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D_{1A}, D_{1B}, and D_{1C} dopamine receptors from *Xenopus laevis*

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ABSTRACT **Three distinct genes encoding members of the D_1 dopamine receptor family were isolated from *Xenopus laevis*. Based on the deduced amino acid sequence, two of the receptors (*Xen* D_{1A} and *Xen* D_{1B}) appear to be homologues of mammalian D_1/D_{1A} and D_5/D_{1B} receptors. The third receptor, termed *Xen* D_{1C}, displays equal overall amino acid and nucleotide sequence identity (≈55%) with mammalian D_{1A} and D_{1B}/D_5 receptors. In agreement with their structural similarities, *Xen* D_{1A} and D_{1B} receptors, when expressed in COS-7 cells, displayed pharmacological profiles that paralleled those of their mammalian counterparts, with dopamine and 2-amino-6,7-dihydroxytetralin exhibiting 10-fold higher affinity for D_{1B} than for D_{1A}. The *Xen* D_{1C} receptor displayed an overall rank order of potency and pharmacological profile clearly indicative of a D_1-like receptor, with individual affinities for most agonists higher than those for either *Xen* or mammalian D_1/D_{1A} and D_5/D_{1B} receptors, whereas antagonist K_i values were intermediate to those for the D_1/D_{1A} and D_5/D_{1B} receptors. All three receptors stimulated adenylate cyclase activity in response to dopamine or SKF-82526. *Xen* D_{1A}, D_{1B}, and D_{1C} receptor mRNAs were differentially distributed, with all three receptors expressed in brain and only D_{1B} and D_{1C} receptors expressed in kidney. The existence of a receptor which lacks appreciable overall sequence similarity to, but displays pharmacological homology with, mammalian D_1-like receptors lends strong support to the contention that additional mammalian D_1-like receptor gene products may exist to allow for the expression of the full spectrum of D_1-like dopamine receptor-mediated events.**

D_1 dopamine receptors have been classically defined, on the basis of pharmacological and biochemical grounds, by their ability to bind and respond to selective dopaminergic ligands, particularly to the benzazepine class, and by coupling to second-messenger systems through the stimulation of adenylate cyclase (see refs. 1–4 for reviews). Molecular biological studies have revealed that native D_1 receptors actually comprise two receptor subtypes, D_1/D_{1A} and D_5/D_{1B}, each of which stimulates the activity of adenylate cyclase. These receptors are, however, distinguishable on the basis of their primary structure, pharmacological profiles, mRNA distribution, and chromosomal localization (5–8).

Despite the existence of two distinct D_1 dopamine receptors, biochemical, pharmacological, and behavioral evidence suggests the existence of additional D_1-like receptor subtypes. Thus, D_1 receptor stimulation in both brain and periphery has been shown to activate phospholipase C (9–12), translocate protein kinase C (13), stimulate K^+ efflux (14), and inhibit Na^+/H^+ exchange, all independent of the activity of adenylate cyclase (15–17). Moreover, recent behavioral studies have differentiated agonist specific D_1-like receptor-mediated behaviors from the activity of D_1-stimulated adenylate cyclase (18–22). It is unclear whether all these D_1-like receptor-mediated responses can be accounted for solely by the presence of D_1/D_{1A} and D_5/D_{1B} receptors (23, 24).

We report here the existence of three distinct genes encoding functional members of the D_1 dopamine receptor family from *Xenopus laevis*. These include a classical D_{1A} receptor, a D_{1B} receptor homologue, and a distinct D_1 receptor variant, which we term D_{1C}, displaying unique amino acid, pharmacological, and tissue-specific expression patterns. The cloning of a third type of vertebrate D_1-like dopamine receptor suggests that additional D_1-like gene products may be encoded in the mammalian genome which may account for some of the pharmacological and behavioral events following selective D_1-like dopamine receptor stimulation.¶

