C. The medium was removed, and the reaction was terminated by the addition of perchloric acid containing 0.1 mM unlabeled cAMP, followed by neutralization with KOH. The amount of [3H]cAMP was determined by a two-step column separation procedure according to Salomon (1991).

2.10. Quantitative real-time PCR analysis

To test the effect of E2 on the relative abundance of taDA-R mRNA, the latter was normalized to the amount of an endogenous reference, the 18S subunit of rRNA, by the comparative threshold (CT) cycle method. 18S mRNA was used as an internal standard for the measurements since it is an abundant RNA and its expression is considered stable. The

2.7. Transfection of COS-7 cells

taDA-D2-R (GenBank accession no. AY673985) cloned in the pcDNA 3.1 (Zepto; Invitrogen, San Diego, CA) expression vector (pcDA-R) under the control of the cytomegalovirus (CMV) promoter, was used for all the expression experiments. A COS-7 cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained at 37 °C in DMEM supplemented with 10% FBS, 1% glutamine, 100 μM penicillin, and 100 μg/ml streptomycin (Beith Haemek) under 5% CO₂ until confluence. Twenty-four hours prior to the transfection, COS-7 cells were seeded in 12-well culture plates (Nunc; Roskilde, Denmark) at a density of 2 x 10⁵ to 4 x 10⁶ cells/well. Co-transfection of pcDA-R (5 μg/plate), pCRE-luciferase reporter plasmids (1 μg/plate; pCRE-LUC; Clontech) and pCMV-β-galactosidase (1.2 μg/plate; pCMV-β-gal) was carried out using FuGENE 6.0 reagent (Roche) according to Jacobsen et al. (2004).
18S rRNA levels were observed to have a greater uniformity than those of other commonly used internal standards, such as β-actin (Ambion Technical Bulletin No. 151, 1997). The 18S levels proved to be stable for other teleost fish and did not vary during different reproductive stages reflecting different estradiol levels (Red Seabream (Pomatoschistus microps) (Gen et al., 2000); Blue gourami (Trichogaster trichopterus) (Jackson et al., 1999); European sea bass (Dicentrarchus labrax) (Halm et al., 2004); Russian Sturgeon (Acipenser gueldenstaedtii) (Hurvitz et al., 2005), and tilapia (Levavi-Sivan et al., 2004)).

A detailed description of the real-time, quantitative PCR procedure used can be found in Levavi-Sivan et al. (2004). Serial dilutions were prepared from a pituitary cDNA sample and the efficiencies of the taDA-R and 18S amplifications were compared by plotting ΔCt versus log(template) according to the method described by PE Applied Biosystems (Perkin-Elmer, Foster City, CA). Linear regressions of the plots showed an R² value and slope of 0.982 and −3.37, respectively, for 18S ribosomal RNA, and 0.986 and −3.37, respectively, for taDA-R. Pituitaries were collected from E2-injected male tilapia (see Section 2.1). Total RNA and cDNA were prepared as already described. Gene-specific primers used for the real-time PCR were designed using Primer Express 2.0 software. The primers used for taDA-R amplified a 189 bp product corresponding to nucleotides 919–939 and 1100–1120 (d2-919F and d2-1120R, respectively; Table 1). Primers for 18S rRNA (18S-660F and 18S-897R, GenBank accession no. AF497908; Table 1) amplified a 256 bp product.

The PCR mixture consisted of 2 μl diluted cDNA sample, 300 nM of each primer, and 10 μl Mastermix for Syber Green I (Eurogentec, Seraing, Belgium) in a final volume of 20 μl. Amplification was carried out in an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) under the following conditions: initial denaturation at 94 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 15 s, annealing-extension at 60 °C for 1 min, and then a final extension at 60 °C for 20 min. taDA-R and 18S rRNA cDNAs were amplified simultaneously in separate tubes, in the same fish. The taDA-R cDNA is 1640 bp in length, including the 5′- and 3′-UTR. The ORF reads through nt 167–1573 and encodes a 469-amino-acid peptide. This sequence was submitted to GenBank under accession no. AY673985.

