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Cloning and Characterization of a Truncated Dopamine D1 Receptor from Goldfish Retina: Stimulation of Cyclic AMP Production and Calcium Mobilization

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SUMMARY

Receptors for dopamine are present on horizontal cells of fish retina that are linked to the activation of adenylate cyclase. In the present study, the goldfish (*Carassius auratus*) gene that encodes these receptors, referred to as gfD1, was isolated and analyzed. A single open reading frame within the gfD1 gene encodes a protein of 363 amino acids that is highly homologous with dopamine D1 receptors from rats and humans. Interestingly, the carboxyl terminus of gfD1 lacks 80 amino acids that are present in the mammalian receptor sequences. RNA analysis using the polymerase chain reaction demonstrated that the gene is expressed in the goldfish retina and is intronless within the coding region. The fact that gfD1 encodes a dopamine D1 receptor was demonstrated through pharmacological analysis of transfected cells. Both the gfD1 receptor and the human D1 receptor expressed in mammalian cells had high affinity for SCH-23390 and other D1-specific ligands. In addition, the gfD1 receptor and the human D1 receptor were able to stimulate the accumulation of cAMP in response to SKF-38393 or dopamine. Interestingly, stimulation of both the gfD1 and human receptors with dopamine also resulted in an increase in intracellular Ca^{2+}. Finally, long term pretreatment of transfected cells with dopamine resulted in the desensitization and down-regulation of both the goldfish and human receptors.

Dopamine is a major neurotransmitter of the vertebrate central nervous system and is involved in a variety of physiological processes. The effects of dopamine are mediated by a number of receptor subtypes. These receptor subtypes were initially differentiated by their ability to activate adenylate cyclase (D1 receptors) or inhibit the activation of adenylate cyclase (D2 receptors) (see Ref. 1 for review). More recently, molecular cloning has led to the identification of at least six mammalian genes that encode dopamine receptor subtypes, referred to as D1A (2–5), D1B (6), and D2-D5 (7–10). These receptors can be classified as "D1-like" (D1A, D1B, and D5) or "D2-like" (D2, D3, and D4) based upon both sequence conservation and pharmacological similarities (see Ref. 11 for review).

In the teleost retina, dopamine is present in the interplexiform cells and these cells make synapses with horizontal cells present in the outer plexiform layer (12). Horizontal cells respond to dopamine in several ways (13). Many studies have shown that horizontal cells accumulate cAMP in response to dopamine treatment, indicating that these cells possess D1 or D1-like receptors (14–17). More recently, activation of the teleost D1 receptor has been reported to induce neurite retraction via activation of phospholipase C and protein kinase C (18). This activation has also been reported for mammalian D1 receptors present in kidney (19, 20) and striatum (21), although the latter has been controversial (22). Finally, D1 receptors that activate adenylate cyclase also appear to be involved in the release of growth hormone from goldfish pituitary (23). Pituitary D1 receptors have not been described in mammals.

To begin to understand the pharmacological effects of dopamine at D1 receptors, including putative subtypes, in teleosts, we now report the isolation of a gene from goldfish that encodes a dopamine D1 receptor that is transcribed in retina. This gene is the first nonmammalian D1 receptor to be described, and sequence and pharmacological analyses demonstrate a high level of conservation of both structure and function among vertebrate D1 receptors.

Materials and Methods

Isolation and sequencing of the goldfish dopamine D1 receptor gene. Standard molecular biology techniques have been described

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ABBREVIATIONS: PCR, polymerase chain reaction; RT, reverse transcription; SSC, standard saline citrate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

