0026-895X/93/050918-08$03.00/0

MOLECULAR PHARMACOLOGY, 44:918-925

Cloning and Characterization of the Opossum Kidney Cell D1 Dopamine Receptor: Expression of Identical D1A and D1B Dopamine Receptor mRNAs in Opossum Kidney and Brain

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Received May 4, 1993; Accepted August 20, 1993

SUMMARY

Opossum kidney cells are an established epithelial cell line which is often studied as a physiological model system of renal proximal tubule function, and which has also been shown to possess dopamine receptors. To identify dopamine receptor subtypes present in renal tissue, as well as to explore the usefulness of opossum kidney cells for the study of D1 dopamine receptors and renal dopaminergic physiology, we have undertaken the cloning and characterization of the dopamine receptor expressed in this cell line.

In the brains of rats and humans, two different subtypes of D1 dopamine receptors, $D1_A$ and $D1_B$, have recently been characterized (1). The OK cell D1 receptor message is 4500 bp long and exhibits extensive homology with the rat and human $D1_A$ subtypes of dopamine receptors. Pharmacological experiments were performed on COS-7 cell membranes transiently transfected with this cDNA. Binding properties were compared with those reported for OK cell membranes, and comparison experiments were performed in parallel with the human $D1_A$ expressed transiently in the same system.

Molecular techniques including Northern blotting, *in situ* hybridization, and RNase protection analysis were used to study the expression pattern of the OK cell D1 receptor message. Expression of both $D1_A$ and $D1_B$ subtypes was detected in both the opossum brain and the opossum kidney, however, the OK cell line expresses exclusively the $D1_A$ receptor subtype.

Dopamine, an important neurotransmitter in the central nervous system, has also been shown to play a role in the physiology of peripheral tissues such as the vasculature and kidney (1, 2). Dopaminergic effects in the periphery, as in the brain, are mediated by specific GTP-binding protein-coupled receptors. Dopamine receptors outside of the central nervous system were extensively characterized by physiological studies of arterial vasodilation, and they have been classified as either DA1 or DA2 based on potency series of agonists and antagonists (3). Although this system of nomenclature differs from the original D1/D2 classification based on receptor coupling that arose from studies of rat brain homogenates, DA1 and D1 as well as DA2 and D2 appear to have analogous properties (4). Physiological as well as ligand binding studies have identified dopamine receptors in both the renal vasculature and the proximal section of the nephron (5–7). In the renal cortex of placental mammals, dopamine has been shown to function in a paracrine manner to produce natriuresis and diuresis (8). These effects are thought to result from an inhibitory effect of dopamine on sodium reuptake within the tubule caused by dopaminergic inhibition of the Na^+/H^+ antiporter and Na^+/K^+ ATPase (9, 10). Dopamine receptors in renal tubules have been shown to bind D1-selective drugs and mediate activation of both adenylyl cyclase production of cAMP, and phospholipase C-mediated production of inositol trisphosphate (11, 12).

Some studies, however, have suggested that there might be pharmacological differences between the dopamine receptors expressed in renal and vascular tissues and those expressed in the central nervous system (13). Several drugs, most notably (+)-sulpiride, fluphenazine, and lisuride, have been reported to display pharmacological properties which differ between an assay based on the *in vivo* measurement of renal vasodilation in dogs and an assay based on stimulation of adenylyl cyclase in rat basal nuclei homogenates (14). Similarly, Northern blot analysis of human and rat kidneys has failed to demonstrate expression of either of the cloned D1 receptor subtypes in these tissues (reviewed in Ref. 1).

OK cells are an epithelial cell line which is derived from the kidney of a North American opossum, *Didelphis virginiana*, and which possesses various characteristics of proximal tubules

This work was supported in part by National Institutes of Health grants NS15976, MH 40159, and MH 44211 to M.G.C. S.R.N. is a student in the Medical Scientist Training Program supported by National Institutes of Health grant 5T32GM07171-18.

ABBREVIATIONS: RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; bp, basepair; kb, kilobase(s); D_5, human homology of the DI_B dopamine receptor.

(15). These characteristics include Na^+-coupled uptake mechanisms for amino acids and hexoses, expression of parathyroid hormone receptors, parathyroid hormone-regulated Na^+ and phosphate transport, and expression of *Escherichia coli* enterotoxin receptors which have previously been shown within the opossum kidney to only be expressed on cells in the proximal tubule (15, 16). Consequently, these cells have become an important *in vitro* model system for proximal tubular physiological studies. Previous work in our laboratory has shown that this cell line expresses dopamine receptors which have a D1-like pharmacological profile and are coupled to adenylyl cyclase (17).

We report here the cloning and sequence of a dopamine receptor from OK cells which is highly homologous to the human and rat striatal $D1_A$ receptors, pharmacologically similar to these receptors, and coupled to stimulation of adenylyl cyclase. Although initial characterizations had failed to localize the $D1_A$ receptor from rats and humans in the renal cortex, the OK cell dopamine receptor is expressed in both the brain and renal cortex of the opossum. The high degree of sequence identity of this receptor with the $D1_A$ receptor described in placental mammals suggests that it is the opossum homolog of this subtype.

Materials and Methods

DNA/RNA techniques. Previously published procedures for work with nucleic acids were used without modification for Northern analysis and routine DNA manipulations (18, 19). RNA was prepared by centrifugation through a cushion of CsCl (18).