MATERIALS AND METHODS

Isolation of *Xenopus* Genomic Clones. A *X. laevis* genomic library (Stratagene) was screened first with a ^{32}P-labeled human D_1 dopamine receptor DNA fragment (25) corresponding to transmembrane domains 2–5. About 5×10^5 independent clones were screened under the following conditions: duplicate nylon filters (DuPont/NEN) were hybridized at 42°C in a solution (26) containing ^{32}P-labeled *Bgl* II–*Hin*dIII D_1 fragment (2×10^6 cpm/ml). Filters were washed once in 2× standard saline citrate (SSC)/1% SDS for 15 min at 60°C and then once in 1× SSC/1% SDS for 15 min at 60°C. The filters were stripped and were subsequently reprobed under identical stringency conditions with a ^{32}P-labeled D_5 dopamine receptor fragment (27) encoding transmembrane domains 2–5. One hybridizing clone, a 2.5-kb *Bam*HI fragment (*Xen* 1) was unique to only the D_1 screening, while two other clones, a 2.8-kb *Acc* I and a 3.0-kb *Pst* I fragment (*Xen* 2 and *Xen* 6, respectively), were identified by both D_1 and D_5 screenings, although *Xen* 6 hybridized poorly to the D_5 probe. Following subcloning into pBluescript SK(−) (Stratagene), both strands were sequenced by the Sanger dideoxy chain-termination method with 7-deaza-dGTP and Sequenase (United States Biochemical) and either specific internal primers (Biotechnology Service Centre HSC, Toronto) or T7/T3 primers.

Cell Culture and Expression. COS-7 monkey cells were grown on 150-mm plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C, 5% CO_2. The three genomic dopamine receptor clones were subcloned into the expression vector pCD-PS for transient expression (25). COS-7 cells were transfected with the CsCl gradient-purified constructs by electroporation (26).

[^{3}H]SCH-23390 Binding Assays. Membranes prepared from harvested COS-7 cells grown on 150-mm plates were suspended in buffer at a protein concentration of 30–50 μg/ml

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¶The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U07863 (D_{1A}), U07864 (D_{1B}), and U07865 (D_{1C})].

and assayed for [^{3}H]SCH-23390 (NEN; 85.5 Ci/mmol; 1 Ci = 37 GBq) binding activity (25). The data were analyzed by nonlinear least-squares fitting with the computer program KALEIDAGRAPH (Abelbeck Software, Reading, PA).

cAMP Accumulation. COS-7 cells transiently transfected with the D_{1A}, D_{1B}, and D_{1C} clones or untransfected cells were grown in 24-well plates. After 72 hr, the wells were washed once with prewarmed (37°C) Dulbecco's modified Eagle's medium containing 0.5 mM 3-isobutyl-1-methylxanthine and 1 μM propranolol. Cells were incubated in 0.4 ml of the aforementioned medium in the presence or absence of 1 μM SCH-23390 for 15 min at 37°C, 5% CO_2. Either dopamine (10 μM) or SKF-82526 (1 μM) was added for an additional 15 min of incubation at 37°C, 5% CO_2. Reactions were stopped by the addition of 500 μl of 0.2 M HCl, the cellular debris was pelleted by centrifugation at 500 × *g*, and supernatants (25 μl) were assayed for cAMP formation by radioimmunoassay (Amersham).

Reverse Transcription–PCR Analysis of D_{1A}, D_{1B}, and D_{1C} Receptor mRNA. Total *Xenopus* brain and kidney RNAs were isolated by using TRISOLV (Biotecx Laboratories, Houston). Samples (≈1 μg) of brain and kidney total RNA were treated with DNase for 15 min at room temperature prior to first-strand cDNA synthesis with 25 pmol of oligo(dT) (Perkin–Elmer/Cetus) and 200 units of Superscript reverse transcriptase (BRL). The single-stranded cDNA was then subjected to 30 cycles of PCR amplification (94°C, 1 min; 58°C, 1.5 min; 72°C, 1.5 min) using 0.5 μg of two receptor-specific internal oligonucleotide primers encompassing the carboxyl tail regions of the three dopamine receptor clones [nucleotides: D_{1A}, 920–943 (5′–3′) and 1406–1383 (3′–5′); D_{1B}, 988–1014 (5′–3′) and 1401–1375 (3′–5′); D_{1C}, 856–888 (5′–3′) and 1464–1441 (3′–5′)] and 2 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus). A DNase control group in which reverse transcriptase was not added was processed in parallel. Amplified products were electrophoresed in a 1.5% agarose gel, transferred to nylon membrane for Southern blot analysis, probed with γ-^{32}P end-labeled oligonucleotides internal to the flanking primers [D_{1A}, 1141–1164 (5′–3′); D_{1B}, 1306–1329 (5′–3′); D_{1C}, 1089–1112 (5′–3′)], and washed in 2× SSC/1% SDS for 15 min at room temperature and then for 15 min at 42°C.