Hydropathy analysis of the taDA-R amino-acid sequence revealed the presence of seven putative TM domains. The taDA-R contains four N-glycosylation sites, three at the extracellular amino terminus and one on the second extracellular loop. The two cysteine residues on the second and third extracellular loops (C125 and C184), which are present in mammalian GPCR, are also present in the taDA-R. taDA-R also contains the conserved amino acids in TMII, III, V and TMVI, five PKC and two PKA putative phosphorylation sites, and the palmitoylation site situated at the carboxy terminus of the receptor (Fig. 1). taDA-R shares additional structural features with D2-like versus D1-like receptors, including a relatively long third intracellular loop and a short carboxy terminus (Figs. 1 and 2). The calculated molecular weight of taDA-R is 56,600 and the molecule has an isoelectric point of 9.46.

The taDA-R shows the highest similarity (93.6%) with the DA-R of mullet (Mugil cephalus), recently cloned in our lab (GenBank accession no. AY673984; Fig 2). taDA-R also shows high similarity (82–89%) with other piscine DA-Rs, more than 70% similarity with the Xenopus, mouse and turkey D2 receptors, and only 53.5%, and 44.3% with mouse D1 and D4, respectively (Table 2). In contrast, only 37% similarity was found to the tilapia D1-like receptor. Sequence comparison of the taDA-R with known D1-like and D2-like receptors suggests that taDA-R falls within the D2 subfamily (Fig 2, Table 2). Sequence alignment with non-mammalian and mammalian vertebrate DA-Rs reveals
Fig. 1. Nucleotide and deduced amino-acid sequences of the tilapia dopamine D₂ receptor. Deduced amino-acid sequence numbering begins with the first methionine of the open reading frame, and is to the right of each line. Nucleotide numbers are to the left of each line. The start and stop codons are shaded in grey, the putative transmembrane domains are underlined, the putative N-glycosylation sites are underscored by triangles, the putative protein kinase A phosphorylation sites by diamonds, the putative protein kinase C phosphorylation sites by squares and the palmitoylation site by a circle. This sequence has been deposited in the GenBank nucleotide database, under Accession No. AY673985.
Fig. 2. Alignment of the amino-acid sequence of the tilapia dopamine receptor with those of other vertebrates. Dashed amino-acids represent residues that are identical in all compared sequences, and gaps introduced for optimal alignment are indicated by dots. The putative transmembrane domains are boxed and numbered. The sequences were extracted from the GenEMBL and Swiss-Prot databases, and the accession nos. are: tilapia (Oreochromis niloticus) D2, AAU87971; mullet (Mugil cephalus) D2, AAU87970; trout (Oncorhynchus mykiss) D2-1, CAC87873; trout D2-2, CAC79663; mouse (Mus musculus) D2, DYMSD2.

A phylogenetic tree of vertebrate DA-Rs was generated using the neighbor-joining method (Fig. 3). The constructed tree showed that the vertebrate DA-Rs identified to date fall into four distinct lineage groups. One lineage includes the dopamine D1 receptors, the second the dopamine D4 of teleosts and mammals, the third the dopamine D3 receptors, and the last and largest lineage contains the dopamine D2 receptors (Fig. 3). The taDA-R was clearly aligned with all vertebrate D2-like receptor sequences cloned to date.

To determine the size of the taDA-R, mRNA was determined by Northern blot analysis on total RNA extracted from tilapia pituitary. We used taDA-R cDNA from close to the start site to TMIII (nt 27–463), as a probe, and found a single mRNA of approximately 3 kb (Fig. 4). The presence of only one band verified that this probe recognizes only one type of taDA-R. Several hybridizing bands were observed in the lanes con-
Table 2  
Amino-acid identity (%) between the dopamine receptor of tilapia and those of other vertebrates

<table>
<thead>
<tr>
<th>Species</th>
<th>Identity (%)</th>
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</table>

Semi-quantitative RT-PCR analysis was performed in an attempt to characterize the tissue-specific expression pattern of taDA-D2-R. The PCR amplification yielded higher levels in the anterior part of the brain, which contains the olfactory...
Fig. 4. Twenty micrograms of genomic DNA from each fish were restricted with enzymes (E-EcoRI, B-BamHI, H-HindIII). Southern blot analysis was performed under high-stringency conditions using 32P-labeled taDA-D2-R from TM-I to TM-IV as a probe. M refers to the molecular marker.

bulb and the telencephalon, than in other parts of the brain (Fig. 6). taDA-D2-R was expressed in the testis, ovary, spleen, and pituitary, whereas no product was found in the heart, kidney or muscle (Fig. 6).

