AJH 1990; 3:29S–33S

Molecular Characterization of Dopamine Receptors

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The D_1 and D_2 dopamine receptors have been biochemically characterized using specific probes based on the subtype selective antagonists SCH 23390 and spiperone, respectively. The D_2 dopamine receptor was identified from several tissues by photoaffinity labeling and was purified from bovine anterior pituitary to homogeneity using a combination of affinity, lectin and hydroxylapatite chromatography. A complementary DNA (cDNA) encoding a rat brain D_2 dopamine receptor has been cloned via low stringency hybridization using a portion of the β_2-adrenergic receptor gene as a probe. Photoaffinity crosslinking and affinity chromatography have also been used to identify and purify the rat brain D_1 dopamine receptor. Am J Hypertens 1990; 3:29S–33S

KEY WORDS: **Dopamine, receptors, protein purification, molecular biology, photoaffinity labeling.**

The mechanisms by which dopamine exerts its diverse effects have been intensely studied over the past thirty years due to dopamine's biological significance and its relationship to several important clinical disorders, such as schizophrenia, Parkinson's disease and hyperprolactinemia. Since the recognition of multiple dopamine receptor subtypes,[1,2] there has been great interest in the nature of these two distinct receptor subtypes with regard to structure and function. Our studies have focused on the molecular characterization of the D_1 and D_2 dopamine receptors, using techniques of photoaffinity labeling, protein purification and molecular biology.

CHARACTERIZATION OF D_2 DOPAMINE RECEPTORS

To characterize a receptor biochemically it is important to be able to identify specifically the receptor protein. Photoaffinity labeling can be used to identify a receptor in membrane preparations in which it is only a minor protein constituent. [^{125}I]*N*-(*p*-Azidophenethyl)spiperone ([^{125}I]N_3-NAPS), a derivative of the high affinity D_2 antagonist spiperone, was developed in our laboratory as a high affinity, high specific activity (~2200 Ci/mmol) photoaffinity probe for the D_2 receptor.[3,4] [^{125}I]N_3-NAPS labeled a band of $M_r \approx 80{,}000$ to 120,000 in a variety of tissues (corpus striatum, anterior and neurointermediate pituitary, retina) from several species, as visualized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Competing ligands blocked the labeling with appropriate D_2 pharmacology. Depending upon the tissue source, various nonspecifically labeled bands are also seen; labeling of these peptides is not blocked by D_2 or other receptor ligands.

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This work was supported in part by National Institutes of Health Grant NS 19576. Dr. Bates and Dr. Gingrich were supported by National Institutes of Health Medical Scientist Training Program Grant P32GM 07171. Dr. Falardeau was supported by a fellowship from the Fonds de la Recherche en Santé du Québec and Dr. Senogles by National Institutes of Health fellowship NS-07922.

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0895-7061/90/$3.50

Photoaffinity labeling, while a useful technique, is limited because the labeled peptides are not pure. Purification of the receptor is required for many structural and functional studies. We recently reported the purification of the D_2 dopamine receptor to apparent homogeneity from bovine anterior pituitary.[5] Purification from this tissue requires an approximately 33,000-fold enrichment of receptor activity. This was achieved by solubilization of membrane-bound receptors in the detergent digitonin followed by successive steps of affinity, lectin, and hydroxylapatite chromatography. The most important step in the purification procedure was the affinity chromatography step, which enriched the receptor approximately 1500-fold. This step used Sepharose-immobilized (carboxymethylene)oximino spiperone (CMOS),[6] with elution using the D_2 selective antagonist haloperidol. Following this step, reconstitution of the digitonin-solubilized receptor into phospholipid vesicles (a process which is 60 to 70% efficient) is required to observe ligand binding affinities similar to those observed for membrane preparations; [^{3}H]spiperone binding to reconstituted receptors was thus used to measure D_2 receptor activity. Interestingly, the affinity-purified receptor displayed biphasic, guanine nucleotide-sensitive agonist competition curves upon reconstitution due to copurification of a novel guanine nucleotide-binding protein (G protein), a pertussis toxin substrate of $M_r \approx 40{,}000$.[7] After elution from affinity chromatography, the receptor was further purified using immobilized *Datura stramonium* (DSA) lectin. Hydroxylapatite chromatography achieved the final purification.

