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Alternative splicing and dopamine D2 receptor diversity

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ALTERNATIVE SPLICING AND DOPAMINE D2 RECEPTOR DIVERSITY

by

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Abstract

ALTERNATIVE SPLICING AND DOPAMINE D2 RECEPTOR DIVERSITY

by

Lenore Anne Snyder

Adviser: Professor James Roberts

Molecular cloning has led to the identification of three genes, termed D2, D3, and D4, which encode receptors of the dopamine D2 family. Alternative mRNA processing of these intron-containing genes generates a diversity of dopamine receptors and truncated receptor variants.

In the present studies, an mRNA variant of the originally published D2 receptor was identified by polymerase chain reaction. The receptor encoded by this transcript (which we named D2L) differed from the reported D2 receptor (renamed D2S) by the presence of an additional 29 amino acids within the third cytoplasmic loop. The reported D2 receptor gene structure indicated that D2L and D2S were alternative mRNA splice variants.

To gain insight into the biological roles of the isoforms, the distribution and relative abundance of D2L and D2S mRNA were mapped in the rat brain and pituitary using a quantitative solution hybridization/ nuclease protection assay. The results of this study indicated that both isoforms were present in all tissues examined, with D2L mRNA in greater abundance.

To investigate the signaling of D2L and D2S, the two receptors were expressed in AtT20 and GH3 cells. The modulation of calcium influx was studied using the calcium sensitive fluorescent dye, fluo-3. In both cell types, the activation of either isoform led to a reduction in calcium influx stimulated by the L-type calcium channel agonist BayK 8644. The responses mediated by both isoforms were similarly attenuated by pertussis toxin , indicating coupling to G-proteins of the Go/Gi family.

Using PCR, a variant of the rat D3 receptor was identified in which the region encoding the first extracellular loop and the third transmembrane domain was deleted. The reported gene structure of the rat D3 receptor indicated that this variant was produced by alternative mRNA splicing. The identified transcript encoded a truncated form of the D3 receptor containing the amino terminal portion of the receptor through the second transmembrane domain, followed by 19 novel amino acids. A similar deletion variant of the D3 receptor was identified in the human. The biological significance of these D3 receptor variants has not been determined.

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CHAPTER 1

Introduction

The dopaminergic system of the mammalian CNS modulates emotional tone, motor control, and prolactin secretion. The abnormal regulation of this neurotransmitter system is implicated in the etiology and treatment of numerous disorders, including schizophrenia (Seeman and Lee, 1975; Seeman et al., 1984, for review see Seeman, 1987), Parkinson's disease (Lee et al., 1978), tardive dyskinesia (Gerlach, 1988), hyperprolactinemia (Weiner and Ganong, 1978), alcoholism (Blum et al., 1990) and drug addiction (Barnes, 1988).

The effects of dopamine are mediated through receptors that have been pharmacologically classified as either D1 (receptors which stimulate adenylate cyclase) or D2 (receptors which inhibit or do not effect adenylate cyclase). Although these receptors have been intensively studied for nearly two decades, it has only recently been demonstrated by molecular cloning that there are multiple genetically encoded subtypes of the D1 and D2 receptors. The pharmacological subtypes of the D1 and D2 are represented by two dopamine receptor subfamilies. The cloned D1, D1B(rat)/D5(human) receptors comprise the "D1" subfamily. The cloned D2L, D2S, D3, and D4 comprise the "D2" receptor subfamily. D2L and D2S refer to receptor isoforms which are generated by alternative mRNA splicing (see below). The terms "D1" and "D2" will be used to refer to the two classes of dopamine receptors in this report.

It is now appreciated that the genes encoding the dopamine receptors belong to a large gene superfamily that includes genes for multiple adrenergic, serotonergic, and muscarinic acetylcholine receptors, as well as the opsins and many peptide receptors. The receptors encoded by these genes are characterized by seven membrane spanning domains and by coupling to intracellular effectors via guanine nucleotide binding proteins

(for review see Probst et al., 1992). Although related, the "D1" and "D2" receptor families have distinct characteristics. The genes encoding the D1 and D5 receptor are closely related to each other, as are the genes encoding the D2(L&S), D3 and D4 receptors. Although the dopamine receptors share a common natural ligand, the "D1" receptor genes appear to be more closely related to the ß2-adrenergic receptor genes, while the "D2" receptor genes more closely resemble the serotonin receptor genes. The D2 receptor genes are distinguishable by the presence of introns within their protein coding regions. Alternative mRNA splicing gives rise to the D2L and D2S receptor variants of the D2 gene, which differ by the inclusion or exclusion of a 29 amino acid (aa) segment. Alternative mRNA splicing also gives rise to several truncated forms of the D3 receptor in the rat and human (Giros et al., 1991, Snyder et al., 1991b; Nagai et al., 1993) and to a full length receptor variant in the rat (Pagliusi et al., 1993).

My dissertation research has focused on the D2 receptor subfamily. Through this research I have accomplished two aims.

1. New members of the D2 receptor family, including the D2L splice variant (chapter 1), and a truncated form of the D3 receptor (chapter 3), have been identified.
2. The localization and coupling characteristics of the D2L and D2S isoforms have been studied. This work includes a solution hybridization/nuclease protection study in which the distribution of the D2L and D2S mRNAs in the rat brain and pituitary was determined (chapter 2), and a functional study in which the coupling of D2L and D2S to intracellular calcium levels was examined (chapter 4).

CHAPTER 2

Background & Significance

Overview of D2 receptor anatomy

The distribution of D2 receptors in the mammalian brain has been mapped by ligand binding techniques. In the rat, D2 receptor autoradiography has revealed high receptor densities in the caudate-putamen, accumbens, olfactory tubercle, islands of Calleja, and neurointermediate lobe of the pituitary, with intermediate receptor densities in the substanstia nigra, entorhinal cortex, septum, anterior pituitary, hypothalamus and anterior pituitary (Boyson et al., 1986; Charuchinda et al., 1987; Dubois et al., 1986; Gehlert and Wamsley, 1985). In general, the pattern of D2 receptor binding matches dopamine inervation. Since D2 receptor mapping studies preceded the cloning of specific D2 receptor subtypes, and the ligands used were not subtype specific, these studies do not provide information on the distribution of specific D2 receptor subtypes. The relative distribution of D2S, D2L, D3 and D4 subtypes will remain undetermined until specific antibodies are developed or until specific ligands are identified. Investigators have mapped the mRNA distributions of the identified D2 receptor subtypes by a variety of techniques, including; northern blotting, quantitative PCR, solution-hybridization/nuclease protection, and in-situ hybridization (Mengod et al. 1989; Snyder et al., 1991; Sokoloff et al., 1990; VanTol et al., 1991). From these studies several generalizations can be made; 1. the mRNA of the D3 and D4 receptors is concentrated in limbic areas of the brain, while the D2(L&S) receptor mRNA, although widely distributed, is more concentrated in the basal ganglia; 2. the expression of D2(L&S) mRNA is much higher than that of D3 or D4 receptor mRNA, such that in rat brain regions where D3 and D4 mRNAs are relatively well expressed, the quantity of D2(L&S) mRNA is 1-2 orders of magnitude higher. It has been suggested that neuroleptics (i.e.

clozapine) with a greater affinity for the D3 or D4 receptors may be less likely to cause motor side effects due to the relative absence of these subtypes from the basal ganglia.

The distribution of D2L and D2S receptors, and mRNA, has been investigated (DalToso et al., 1989; McVittie et al., 1991; Monsma et al., 1989; Rao et al., 1990; Snyder et al., 1991). Because D2L and D2S are pharmacologically identical, it is impossible to determine the relative amounts of these receptors by ligand binding. Antibodies have been raised that recognize the novel 29 amino acid segment of the D2L, or a region common to both isoforms. Using this strategy, it has been reported that the majority of the D2 receptors in the rat striatum are D2L (McVittie et al., 1991).