RESULTS AND DISCUSSION

An approach based on homology probing and multiple subtractive screenings was used to clone three distinct members of the D_1 dopamine receptor family. Sequence analysis indicated that all three clones shared strong amino acid sequence homology to mammalian D_1-like dopamine receptors. A putative initiation methionine codon with predicted Kozak sequence (28) was followed by long open reading frames uninterrupted by intronic sequences for all three clones. *Xen* 1, 2, and 6 contained open reading frames of 1353, 1371, and 1395 nt, encoding proteins with calculated molecular masses of 50,516, 51,622, and 52,606 Da, respectively.

The deduced amino acid sequences of the isolated clones revealed that *Xen* 1 may be an orthologue of the mammalian D_1/D_{1A} receptor and *Xen* 2, the equivalent of the D_5/D_{1B} receptor (Fig. 1). *Xen* 1 shared highest overall amino acid sequence identity (80%) with the human D_1 receptor and showed only 54% and 52% identity with human D_5 and rat D_{1B} receptors, respectively. *Xen* 2 displayed the highest

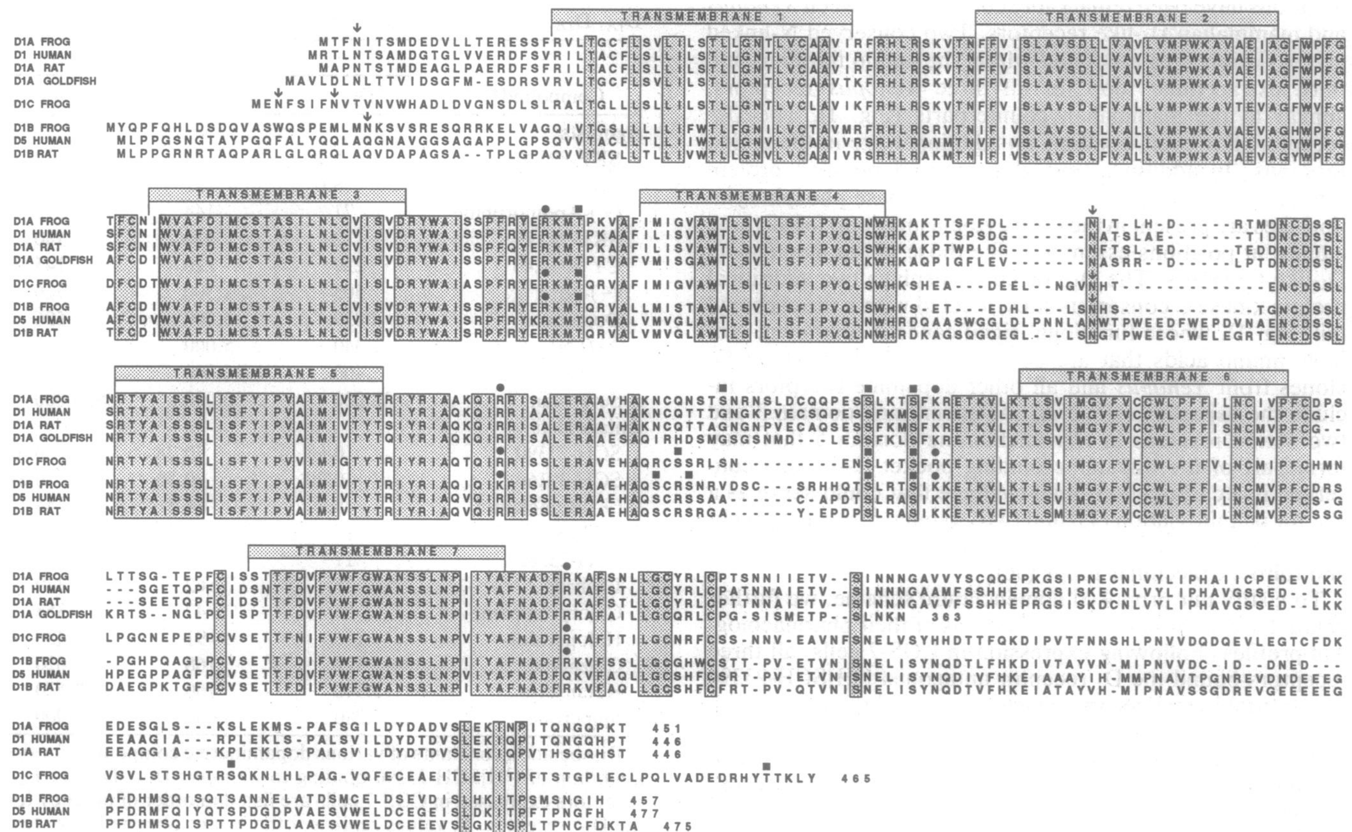

FIG. 1. Alignment of the deduced amino acid sequence (single-letter symbols) of *Xenopus* D_{1A}, D_{1B}, and D_{1C} receptors with other members of the D_1 dopamine receptor subfamily. Boxed and shaded regions indicate amino acid residues absolutely conserved among all D_1-like receptors represented. Positions of seven putative transmembrane domains are demarcated above the amino acid sequence. Putative N-linked glycosylation sites are indicated by arrows. Potential cAMP-dependent protein kinase (●) and protein kinase C (■) phosphorylation sites are indicated. Sequence sources are as follows: human D_1 (25, 29), rat D_{1A} (29), goldfish D_{1A} (30), human D_5 (26, 31, 32), and rat D_{1B} rat (23).

sequence identities, 66% and 65%, with the rat D_{1B} and human D_5 receptors, respectively, and only 55% identity with the human D_1 receptor. Although *Xen* 2 displayed greater sequence divergence from mammalian D_5/D_{1B} receptors than either the rat D_{1B} receptor from the human D_5 receptor (≈82%) or *Xen* 1 from mammalian D_1/D_{1A} receptors (≈80%), the low overall percent homology of *Xen* 2 to its mammalian counterparts is well in accord with the predicted (33) accelerated rate of evolutionary mutation exhibited by D_5/D_{1B} receptors (≈1% per 8×10^6 years) compared with D_{1A} receptors (≈1% per 1.2×10^7 years). Consistent with the