previously (24). A goldfish (*Carassius auratus*) genomic library in the phage Lambda GEM-11 (Promega) was generously provided by Dr. Gregory Warr (Medical University of South Carolina). Approximately 250,000 plaques from the library were screened on duplicate Hybond-N filters (Amersham). A DNA fragment encoding part of the putative rat catecholamine receptor G-36 (25), now known to encode the rat dopamine D1 receptor (4), was obtained by PCR, gel purified, and radiolabeled by random priming (Boerhinger-Mannheim). After prehybridization, the filters were incubated with the probe at 42° in hybridization buffer (50% formamide, 6× SSC, 1.0% sodium dodecyl sulfate, 100 μg/ml denatured salmon sperm DNA, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate). After 24 hr, the filters were washed twice for 30 min in 0.2× SSC, 0.5% sodium dodecyl sulfate, at 50°. A single positive clone, gfD1, was obtained and plaque purified. Purified phage DNA was mapped and the insert was >15 kilobases. An open reading frame contained within a *Pst*I fragment of approximately 4.7 kilobases was completely sequenced on both strands in Bluescribe (Stratagene) and the sequence was highly homologous to that of the human dopamine D1 receptor.

mRNA analysis. Total mRNA was isolated from dark-adapted goldfish (*C. auratus*) retina (40 pairs, approximately 2 g) using an RNA isolation kit (Stratagene). The resulting material (approximately 600 μg) was dissolved in RNase-free water and stored at −70°. Before PCR, 6.5 μg of RNA were treated with 3 units of RQ1 DNase (Promega) in 50 μl for 15 min at 37°, extracted with phenol, and precipitated with ethanol. This RNA was used for RT-PCR (Perkin-Elmer) using random primers for cDNA synthesis, according to the supplied protocol. Both RT-PCRs and genomic PCRs and conditions were as suggested, except that the reactions contained 20 pmol of each primer and 50 μM trimethylammonium chloride (26).

Expression in transfected cells. A 2.1-kilobase *Bam*HI fragment containing the goldfish D1 receptor open reading frame was subcloned into the eukaryotic expression plasmid RcCMV (Invitrogen) and used to transfect COS-7 cells as described previously (27). The subcloning of the human D1 receptor has also been described previously (27). HEK-293 cells (American Type Culture Collection CRL1573) were grown and maintained in minimal essential medium (HEPES buffered) containing 10% fetal bovine serum and antibiotic/antimycotic solution. HEK-293 cells were transiently transfected with DNA (20 μg of DNA/3.5 × 10^6 cells) using a modified calcium phosphate procedure (28). Cells were used in either binding assays or functional assays 72 hr after transfection.

Pharmacological assays. Receptor binding assays using membranes from transiently transfected cells were performed as described previously (27). For saturation binding analysis, each concentration of radioligand was assayed in triplicate and each saturation curve was repeated at least three times. For competition binding analysis, each competitor concentration was assayed in triplicate and each displacement curve was repeated at least three times. The data shown for each competitor have been normalized as percentage of specific binding and averaged. Analysis of the binding data was performed with Accufit Saturation Two Sites software and Accufit Competition software (Lundon Software, Inc.). Detailed procedures used to assay cAMP have been described previously (27). Intracellular calcium was measured as described previously (29), with modifications. Transfected HEK-293 cells (2–3 ml of cell pellet) were resuspended in 50 ml of minimal essential medium containing 0.4% bovine serum albumin, 250 μl of 1 mM indo-1/acetoxymethyl ester (Molecular Probes) in 100% dimethylsulfoxide were added, and the suspension was incubated for 1 hr at 37°. Cells were then resuspended in assay buffer (Dulbecco's phosphate-buffered saline, pH 7.4, 0.1% bovine serum albumin, 0.1% glucose) at 2–4 × 10^6 cells/ml. Intracellular calcium levels were monitored with a spectrofluorimeter (SLM 8000C) at settings of 350-nm excitation and 405- and 480-nm emissions and were calibrated as described previously (30).

Results

Isolation of the gfD1 cDNA clone. A cDNA probe encoding a portion of the rat dopamine D1 receptor was used to screen a goldfish (*C. auratus*) genomic library and a single positive clone, gfD1, was obtained. Southern blot analysis of the insert indicated that the probe was completely contained within a 4.7-kilobase *Pst*I fragment and that the sequences encoded by the rat probe were probably not interrupted by introns. It is known that the genes that encode the human and the rat D1 receptors are intronless within the coding regions (2, 4). The *Pst*I fragment was subcloned and mapped further (Fig. 1). Finally, it was determined that the rat probe was contained within a single 2.0-kilobase *Bam*HI fragment, and the open reading frame was completely sequenced on both strands (Fig. 1).