Overview of D2 receptor function

Classically D1 and D2 receptors have been distinguished functionally by their ability to modulate adenylate cyclase in opposite directions, with D1 receptors stimulating adenylate cyclase, and D2 receptors inhibiting it. D2 receptors also been shown to inhibit calcium influx, potentiate potassium efflux, attenuate IP_3 production, and to lower arachidonic acid levels (for review see Vallar & Meldolesi, 1989; Canonico, 1989; Florio et al., 1992). In all cases where it has been examined, the effects of dopamine on various types of cell signalling have been sensitive to pertussis toxin, indicating coupling to G-proteins in the G_i /G_o family. Indeed, both G_i and G_o have been observed to co-purify with the D2 receptor (Elazar and Fuchs, 1989). Presently, one G_o α-subunit, and three different G_i a subunits (α_{i1}, α_{i2}, α_{i3}) have been identified. Artificial membrane reconstitution techniques have been utilized to study the coupling of bovine D2 receptors purified from the anterior pituitary to G-proteins purified from bovine cortex (Senogles et al., 1990). It was observed that the affinity of purified D2 receptors to G_{i2} was ~ 10-fold higher than the affinity of purified D2 receptors to either G_{i1} or G_{i3}. The rate at which D2 receptors catalyzed the exchange of GDP for GTP on the G-proteins (termed G-protein activation) was also examined. It was also noted that the maximal rate of D2 receptor

mediated activation of either G_{i1} or G_{i3} was only 50% that of G_{i2}. In vivo, the affinity of a particular receptor for certain G-proteins may not be the only factor influencing coupling. The relative abundance of specific G-protein subtypes is also likely to play a significant role in determining which receptor/G-protein interactions predominate, and ultimately, which types of intracellular signals are observed. Studies suggest that G_o and G_{i3} play important roles in the signal transduction of D2 receptors in different cell types. In lactotrophs from lactating rats, antibodies to G_o attenuated the inhibition by dopamine of two different Ca^{+2} currents in patch clamp recordings, while G_{i3} antibodies blocked the induction by dopamine of two different K^+ channel currents (Lledo et al., 1992). Missale et al. (1991) observed that the treatment of GH3 cells with epidermal growth factor, leads to the induction of D2 receptors (D2L and D2S), as well as G_{i3}. In this report, the strong coupling of D2 receptors to potassium channels, and the absence of coupling to adenylate cyclase, was attributed to the unusually high levels of G_{i3} induced by EGF.

The signal transduction of specific D2 receptor subtypes has not been fully elucidated. In order to study the function of D2 receptors subtypes, investigators have transfected the respective cDNAs into cell lines that do not normally express D2 receptors. Analysis of these cell lines has demonstrated that the D2S receptor can modulate a variety of intracellular responses. In transfected pituitary GH4-C1 cells, D2S activation mediated the attenuation of cAMP levels (Albert et al., 1990) and of intracellular calcium levels (Vallar et al., 1990). In transfected Ltk^- fibroblasts, D2S activation mediated the attenuation of cAMP levels (Bates et al., 1991; Neve et al., 1989) and increased IP_3 production (Vallar et al., 1990). In transfected GH3-C1 cells, D2S receptor activation also caused cell hyperpolarization ,which indicated that D2S activation probably increased K^+ efflux (Vallar et al., 1990). In comparing the coupling of D2S in different transfected cell lines, Vallar et al. noted that they were able to stimulate IP_3 production in Ltk^- cells but not in GH4-C1 cells, even though the levels of receptor expressed in each cell line was similar.

The analysis of D2S signal transduction in cell lines leads to two conclusions. Within a single cell type, D2S can couple to more than one signal pathway, (perhaps through interaction with different G-proteins). In two different cell types, D2S is not necessarily coupled to the same signal pathways, possibly due to differences in the signal transduction components present -i.e. receptors, G-proteins and intracellular effectors. A third important point can be made from the analysis of D2S in cell lines. The signal transduction observed in cell lines may be very different from the signal transduction observed in the neurons or endocrine cells in which the receptors were originally studied. An example of this is the modulation of IP_3 production by transfected D2 receptors in the fibroblast derived cell lines, Ltk^- and CHO-K1. Prior to the expression of D2S in Ltk^- cells, it was generally believed that D2 receptors were either not coupled to IP_3 or that they were coupled to the inhibition of IP_3 production. In lactotrophs from female rats, dopamine had been reported to inhibit IP_3 production (Journot et al., 1987; Vallar et al, 1988, Enjalbert et al., 1990) or to inhibit calcium mobilization characteristic of IP_3 production (Malgaroli et al., 1987). The differences in signalling observed in lactotrophs and in fibroblast cell lines is likely to reflect differences in signal transduction components present. However, it should not be concluded that the unusual signalling of D2 receptors observed in these cell lines does not occur *in vivo*. It is interesting to note that there is one report in which D2 receptor activation in lactotrophs has been correlated with a rise in intracellular calcium characteristic of IP_3 (Winiger et al., 1987). In individual fura-2 loaded lactotrophs from male rats, dopamine stimulation caused a rise in intracellular calcium (typical of IP_3 production) in ~40 of the lactotrophs examined. In ~60% of the lactotrophs examined, dopamine caused a decrease in intracellular calcium levels. The (apparent) stimulation of IP_3 production in this study, but not in the other lactotroph studies mentioned above, may be due to differences in the populations of lactotrophs selected for study, or due to differences in the conditions under which the cells were cultured. Note, lactotrophs from female rats were examined in the studies

reporting decreases in IP_3, or decreases in IP_3 mobilized calcium. Lactotrophs from male rats were examined in the study reporting a rise in intracellular calcium characteristic of IP_3 production. *In vivo*, the signalling of a particular receptor in a praticular cell type may be dependent on the physiological state of the cell. Differences in hormonal and nutritional states could potentially affect the signalling of a receptor by altering the expression of G-proteins and intracellular effectors.

Basis for potential differences in the function of D2L and D2S

As noted earlier, the D2L and D2S receptors represent alternative mRNA splice variants that vary respectively by the inclusion or exclusion of a stretch of 29 amino acids within the third cytoplasmic loop. Although functional differences between the two isoforms have not been established, their biological importance is suggested by the preservation of the alternative splice site throughout mammalian evolution. The third cytoplasmic loop has been implicated in the G-protein coupled signal transduction of the D2S receptor (England et al., 1991). Structure/function analysis of the third cytoplasmic loop of related neurotransmitter receptors (including the adrenergic, serotonergic, and muscarinic acetylcholine receptors) indicates that this intracellular domain plays a role in determining the specificity of G-protein interactions, and in kinase mediated receptor desensitization (for review see Probst et al., 1992). Thus, it is possible that the inclusion or exclusion of 29 amino acids in the third cytoplasmic loop of D2L and D2S confers differences in these functional parameters. The role of the third cytoplasmic loop in G-protein coupling and desensitization will be discussed below, along with recent data concerning these processes in D2 receptors.

Coupling

Short stretches of the membrane proximal regions of the third cytoplasmic loop and possibly the carboxy-terminus appear particularly critical in determining the specificity of G-protein coupling for many receptors. These regions may form clustered amphipathic

α-helices that cooperatively interact to efficiently bind and activate G-proteins during agonist stimulation (for review see Probst et al., 1992). It should be emphasized however, that other intracellular regions and/or amino acid residues (i.e. DRY) have been implicated in efficient signal transduction. The role of non-membrane proximal regions of the third cytoplasmic loop in modulating G-protein coupling has not been thoroughly examined.

To date, several studies have been reported in which the G-protein coupled signal transduction of D2L and D2S was compared. In pituitary GH3 cells, it was observed that either isoform could mediate repression of the rat prolactin promoter, although no differences between D2L and D2S were noted (McChesney et al., 1991). In two fibroblast derived cell lines, CHO-K1 and Ltk$^-$, it was demonstrated that dopaminergic stimulation of cells expressing either D2L or D2S resulted in calcium mobilization characteristic of IP_3 turnover (Hayes et al., 1992; Liu et al. 1992). In these studies, no differences were observed in the ability of D2L and D2S to mobilize calcium. In contrast, several studies have indicated differences in the ability of D2L and D2S to inhibit cAMP accumulation (Hayes et al., 1992; DalToso et al., 1989, Montmayeur and Borrelli, 1991). In the CHO-K1 cell line (derived from hamster ovarian fibroblasts) and in the 293 cell line (derived human embryonic kidney), D2S was observed to be more effective than D2L at inhibiting the accumulation of cAMP (Hayes et al., 1992; DalToso et al., 1989). In the JEG-3 cell line (derived from human chorionic cells), it was observed that both isoforms could attenuate transcription of a reporter gene containing a cAMP regulatory element, indicating indirectly that both isoforms were capable of lowering intracellular cAMP levels (Montmayeur and Borrelli, 1991). However, at all dopamine concentrations, D2S caused a greater suppression in cAMP mediated transcription than D2L, and D2S was much less sensitive to pertussis toxin than D2L, suggesting that D2L and D2S were differentially coupled to the G-proteins present in JEG-3 cells. In a follow-up study it was observed that the differences in D2L and D2S coupling were

abolished by the cotransfection of the $G_{i\alpha 2}$ subunit, which suggests that in JEG-3 cells, the inhibition of adenylate cyclase by D2L is preferentially coupled via the G-protein, G_{i2} (Montmayeur et al., 1992). In summary, differences in the coupling of D2L and D2S to the inhibition of cAMP accumulation have been observed in several transfected cell lines, however, the differential coupling of the isoforms to other signal transduction systems has not been investigated.