Several criteria suggest that the purified protein represents the D_2 receptor. First, the specific activity of the purified reconstituted material (~5.3 nmol/mg protein) is close to the theoretical activity (~8.3 nmol/mg protein). Second, the reconstituted purified protein displays the pharmacological and biochemical properties expected for the D_2 receptor, including high affinity ligand binding and G protein coupling. Finally, a radioiodinated bromoacetyl derivative of NAPS specifically alkylated purified D_2 dopamine receptors.[5] The M_r of the protein stained and affinity alkylated material is 120,000. The purified preparations can be used for biochemical studies and to generate peptide sequences for cloning.

Important structural and functional information regarding the G protein-coupled receptors and the recognition of a gene family for these receptors has been obtained from the use of the techniques of molecular biology.[8] This family, which includes adrenergic, muscarinic, serotonin, and peptide hormone receptors and the visual opsins, is characterized by the presence of seven hydrophobic, presumably membrane-spanning, domains in the receptor protein. Two experimental approaches have been taken to identify clones for these receptors: first, large-scale purification, microsequencing of chemically- or proteolytically-derived peptides, and screening of complementary DNA (cDNA) or genomic libraries with oligonucleotide probes designed using the peptide sequence; and, second, low-stringency screening of libraries with full- or partial-length probes from other G protein-coupled receptors, with functional identification of the resulting clones by pharmacological, biochemical, or electrophysiological means.

Using the first approach, purified bovine anterior pituitary D_2 receptor was cleaved by cyanogen bromide and pepsin. The peptides were separated by reverse phase high performance liquid chromatography (HPLC), and individual fractions were subjected to gas phase microsequencing. Partial amino acid sequences so obtained are currently being used to isolate a cDNA clone for the purified protein.

The second approach, low-stringency screening using probes based on other receptors, has been used as well. A partial length rat genomic clone was isolated at low stringency using a probe derived from the hamster β_2-adrenergic receptor gene. This clone was then used to isolate a rat brain cDNA which was found to code for a D_2 dopamine receptor.[9] The cDNA codes for a protein of 415 amino acids which is homologous to the other G protein-coupled receptors which have been sequenced (Figure 1A). The sequence includes three consensus sites for asparagine-linked glycosylation in the amino-terminal portion of the protein, a large third intracellular loop of 125 amino acids, and a short carboxyl-terminal tail of fourteen amino acids. Northern blot analysis in various tissues demonstrated the presence of mRNA hybridizing to the clone in brain, particularly in the basal ganglia, and in the neurointermediate and anterior lobes of the pituitary.[9] When expressed in mammalian cell lines, this receptor displayed appropriate D_2 pharmacology by ligand binding studies using [^{3}H]spiperone.[10] Furthermore, it coupled to the inhibition of adenylyl cyclase in these cells, as measured by inhibition of basal adenylyl cyclase activity[10] or of cyclic AMP (cAMP) accumulation stimulated by forskolin (FSK) or hormone stimulators of adenylyl cyclase. As seen in Figure 1B, dopamine completely inhibited FSK (1 μmol/L)-stimulated cellular cAMP levels in a dose-dependent manner, with an IC_{50} of 4.3 nmol/L.

Photoaffinity labeling of membranes from various cell lines expressing the rat brain D_2 receptor results in the identification of peptides of $M_r \approx 74{,}000$, compared to the calculated molecular weight of 47,064 daltons (based on the amino acid sequence) and to the broad band of $M_r \approx 94{,}000$ to 120,000 identified for rat anterior pituitary or corpus striatum (Figure 1C). The difference between the M_r predicted by the amino acid sequence and that observed can presumably be explained by glycosylation of the expressed receptor; the broad band seen in photoaffinity labeling is consistent with