Protein sequence of gfD1. The open reading frame of gfD1 encoded a protein that was highly homologous to the human (75%) and rat (75%) dopamine D1 receptors (Fig. 2), especially within the transmembrane regions (human, 92%; rat, 91%). Interestingly, whereas the human and rat D1 receptors contain long (115 amino acids) and highly homologous (90%) cytoplasmic tails following transmembrane seven, the open reading frame of gfD1 terminates 34 amino acids after transmembrane seven. We therefore considered the possibility that the gfD1 termination codon was in fact present in an intron and that the carboxyl terminus of the protein was encoded by a downstream exon.

Evidence that the gfD1 coding sequence is intronless. An experiment using PCR was performed to show that the termination codon was present within an exon and not removed by a splicing event. Primers 5′C and 3′UT were made (Fig. 3A) such that 5′C was within the coding region and 3′UT was just 3′ to the termination codon. This primer set produced a PCR product of the predicted size when goldfish genomic DNA was used as a template (Fig. 3B, *lane 7*). If the termination codon, but not primer 3′UT, was within an intron, then the product produced by this primer set using RNA as a template would be smaller than the genomic product. If the 3′UT primer was within an intron, then no product would be produced from RNA. In fact, this primer set produced a product of identical size when either genomic DNA or RNA was used as a template (Fig. 3B, *lane 3*), thus demonstrating that the termination codon was not within an intron and that the open reading frame of gfD1 was intronless. Appropriate controls were performed to show 1) that the product produced from RNA was not the result of contaminating genomic DNA, because synthesis of the product was dependent upon reverse transcriptase (Fig. 3B, *lane 5*), and 2) that the expected results were obtained with primers 5′C and 3′C, two primers that were contained within the open reading frame (Fig. 3B, *lanes 2, 4, 6,* and *8*).

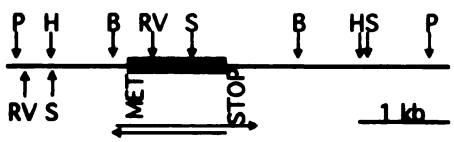

Fig. 1. Restriction map of a 4.7-kilobase *Pst*I genomic fragment that encodes the gfD1 open reading frame. *Hatched box*, intronless coding region. The locations of the initiator methionine (*MET*) and stop codon (*STOP*) are noted. *Horizontal arrows*, sequenced regions. All restriction sites are shown for the following enzymes: *P*, *Pst*I; *H*, *Hind*III; *B*, *Bam*HI; *RV*, *Eco*RV; *S*, *Sac*I.