view that *Xen* 2 is a D_5/D_{1B} species homologue is the fact that at the nucleotide level *Xen* 2 is more homologous (66%) to the human D_5 receptor and its two pseudogenes (64% and 65%) than either *Xen* 1 (≈53%) or *Xen* 6 (59% to D_5; 48% and 59% to two pseudogenes). At the amino acid level, *Xen* 6 appears distinct, displaying equivalent overall sequence identities to either the rat D_{1B} (56%) or human D_1 and D_5 receptors (54%). Within the putative transmembrane domains *Xen* 1, 2, and 6 exhibit >92% identity with mammalian D_1-like receptors. Regions of significant sequence divergence between these receptors were particularly evident in the amino termini, third intracellular and extracellular loops, the fourth extracellular domain, and carboxyl-terminal tails. Based on the strong overall sequence homologies to either the mammalian D_1/D_{1A} or D_5/D_{1B} receptors, aligned in Fig. 1, we propose to term the proteins encoded by the *Xen* 1, *Xen* 2, and *Xen* 6 clones the D_{1A}, D_{1B}, and D_{1C} receptors, respectively, in line with the nomenclature scheme originally suggested for multiple rat D_1 receptors (8, 23).

Consensus sequences for putative posttranslational modifications have been remarkably conserved between *Xenopus* and mammalian D_1-like receptors. Two conserved N-linked glycosylation sites are found within the amino terminus and third extracellular loop for all three *Xenopus* receptors, similar to the mammalian counterparts (Fig. 1). The D_{1C} receptor, however, contains an additional site in the amino terminus. In addition, several conserved putative protein kinase C and cAMP-dependent protein kinase phosphorylation sites are present in the third cytoplasmic loop for all three clones, at positions essentially like those in the mammalian D_1-like receptors. The D_{1C} receptor displays two additional protein kinase C consensus sites, located within the carboxyl tail (Fig. 1), whose functional significance is unknown. Notable amino acids that are conserved among the three D_1 clones from *Xenopus* and all other dopamine receptors include the aspartic residue in transmembrane domain 3 and two serine residues in transmembrane domain 5 that may form part of the dopamine binding pocket (34). Also conserved is a carboxyl-terminal cysteine that could serve as a site for palmitoylation (35) and two cysteine residues thought to take part in disulfide bond formation between the second and third extracellular loops (36).