RNase protection analysis. The *Kpn*I-*Bam*HI fragment of the OK cell D1 receptor and the *Bpm*I-*Xho*I fragment of the opossum $D1_B$ receptor subtype were subcloned into Bluescript and used to prepare single-stranded RNA antisense probes labeled with [α-^{32}P]CTP. These probes were hybridized with 50 μg of total cellular RNA prepared from opossum brain (the entire right hemisphere), kidney (a wedge of tissue composed of about two-thirds cortex and one-third medulla), or the OK cell line. Hybridization was performed at 50° overnight, and the mixtures were digested by RNase A and RNase T1 for 1.5 hr at 30° as described (19). Digests were analyzed on a 7 M urea 6% acrylamide sequencing gel, and band intensities were quantitated using a PhosphorImager and ImageQuant™ software (Molecular Dynamics).

Preparation of the full-length gene construct. The full-length cDNA construct was prepared from DNA fragments obtained by several methods. An OK cell cDNA library (20) (a generous gift from D. Bylund) was screened (18) with a nick-translated probe derived from the human $D1_A$ *Nco*I-*Hin*dIII fragment covering from the beginning of the coding region to the middle of the fifth transmembrane region (21). This yielded one positive hybridizing clone with a 960-bp insert. By comparison with the human $D1_A$ receptor, this insert extended from the 5′-untranslated sequences to the beginning of the TM V at a site 80-bp downstream of the *Sac*I site (nucleotide 504) which is underlined in Fig. 1B. A RACE protocol (22) was performed in which a primer of sequence (5′ GACTCGAGTCGACATCGT_{17} 3′) was used to synthesize the first strand cDNA from a sample of 5 μg of OK cell total cellular RNA using reagents obtained commercially (Invitrogen). Using an oligo primer from TM IV (5′ TGCTTGGACTTTGTCTGTGTTG 3′) and the 5′ half of the above primer (5′ GACTCGAGTCGACATCG 3′), one round of RACE PCR was performed for 35 cycles of 1 min at 93°, 1 min at 55°, and 2 min at 72°, followed by 1 cycle of 10 min at 72°, and amplified products were gel purified and subcloned into the pCR 1000 TA cloning system (Invitrogen). All of the transformants obtained were truncated and none contained the termination codon of the coding sequence or the 3′ primer. The longest fragment obtained extended to nucleotide 1326, within 15 bp of what turned out to be the location of the termination codon. This piece was spliced to the fragment obtained from the cDNA library using the *Sac*I site and a restriction site in the polylinker 3′ to the truncation site. To obtain the remaining sequences, a primer from TM VI (5′ CATATTGAACCGCATGGTACC 3′) was used with the RACE primer described above for a second round of RACE PCR, and the PCR product was sequenced directly without subcloning, as described below. Using this sequence information, a final primer (5′ GGATCCGAATTCAGGATTCATCTTACG 3′) complementary to the messenger strand and adjacent to the termination codon was used with the TM VI oligo to prepare the 3′-most piece of the construct by PCR. This piece was cloned in using the *Bam*HI site at nucleotide 1225 and the *Bam*HI site engineered into the primer. The correct orientation was identified using the *Eco*RI site adjacent to the *Bam*HI site within the primer.

Sequencing. Sequencing was performed by the dideoxy method of Sanger *et al.* (23), with commercially obtained reagents (Pharmacia). Sequencing directly on DNA templates obtained by PCR was performed by a combination of previously published protocols (24, 25). Briefly, commercially obtained reagents (Pharmacia) were supplemented with 0.5% NP-40 detergent (Sigma). An amount of 0.25 pmol of double-stranded template in annealing buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 50 mM NaCl, 7 mM $MgCl_2$, 15 mM dithiothreitol, 0.5% NP-40) was combined with 20 pmol of primer and heat denatured in a boiling water bath for 3 min and then snap cooled in a dry ice/methanol bath. Labeling was allowed to proceed for 45 sec at room temperature followed by termination for 5 min at 37°. An aliquot of 2 μl of chase solution containing 1 μM dNTPs, 50 mM NaCl, and 0.5% NP-40 was added, and the reaction was allowed to proceed 5 min at 37°. The reaction was then stopped with 6 μl of stop solution, and the reactions were electrophoresed on a 6% acrylamide denaturing gel. Sequences were analyzed using software from the Genetics Computer Group.

Cellular expression. The full-length expression construct was subcloned behind the CMV promoter in the pCMV5 expression vector (21). This construct was then used to transiently transfect African green monkey kidney cells (COS-7) by the DEAE-dextran procedure (26), or 293 cells by the calcium phosphate procedure (26).

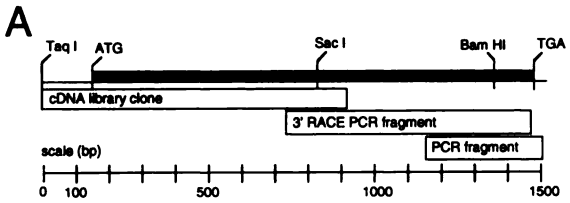
Adenylyl cyclase study. Adenylyl cyclase activity in 293 cells was measured 72 hr after transfection. Crude membranes from transfected cells were prepared and resuspended to ~1.25 mg of protein/ml in assay buffer (75 mM Tris-HCl, pH 7.2; 5 mM $MgCl_2$; and 2 mM EDTA, pH 8.0). Enzymatic activity was measured in a final volume of 50 μl according to a previously described method (17).