Fig. 5. Northern blot analysis of taDA-D2-R. Total RNA from female tilapia pituitary was separated by agarose-formaldehyde gel electrophoresis, transferred, and hybridized using DIG-labeled taDA-D2-R from TM-I to TM-IV as a probe at high stringency. The positions of the 28S and 18S rRNAs are indicated on the left.

Fig. 6. RT-PCR analysis for the detection of taDA-D2-R expression in various tilapia tissues. Amplification products of the first-strand cDNA from various tissues were obtained with primers D2-27F and D2-463R. The brain sections were as follows: Anterior: olfactory bulb and telencephalon; mid: diencephalons, optic tectum and cerebellum; posterior: medulla oblongata and spinal cord.

3.4. Identification of alternatively spliced transcripts

In mammals, the D2 receptor subtype exists in two isoforms generated by alternative splicing of the pre-mRNA. These two proteins are identical except for the third cytoplasmic loop, where there is an insertion of 29 amino acids in D2L relative to the D2S (Monsma et al., 1989). To examine the possible presence of such an alternative splicing in tilapia, we performed RT-PCR using a primer set that spans the third cytoplasmic loop. Only one band was evident, corresponding to D2L, and no evidence for alternative splicing could be found (data not shown). It is concluded, therefore, that unlike the situation in mammals, only a single form of dopamine D2 receptor mRNA is present in tilapia.

3.5. Luciferase accumulation mediated by taDA-D2-R in COS-7 cells

taDA-D2-R was transiently expressed in COS-7 cells together with the reporter construct, CRE-LUC. Functional assessment of the taDA-D2-R in COS-7 cells revealed that, similar to the corresponding mammalian homolog, these proteins are coupled to inhibitory G proteins. In the presence of 20μM forskolin, transfected cells demonstrated 16- to 18-fold stimulation of cAMP, reflected by an elevation in luciferase activity. The addition of the specific D2 dopamine agonists, quinpirole or bromocriptine, led to a marked decrease in forskolin-induced cAMP levels, reflected by a concomitant reduction in luciferase activity (Fig. 7). The two agonists used yielded concentration-dependent inhibition of the forskolin-stimulated luciferase induction with approximately the same maximal percentage of inhibition. However, the potency of taDA-D2-R for bromocriptine was higher than that for quinpirole, as reflected in their EC50 values (9.7
Fig. 7. The effect of dopamine receptor agonists on forskolin-stimulated luciferase expression in COS-7 cells co-transfected with taDA-D2-R and CRE-LUC. Cells were incubated with 20 μM forskolin in the presence of quinpirole or bromocriptine at the indicated doses. Values were calculated as % luciferase expression in the presence of forskolin and absence of agonist. The curves were constrained to a maximum of 100%.

and 130 pM for bromocriptine and quinpirole, respectively; Fig. 7).

3.6. Quinpirole stimulation of taDA-D2-R results in Gαi/o-mediated signaling

To determine which G proteins are involved in the signal transduction of taDA-D2-R, we incubated COS-7 transfected cells with CTX or PTX. In the absence of PTX, an established selective inhibitor of Gαi/o-mediated signaling (Spiegel et al., 1992), administration of 20 μM forskolin resulted in a mean 2.01 ± 0.2-fold increase in AC activity over basal, and co-incubation with 10 μM quinpirole produced around 40% inhibition of this forskolin-stimulated level (Fig. 8; Control). Preincubation with PTX (130 ng/ml) increased the forskolin-stimulated levels of AC activity. However, preincubation with PTX abolished the quinpirole-mediated inhibition of forskolin stimulated expression (Fig. 8; +PTX). The quinpirole-mediated inhibition of forskolin-stimulated expression, in the presence of PTX, was antagonized by the dopamine antagonist haloperidol. These results suggest that Gαi/o is involved in the signal transduction of taDA-D2-R.