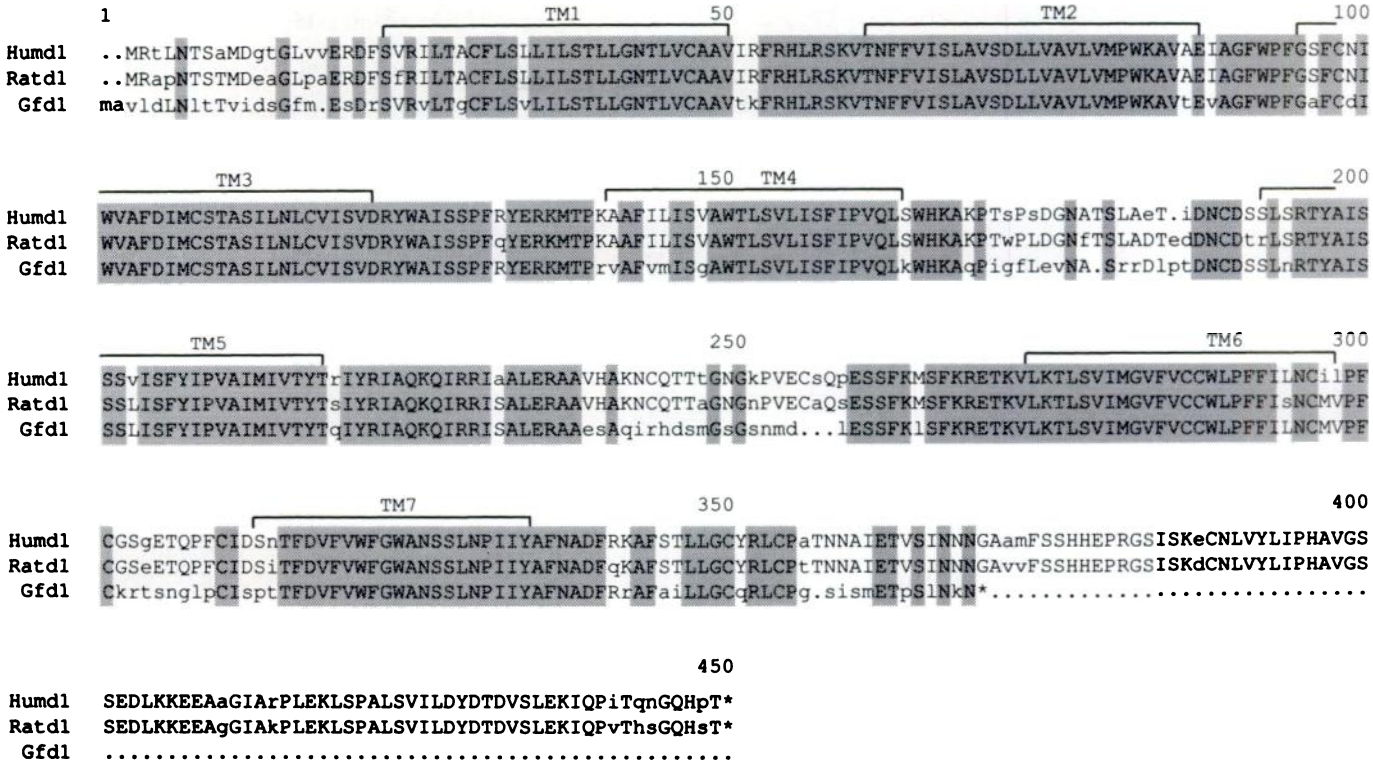

Fig. 2. Alignment of the amino acid sequences of dopamine D1 receptors from humans (2–4) (*Humd1*) and rats (3, 5) (*Ratd1*) with the gfD1 sequence. *TM*, transmembrane regions. Amino acids common to all three sequences are *shaded*. *Upper case letters*, amino acids that are conserved in at least two of the sequences.

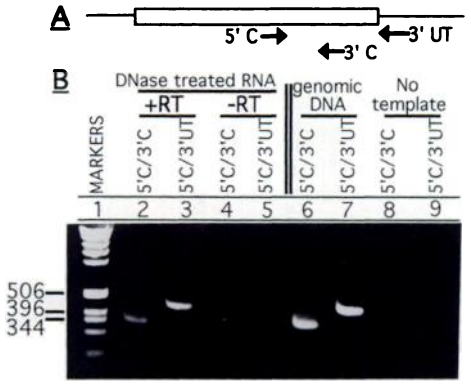

Fig. 3. Evidence that the gfD1 mRNA transcript is intronless within the coding region and is transcribed in retina. A, The locations of oligonucleotide primers used for PCR are shown relative to the genomic sequences that encode the gfD1 open reading frame (*box*). Two primers (5′C and 3′C) are located within the open reading frame and one (3′UT) is located just 3′ to the termination codon (5′C, GGCACGACAGCATGGGC; 3′C, TATAGAGCTCGGACAGAGTCTCTGGCATC; 3′UT, CTGAGAGCTCTTGCTTTGCTTGGCAAAGG). B, The products of various PCRs were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide. Two primer sets, 5′C/3′C and 5′C/3′UT, were used in RT-PCRs containing RNA from goldfish retina (*lanes 2-5*) or in PCRs containing goldfish genomic DNA (*lanes 6* and *7*) or no template (*lanes 8* and *9*). RT-PCRs were run with (*lanes 2* and *3*) or without (*lanes 4* and *5*) reverse transcriptase, to show that the RNA was not contaminated with genomic DNA. Size standards (in bases) (BRL 1-kilobase DNA ladder) are also shown (*lane 1*).