Ligand binding. Saturation and competition assays were performed as previously described (26). Drugs were obtained from the following sources: *cis*-flupenthixol (Lundbeck, Denmark), SCH 23390 (Schering Plough, Bloomfield, NJ), (+)-butaclamol, dopamine-HCl, haloperidol, 5,6-ADTN, SCH 23388, (−)-butaclamol, 6,7-ADTN, bulbocapnine (Research Biochemical Industries), fenoldopam, and SKF 38393 (Smith, Kline & French). ^{125}I-labeled SCH 23982 was purchased from New England Nuclear or prepared by iodination with chloramine T (27). Binding on tissue slices was performed as described previously (28).

***In situ* hybridization.** Opossums obtained from a local supplier were killed by injection of an overdose of sodium pentobarbital. Brains and kidneys were frozen in isopentane precooled in dry ice, and 15-μm slices were cut in a cryostatic microtome and transferred to silylated slides (Onasco). Single-stranded RNA probes were made using RNA polymerase promoters flanking a 792-bp fragment of the cDNA cloned into pSP 72 (bp −147 to 582). Hybridization and washing was as reported previously (28) and included treatment with RNase A to eliminate any signal from homologous but nonidentical sequences. Slides were exposed to x-ray film then dipped in a photographic emulsion (NTB-2, Kodak).

Results

OK cells are an epithelial cell line established from the opossum kidney that resemble the mammalian proximal tubule



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      -295 GATTAGAACTCTTGTGGGACTTGGGTTCAATTTCAAAACTATTACCTGTAGGACTTTGGAAAGTCACA
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TCGATTCTCTGACTTAGGTTGCAAATTATAAATACATATCTAAAAATTTGCTGCCAGTTGTTATTGGAATACAGACATC
TATATAAAAGAGAAGACATTTCAACAGAAGCTCTAGATCAACTTGGTGAAATATACAATAGAGACATA
                                                                ATG CCC      6
                                                                MET Pro      2

TTG AAT GAC ACA ACT ATG GAC AGA AGA GGG CTG GTA GTG GAA AGG GAC TTC TCC TTC CGC     66
Leu Asn Asp Thr Thr MET Asp Arg Arg Gly Leu Val Val Glu Arg Asp Phe Ser Phe Arg     22

ATC CTT ACT GCC TGC TTC CTT TCA CTA TTG ATC TTG TCT ACA CTT TTG GGA AAT ACT TTG    126
Ile Leu Thr Ala Cys Phe Leu Ser Leu Leu Ile Leu Ser Thr Leu Leu Gly Asn Thr Leu     42

GTG TGT GCA GCT GTC ATC AGG TTC CGC CAC CTA AGG TCC AAG GTG ACT AAT TTC TTT GTC    186
Val Cys Ala Ala Val Ile Arg Phe Arg His Leu Arg Ser Lys Val Thr Asn Phe Phe Val     62

ATC TCG TTG GCT GTT TCT GAC CTC TTA GTA GCT GTC TTG GTC ATG CCC TGG AAA GCT GTT    246
Ile Ser Leu Ala Val Ser Asp Leu Leu Val Ala Val Leu Val MET Pro Trp Lys Ala Val     82

GCA GAG ATT GCG GGT TTC TGG CCC TTT GGC TCC TTC TGC AAT ATC TGG GTG GCA TTT GAT    306
Ala Glu Ile Ala Gly Phe Trp Pro Phe Gly Ser Phe Cys Asn Ile Trp Val Ala Phe Asp    102

ATC ATG TGT TCT ACG GCC TCC ATT CTA AAC TTA TGT GTC ATC AGT GTT GAT AGA TAT TGG    366
Ile MET Cys Ser Thr Ala Ser Ile Leu Asn Leu Cys Val Ile Ser Val Asp Arg Tyr Trp    122

GCT ATT TCC AGT CCC TTT CGC TAT GAG AGA AAA ATG ACC CCC AAG GCA GCC TTC ATT TTG    426
Ala Ile Ser Ser Pro Phe Arg Tyr Glu Arg Lys MET Thr Pro Lys Ala Ala Phe Ile Leu    142

ATC AGT GTT GCT TGG ACT TTG TCT GTG TTG ATT TCC TTC ATT CCA GTA CAG TTG AAT TGG    486
Ile Ser Val Ala Trp Thr Leu Ser Val Leu Ile Ser Phe Ile Pro Val Gln Leu Asn Trp    162

CAC AAG GCC AGA CCC CTG AGC TCA CCA GAT GGG AAT GTT AGT TCC CAA GAT GAG ACA ATG    546
His Lys Ala Arg Pro Leu Ser Ser Pro Asp Gly Asn Val Ser Ser Gln Asp Glu Thr MET    182

GAC AAC TGT GAC TCT AGC CTG AGC AGG ACA TAT GCC ATC TCT TCT TCT CTT ATT AGC TTT    606
Asp Asn Cys Asp Ser Ser Leu Ser Arg Thr Tyr Ala Ile Ser Ser Ser Leu Ile Ser Phe    202

TAC ATT CCA GTG GCT ATC ATG ATA GTC ACA TAC ACG AGG ATC TAC AGG ATT GCA CAG AAG    666
Tyr Ile Pro Val Ala Ile MET Ile Val Thr Tyr Thr Arg Ile Tyr Arg Ile Ala Gln Lys    222

CAA ATA AGA CGA ATC TCA GCT TTG GAG AGA GCT GCC GTT CAT GCC AAG AAC TGC CAG AAC    726
Gln Ile Arg Arg Ile Ser Ala Leu Glu Arg Ala Ala Val His Ala Lys Asn Cys Gln Asn    242