To justify our proposed nomenclature scheme, we further characterized these receptors in terms of their pharmacological profiles. Following expression in COS-7 cells, all three receptors bound the D_1 receptor antagonist [^{3}H]SCH-23390 in a saturable manner to a single class of binding sites with high affinity and with estimated dissociation constants (K_d values) of 250, 350, and 80 pM for the D_{1A}, D_{1B}, and D_{1C} receptors, respectively. Saturation analysis revealed receptor densities (B_{max} values) that were on average 15, 7, and 0.6 pmol/mg of protein for D_{1A}, D_{1B}, and D_{1C}, respectively. [^{3}H]SCH-23390 binding to membranes of COS-7 cells expressing D_{1A}, D_{1B}, or D_{1C} receptors was inhibited by various dopaminergic agonists and antagonists, in a stereoselective, concentration-dependent, and uniphasic manner (as indexed by Hill coefficients close to unity) with a pharmacological profile clearly indicative of a D_1 receptor. Estimated K_i values for these agents are listed in Table 1.

One unique distinguishing pharmacological feature between mammalian D_1-like dopamine receptors is the inherent ability of the D_5/D_{1B} receptor to display higher affinity for the endogenous neurotransmitter dopamine than D_1/D_{1A} (23, 27, 31, 32). Consistent with our proposed classification of these receptors based on amino acid sequence homologies, the *Xen* D_{1A} receptor displayed an affinity for dopamine (≈4 μM) 10-fold less than that of the *Xen* D_{1B} receptor (≈500 nM) (Fig. 2*A*), paralleling their mammalian counterparts. Moreover, like the mammalian D_5/D_{1B} receptor, 2-amino-6,7-dihydroxytetralin exhibited a 10-fold higher affinity for the *Xen* D_{1B} receptor than for the *Xen* D_{1A} receptor. Most antagonists, particularly butaclamol and α-flupentixol, exhibited lower affinities for the D_{1B} receptor than for the D_{1A} receptor, similar to those seen with the rat D_{1B} and human D_5 receptor. Estimated K_i values for the inhibition of [^{3}H]SCH-23390 binding to *Xen* D_{1A} and D_{1B} receptors by a series of compounds were highly correlated to K_i values obtained on the human D_1 and D_5 receptors, respectively, with a virtual one-to-one correspondence in drug affinities.

The D_{1C} receptor, however, displayed pharmacological characteristics consistent with both types of receptors, with an observed affinity for dopamine (K_d ≈800 nM) somewhat intermediate to the affinities of D_1 and D_5 receptors. Similar results were obtained for 2-amino-6,7-dihydroxytetralin. All other agonists displayed somewhat higher affinity for the D_{1C} receptor than for the D_{1A} or D_{1B} receptor. Most antagonists exhibited affinities at the D_{1C} receptor intermediate to those for the D_{1A} and D_{1B} receptors (Table 1). Comparison of the

Table 1. K_i values for inhibition of [^{3}H]SCH-23390 binding to D_{1A}, D_{1B}, and D_{1C} receptors expressed in COS-7 cells

Compound*	K_i, nM		
	D_{1C}	D_{1A}	D_{1B}
Agonists			
SKF-82526	5.2	14	12
SKF-81927	8.0	12	17
(−)-Apomorphine	71	144	213
SKF-38393	72	136	130
CY 208-243	229	226	145
(−)-NPA	505	1,300	716
Dopamine†	839	3,800	506
ADTN†	1,340	5,000	332
Noradrenaline	5,400	>16,000	>50,000
Serotonin	>25,000	>50,000	>50,000
Antagonists			
SCH-23390	0.08	0.28	0.65
SCH-39166	0.20	0.50	0.82
(+)-Butaclamol†	8.2	4.5	44
α-Flupentixol†	12	4.5	81
SCH-23388	17	32	24
Lisuride	35	62	379
Chlorpromazine	65	47	358
Haloperidol	116	91	430
Clozapine	522	168	620
Ketanserin	1,340	760	2,570
Spiperone	2,280	5,600	>10,000

Dissociation constants of various dopaminergic compounds for the inhibition of [^{3}H]SCH-23390 binding to *Xenopus* D_{1A}, D_{1B}, or D_{1C} receptors expressed in COS-7 cells are listed in order of potency for the D_{1C} receptor. K_i values represent the means obtained from two to three independent experiments, each conducted in duplicate, which varied by <15%.

*ADTN, (±)-2-amino-6,7-dihydroxytetralin; NPA, (−)-*N*-propylapomorphine.