The RNA used for the PCR experiment shown in Fig. 3 was isolated from the retina of goldfish. The fact that PCR products were obtained from this RNA using primers encoding gfD1 sequences demonstrated that gfD1 was transcribed in goldfish retina. In addition, a single fragment was detected by Southern blot analysis of goldfish genomic DNA at high stringency using a gfD1 probe, indicating that gfD1 was most likely encoded by a single gene (data not shown). It appeared likely, then, that gfD1 encoded the well characterized goldfish retina dopamine D1 receptor.

Evidence that gfD1 encodes a dopamine D1 receptor. The pharmacology of the receptor encoded by gfD1 was investigated after transient expression of gfD1 in eukaryotic cells. Membranes of transfected COS-7 cells were used for saturation and competition binding experiments using the D1-specific antagonist [^{3}H]SCH-23390. The gfD1 receptor bound SCH-23390 with high affinity; in fact, the affinity was consistently higher for the gfD1 receptor (K_d = 0.13 nM, B_{max} = 1–5 pmol/mg of protein) than for the human D1 receptor (K_d = 0.70 nM, B_{max} = 0.4–1.0 pmol/mg of protein). The ability of various dopaminergic agonists and antagonists to compete for SCH-23390 binding sites was also analyzed and affinity values for these ligands were determined (Table 1). A comparison of the affinities of these compounds for the gfD1 receptor and the human D1 receptor showed that the gfD1 receptor was a dopamine D1 receptor.

Activation of dopamine D1 receptors leads to an increase in intracellular cAMP accumulation (1). Therefore, the gfD1 receptor and the human D1 receptor were separately transfected into HEK-293 cells and the ability of agonists to increase cAMP accumulation in these cells was assayed. Treatment with either the endogenous ligand dopamine or the D1-specific ligand (*R*)-(+)-SKF-38393 resulted in a dose-dependent increase in cAMP accumulation in cells expressing either the gfD1 receptor or the human D1 receptor (Fig. 4). The EC_{50} values for dopamine and

TABLE 1

Affinities of ligands for human and gfD1 receptors expressed in COS-7 cells

Values are means of *n* experiments done in triplicate, with <5% sample variation.

	Affinity	
	Human D1	Goldfish D1
	nM	
Antagonists		
SCH-23390	0.58 ($n = 3$)	0.15 ($n = 4$)
SCH-23388	276 ($n = 2$)	178 ($n = 2$)
(*cis*)-Flupentixol	0.84 ($n = 4$)	1.74 ($n = 3$)
(*trans*)-Flupentixol	89.7 ($n = 3$)	185.7 ($n = 3$)
Agonists		
(*R*)-(+)-SKF-38393	94.5 ($n = 3$)	149.0 ($n = 3$)
(*S*)-(−)-SKF-38393	9,820 ($n = 3$)	13,930 ($n = 3$)
Dopamine	1,126 ($n = 3$)	906 ($n = 3$)