ACT ACT GGG AAT GGG GCA AAT GTG GAG TGT TCC CAG CCA GAA AGT TCC TTC AAG ATG TCC    786
Thr Thr Gly Asn Gly Ala Asn Val Glu Cys Ser Gln Pro Glu Ser Ser Phe Lys MET Ser    262

TTC AAG AGA GAA ACC AAA GTT TTA AAG ACT CTG TCA GTG ATC ATG GGA GTG TTT GTA TGC    846
Phe Lys Arg Glu Thr Lys Val Leu Lys Thr Leu Ser Val Ile MET Gly Val Phe Val Cys    282

TGC TGG CTA CCT TTT TTC ATA TTG AAC TGC ATG GTA CCC TTC TGT GAA TCT GAT TTG CCT    906
Cys Trp Leu Pro Phe Phe Ile Leu Asn Cys Met Val Pro Phe Cys Glu Ser Asp Leu Pro    302

TCT GGG GAA ACA AAA CCC TTC TGT ATT GAT TCT ATT ACC TTT GAT GTT TTC GTG TGG TTT    966
Ser Gly Glu Thr Lys Pro Phe Cys Ile Asp Ser Ile Thr Phe Asp Val Phe Val Trp Phe    322

GGA TGG GCA AAT TCC TCA CTG AAC CCT ATC ATT TAT GCC TTT AAT GCT GAC TTC CGA AAG   1026
Gly Trp Ala Asn Ser Ser Leu Asn Pro Ile Ile Tyr Ala Phe Asn Ala Asp Phe Arg Lys    342

GCA TTT TCT ACT CTC TTA GGA TGC TAC AGG CTC TGT CCC ACT GCC AAC AAT GCA ATA GAG   1086
Ala Phe Ser Thr Leu Leu Gly Cys Tyr Arg Leu Cys Pro Thr Ala Asn Asn Ala Ile Glu    362

ACA GTT AGC ATC AAC AAT AAT GGG GCT GTG TTT TCA AGC CAT CAT GAG CCC AGA GGG TCC   1146
Thr Val Ser Ile Asn Asn Asn Gly Ala Val Phe Ser Ser His His Glu Pro Arg Gly Ser    382

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Ile Ser Lys Asp Cys Asn Leu Val Tyr Leu Ile Pro Gln Ala Val Thr Ser Arg Asp Pro    402

AAG AAG GAA GAA GGT GGA GGA TCC AAG CCA TTG GAG AAA ACC TCT CCA GCT TTA TCT GTC   1266
Lys Lys Glu Glu Gly Gly Gly Ser Lys Pro Leu Glu Lys Thr Ser Pro Ala Leu Ser Val    422

ATT TTG GAT TAT GAA GTT GAT CTA TCT TTG GAA AAG ATT AAC CCC ATC ACA CAG AAT GGA   1326
Ile Leu Asp Tyr Glu Val Asp Leu Ser Leu Glu Lys Ile Asn Pro Ile Thr Gln Asn Gly    442

CAA CAC AAG ACC TGA ACCGTAAGATGAATCCTGTAAAAATATGCTAGTGAAAACAAAAAACAAAAACCTTCTGA   1400
Gln His Lys Thr *** (446 aa)          GAATTCGATCC   1370
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Fig. 1. Assembly strategy of the full-length expression construct, and the nucleotide and deduced amino acid sequences of the OK cell D1 receptor. A, Partial fragments of message obtained from cDNA library screening and PCR were spliced together at the *Sac*I and *Bam*HI restriction sites, then subcloned together into the expression plasmid pCMV5. B, Boxed restriction sites (*Taq*I at nucleotide −147 and *Bam*HI at nucleotide 1358) indicate the limits of the region inserted into the pCMV5 expression vector; the *Bam*HI at nucleotide 1358 was engineered into the PCR primer used to amplify the final fragment of the construct. The nucleotide sequence adjacent to this *Bam*HI site in the oligonucleotide primer used for amplification of the 3′ extremity of the construct is shown on the line below the continuous sequence of the opossum DNA to indicate where it differed from the wildtype sequence. Other important restriction sites (*Sac*I at nucleotide 504, *Kpn*I at nucleotide 883, and *Bam*HI at nucleotide 1225) are *underlined*. The initiation codon and termination codon of a 14-codon open reading frame in the 5′-untranslated region are *double underlined*.

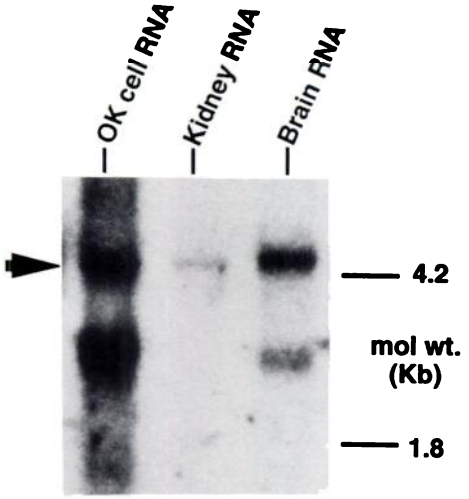

Fig. 2. Northern blot analysis of the OK cell line and opossum tissues. Total cellular RNA from the OK cell line (40 μg), OK (40 μg), and opossum brain (10 μg) was separated on a 1.2% agarose formaldehyde gel, transferred to a nitrocellulose membrane, and probed with a nick-translated full-length fragment of the OK cell D1 receptor clone. Exposure was for 16 days at −70°.

and have been shown to possess D1 dopamine receptors (16, 17). To identify the dopamine receptor subtype expressed in kidney, we screened a cDNA library prepared from OK cell mRNA with a probe from the 5′ end of the coding block of the gene for the human $D1_A$ receptor. Using the partial clone obtained from this screening and fragments generated by PCR as described in Materials and Methods, a full-length sequence of the OK cell D1 dopamine receptor was constructed. Fig. 1A shows the overlap between the various subclones and the enzyme restriction sites used to splice them into one construct. Fig. 1B shows the nucleotide sequence and deduced amino acid sequence for the coding region of the putative receptor message.