†Compound that distinguishes mammalian D_1/D_{1A} from D_5/D_{1B} receptors.

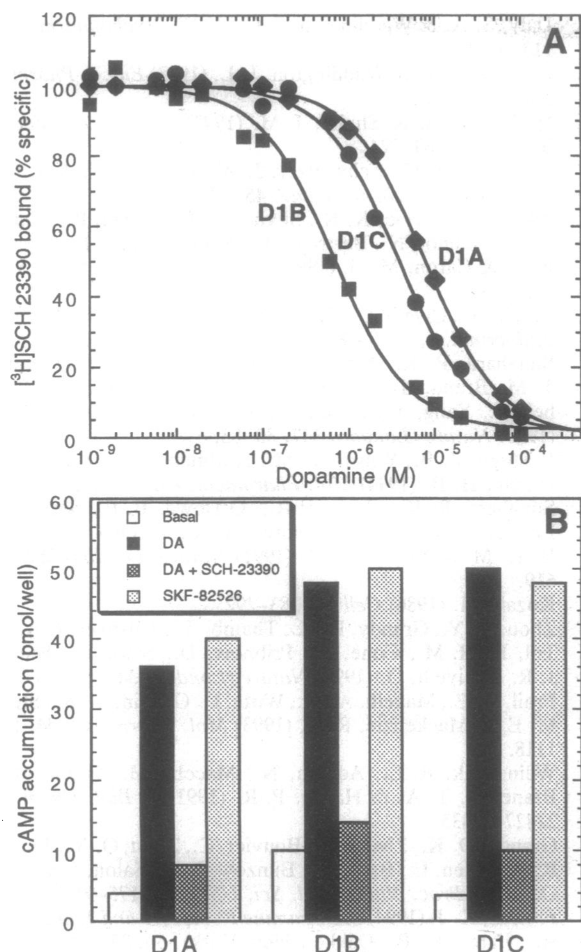

FIG. 2. Dopamine affinity and second-messenger activation of *Xenopus* D_1-like receptors. (*A*) COS-7 cell membranes expressing either D_{1A}, D_{1B}, or D_{1C} receptors were incubated with ≈300 pM [^{3}H]SCH-23390 in the presence of the indicated concentrations of dopamine and assayed for receptor activity. Estimated K_i values for dopamine at each of these receptors are listed in Table 1. Data are representative of three independent experiments, each conducted in duplicate. (*B*) cAMP accumulation in COS-7 cells transiently expressing the D_{1A}, D_{1B}, and D_{1C} receptors. Approximately 2.5×10^5 cells were incubated with 10 μM dopamine (DA) or 1 μM SKF-82526 and assayed for cAMP content by radioimmunoassay. Dopamine-stimulated cAMP production was prevented by the specific D_1 antagonist SCH-23390 (1 μM). Basal cAMP ranged from 3 to 8 pmol per well. Stimulation of adenylate cyclase by 100 μM forskolin resulted in levels of cAMP >120 pmol per well. Data are representative of three independent experiments, each conducted in duplicate.

K_i values of various agonists and antagonists revealed that the *Xen* D_{1C} receptor displayed higher affinities for agonists and lower affinities for most nonselective D_1 antagonists than the human D_1 receptor. In contrast, comparison of the K_i values for these agents at the D_{1C} receptor with those at the human D_5 receptor revealed a closer correspondence in estimated affinity values between these two receptors, albeit with some agonists, such as dopamine and apomorphine, clearly lying outside the line of eqimolarity. As none of the receptors displayed guanine nucleotide-sensitive high-affinity forms (data not shown), it is possible to directly compare agonist affinities of these receptors with those of their mammalian counterparts expressed in the same cells and assayed under similar conditions.

Despite the distinct pharmacological profile of the D_{1C} receptor, all three receptors were found to couple to the same second-messenger system when expressed in COS-7 cells. Dopamine (10 μM) and the selective D_1 agonist SKF-82526 (1 μM) stimulated the production of cAMP 5- to 10-fold over basal levels, whereas pretreatment with the D_1 receptor antagonist SCH-23390 (1 μM) virtually abolished the stimulatory effect of dopamine (Fig. 2*B*). However, no consistent stimulation of phosphatidylinositol turnover was seen with dopamine or SKF-82526 up to 100 μM. It is unknown whether this lack of response in COS-7 cells is due to the inappropriate complement of subtype-specific G-protein α or βγ subunits needed for specific *Xen* D_1-like receptor stimulation of phospholipase C or due to the lack of the appropriate molecular form of the enzyme. Coexpression studies with these proteins in COS-7 cells may reveal receptor-specific functional coupling to phospholipase C and phosphatidylinositol hydrolysis (37, 38).