Desensitization

Desensitization refers to the attenuation of receptor function that occurs during persistent agonist stimulation. Phosphorylation of cytoplasmic residues has been identified as an important mechanism in the desensitization of many cell surface receptors (for review see Sibley et al., 1987). For several G-protein coupled receptors (GPCRs) including rhodopsin, the M4-muscarinic receptor, and several adrenergic receptors, it has been demonstrated that phosphorylation leads to the rapid (within minutes) uncoupling of these receptors from their signal transduction systems (Hargrave et al., 1982; Benovic et al., 1989; Benovic et al., 1987; Kwatra et al., 1989). In general, the carboxy-terminus and third cytoplasmic loop of the GPCRs are rich in serine and threonine residues, and it has been demonstrated that these regions are involved in the desensitization of GPCRs (for review see Probst et al., 1992). The D2L and D2S receptors lack serine and threonine residues in their carboxy-termini. There are however, many serine and threonine residues present in the third cytoplasmic loop. In the 29 amino acid segment unique to D2L, there is one threonine, and two serines. These amino acid residues may represent potential phosphorylation sites.

Agonist desensitization of D2 receptor signal transduction has been reported to occur in fibroblast Ltk^- cells transfected with D2S (Bates et al., 1991) and in retinal Y-79 cells which express native D2 receptors (Barton et al., 1991). In CHO cells transfected with either D2L or D2S, it was demonstrated that activation of kinase C, but not kinase A, leads to phosphorylylation of both isoforms (Olinger et al., 1991). The differential

desensitization of D2L and D2S has been demonstrated in transfected Ltk$^-$ cells (Liu et al. 1992). In this study, prior activation of kinase C preferentially inhibited calcium mobilization by D2S, but had little effect on calcium mobilization by D2L.

Summary

Molecular cloning techniques have led to the identification of genes comprising the D2 receptor family. These genes encode the D2(L&S), D3, and D4 receptors. The cloning of these genes represents a significant advance towards understanding the of the biological roles of these receptors. The presence of introns within the coding regions of these genes suggests that alternative mRNA splicing of primary transcripts plays an important role in creating D2 receptor diversity. The identification of D2 receptor subtypes generated by alternative splicing, along with the anatomical localization and the functional characterization of these receptor subtypes, will be required in order to fully elucidate the biology of the mammalian dopaminergic system.

CHAPTER 3

The Identification of a D2 Receptor Subtype by Polymerase Chain Reaction Cloning

Abstract

A rat brain D2 receptor has been identified (Bunzow et al., 1988). Based on the published sequence, we designed oligonucleotides for PCR cloning. Oligo(dT) primed cDNA was synthesized from weanling rat brain RNA. When used as a PCR template, with oligos spanning the coding region, two distinct bands were observed on agarose gels. One band was the expected size of 1250 base pairs (bp). An unexpected second band, ~100 bp larger was also seen. Both bands hybridized to an internal oligonucleotide probe. Restriction mapping of these PCR products indicated that the ~100 bp segment was located between nucleotides 608 and 774 of the published D2 coding sequence. A second pair of oligonucleotide primers flanking this region was synthesized and used in PCR with rat brain cDNA template. Two products were obtained which varied in size by ~100 bp. Sequence analysis of the larger product revealed a 306 bp fragment that contained an 87 bp piece flanked by sequence matching that of the published D2 receptor. The novel 87 bp piece was inserted between nucleotices 723 and 724 of the published D2 receptor coding region (Fig. 2). These results suggest that a second D2 receptor subtype exists, which appears to be a mRNA splice variant of the D2 receptor identified by Bunzow et al. (Fig. 3).

Introduction

The first dopamine receptor sequence was reported in 1988 by Bunzow et al. working in the laboratory of Olivier Civelli. These investigators isolated a rat brain cDNA from a library screened at low stringency with the β2-adrenergic gene as a probe. Homology to

rhodopsin and other cloned neurotransmitter receptors, including the adrenergic, serotonergic, and muscarinic acetylcholine receptors, identified it as a member of the G-protein coupled receptor (GPCR) family. These receptors all contain seven putative membrane spanning α-helices, and are coupled via G-proteins to signal transduction.

Based on the published D2 sequence, we designed oligonucleotides for polymerase chain reaction (PCR) cloning. Suprisingly, when these primers were used in reactions with rat brain cDNA as a template, two PCR products were observed. One product corresponded to the reported D2 receptor. A second product, we have identified as a mRNA splice variant of the reported D2 receptor. Some of the results presented here have appeared in earlier reports from this laboratory (Snyder et al., 1989; Autelitano, Snyder et al. 1989).

Methods

Total RNA was isolated from the brains of 14 day old rats using guanidinium isothiocyanate and lithium chloride as previously described (Cathala et al, 1983). RNA prepared from E2 induced prolactinomas (Wiklund et al., 1981) was donated by Dr. Stuart Sealfon. To obtain template for PCR, first strand cDNA was synthesized from total RNA using oligo(dT) as a primer and MMLV reverse transcriptase (BRL).

Six different oligonucleotides were synthesized for PCR cloning.

SD101: 5'-GGTAACTGTAATACGACTCACTATAGGGCGAATTCCAATGG-
ATCCACTGAACCTGTCCT-3

AD101: 5'-CTGCAGAAGCTTTTTTTTTTGCAGACTCAGCAGTGCAAGAT-
CTTC-3'

SD400: 5'-TGGATCCACACCAGCTCACTCTCCCTGATCCAT-3'

AD100: 5'-AGAATTCTGTAGAGGACTGGTGGGATGTTGCAATC-3'

SD582: 5'-GAGCTCGGATCCTATACCGGGTCCTCTCTGG-3

AD800: 5'-GAGCTCGGATCCTATACCGGGTCCTCTCTGG-3'

PCR reactions contained approximately 100 ng of cDNA, 1mM primers, 0.3 mM nucleotides, 1U Taq polymerase (Amplitaq, U.S. Biochem. Corp., Cleveland, OH), 16 mM amonium sulfate, 2 mM $MgCl_2$, 67 mM Tris (pH 8.8), and 200 ug/mg bovine serum albumin. Fourty cycles of PCR were performed in a programmable heat block (Coy Lab. Products, Ann Arbor, MI). Each cycle consisted of 1min at 94 C, 1 min at 55 C, and 2 minutes at 74 C. The resulting PCR products were phenol/chloroform extracted, and percipitated in EtOH. Products were initially visualized by electrophoresis through 1% agarose gels, followed by ethidium bromide staining. PCR products obtained with the primer set SD101 and AD101, were resolved on agarose gels, Southern blotted, and hybridized to the ^{32}P2-dATP labeled PCR product obtained from the primer set SD400 and AD100. For restriction mapping gel purified PCR products were digested with Sac1, Acc1, or EcoRII for 1 h at 37 C. The digests were end labeled with ^{32}P2-dATP and ^{32}P2-dCTP using the Klenow fragment. The labeled digests were electrophoretically resolved through 5% polyacrylamide gels. The gels were mounted on filter paper, dried, and exposed to X-ray film for 1 h. For the purpose of subcloning, EcoRI linkers were ligated to gel purified PCR products, followed by digestion with EcoRI for 2 h at 37 C, and ligation into EcoRI digested Bluescript KS$^+$ (Stratagene). Sequence analysis of PCR products subcloned into Bluescript KS$^+$ was performed using Sanger dideoxy chain termination. Restriction digests, end labeling, electrophoresis, Southern blots, and sequencing, were performed using standard protocols (Sambrook et al., 1989).

Results

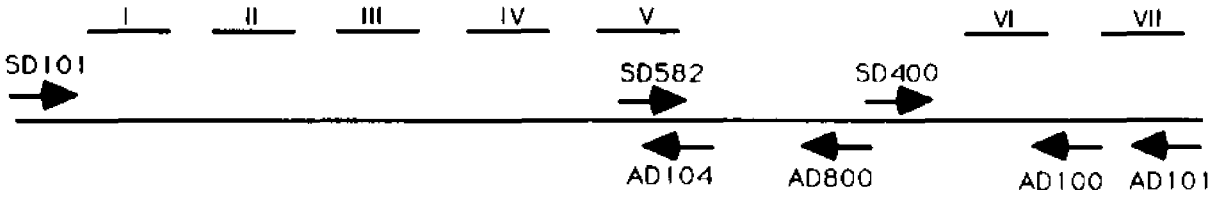
The regions of the D2 receptor to which oligonucleotide primers were synthesized are shown in Fig. 1A, while the results obtained from PCR analysis of the rat D2 receptor are summarized in Fig.1B. Oligonucleotides (25-mers) matching the coding ends (SD101 and AD101) of the Civelli D2 receptor sequence were synthesized and used as primers in polymerase chain reactions (PCR) with template cDNA synthesized from weanling rat

brain. When the reaction products were electrophoresed on agarose gels, two bands were observed. One band of 1245 bp corresponded to the expected size of the D2 receptor coding region reported by Bunzow et al. (1988). The second band appeared to be approximately 100 bp larger. Only the larger of the two bands was observed when the same reaction was run with cDNA template synthesized from rat prolactinoma. A second set of oligonucleotide primers (SD400 and AD100) was synthesized to use in PCR reactions to evaluate the possibility of alternative splicing at the site of the reported intron. (At the time these experiments were performed, only a single intron had been reported by Bunzow et al. in the D2 receptor gene). However, PCR with this primer set yielded only a single product when either rat brain cDNA or rat prolactinoma cDNA as template. Together these results suggested the existence of a second tissue specific D2 subtype with an extra ~100 bp, and that the extra segment was not located within the 330 bp D2 fragment generated by the oligonucleotide primers SD400 and AD100 (i.e. not between nucleotides 815 and 1145 of the coding region of the reported D2 receptor sequence). To further localize the receptor region containing the extra bp, an antisence primer to the putative fifth transmembrane (TM-5) domain was synthesized (AD104) and used in PCR experiments with the primer SD101 to generate (approximately) the 5' half of the receptor coding region. In reactions using either rat brain cDNA or prolactinoma cDNA as a template only a single PCR product was observed, indicating that the extra bp were located downstream of the sequence encoding TM-5. This result, along with the results of the previous PCR experiments indicated that the extra bp were located between TM-5 and TM-6.