Northern blots performed on total cellular RNA from OK cells reveal a hybridizing band around 4500 bp and an inconsistent band which appears around 2700 bp in some preparations (Fig. 2).

To characterize the protein coded for by the corresponding messenger RNA, we performed transient transfections of the full-length expression construct into African green monkey kidney epithelial (COS-7) cells. Membranes from transfected cells had >10-fold increase in binding of ^{125}I-SCH 23982 compared with untransfected cells. The K_d for the radioligand was determined by saturation binding curves to be 0.15 nM (Fig. 3A). Radioligand binding could be competed for by dopaminergic ligands with an order of potency and stereoselectivity characteristic for a receptor of D1 pharmacology (Fig. 3B, Table 1). A comparison with the published K_d values for antagonist competition of ^{125}I-SCH 23982 from OK cell membranes showed a strong correlation (Fig. 3B) (17).

To determine whether the receptor from OK cells was the same or a different subtype from the central nervous system receptor, we performed transient transfections of the human $D1_A$ receptor in parallel with transient transfections of the OK cell receptor. Both cDNAs were transfected into COS-7 cells, and at least three independent assays were performed in parallel. The mean of the K_d values obtained for the two receptors were compared for statistical significance using Student's *t* test. The order of potency of drugs tested was the same for both receptors. In general, the K_d values obtained were quite similar, although small but significant differences in affinity existed for most drugs. In particular, drugs of the benzazepine class showed consistently higher affinity (4- to 9-fold) for the opossum receptor than for the human $D1_A$ (Table 1).

The receptor message isolated from OK cells conferred dopamine-stimulated adenylyl cyclase activity on membranes from transiently transfected 293 cells (Fig. 3C). However, this cDNA did not confer dopamine-linked phosphatidylinositol turnover to membranes from transiently transfected COS-7 cells, although an α_{1B} adrenergic receptor cDNA transfected and assayed in parallel did stimulate phosphatidylinositol hydrolysis (data not shown).

To determine the distribution of the OK cell D1 receptor message expression we examined opossum kidney and brain by an RNase protection assay and *in situ* hybridization. Hybridization of a probe complementary to the OK cell D1 receptor message with total cellular RNA prepared from either opossum brain or opossum kidney protected the appropriate 341-bp fragment in both tissues as well as in RNA from the OK cell line. Similarly, use of a 243-bp probe complementary to the opossum $D1_B$ receptor subtype which was isolated by PCR from opossum brain RNA[1] protected the appropriate fragment in opossum brain and OK RNA but no signal was detected in OK cell RNA (Fig. 4). A phosphorimager was used to quantitate the intensities of the protected bands in arbitrary relative units where the level of expression of the opossum $D1_A$ in OK cells was set at 1 unit. Total cellular RNA from the kidney contained virtually identical levels of the $D1_A$ and $D1_B$ subtypes (0.27 and 0.26 units,respectively), whereas total cellular RNA from the whole brain contained ten times more $D1_A$ than $D1_B$ (9.0 and 0.95 units respectively), as is found in rat and human (1). Expression of the $D1_A$ mRNA in the brain is therefore at least 30-fold higher than in kidney.

In situ hybridization was very useful for localizing the regions expressing the OK cell D1 receptor in the brain, but was not very informative on the localization of message containing cells in the kidney due to the low signal to noise ratio. In the brain, classical regions of $D1_A$ receptor expression were detected by the OK cell D1 ($D1_A$) probe. These included strong expression in the caudate-putamen, the nucleus accumbens, and the olfactory tubercle, and weaker expression in the deep cortical layers (28, 29). A specific signal was also detected in the mamillary bodies and the pontine nucleus (Fig. 5A). Receptor autoradiography with ^{125}I-SCH 23982 showed binding in the caudate-putamen, nucleus accumbens, olfactory tubercle,and substantia nigra (Fig. 5C). These results agree with autoradiographic studies of rat brain (29, 30). Hybridization in the kidney appears confined to a weak signal stronger in the renal cortex than the renal medulla (Fig. 5E). Examination of slides dipped in photographic emulsion reveals precipitation of silver grains primarily over tubular elements with little to no precipitation over the glomeruli (data not shown).

Discussion

OK cells have become an important physiological model system for studies of functions of the renal proximal tubule. These cells have been used to study the effects of parathyroid hormone on sodium and phosphate transport (31), and have

[1] S. R. Nash and M. G. Caron, unpublished observations.