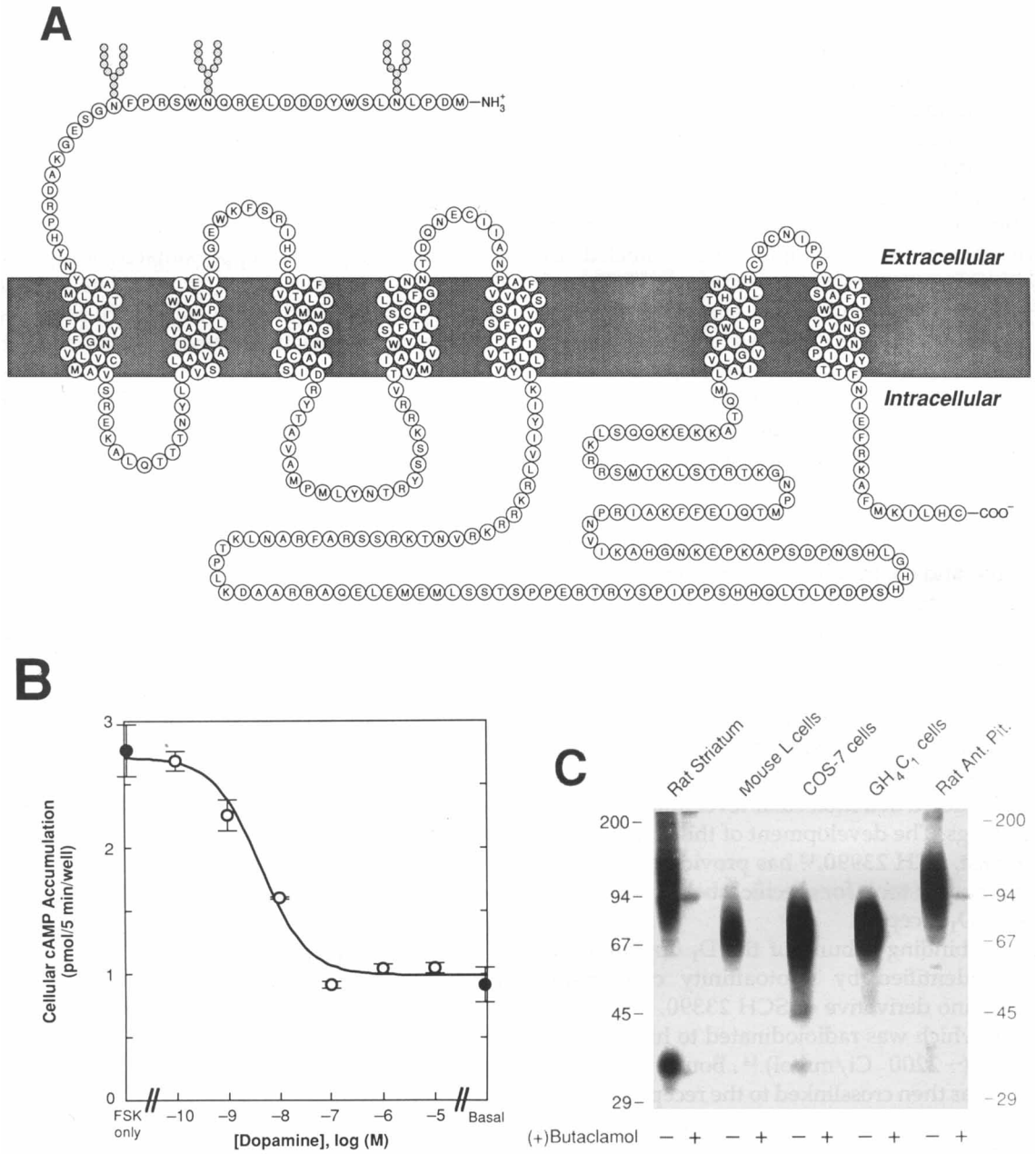

FIGURE 1. (A) *Amino acid sequence of the rat brain D_2 dopamine receptor,[9] showing its predicted organization in the plasma membrane and potential glycosylation sites. Single letter abbreviations for the amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.* (B) *Dopaminergic inhibition of forskolin (FSK)-stimulated cyclic AMP (cAMP) levels in mouse L cells stably expressing the rat brain D_2 dopamine receptor. A modification of the method of Liggett et al[16] was used. Mouse L cells stably expressing the rat brain D_2 receptor were grown on 24-well culture plates, washed twice with phosphate-buffered saline, and incubated at 37°C in the presence of experimental compounds in medium containing 0.1% ascorbate. After 5 min, the assay was terminated by the addition of perchloric acid. After neutralization, centrifugation, and acetylation, the cAMP content of the samples was measured by radioimmunoassay. Graph shows mean ± standard error of the mean for triplicate assays from one experiment. The data were fit using the program ALLFIT, which models using a four-parameter logistic equation.[17]* (C) *Photoaffinity labeling of D_2 dopamine receptors in rat tissues and in cell lines expressing the cloned rat brain receptor. The rat brain D_2 dopamine receptor was expressed stably in mouse L cells and rat GH_4C_1 cells and transiently in COS-7 cells. [^{125}I]N_3-NAPS (~150 pmol/L) was incubated in the dark with crude membrane preparations from the tissue or cell line of interest, in the absence (–) or presence (+) of the dopaminergic antagonist (+)butaclamol to define nonspecific labeling. Following washing and resuspension in a small volume, the membranes were photolyzed for 90 seconds using a high intensity lamp to cause covalent incorporation of the ligand to the receptor. Following further washing, the membrane samples were then electrophoresed on SDS-PAGE. The gels were dried and exposed to x-ray film using intensifying screens. Autoradiographs are shown.*