Restriction digest mapping was also performed to localize the receptor region containing the extra ~100 bp (data not shown). The two PCR products generated with the oligos SD101-AD101 using rat brain cDNA as a template, were individually purified from agarose gels, digested with restriction enzyme, end labeled, electrophoresed through 5% polyacrylamide gels, and exposed to X-ray film. Restriction enzymes predicted to cut

within the published D2 sequence were used (EcoRII, Acc1, and Sac1). Size analysis of the fragments generated by the enzymes AccI, and SacI, indicated that the extra bp were located between nucleotides 608 and 774 of the published D2 coding sequence.

A.



B.

primers	brain cDNA	prolactinoma cDNA
SD101/AD101	2 bands, 1245 bp and 1332 bp	1 band, 1245 bp
SD400/AD100	1 band, 330 bp	1 band, 330 bp
SD101/AD104	1 band, 610 bp	1 band, 610 bp
SD582/AD800	2 bands, 218 bp and 305 bp	1 band , 305 bp

Figure 1. PCR analysis of the rat D2 receptor. A) Primers for PCR analysis. The coding region of the rat D2 receptor is represented by a solid line, above which the approximate locations of the transmembrane domains are indicated by Roman numerals. Primers are indicated by arrows: arrows pointing to the right denote sence primers, arrows pointing to the left denote antisense primers. B) PCR products obtained from two different cDNA templates; analysis by agarose gel electrophoresis.

Based on the results obtained from restriction digest mapping and the earlier PCR experiments, a set of 25-mer oligonucleotide primers (SD582 and AD800) was synthesized and used in polymerase chain reactions to generate portions of the D2 receptor lying between nucleotides 582 and 800 of the published D2 coding sequence (Fig. 1A). When rat brain cDNA was used as a template, two PCR products were observed on agarose gels; a band of 219 bp, corresponding to the published D2 receptor, and a second band ~100 bp larger (Fig. 1B). Using prolactinoma cDNA as a template, a single PCR product of ~319 bp was observed (Fig. 1B). This band corresponded to the larger of the two products observed in PCR reaction using rat brain cDNA as a template. The larger band (~319 bp) was cut out and subcloned into the plasmid, Bluescript KS$^+$, and sequenced. Sequence analysis revealed a 306 bp fragment related to the reported D2 receptor. The 306 bp fragment contained an 87 bp piece flanked by sequences that exactly matched that of the D2 receptor reported by Bunzow et al. The novel 87 bp piece was inserted between nucleotides 723 and 724 of the published D2 receptor coding sequence (Fig. 2). Full length clones generated in PCR reactions with the primers SD101/AD101 were also sequenced (McChesney et al., 1991).

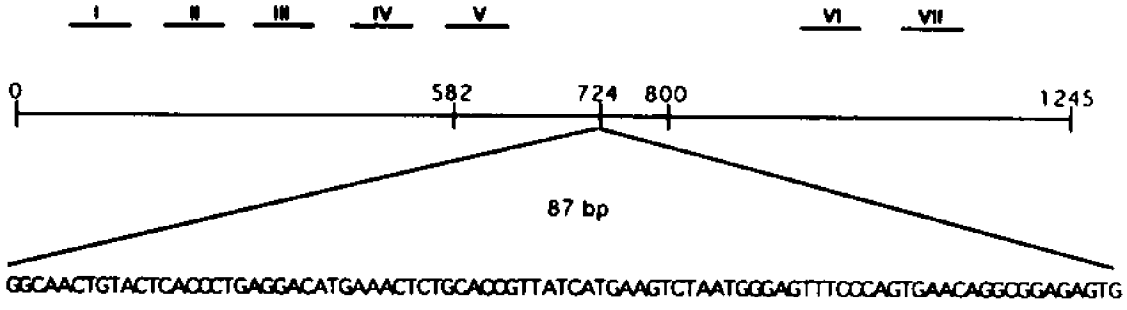


Figure 2. Sequence of the 87 base exon and location within the coding region of the rat D2 mRNA. The approximate location of transmembrane domains are indicated by bars with Roman numerals.

Discussion

Our results indicated that a second variant of the D2 receptor cloned by Bunzow et al. exists in the rat brain and pituitary. This variant containes an extra 87 bp located in the domain corresponding to the third cytoplasmic loop. We called this new variant D2L, because it was 29 amino acids longer than the D2 receptor originally cloned (which we called D2S). Subsequent to our own studies, D2L was reported by several other laboratories (Chio et al., 1990; DalToso et al., 1989; Giros et al., 1989; Miller et al., 1990; Monsma et al, 1989; O'Malley at al., 1990; Selbie et al., 1989). Expression of D2L mRNA in transfected cell lines indicated that it encodes a functional receptor (Chio et al., 1990; DalToso et al., 1989; Monsma et al., 1989; Giros et al., O'Malley at al., 1990). The D2L transcript that we identified is generated by alternative mRNA splicing, indicating the existence of other introns in addition to the single intron reported by Bunzow et al. This was also confirmed in subsequent studies. Reports on the gene structure of the D2 receptor indicated that the 87 bp segment represented an exon, and that the 87 bp exon was indeed flanked by two introns (Grandy et al, 1989; O'Malley et al., 1990; Gandelman et al., 1991; Araki et al., 1992). These features indicated that D2L and D2S were in fact, alternative mRNA splice variants.

The results of our study suggested that the relative abundance of D2L and D2S mRNA varied considerably in the brain and pituitary of the rat. In PCR reactions using (pituitary derived) prolactinoma cDNA as a template, only products representing D2L were observed. In contrast, using cDNA derived from whole rat brains (15 day old), products representing both D2L and D2S were observed. These results prompted us to examine the distribution and relative abundance of the two species in other areas of the rat brain (Snyder et al., 1990, -see Chapter 2). It will be important to determine whether the relative abundance of D2L and D2S mRNA can be regulated. If so, this may represent a mechanism by which D2 receptor level (and/or activity) is controlled. The regulation these transcripts may play a role controlling dopaminergic activity during development,

and in the adult animal. In humans, aberrant regulation of these transcripts may be involved in the etiology and/or treatment of certain neurological disorders involving the dopaminergic system, including schizophrenia and Parkinson's disease.

CHAPTER 4

Distribution of Dopamine D2 Receptor mRNA Splice Variants in the Rat Brain by Solution Hybridization/ Protection Assay

Abstract

We investigated the distribution of the two dopamine D2 receptor mRNA splice variants in the rat using a sensitive and quantitative solution hybridization/nuclease protection assay. In all brain and endocrine regions studied, both splice variants were detected and the mRNA of the longer form, D2L, was more abundant than that of the shorter form, D2S. The lowest percentages of D2S mRNA were found in the pituitary and adrenal glands.

Introduction

The cloning of a rat D2 receptor (D2R) cDNA led rapidly to the discovery in all species characterized (Chio et al., 1990; DalToso et al., 1989; Giros et al., 1989; Miller et al., 1990; Monsma et al, 1989; O'Malley at al., 1990; Selbie et al., 1989; Snyder et al, 1990) that there exist two different D2R mRNAs. The D2R, like other G-protein coupled receptors, has 7 transmembrane domains. The two D2R mRNA forms, which represent splice variants of a single D2R gene, differ only in the region encoding the putative third cytoplasmic loop. The long form of the receptor mRNA (D2L) contains an 87 bp insert derived from a separate exon not present in the form first isolated (D2S). The functional significance of the insertion of 29 amino acids into a region postulated to be involved in G-protein coupling remains to be elucidated.