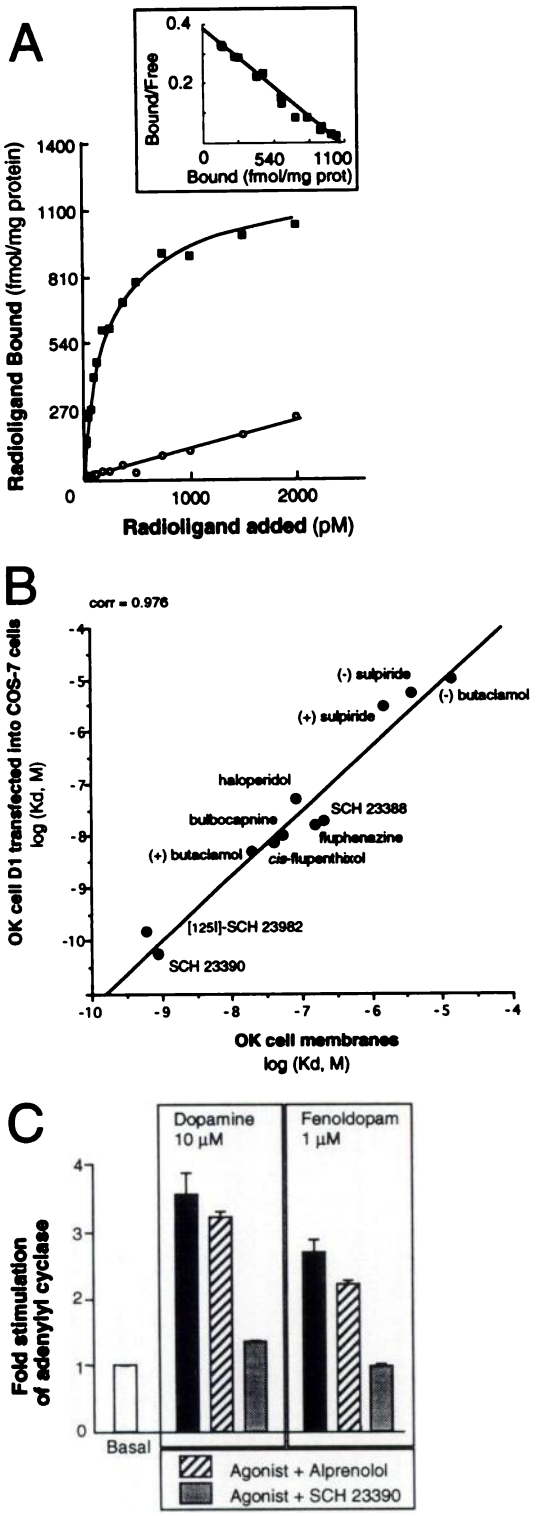

Fig. 3. Pharmacological characterization of the OK cell D1 dopamine receptor transfected into COS-7 or 293 cells. A, Saturation binding of ^{125}I-SCH 23982 to OK cell D1 dopamine receptor transfected COS-7 cell membranes, performed as described in Materials and Methods. Data from a representative experiment performed in triplicate are shown (n = 3): ■, total binding; ○, nonspecific binding. *Inset*: Scatchard transformation of the saturation data. B, Comparison of the cloned OK cell D1 receptor to the D1 receptor characterized in OK cell membranes (17). The log of the K_d values obtained from competitive binding experiments on the OK cell D1 receptor transfected into COS-7 cells (see Table 1) were compared with the log of previously published K_d values. The correlation coefficient of the least squares determined line was 0.976. C, Cultures of 293 cells were transfected with the OK cell D1 receptor cDNA, and adenylyl cyclase assays were performed as described in Materials and Methods. Membrane-bound adenylyl cyclase was stimulated with either dopamine (10 μM) or fenoldopam (1 μM) in the absence or presence of alprenolol (1 μM) or SCH 23390 (1 μM). A control sample stimulated with forskolin gave a 7.3-fold stimulation over basal. Two independent experiments were performed in triplicate.

TABLE 1

Pharmacological characterization of the cloned OK cell D1 dopamine receptor and comparison to the human D1$_A$ dopamine receptor

Values are means ± standard error for three to six independent experiments performed in triplicate. Values for ^{125}I-SCH 23982 were determined by four saturation binding experiments performed in duplicate. Data were modeled using the least squares fitting program SCATFIT as described previously (17). Comparisons with the human D1$_A$ receptor were performed in at least three parallel, independent experiments. N.D. = not determined. An asterisk (*) indicates that the difference in K_d values between the opossum and human receptors are statistically significant ($p \leq 0.025$) by Student's t test.

Drug	K_d OK cell D1	K_d human D1$_A$	Ratio opossum:human
	nM	*nM*	
Antagonists			
^{125}I-SCH 23982	0.15 ± 0.01	1.1 ± 0.12	0.14*
R(+)-SCH 23390	0.059 ± 0.008	0.29 ± 0.07	0.20*
(+)-Butaclamol	5.0 ± 1.3	2.9 ± 0.7	1.7
cis-Flupenthixol	7.3 ± 1.8	21 ± 4	0.35*
Bulbocapnine	10 ± 1.5	35 ± 8	0.28*
Fluphenazine	16 ± 5.9	28 ± 10	0.57
S(−)-SCH 23388	19 ± 2.6	170 ± 16	0.11*
Haloperidol	50 ± 10	38 ± 7	1.3
R(+)-Sulpiride	2,940 ± 840	N.D.	N.D.
S(−)-Sulpiride	5,300 ± 580	N.D.	N.D.
(−)-Butaclamol	10,700 ± 820	18,000 ± 1,000	0.59*
Agonists			
Fenoldopam	1.9 ± 0.5	11 ± 3.0	0.16*
Lisuride	14 ± 1.3	30 ± 3	0.47*
SKF 38393	29 ± 2	120 ± 12	0.24*
Dopamine	2,200 ± 140	6,700 ± 680	0.33*
6,7-ADTN	3,400 ± 940	N.D.	N.D.
5,6-ADTN	7,500 ± 1500	N.D.	N.D.