We incubated COS-7-transfected cells with CTX (20 μg/ml) in order to irreversibly activate the endogenous Gs. In these CTX-incubated cells, quinpirole did not change AC activity (Fig. 8; +CTX), suggesting that Gs is probably not involved in the quinpirole effect.

3.7. Effect of E2 on taDA-D2-R mRNA levels in vivo

To examine the effect of E2 on the transcription of taDA-D2-R, mature male tilapia (60.2 ± 3.13 g BW; GSI 0.121 ± 0.02%) were injected with graded doses of E2 (250 or 500 μg/kg BW) every other day for 6 days. mRNA levels of taDA-D2-R increased up to 2.9-fold at 500 μg/kg (Fig. 9).

4. Discussion

This is the first report of cloning a functional DA-R from the pituitary of a perciform fish. Dopamine is a major neurotransmitter in the central nervous system that is involved in the control of locomotor activity, learning, behavior, emotion, and neuroendocrine secretion.

The DA-R from the pituitary of tilapia cloned in this study is a member of the superfamily of GPCRs. It spans 469 residues, not much different from that of the mouse D2 (444 aa), or fugu D2 (463 aa). The receptor has seven TM-spanning helices that form a ring-like hydrophobic pocket surrounded by three intra- and four extracellular loops. The extracellular amino terminus of the taDA-R contains three putative N-glycosylation sites, similar to the mammalian D2 recep-
tor, which represents a highly glycosylated neural receptor. However, removal of the N-linked oligosaccharide from the receptor did not change the rank order of potency of agonist and antagonist compounds in competing for [³H]piperon binding to crude membrane fractions (Clagett-Dame and McKelvey, 1988). The receptor contains the two conserved cysteine residues on the second and third extracellular loops that are found at the same positions in the mammalian D2, where they form a disulfide bond that stabilizes receptor conformation (Missale et al., 1998). The conserved amino acids in TMII (aspartate), TMIII (aspartate), TMV (two serines), and TMVI (phenylalanine) that define the narrow pocket for agonist binding (Simpson et al., 1999) are also found in taDA-R. Both PKA putative phosphorylation sites that are present in the mammalian receptor are present in its tilapia counterpart as well (Ser348, Thr376). However, of the five potential phosphorylation sites for PKC, three are at the same positions as in the mammalian receptor (Ser388, Thr396) while two sites are unique for the tilapia receptor (Ser243, Ser298). The role of IP₃ as a mediator of dopamine action on mammalian lactotrophs is unclear (Ben-Jonathan and Hnasko, 2001), making it important to compare the role of this pathway in transducing the effect of dopamine in tilapia. taDA-R shares additional structural features with D2-like versus D1-like receptors, including a relatively long third intracellular loop and a short carboxy terminus. All seven predicted TM domains are highly conserved between the tilapia, mullet, Xenopus and mammalian D₂ receptors, as are the first and second intracellular loops, the first extracellular loop and the amino- and carboxy-terminal domains of the receptors. The putative TM regions, thought to provide the binding pocket for neurotransmitters (Simpson et al., 1999), are especially well conserved between tilapia and the mammalian D₂ receptors (100% identity at the amino-acid level). This degree of identity is remarkable since two receptors for another neurotransmitter (GnRH) cloned from the same fish showed only 41–72% identity in their TM-domains (Levavi-Sivan et al., 2004; Levavi-Sivan and Avitan, 2005). The broadest divergence between the piscine DA-R cloned in this study and its mammalian counterparts is in the middle portion of the third intracellular loop. As the third intracellular loop is the site of G-protein coupling (Ilan et al., 2002; O’Dowd et al., 1988), a comparison of G-protein coupling in piscine versus mammalian D₂ receptors is called for. Recognizing the conserved and divergent regions of the molecule should enable delineation of the functionally important domains of the receptor. Moreover, additional study should clarify the extent to which fish can be used to enhance our understanding of the molecular mechanisms underlying DA-R mediated function, and pathophysiology in higher organisms.