To begin investigating the biological significance of the two D2R forms, we were interested in determining the anatomical distribution and the relative abundance of the D2L and D2S mRNAs in the rat. Because the two splice variants are identical except in

one small region, accurate measurement of each mRNA is technically difficult. Indeed, preliminary reports on the relative abundance of the two forms have been inconsistent. By northern blotting (Selbie et al., 1989) or in situ hybridization histochemistry (Dal Toso et al., 1989; Monsma et al., 1989), D2S mRNA was undetectable in the brain. Using polymerase chain reaction (PCR), one group did not detect D2S mRNA (Selbie et al., 1989), while two others (Monsma et al., 1989; O'Malley et al., 1990), while detecting D2S mRNA in all brain regions, found quite different abundances of D2S mRNA relative to D2L mRNA. For example, in the midbrain and olfactory bulb, O'Malley et al. reported that D2S was 66% and 47% of the total D2R mRNA respectively, while Monsma et al. found D2S represented 20-25% and 12% in the two regions. By PCR analysis, the reported percentage of D2S mRNA in the basal ganglia/striatum also varies widely: 10-20% (Dal Toso et al., 1989), 20-25% (Monsma et al., 1989), and 40% (O'Malley et al., 1990). The reported discrepancies in detecting D2R mRNA and in measuring the relative abundance of the two forms, are most likely due to limitations in the methods used in reliably quantifying low levels of mRNA. Because the solution hybridization/nuclease protection (SHNP) approach can be adapted to measure accurately low levels of mRNA splice variants (Blum, 1989), we developed this assay for the D2R.

The results presented in this report have been published (Snyder at al., 1990).

Methods

Six-month old male Sprague-Dawley rats were sacrificed by decapitation and the olfactory bulbs, pituitary anterior lobe (AL), neurointermediate lobe (NIL) and adrenal glands were removed. The brains were chilled, sectioned in a coronal rodent brain matrix and 10 brain regions were dissected using a microdissection atlas as a guide (Palkovitz & Brownstein, 1988). The midbrain dissection included the substantia nigra and ventral tegmental area. The hypothalamus dissection contained mainly the tuberal and posterior regions. The entire cortex was pooled. The dissections were snap frozen in liquid

freon/dry ice and stored at -80 C until RNA extraction. To prepare RNA for SHNP assays, individual tissue dissections were thawed on ice and homogenized in cold 0.3 AT buffer (10 mM Tris-Cl pH 8.0, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM DTT, O.3 M sucrose, 0.15% Triton X-100). The homogenate was layered over 0.4 AT buffer (0.4 M instead of 0.3 M sucrose) in a 1.5 ml microcentrifuge tube. After centrifugation for 10 minutes at 2500 g, the supernatant was removed, mixed with 1/10 volume of 10X SET (10% SDS, 50 mM EDTA, and 100 mM Tris-Cl, pH 8.0. Proteinase K was added to 100 ug/ml followed by incubation for 1 hour at 45 C. The samples were phenol/chloroform (1:1) extracted and precipitated with 0.7 ml isopropanol. Total cytoplasmic RNA was quantified by determining the A_{260} absorbance.

To obtain template for PCR, first strand cDNA was synthesized using oligo(dT) as a primer and MMLV-reverse transcriptase (BRL). D2R clones were isolated by PCR using rat brain cDNA as a template and oligonucleotides designed from the original rat D2R sequence (Bunzow et al., 1988; Snyder et al., 1989). Clones of 219 bp (D2S-219) and 306 bp (D2L-306), representing D2S and D2L, respectively, were subcloned into Bluescript KS^+ (Stratagene) and used to generate riboprobe and/or standards. Antisense RNA for riboprobe, and sense RNA for standards were synthesized by T7 and T3 RNA polymerase, respectively. Riboprobes were synthesized with ^{32}P-rUTP to a specific activity of approximately 1.2 X 10^9 cpm/ug. Each experiment consisted of a set of sense strand standards, (ranging from 0.5 - 10 pg) along with samples of tissue extracted RNA. Hybridization was performed with antisense D2S-219 riboprobe. Sense transcript derived from both subclones served as standards. Standards and samples were hybridized to riboprope in individual microcentrifuge tubes, in a total volume of 30 ul buffer (80% formamide, 40 mM PIPES pH 6.7, 0.4 M NaCl, 1 mM EDTA). Hybridization to 0.5 ng (antisense) riboprobe was carried out for 16 hours at 45 C, after which non-hybridized RNA was degraded by incubation with 160 ug RNAse A for 1 h at 37 C. This was followed by a 1 hour incubation with 100 ug proteinase K. The samples were

phenol/chloroform extracted and precipitated with 10 ug RNAse-free yeast RNA and 0.5 ml cold isopropanol. After washing the pellet with 70% ethanol to remove salt, the entire sample was dissolved in 10 ul H20, 2 ul dye (15% Ficol in H_2O, 0.25% xylene cyanol, 0.25% bromphenol blue). Samples were electrophoresed through a 5% polyacrylamide gel. Gels were dried and exposed to X-ray film overnight. Protected bands at 143 and 219 bp, representing D2L and D2S respectively, were excised, counted in a scintillation counter. The amount of each D2R form determined by comparison with the standard curve.

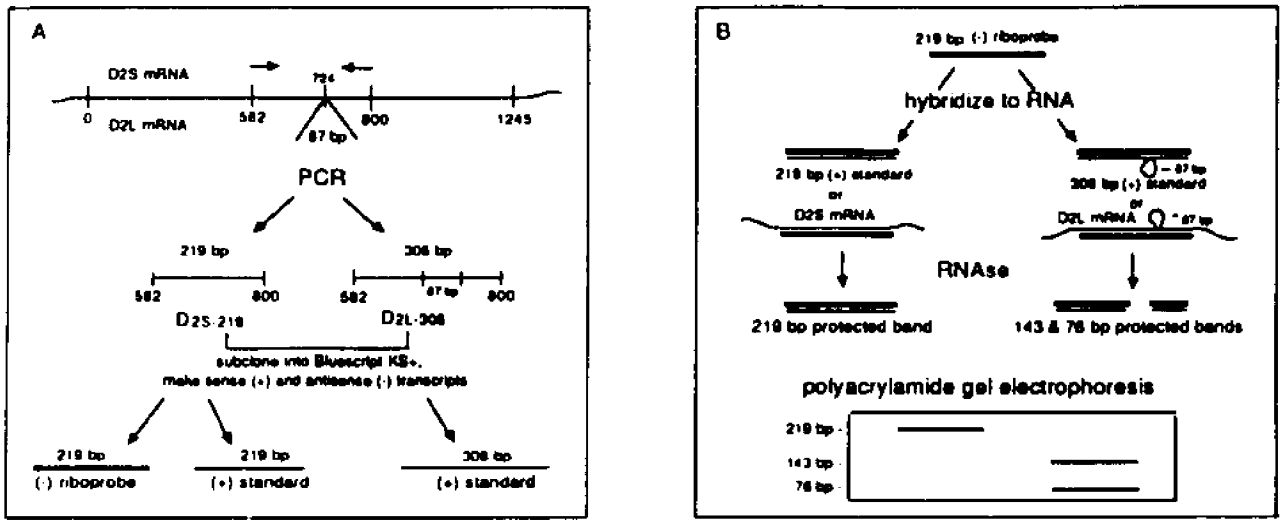


Figure 1. A) Isolation of D2R clones and synthesis of riboprobe and standards for SHNP assays. Arrows indicate the location of PCR primers within the D2R coding region originally described by Bunzow et al. Using rat brain cDNA as a template, two clones of 219 and 306 bp were isolated via PCR, representing fragments of the D2S and D2L receptors, respectively. Subclones of the two PCR products were used to synthesize antisense (-) riboprobe or sense(+) standards. B) Schematic of SHNP assay. 32P-labeled (-) riboprobe was hybridized in solution to samples of total RNA (extracted from tissues) or to varying picogram amounts of standard. D2S mRNA (or 219 bp standard) protected a complementary 219 bp fragment of (-) riboprobe from digestion by RNAse. Hybridization of riboprobe to D2L mRNA (or 306 bp standard) led to the protection of 143 and 76 bp fragments flanking the (uncomplementary) 87 bp exon. Thus D2S mRNA is represented on polyacrylamide gels by a single band, while D2L mRNA is represented by two bands.

Results

As can be seen in Fig. 2, D2S mRNA gave a single protected band of 219 bp whereas D2L mRNA generated two bands of 143 and 76 bp. Both D2S and D2L mRNA were detected in all regions studied. In all regions, D2L was the more abundant form (Fig 3). D2S mRNA ranged from 0.070 ± 0.004 attomoles/ug RNA in the cerebellum and adrenal gland to 6.11 ± 0.24 attomoles/ug RNA in the caudate putamen. D2L mRNA ranged from 0.18± 0.01 attomoles/ug RNA in the cerebellum to 37.2± 2.5 attomoles/ug in the neurointermediate lobe. D2S mRNA represented 12%, 15%, and 20% of total D2R mRNA in the NIL, AL and adrenal gland respectively. D2S mRNA ranged from 24% to 34% in the brain regions assayed.