[a] ADTN, (±)-2-amino-5,6-dihydroxy-1,2,3,4,-tetrahydronaphthalene.

been shown to possess various metabolic characteristics of the renal proximal tubule (15). Additionally, these cells possess D1 dopamine receptors that stimulate adenylyl cyclase (17). We report here the cloning and characterization of a dopamine receptor from these cells, and demonstrate that the message for this receptor subtype is expressed in both "central" and "peripheral" locations. It is likely that the dopamine receptors of placental mammals (*e.g.*, rat, human) are also expressed in this manner.

The receptor we describe here is quite similar to the previously characterized rat and human D1$_A$ receptors (1). Computer-assisted alignment of the nucleotide sequences from the coding regions of this receptor message revealed 78.6% and 79.3% identity to the coding sequences of the rat and human D1$_A$ receptor messages, respectively. For comparison, the human and rat nucleotide sequences are 86.7% identical. At the nucleotide level, the rate of 79% conservation between opossum and human agrees well with the rate of 77% conservation observed between the nucleotide sequences of opossum and human hemoglobin β-chain gene homologs (32). Structurally, the level of amino acid conservation is quite high among the three sequences. The OK cell D1 receptor-deduced amino acid sequence is 87.0% and 87.4% identical with the rat and human

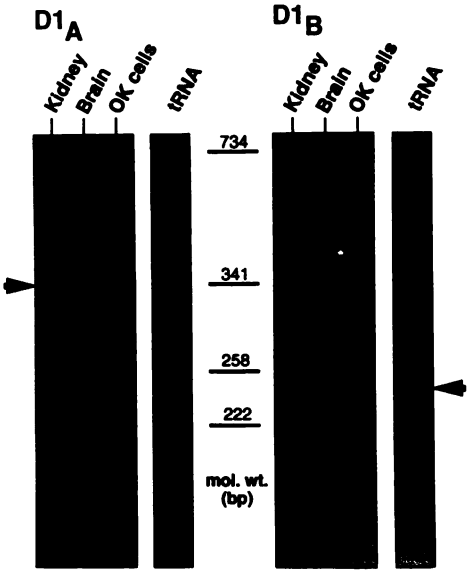

Fig. 4. RNase protection of RNA from opossum tissues and the OK cell line. Specific fragments of appropriate size (marked by *arrows*) could be protected in both opossum kidney and opossum brain RNA by high specific activity single-stranded RNA probes. The *upper band* in each lane (454 bp in $D1_A$ lanes and 308 bp in $D1_B$ lanes) is the undigested probe. Opossum brain RNA was prepared from the entire right hemisphere without dissection, and opossum kidney RNA was prepared from a wedge of tissue containing approximately two-thirds renal cortex and one-third renal medulla. The quantity of total cellular RNA used for each sample was 50 μg. The gel was exposed overnight.

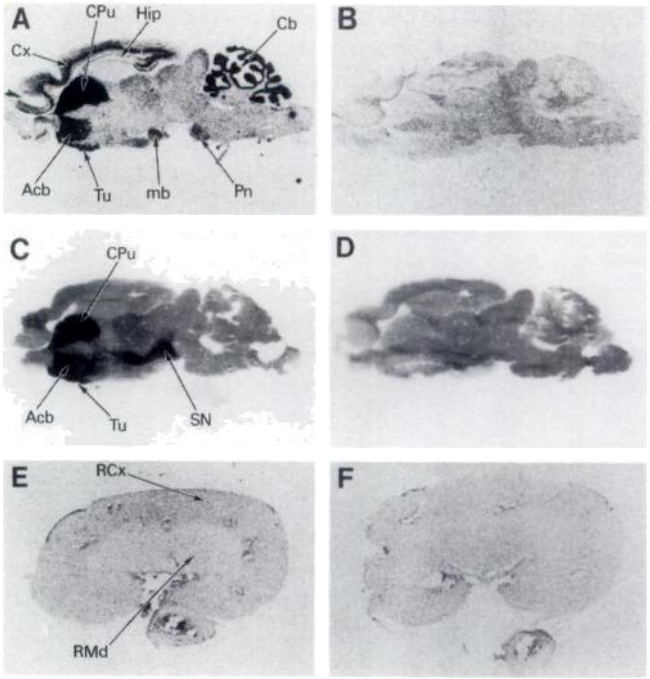

Fig. 5. *In situ* hybridization and receptor autoradiography in opossum tissues. A and B, Sagittal sections near the midline of an opossum brain were hybridized to either the specific antisense strand probe (A) or the nonspecific sense strand probe (B). C and D, Receptor autoradiography using ^{125}I-SCH 23982 as radioligand in the absence (C) or presence (D) of 10 μM *cis*-flupentixol to define nonspecific binding. E and F, Transverse sections through the opossum kidney were hybridized to either the specific antisense strand probe (E) or the nonspecific sense strand probe (F). *Acb*, accumbens; *Cb*, cerebellum; *Cx*, cortex; *Hip*, hippocampus; *mb*, mamillary body; *Pn*, pontine nucleus; *RCx*, renal cortex; *RMd*, renal medulla; *Sn*, substantia nigra; *Tu*, olfactory tubercle.

$D1_A$ receptor sequences overall, although identity in the putative transmembrane domains is >97%. At the amino acid level the rat $D1_A$ receptor is 91% identical to the human $D1_A$ receptor (33). Important features already identified in the human and rat $D1_A$ receptor sequences are likewise highly conserved including glycosylation sites in the N-terminal and second extracellular domains, putative GTP-binding protein coupling regions, as well as the consensus sequences for protein kinase A- and protein kinase C-mediated phosphorylation sites. The 5′-untranslated region contains a 14-codon open reading frame with a poor Kozak's translation start sequence (34). This sequence is analogous, but not homologous, to a similar short open reading frame that was found in the 5′-untranslated of the β_2-adrenergic receptor (35). However, as yet, no function has been described for this feature.