A recent phylogenetic analysis of the GPCRs showed that they segregate into five subfamilies (Fredriksson et al., 2003). The DA-R belongs to the amine receptor cluster of the α group of the rhodopsin subfamily (Fredriksson et al., 2003). Molecular cloning confirmed that D1 and D2 receptors constitute two different classes of DA-Rs, as revealed by a phylogenetic tree. Three subtypes of D2 receptors have been isolated in jawed vertebrates (D₂L, D₂S, and D₂x), and are differentially clustered in the phylogenetic tree of the DA-Rs. The taDA-R was clearly aligned with all currently available cloned vertebrate D₂-like receptor sequences, and hence it was designated taDA-D₂-R.

Northern blot analysis of total RNA revealed the presence of only a single taDA-D₂-R mRNA species in the pituitary of tilapia, of approximately 3 kb. This mRNA is the only piscine DA-D₂-R cloned to date, and it is longer than those described in rat or turkey, where a major band of 2.4–2.5 kb has been detected (Bunzow et al., 1988; Schnell et al., 1999).

It is interesting to note that when the genomic DNA of tilapia and mullet was treated with EcoRI or HindIII, the restriction-fragment lengths were similar, suggesting that the gene organization of the dopamine D₂ receptor in these two fish is similar. The fact that the DNA probe used in the genomic Southern analysis recognized several bands in the genomic DNA of catfish suggests that all the Teleostei dopamine D₂ receptors have a high degree of similarity.

A fundamental aspect of dopamine function in the whole organism is the localization of its receptors in the various areas of the nervous system or at the body’s periphery. There is ample evidence in the literature of the dopamine D₂ receptor’s expression in extrapituitary tissues. Accordingly, we found the taDA-D₂-R not only in the pituitary and brain but also in muscle, gill, ovary and tests.

It is well established that alternative splicing of GPCRs may selectively affect the patterns of tissue- or cell-specific expression without altering receptor-mediated signaling. In the mammalian D₂ receptors, the short isoform is expressed in presynaptic dopaminergic cell bodies and functions as an autoreceptor, thus controlling dopamine release. In contrast, the long form is localized to post-synaptic sites, where it mediates locomotor as well as other physiological functions (Lindgren et al., 2003; Usiello et al., 2000). In this study, even after many cycles of PCR at a wide range of annealing temperatures, using a primer set that spans the third intracellular loop, a second band could not be detected. Hence, we found no evidence of tilapia D₂-receptor-gene-transcript splicing. In Xenopus, as well as in carp retina, using specific primers flanking the third intracellular loop, only one isoform of the D₂ receptor was found (Hirano et al., 1998; Martens et al., 1993, respectively). From the 29 amino acids that differ between mouse D₂α and D₂β, 27 were found to be similar to the taDA-D₂-R. Since both taDA-D₂-R and trout DA-R resemble the D₂α subtype more closely than the D₂β subtype, it may be assumed that agonists and antagonists for post-synaptic DA-Rs will more efficiently inhibit LH release from fish pituitaries and be a better choice for use in spawning induction in fish. In view of the possible functional differences between the two isoforms in mammals, it is remarkable that both Xenopus and teleosts lack alternative splicing. In this context, it should be noted that two isoforms of the D₂ DA-R, differing in three amino acids, were found in trout, but the site of the alternative splicing was the first extracellular loop.
Co-expression of taDA-D2-R and CRE-LUC in COS-7 cells conferred, on the receptor, the ability to effectively and consistently inhibit forskolin-stimulated cAMP accumulation, as reflected by a decrease in luciferase activity. This is in agreement with the results attained by transfection of several mammalian D2 DA-Rs, in various cell lines (Obadiah et al., 1999; Robinson and Caron, 1997). This further corroborates the classification of the newly cloned receptor among the D2-type receptors. Although the taDA-D2-R has classic characteristics as a D2-type receptor, its potential for inhibiting AC, as determined by either cAMP accumulation (Levavi-Sivan et al., 2003) or by luciferase as a reporter gene (Figs. 7 and 8), is much less potent than the classic mammalian D2 receptors. The maximal inhibition of forskolin-stimulated AC activity, in response to quinpirole, of the mouse D2 receptor is 80% (Obadiah et al., 1999), whereas that of the human D2 is higher than 90% in response to bromocriptine (George et al., 1998; Kemp et al., 1999). However, the maximal response of taDA-R with either quinpirole or bromocriptine was less than 50%. This may explain why, based on signal-transduction tests, it was previously suggested that fish DA-Rs are not exactly similar to mammalian dopamine D2-type receptors (tilapia (Levavi-Sivan et al., 1995); trout (Vacher et al., 2003) and goldfish (Omeljanjuk et al., 1989)). However, the ED50 values found in this study for tilapia were in the picomolar range (9.7 and 130 pM for quinpirole and bromocriptine, respectively), while the ED50 values found in mammals are in the nanomolar range (reviewed by Ben-Jonathan, 1985; Ben-Jonathan and Hnasko, 2001).