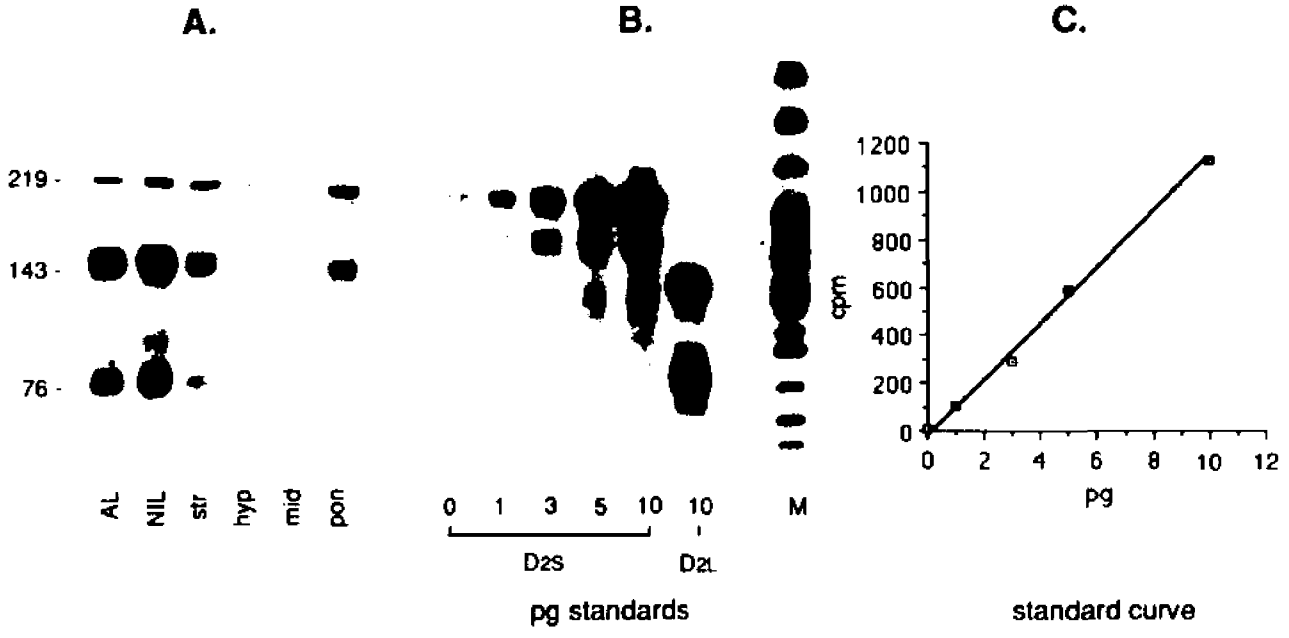


Figure 2. Autoradiogram of a SHNP assay with rat RNA samples (A), synthetic D2L and D2S standards (B) and the graph of scintillation counts for D2S standards (C). AL, pituitary anterior lobe; NIL, pituitary neurointermediate lobe; str, caudate/putamen; hyp, hypothalamus; mid, midbrain; pon, pons/medulla; M, marker (Msp I digested PBR322). Standard curve $r^2 = 1$.

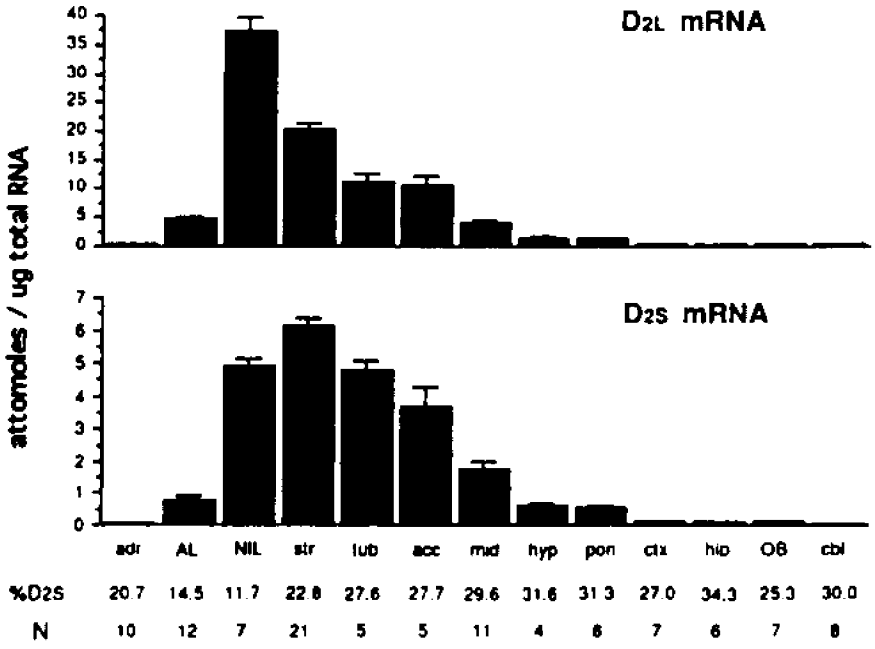


Figure 3. Levels of D2S and D2L mRNA. The % D2S of the total D2R mRNA and the number (N) of samples assayed is shown below the graphs. Data represent the means ± S.E.M. of two experiments. In cases where the error bars were not resolved (pon, ctx, hip, OB, and cbl), the SE was less than 11.5% of the mean. AL, pituitary anterior lobe; NIL, pituitary neurointermediate lobe; str, caudate/putamen; tub, olfactory tubercle; acc, accumbens; mid, midbrain; hyp, hypothalamus; pon, pons/medulla; ctx, cortex; hip, hippocampus; OB, olfactory bulb; cbl, cerebellum.

Discussion

In the NIL, D2R mRNA has been localized to melanotrophs of which there are approximately 2.5 X 10^5. Assuming uniform cellular distribution, it can be calculated from our data that each melanotroph is expressing approximately 24 copies/cell of D2S mRNA and 180 copies/cell of D2L mRNA. The relative abundance of D2R mRNA in most tissues examined, corresponds to D2R levels as reported in previous ligand binding studies (Bouthenet et al, 1987; Boyson et al, 1986; Charuchinda et al, 1987; Dubois et al, 1986; Gehlert et al, 1984; Nock et al, 1986; Palacios & Pazos, 1987). In the NIL, however, the D2R mRNA level is nearly 2-fold that found in the striatum, whereas receptor levels in the NIL have been reported to be lower (Gehlert et al, 1984; Lightman et al., 1982; Palacios & Pazos, 1987). The total D2R mRNA in the NIL might be translated less efficiently or alternatively, the receptor turnover is more rapid than in the striatum. Of note, the excess total D2R mRNA in the NIL relative to the striatum consists entirely of D2L, with the D2S mRNA in the NIL being, in fact, less abundant than that in the striatum (Fig. 3). These findings raise the possibility that D2S mRNA, while less abundant than D2L mRNA, contributes disproportionately to D2R levels. This could be the case if D2S mRNA were translated more efficiently, or the D2S receptor were more stable than the D2L receptor. If the translational efficiency or stability of the two forms differ, changing the relative proportion of the two splice variants could provide a mechanism to alter the level of receptor expression. It has been demonstrated that D2R mRNA is increased by receptor blockade in the NIL (Autelitano et al, 1989) and in the striatum (LeMoine et al., 1990). It will be interesting to learn if this regulation differentially affects the two splice forms.

In summary, we have used a sensitive, quantitative assay to measure levels of the two D2R mRNAs accurately. We detect both D2L and D2S mRNA in all areas studied, and we find that the ratio of the two forms in the brain is relatively constant.

CHAPTER 5

The Identification of Alternative Transcripts of the Rat and Human Dopamine D3 Receptor

Abstract

A cDNA for the rat dopamine D3 receptor (D3R) containing a 113 bp deletion has been isolated. The segment deleted, encompassing the first extracellular loop and third transmembrane domain, alters the reading frame, introducing 19 amino acids not found in the full length receptor followed by a premature stop codon. The novel mRNA encodes a 109 amino acid protein containing two putative transmembrane domains. A similar variant cDNA for the human D3 receptor has also been identified.

Introduction

The dopamine D_3 receptor (D3R), a dopamine receptor subtype which is concentrated in mesolimbic structures, has recently been identified by molecular cloning (Sokoloff et al., 1990; Giros et al., 1990). In contrast with most G-protein coupled receptors which are intronless, the D3R gene contains at least six exons and five introns (Sokoloff et al., 1990; Giros et al., 1991). The D2R gene, which has a similar structure, has been found to give rise to two different mRNA species through alternative splicing, both of which encode functional receptors (Grandy et al., 1989; Monsma et al., 1989; O'Malley et al., 1990; Gandelman et al., 1991; Snyder et al., 1991b).