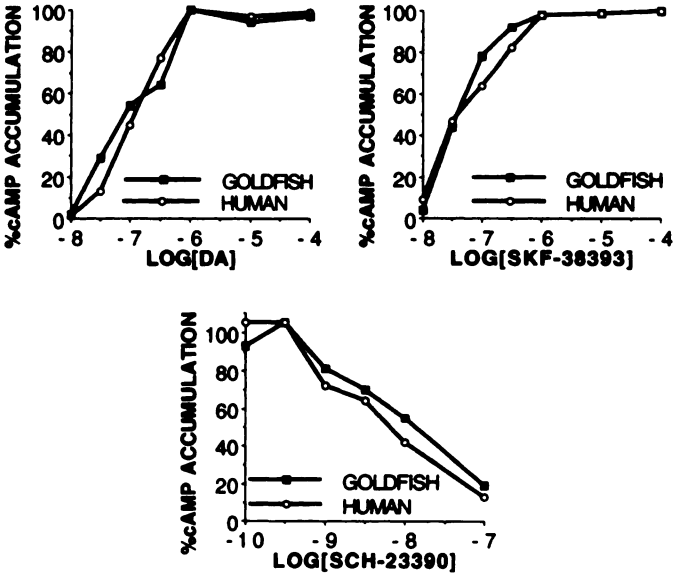

Fig. 4. Agonist-induced receptor stimulation of adenylyl cyclase in HEK-293 cells expressing the human or goldfish dopamine D1 receptor. Transfected cells were incubated with various concentrations of dopamine (*DA*) (*top left*) or (*R*)-(+)-SKF-38393 (*top right*), and the amount of cAMP was measured. The accumulation of cAMP caused by 10 μM dopamine was reversed in a dose-dependent manner by the antagonist SCH-23390 (*bottom*). Each *curve* is an average of at least three experiments. Maximal cAMP values (in pmol/well) for human and goldfish, respectively, were 67 and 53 (*top left*), 56 and 40 (*top right*), and 25 and 30 (*bottom*). Basal cAMP values varied between 2 and 5 pmol/well. The mean EC_{50} or IC_{50} values (in nM) for human and goldfish, respectively, were 119 ± 13 and 102 ± 14 (*top left*), 52 ± 4 and 39 ± 5.3 (*top right*), and 5.8 ± 0.4 and 10.2 ± 0.75 (*bottom*).

(*R*)-(+)-SKF- 38393 were similar between the gfD1 and human receptors. The increase in cAMP accumulation that resulted from dopamine treatment of cells expressing either gfD1 or human D1 receptors was reversed in a dose-dependent manner with SCH-23390 (Fig. 4), further indicating that the functional effects observed were a result of D1 receptor activation. Because the gfD1 receptor binds the standard D1 receptor compounds and because activation of this receptor results in an increase in cAMP accumulation, gfD1 is a dopamine D1 receptor.

Increased intracellular Ca^{2+} levels resulting from activation of the D1 receptor. Transfected HEK-293 cells expressing the human or goldfish D1 dopamine receptor were used to test the ability of dopamine to increase intracellular Ca^{2+} levels. Stimulation of the cells transfected with either the human or the goldfish receptor resulted in large increases in intracellular Ca^{2+} (Fig. 5). This increase was blocked by the D1-specific antagonist SCH-23390 but not by the D2-specific antagonist spiperone (Fig. 5), thus indicating that the increase was due to the activation of the D1 receptor. Neither untransfected cells nor "mock-transfected" cells (cells that were exposed to the transfection protocol) showed an increase in intracellular Ca^{2+} in response to dopamine (data not shown). Thus, activation of both the human receptor and the gfD1 receptor resulted in both the accumulation of cAMP and an increase in intracellular Ca^{2+} when receptors were expressed in HEK-293 cells.

Long term desensitization of the goldfish and human D1 receptors. Both the goldfish and the human D1 receptors were tested for their ability to undergo long term desensitization. Transfected HEK-293 cells were incubated with or without (control) 10 μM dopamine for 24 hr before cAMP assays or binding assays. After the 24-hr pretreatment, a dose-dependent increase in cAMP accumulation was observed for both the goldfish and the human D1 receptors (Fig. 6, *top*). Maximal cAMP values for goldfish were 139 (control) and 81 pmol/well (pretreated) (42% decline) and for human were 97 (control) and 62 pmol/well (pretreated) (36% decline). Maximal cAMP values induced by forskolin treatment for goldfish were 267 (control) and 236 pmol/well (pretreated) and for human were 235 (control) and 222 pmol/well (pretreated). Basal cAMP values varied between 2 and 5 pmol/well. Therefore, for both the goldfish and human receptors the maximal accumulation of cAMP in cells was decreased by pretreatment with dopamine. Furthermore, the effect on the EC_{50} values was similar for goldfish and human receptors. The EC_{50} values for goldfish were 25 ± 2 (control) and 247 ± 32 nM (pretreated) and for human were 27 ± 2 (control) and 127 ± 8 nM (pretreated).