We demonstrated that quinpirole augments forskolin-stimulated cAMP accumulation in D2-transfected and PTX-pretreated COS-7 cells. However, cAMP accumulation was not inhibited when Gs coupling was abolished, in the presence of CTX. This would suggest that taDA-D2-R can couple with a member of the G/o-protein family. It is very well documented that dopamine inhibits AC activity in the anterior pituitary in vivo and in cultured lactotrophs in vitro through coupling to PTX-sensitive signaling pathways, implicating its association with the G/o family (Ben-Jonathan and Hnasko, 2001).

Neuroendocrine regulation of LH involves a complex interplay of neurotransmitters (Trotseau et al., 1993; 2000). Among the main monoamines that have an important inhibitory effect on LH release is dopamine (see Section 1). In rainbow trout, such inhibition appears to be positively correlated with the circulating levels of E2 during the preovulatory period (Linard et al., 1995). In both goldfish and rainbow trout, dopaminergic neurons of the anterior part of the ventral preoptic region project to the proximal pars distalis of the pituitary (Kah et al., 1987). Furthermore, these dopaminergic neurons of the preoptic area express the estrogen receptor (Linard et al., 1996a) providing a neuroanatomical substrate for dopamine-estrogen interactions. Moreover, E2 was found to be essential for the maintenance of the dopaminergic inhibition upon LH release in immature or vitellogenic trout (Saligaut et al., 1999), and to modulate the release of gonadotropins in female trout (Vacher et al., 2002). In the current work, we show a remarkable increase in the synthesis of DA-R in the pituitary of tilapia upon stimulation with E2. Taken together, our results suggest that the effect of E2 on the DA-R is part of the negative steroid feedback. A direct effect of E2 on the number of anterior pituitary dopamine binding sites has also been shown in rats (Pasqualini et al., 1986).

It is well documented that fish with strong dopaminergic inhibition, such as cyprinid and tilapia, need co-treatment with GnRH and DA antagonists in order to receive the LH surge that will induce final maturation and ovulation (Yaron, 1995). In vitro experiments have also demonstrated the involvement of D2-like, but not D1-like receptors in the dopaminergic inhibition of gonadotropin secretion directly at the pituitary level in goldfish (Chang et al., 1990), tilapia (Levavi-Sivan et al., 1995), and catfish (Van Asselt et al., 1990). The association of DA-Rs with many neurological disorders has led to the development of many agonists and antagonists. These antagonists are broadly used in the spawning induction of various fish. The cloning and biochemical identification of piscine DA-Rs will help in the discovery and use of more selective drugs for the induction of fish spawning.

In this study, we cloned and functionally analyzed a newly discovered DA-R from the pituitary of tilapia. The molecular characteristics of this receptor in comparison with other D2-type receptors and their isoforms lead to the conclusion that taDA-D2-R should be classified as a D2-type receptor. This conclusion is supported by the following findings: (i) the taDA-D2-R amino-acid sequences share highest homology with known mammalian and piscine D2-like receptors; (ii) the tilapia receptor, similar to the corresponding mammalian D2-like homolog, are coupled to G/o, and (iii) the taDA-D2-R can be fully activated by very low concentrations of the synthetic D2-like receptor drug, bromocriptine. Additional studies should indicate the extent to which fish can be used to enhance our understanding of the molecular mechanisms underlying dopamine-receptor-mediated function, and pathophysiology in higher vertebrates.

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References