We have determined that two forms of the rat D3R mRNA exist. Unlike the D2R isoforms, the novel D3R mRNA (D3R-del) contains a deletion which alters the amino acid reading frame and encodes a truncated protein. This mRNA variant of the rat D3R has recently been reported (Giros et al., 1991). The protein sequence reported by Giros et al. however, is nine amino acids shorter than that predicted from the nucleotide sequence

of our clone (see discussion). To investigate whether the D3R-del form has been conserved evolutionary, we have cloned the corresponding human cDNA. Preliminary results of our work have been published in abstract form (Snyder at al., 1991a). The results of these studies have been published (Snyder at al., 1991a; Snyder et al, 1991b).

Methods

mRNA was purified from Sprague-Dawley rat brain using guanidinium isothiocyanate and lithium chloride (Cathala et al., 1983). Poly-A+ mRNA was selected on an oligo-dT cellulose column (Aviv & Leder, 1972). Human parietal cortex RNA was a generous gift of Dr. William Wallace. Single strand cDNA was synthesized from poly-A+ mRNA using oligo-dT and MMLV reverse transcriptase. The following oligonucleotides were designed for PCR based on the published rat D3R nucleotide or amino acid sequence (Sokoloff et al., 1990):

OS1: 5'(-32)-GCCACTCGAGGGTACCACATTTTGGAGTCGCGTTCCTCTG-3'

OS2: 5'(+1)CCGAGGATCCATGGC(A,T)CC(A,C,T)CT(C,G)AG(C,T)CA(A,G) AT-3'

OS3: 5'(+89)-CACTGGATCCTACTACGCCCTGTC(C,T)TACTG(T,C)GC-3'

OA1: 5'(+400)-CCGCGAATTCGTG(C,T)TG(A,G)TA(A,G)TG(C,G)AC(A,G,T)GGCAT-3'

OA2: 5'(+462)-CTGAGGTACCAAAGCCAGCACCCACACAGCTGTG-3'

OA3: 5'(+1362)-CCACGAATTGAGATCTCGAAGTGGGTAAAGGG-3'

Restriction sites were included at the 5' end of the oligomers to facilitate subcloning. PCR was performed in a programmable heat block as previously described (Autelitano et al., 1989). PCR products were subcloned into the Bluescript KS$^+$ plasmid (Stratagene, La Jolla, CA) for sequencing. Sequences obtained were confirmed in at least three separate subclones. Restriction digests, end labeling, and electrophoresis were performed using standard protocols (Sambrook et al., 1989).

Results

PCR was performed to isolated a full coding length clone of the rat D3R using primers recognizing the N-terminus and 3'-untranslated region. Unexpectedly, two bands were generated, one of the anticipated size, 1385 bp, and a second approximately 100 bp smaller (Fig. 1A). To determine the relationship between them, the two PCR products were purified, digested by restriction enzymes, ^{32}P-labeled, and electrophoresed. As can be seen from the resulting autoradiograph (Fig. 1B), both MspI and BamHI each cut at two sites, resulting in three major bands, two of which were identical in size for each pair of digests, and one of which varied by ~100 bp. The restriction map obtained for the long form corresponded precisely to the map predicted from the EMBL Genebank sequence for the rat D3R (Sokoloff et al., 1990). Comparison of the digests obtained for the two variants suggested that the smaller species contained an approximately 100 bp deletion between +72 and +426.

To confirm the location of the deletion, a series of PCR reactions were performed to generate cDNA fragments of the rat D3R and its smaller form (OS3-OA2; OS1-OA1; OS3-OA1). In all reactions, two products were obtained (data not shown) and these products were subcloned and sequenced (Figs. 2 & 3). Sequence analysis revealed that the D3R-del mRNA contained a 113 bp deletion from +270 to +382. The deleted nucleotides encoded all of the putative first extracellular loop and third transmembrane domain. The deletion altered the reading frame and the predicted sequence contained 19 amino acids in the new reading frame before a stop codon was reached (Fig. 2).

To determine whether the D3R-del mRNA variant was present in the human brain, PCR was performed using the degenerate oligomers OS3 and OA1, with human brain cDNA as a template. As with the rat, two species were identified. The larger cDNA obtained was identical in sequence to the published human D3R (Giros et al., 1990). As found in the rat, the smaller human transcript had a 113 bp deletion at the corresponding

location (Fig. 3). On both sides of the deletion in the human and rat D3R and D3R-del forms, the consensus intron/exon junction sequence, AG/GT, was found.

Discussion

Alternative splicing is emerging as an important mechanism of producing different receptor gene products. Functionally and anatomically distinct glutamate receptor forms, termed "flip" and "flop," are generated by the incorporation of two different short exons (Sommer et al., 1990). $GABA_A$ ß subunit heterogeneity due to alternative splicing has also been reported (Bateson et al., 1991). In the G-protein coupled receptor (GPCR) superfamily, of which the D3R is a member, alternative splicing of the LH receptor gene alters the reading frame, producing a secreted hormone binding protein which lacks transmembrane domains (Tsai-Morris et al., 1990). Two forms of the D2R have been identified which differ by the inclusion of a 29 amino acid segment in the third cytoplasmic loop.

We report the existence of two forms of the D3R mRNA in rat and human. In both species, transcripts have been cloned that have a 113 bp deletion which causes an altered reading frame. These variants appear to be generated by alternative RNA splicing. The existence of three alternatively spliced rat D3R transcripts was recently reported (Giros et al., 1991). One of the variants, which was found in low abundance, contained a small deletion in the second extracellular domain. This transcript retained the usual reading frame. When expressed in COS cells, however, no dopamine binding was obtained. Giros et al. also describe the rat variant we have isolated, which lacks a 113 bp exon. Their sequence, however, has a stop codon where we find ser_{101} (see Fig. 2), and therefore encodes a protein lacking the nineteen carboxy-terminus amino acids predicted by our clone. We have confirmed this sequence in multiple subclones. The discrepancy may be due to the high mutation rate in PCR generated clones (Innis & Gelfand, 1990).

Our finding that the 113 bp deletion has been conserved over the 80 million years of evolution from rodent to man suggests that the encoded variant protein may serve an important cellular function. The novel protein contains only the first two transmembrane domains of the native D3R, and is unlikely to function as a GPCR. Perhaps it serves a regulatory or modulatory role. For some receptors, such as the insulin receptor and the G-protein coupled gonadotropin releasing hormone receptor, dimerization has been reported to be important in cell signaling (Kahn et al., 1982; Gregory et al., 1982). If receptor aggregation plays a role in D3R signalling, one could envision a role for this truncated membrane protein in promoting or inhibiting activation. It has been demonstrated that GPCRs can assemble in-vitro from individually expressed subunits which encode different transmembrane domains (Maggio et al., 1993a; Maggio et al., 1993b). This suggests the possibility that D3R-del may combine with some other GPCR splice variant to form a functional protein. Further studies will be needed to investigate whether co-expression of the truncated D3R-del alters the pharmacology or function of the full length receptor.

Figure 1. Identification of the short rat D3R mRNA variant by PCR and by restriction mapping. A) PCR of coding region. PCR was performed using the OS2-OA3 primers to the amino and carboxy ends of the rat D3R sequence. Following gel electrophoresis through 1% agarose and ethidium bromide staining, two bands were visualized. Markers (M) were BstII digested Lambda DNA. B) Autoradiograph of restriction digests. The two PCR products obtained were excised, purified, cut with either BamH1 or Msp1, [^{32}P] end-labeled using the Klenow fragment. The [^{32}P]-labeled digestion products were electrophoresed through a 6% polyacrylamide gel. (a) and (b) refer to digests of the long and short forms respectively. Markers are Msp1 digested pBR322.

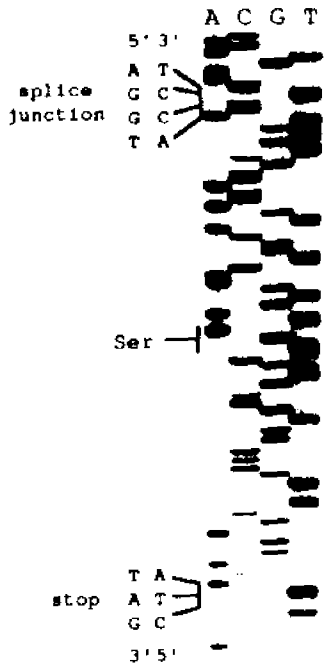


Figure 2. Sequence autoradiograph of the rat D3R-del.