Pretreatment of cells expressing either goldfish or human D1 receptors also resulted in a decrease in the number of binding sites (Fig. 6, *bottom*). In three separate experiments, the decline in B_{max} due to the pretreatment for the goldfish receptor was

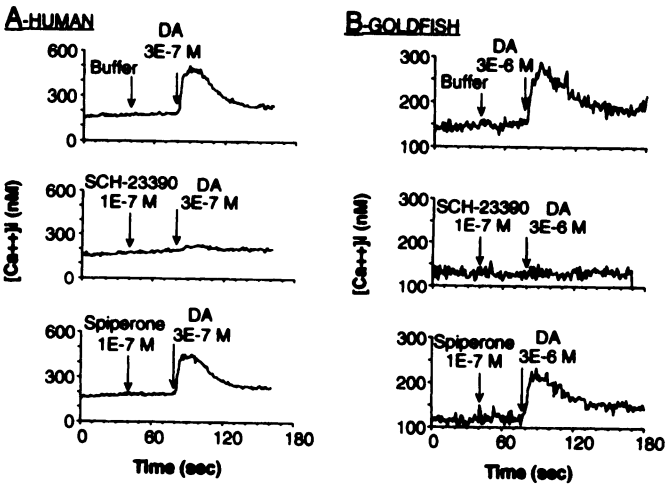

Fig. 5. Dopamine (*DA*)-stimulated increase in intracellular Ca^{2+} levels in cells transfected with the human (A) or goldfish (B) dopamine D1 receptor. The cells were pretreated with buffer (*top*), the D1 antagonist SCH-23390 (*middle*), or the D2 antagonist spipirone (*bottom*), at the indicated concentrations, before treatment with dopamine. The experiment was performed three times and the data shown are from one representative experiment. The potency of dopamine was variable, due to the variable levels of expression between different transient transfections.

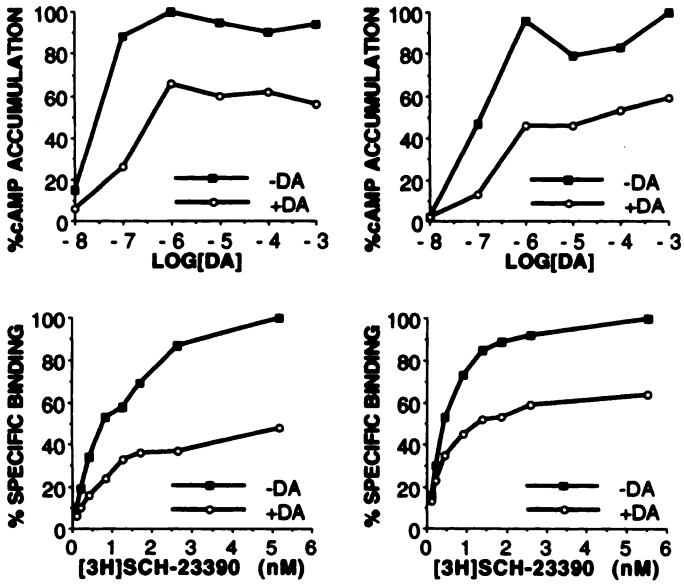

Fig. 6. Desensitization of human and goldfish dopamine D1 receptors, expressed in transfected HEK-293 cells, by a 24-hr pretreatment with 10 μM dopamine. Cells transfected with the human (*left*) or goldfish (*right*) D1 receptor were treated for 24 hr with (+*DA*) or without (−*DA*) 10 μM dopamine before analysis of dopamine-induced cAMP accumulation (*top*) or before saturation binding analysis (*bottom*). The *curves* showing cAMP accumulation are an average of at least three experiments. The binding *curves* are representative of a single experiment. The saturation binding data were normalized to the maximum binding obtained in the absence of dopamine.