Similarly the message size we observe (4.5 kb) is the same found in the rat and human (1). Although the presence of the second smaller (2.7 kb) band might represent alternative splicing of a large intron in the untranslated regions of the message, it is most likely a specific degradation product of the full-length message because the size of this fragment would correspond roughly to the location of spontaneous subcloning truncation artifacts observed during preparation of the construct by RACE PCR.

Comparison of the K_d values determined for the cloned OK cell receptor show a strong correlation with published values that were determined by study of the receptor in OK cells (17). One notable difference between the properties of the receptor we have characterized and the binding observed in OK cell membranes concerns the binding profile of the D1 dopaminergic antagonist SCH 23390. Bates *et al.* (17) demonstrated that a biphasic competition profile is obtained with this drug when competing ^{125}I-SCH 23982 binding from OK cell membranes. We do not observe this phenomenon in the COS-7 cells transfected with the OK cell D1 receptor cDNA (data not shown). Although there is no adequate explanation of this discrepancy at this time, it is clear that the low affinity site described by Bates *et al.* is not an inherent property of the cloned receptor.

Pharmacologically, competitive binding experiments comparing in parallel the human $D1_A$ and OK cell D1 receptors reveal that these two receptors have very similar K_d values for most ligands tested. Previous characterization of canine vascular dopamine receptors using an *in vivo* vasodilation assay had demonstrated that certain drugs such as ergot compounds and (+)-sulpiride had potencies at peripheral receptors different from their potencies to stimulate or inhibit stimulation of adenylyl cyclase in rat basal ganglia homogenates (13, 14). Similarly, haloperidol and fluorinated phenothiazines appeared to have a reverse order of potencies between the two systems (13). Our data do not agree with these observations; however, it is possible that the differences observed in these early physiological comparisons arose from fundamental differences in the experimental systems and not from actual receptor differences. Although some pharmacological differences (most notably the increased affinities observed for benzazepines) are apparent from our parallel comparisons, in view of the high homologies between these proteins, it is likely that the small pharmacological differences observed are due to species variations in protein sequence rather than to an inherent difference in receptor subtype. Similarly to our findings, the recent char-

acterization of a D1 subtype dopamine receptor expressed in cultured rat mesangial cells demonstrated that the receptor of these cells has a pharmacological profile very similar to the rat striatum D1 receptor (36).

Due to the sensitivity of RNase for detecting mismatches within RNA:RNA duplexes, RNase protection analysis and *in situ* hybridization using complementary RNA probes are very specific tests of the expression of a message of interest in a particular tissue. In the opossum, it is clear that the $D1_A$ receptor described here is expressed in both renal and central nervous tissues. These RNase protection experiments suggest that the level of expression in the kidney is at least an order of magnitude less than that in the brain. Because the striatum is a relatively small brain region and cortical tubular elements compose the majority of the renal cortex, the expression of message is probably closer to 2 orders of magnitude less in renal tubular cells than in striatal neurons. It is likely that this low level of message explains why the $D1_A$ subtype was not detected in the kidney at the time it was first described, because relatively small amounts of RNA were examined by Northern hybridization.

The *in situ* hybridization and receptor autoradiography results presented here agree closely with previous studies of the rat brain (28–30). Several small differences from the rat include a stronger signal from the cerebellar granular cells than is reported for rat brain (29), and presence of the $D1_A$ mRNA in opossum mamillary bodies. In the rat, the $D1_B$ but not the $D1_A$ subtype is expressed in the mamillary complex. These differences may represent a difference between these two species in the regulation and expression of these receptor subtypes.

A previously published report of *in situ* hybridization in rat kidney used oligonucleotide probes complementary to the rat $D1_A$ (37). Through digital analysis and interpretation of autoradiograms, they demonstrated a weak signal localized exclusively to the outer medulla with no expression in the cortex. This finding conflicts with the result presented here of a weak, uniform signal throughout the opossum renal cortex. This conflict could be due to the higher specific activity and therefore higher sensitivity of complementary single-stranded cRNA probes. In any case, autoradiographic studies in rat kidney with D1 selective ligands have localized D1 receptors to the proximal tubular elements of the renal cortex (6, 7).

The divergence of the marsupial and placental branches of the mammalian family is commonly placed around 125 million years ago during the Cretaceous geological period, almost twice as long ago as the divergence of primates and rodents (32). The high level of homology among the opossum, rat, and human dopamine receptors, including putative important post-translational modification sites, suggests a conservation of function as well as structure. Because the $D1_A$ receptor subtype has recently been detected in rat proximal tubules by PCR and RNase protection (38), it is likely that the expression of central nervous system dopamine receptors in renal tissue occurs throughout the mammalian family. The OK cell line provides a good system for the future study of $D1_A$ dopamine receptors, because this receptor is stably expressed at reasonable levels, important structural features of the human and rat receptors are conserved within this protein, and these cells express only the $D1_A$ and not the $D1_B$ subtype of dopamine receptors.

Acknowledgments

We would like to thank S. Page and L. Bertrand for their kind assistance with this work; and Drs. M. Tiberi, K. Jarvie, U. Schambra, S. Collins, D. Price, and J. R. Raymond for their generous assistance and valuable advice.

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