Despite the lack of functional differentiation, D_{1A}, D_{1B}, and D_{1C} receptor mRNAs appear to be differentially distributed in central and peripheral tissues. All three receptor mRNAs were found to be expressed in brain but only the D_{1B} and D_{1C} receptors were present in kidney as determined by reverse transcription–PCR analysis (Fig. 3). In contrast, both the D_{1A} and D_{1B} receptors have been detected in opossum kidney (39), whereas the D_{1A} receptor has been detected in the rat proximal tubule by PCR analysis (40). The selective lack of expression of D_{1A} receptor mRNA in *Xenopus* kidney suggests that in frogs, unlike mammals, central and peripheral D_1-like receptor expression is differentiated. Moreover, the expression of the D_{1C} receptor in kidney may reflect the reported difference in pharmacological profiles between central and peripheral D_1-like receptors (ref. 17 and references therein).

The D_{1C} receptor subtype probably did not arise from a duplication event that occurred 25–30 million years ago in *Xenopus* (41), since duplicated genes in this tetraploid organism tend to be structurally more similar than predicted for these three clones. The D_{1A} receptor shares only 59% nucleotide identity with *Xenopus* D_{1B} and D_{1C} receptors, while the D_{1B} and D_{1C} receptors are only 57% identical at the amino acid level and 61% identical at the nucleotide level. This situation is distinct from the two recently cloned dopamine D_2 receptors and other gene pairs from *Xenopus* such as the

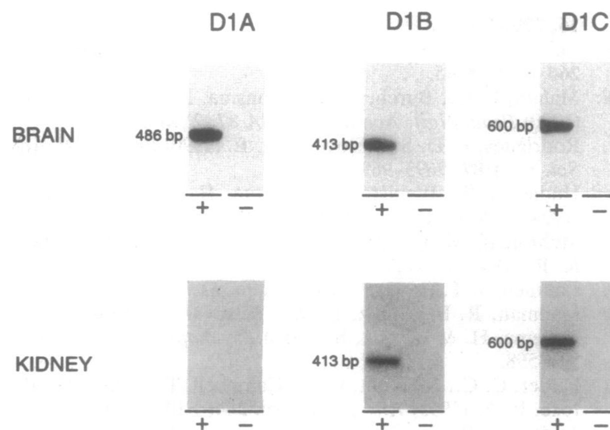

FIG. 3. Tissue-specific D_{1A}, D_{1B}, and D_{1C} receptor mRNA distribution. Samples (1 μg) of *Xenopus* brain and kidney total mRNA were subjected to reverse transcription using oligo(dT) and then to PCR using two additional subtype-specific receptor oligonucleotides (*Materials and Methods*). Amplified cDNA products were Southern blotted and probed with receptor subtype-specific ^{32}P-labeled oligonucleotides internal to the PCR fragments. D_{1A}, D_{1B}, and D_{1C} receptor transcripts of the appropriate sizes were evident in brain. Only D_{1B} and D_{1C} receptor mRNAs could be amplified from kidney. DNase controls with no reverse transcriptase (−) are located to the right of each hybridizing PCR product. Sizes of hybridizing bands (bp) are shown.

preproinsulins, proopiomelanocortins, and proenkephalins which share 92–95% nucleotide and amino acid identity (42–46). The low homologies displayed between the members of the *Xenopus* D_1 receptor family suggest that they diverged prior to the predicted duplication event, in accord with the proposed early evolutionary appearance (≈500 million years) and ancient status of the D_1-like receptor subfamily (33).

The identification of the *Xen* D_{1C} receptor, which lacks appreciable overall sequence similarity to, but displays a pharmacological profile consistent with, mammalian D_1-like receptors may allow, with the use of inter- and intraspecies specific chimeras, the identification of those amino acid residues and structural motifs regulating D_1 ligand binding specificity, signal transduction, and receptor desensitization. Moreover, the existence of the D_{1C} gene may aid in the isolation of mammalian homologues of this or related D_1 receptor variants which have so far eluded detection.

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