37%, 38%, and 54% and for the human receptor was 54%, 31%, and 37%. The actual receptor density varied between 1.5 and 7.0 pmol/mg of protein, depending on the transfection efficiency in each experiment. The average K_d values for goldfish were 0.29 ± 0.05 (control) and 0.30 ± 0.02 nM (pretreated) and for human were 1.45 ± 0.29 (control) and 1.17 ± 0.26 nM (pretreated). Pretreatment had little effect on the affinity of the ligand, although the affinity for the goldfish receptor was higher than that for the human receptor, as previously mentioned. Therefore, both the goldfish and the human D1 receptors undergo desensitization after prolonged pretreatment with agonist, and the decrease in cAMP accumulation observed directly correlates with the loss of receptor binding sites.

Discussion

A gene, gfD1, was isolated from goldfish and shown to encode a dopamine D1 receptor, based upon several criteria. First, the sequence of this receptor was highly homologous with the D1 receptor from rat and human. Second, the gene product bound the D1 antagonist SCH-23390 and the D1 agonist (*R*)-(+)-SKF-38393 with high affinity. Third, the gene product caused the accumulation of intracellular cAMP in response to dopamine or (*R*)-(+)-SKF-38393. In fact, the pharmacology of this D1 receptor was very similar to that of the human D1 receptor.

The pharmacology of the gfD1 receptor was also consistent with the reported pharmacology of the receptor that is present in homogenates of carp retina and activates adenylate cyclase in response to dopamine (15). In addition, the mRNA for gfD1 was present in the retina. Therefore, it is likely that gfD1 encodes the previously described teleost retinal D1 receptor (14–17).

There are indications that D1 receptor subtypes exist in the teleost retina. In studies of carp retina, Van Buskirk and Dowling (17) reported that the affinities of the antagonists haloperidol and (+)-butaclamol were 2–4 orders of magnitude higher for isolated horizontal cells than for homogenates of retina, possibly reflecting different subtypes present among different retinal cell types. In a separate study, Rodrigues and Dowling (18) reported that dopamine induced neurite retraction in retinal horizontal cells via diacylglycerol and protein kinase C, and not cAMP. Mammalian D1 receptors have also been reported to be involved in activation of phospholipase C and protein kinase C in kidney (19, 20) and striatum (21). Novel genes may exist for these receptors, or the known D1 receptor genes may activate multiple signal transduction pathways.

It is therefore interesting that stimulation of the gfD1 receptor resulted in the mobilization of intracellular calcium when receptors were expressed in HEK-293 cells. This result is not exclusive to the goldfish D1 receptor, because the human receptor displayed similar results (31, 32) (Fig. 5). Thus, the D1 receptor can activate multiple signal transduction pathways, although the mechanism of calcium activation requires further investigation.

Dopamine has also been shown to stimulate growth hormone release from the pituitary of goldfish through D1 receptors (23). The release of growth hormone was stimulated by dopamine (ED_{50} = 0.26 μM) or (*R*)-(+)-SKF-38393 (ED_{50} = 0.41 μM) and was reversed by 5 μM SCH-23390 (23). These values are not inconsistent with the values reported here for gfD1 and cannot exclude gfD1 as the receptor that is involved in growth hormone release.

Finally, the conservation of receptor structure may provide insights into the relationship between D1 receptor structure and function. The overall sequence homology between the goldfish and human D1 receptors within the transmembrane regions, the regions that are involved in ligand binding, is 92%. Given this high degree of conservation, it was surprising that the gfD1 receptor lacked the carboxyl-terminal 80 amino acids present in the mammalian D1 receptors. This missing region has been the target of site-directed mutagenesis for the β-adrenergic receptor (for review, see Ref. 33), and findings can be summarized as follows. It appears that this region does not play a major role in the binding of ligands or in receptor activation. However, sites present within this region are phosphorylated in response to agonist treatment, and short term agonist treatment results in receptor desensitization. However, this region does not appear to be involved in receptor down-regulation after long term agonist treatment. The results reported here for the goldfish D1 receptor are consistent with these mutagenesis studies. However, the ability of the gfD1 receptor to undergo short term desensitization and the physiological relevance of the truncated receptor in goldfish retina remain to be determined.

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