the protein being glycosylated. It is noteworthy that the peptides identified in rat anterior pituitary (where D_2 receptors reside on prolactin-secreting cells) migrate differently than those for the cloned rat brain D_2 receptor expressed in rat pituitary GH_4C_1 cells [a prolactin-secreting cell line derived from a radiation-induced pituitary tumor[11]]. This disparity could be due to differential glycosylation of the expressed receptor. Grigoriadis et al[12] have shown that multiple bands labeled by [^{125}I]-N_3-NAPS in canine striatal membranes yield similarly-migrating peptide cores upon enzymatic deglycosylation. Alternatively, this difference may be indicative of multiple subtypes of D_2 dopamine receptors. An important piece of evidence in this regard is that the sequence of peptides obtained from the purified anterior pituitary D_2 receptor does not correspond to any sequence of the cloned rat brain receptor (P. Falardeau et al, unpublished observations). Thus, the cloned rat brain receptor may represent one subtype of D_2 dopamine receptor, and the purified bovine anterior pituitary receptor another form.

CHARACTERIZATION OF D_1 DOPAMINE RECEPTORS

After the distinction between D_1 and D_2 dopamine receptors was made, it was some time before the D_1 receptor could be studied at a molecular level due to the lack of specific drugs. The development of the first D_1 selective antagonist, SCH 23990,[13] has provided a basis for the development of tools for specific labeling and purification of the D_1 receptor.

The ligand binding subunit of the D_1 dopamine receptor was identified by photoaffinity crosslinking using a 3′-amino derivative of SCH 23390, designated SCH 38548, which was radioiodinated to high specific radioactivity (~2200 Ci/mmol).[14] Bound [^{125}I]iodo-SCH 38548 was then crosslinked to the receptor protein using the bifunctional crosslinking agent, *N*-succinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanote (SANPAH). Using this technique a peptide of $M_r \approx$ 72,000 was identified by SDS-PAGE. Labeling of this peptide was blocked with appropriate D_1 dopamine pharmacology. Thus, the ligand binding subunit of the D_1 dopamine receptor can be identified, allowing further molecular characterization of the receptor in membrane preparations.

Another derivative of SCH 23390 was the basis for the development of an affinity chromatography matrix for the purification of the D_1 receptor. SCH 39111, the 4′-amino derivative of SCH 23390 which retains high affinity for the D_1 receptor, was immobilized on Sepharose 6B using an extended spacer arm.[15] SCH 39111-Sepharose specifically bound digitonin-solubilized D_1 receptors from rat corpus striatum. Adsorption could be blocked stereospecifically with enantiomers of SCH 23390 and butaclamol, and elution by various receptor agonists and antagonists was consistent with D_1 pharmacology. The affinity gel adsorbed 75 to 85% of the solubilized receptor, and after washing, 35 to 55% of the bound receptor was recovered by elution with (+)butaclamol, with a purification of 200 to 250-fold from the solubilized receptor preparation. The affinity-purified material had a specific activity of 300 to 375 pmol/mg protein, approximately 40-fold short of theoretical specific activity for completely purified receptor. Coupling of this procedure with several steps of ion exchange and lectin chromatography yielded a purified preparation from rat striatum. Cyanogen bromide treatment of this preparation has yielded peptides from which amino acid sequences have been obtained (J. Gingrich et al., unpublished observations). These sequences are being used in an attempt to isolate a cDNA clone for the D_1 dopamine receptor.

SUMMARY

Over the past several years, significant progress has been made toward the goal of elucidating the molecular characteristics of the D_1 and D_2 dopamine receptors. The D_2 receptor can be specifically radiolabeled and purified to homogeneity. A cDNA encoding a rat brain D_2 receptor has been cloned and expressed. The D_1 receptor can also be specifically radiolabeled, and an affinity chromatography method has been developed as an important step in this receptor's purification. Using the molecular probes developed to study these receptors, work is proceeding to characterize the various subtypes of receptors mediating the effects of dopamine.

ACKNOWLEDGMENT

We thank Dr. Peter Andersen for his critical reading of the manuscript.

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