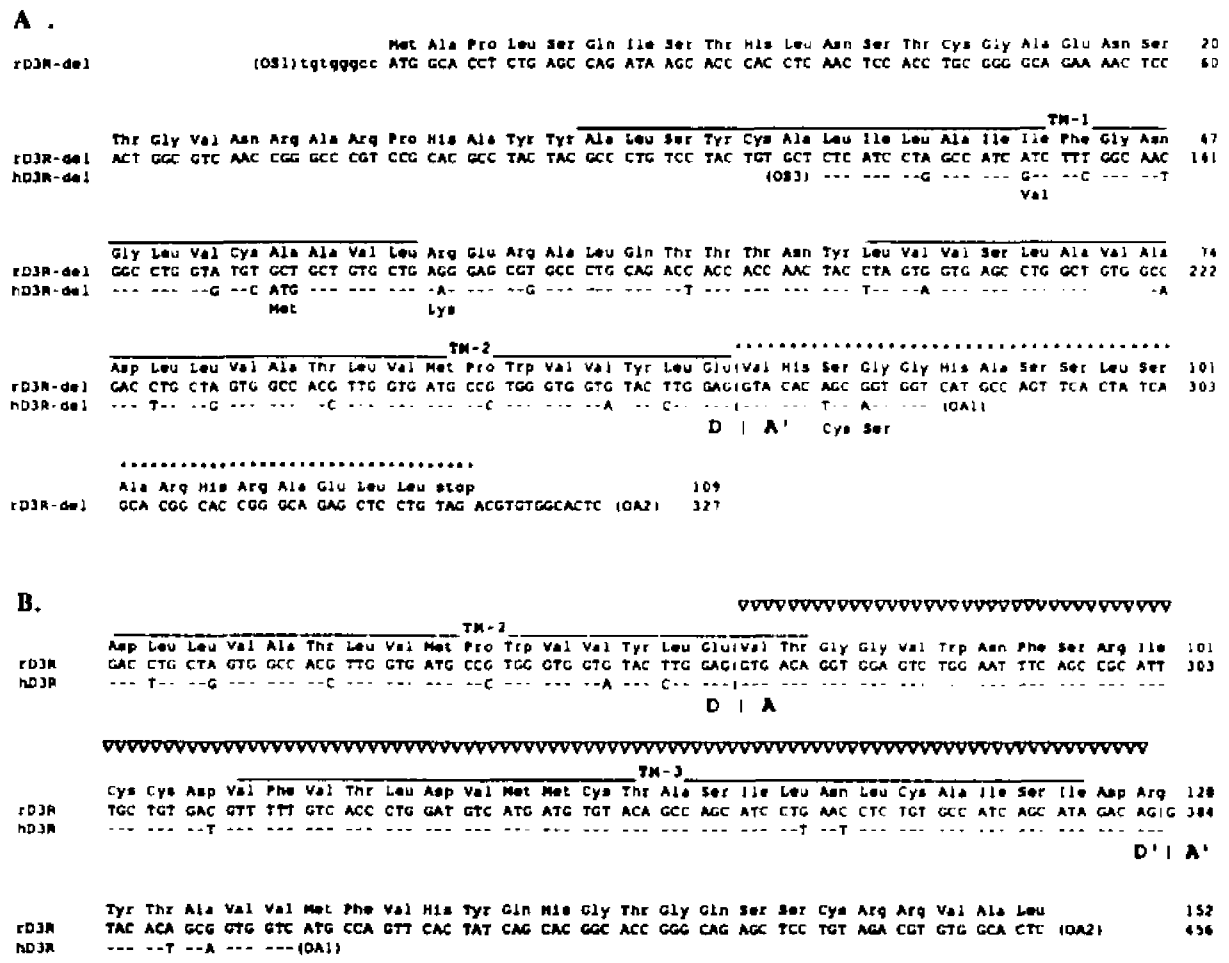


Figure 3. Sequence of rat and human D3R transcripts. The locations of the oligonucleotides used for cloning are denoted within parentheses. Dashed lines indicate nucleotide sequence identity between rat and human. Transmembrane domains are designated by solid bars. Bold type D, D', A, and A' denote consensus splice donor or acceptor sequences. A) Nucleotide and putative amino acid sequence of human and rat D3R-del variants. Vertical bars denote the presumed splice junction site at which 113 bp are deleted, leading to an altered reading frame. The 19 novel amino acids before a stop codon is reached in the rat clone are indicated by asterisks. B) Rat and human D3R sequence encompassing the 113 bp deleted in the D3R-del form. The sequence encodes the previously characterized functional receptor (Sokoloff et al., 1990; Giros et al., 1990). Sequence 5' to transmembrane domain 2 (TM-2) was identical in both splice variants and is omitted.

CHAPTER 6

The Coupling of D2L and D2S to Calcium Influx in AtT20 and GH3 Cell Lines

Abstract

Two dopamine D2 receptors have been identified, D2L and D2S, which vary by the presence or absence of a 29 amino acid segment in the third cytoplasmic loop, a domain implicated in the coupling of G-proteins. In the pituitary, D2 receptors are coupled via pertussis sensitive G-protein to the inhibition of L and T-type calcium channels. They are also coupled to the inhibition of calcium release from intracellular stores by hormones which stimulate IP_3 production. In order to examine the coupling of cloned D2L and D2S receptors to calcium influx and mobilization, stably transfected AtT20 and GH3 cell lines expressing either D2L or D2S were selected. The calcium sensitive fluorescent dye, fluo-3, was used to monitor intracellular calcium levels. Suspensions of fluo-3 loaded cells in cuvets were placed in a spectrofluorimeter equipped with a micro-stirrer. For both AtT20 and GH3 cell lines, the stimulation of D2L and D2S receptors by the D2 agonist quinpirole (QP) was found to inhibit BayK 8644 (BK) stimulated calcium fluorescence. Calcium fluorescence stimulated by K^+ (15 or 30 mM) were also inhibited by QP in cells expressing either isoform. High doses of pertussis toxin (PTX) completely blocked the effect of D2 receptor activation on BK stimulated calcium influx. This result indicates that both isoforms are coupled to the inhibition of calcium influx via G-proteins of the G_o/G_i family. The responses mediated by D2L and D2S were similarly sensitive to PTX. The inhibition of BK stimulated calcium influx by D2 receptor activation was compared to the inhibition of BK stimulated calcium influx by native somatostatin (SS) receptors present in GH3 cell lines. In several different lines expressing D2L or D2S, a maximally effective dose of SS (1 uM) was observed to inhibit BK stimulated calcium fluorescence

to a similar degree as a maximally effective dose of QP (1 uM). When cells from these lines were stimulated by both SS and QP, a lack of additivity of the response to the two drugs was observed. These results suggest that in GH3 cells, D2L, D2S, and SS receptors are coupled to the inhibition of BK stimulated calcium influx via a common G-protein. The effect of D2L and D2S activation on TRH mediated calcium mobilization was also studied in GH3 cells. Stimulation by TRH led to changes in fluorescence characteristic of calcium mobilization by phospholipase C activation. In fluorescence assays this was visualized as a brief "spike" in fluorescence, followed by a prolonged "plateau" phase of fluorescence in which intracellular calcium levels appeared to be moderately elevated. The stimulation of D2L or D2S by QP led to the attenuation of fluorescence during the plateau phase, but not during the initial spike.

Introduction

In the anterior pituitary, it is well established that the actions of dopamine at D2 receptors provide tonic inhibition over prolactin secretion. To overcome domaminergic inhibition, the secretion of prolactin requires the concerted actions of multiple hormones and neurotransmitters acting at the hypothalamus and pituitary. At the cellular level, several hormones, including estradiol, TRH, angiotensin and VIP, are known to act on the lactotroph to directly stimulate prolactin secretion. These hormones generate multiple signals, including cAMP, IP_3, arachidonic acid, and increased electrical activity, to promote secretion by raising intracellular calcium levels. To control prolactin secretion effectively, lactotroph D2 receptors can activate an equally complex array of intracellular signals which act (directly and indirectly) to suppress the elevation of intracellular calcium. These signals include: the inhibition of cAMP accumulation, the inhibition of IP_3 production, the inhibition of calcium influx, and the activation of K^+ efflux. The effects of D2 receptor activation are mediated by coupling to PTX sensitive G-proteins.

The presence of high levels of D2L and D2S in the pituitary is suggested by mRNA analysis (Meador-Woodruff et al., 1989; Snyder et al., 1990). Because there are no pharmacological agents that selectively bind to D2L and D2S, it is impossible to study the subtype specific coupling of these isoforms in the pituitary. Furthermore, there is no evidence to date that the either isoform is expressed exclusively in any tissues or individual cell types. Therefore to study the subtype specific coupling of these receptors it is necessary to transfect individual receptor subtypes into cell lines lacking native D2 receptors. This procedure has been used to examine the coupling of D2L and D2S to the inhibition of cAMP in several different cell lines (Hayes et al., 1992; DalToso et al., 1989, Montmayeur and Borrelli, 1991). There have been two reports in which the coupling of D2L and D2S to the activation of IP_3 production was indicated. In two fibroblast derived cell lines, CHO-K1 and Ltk^-, the stimulation of D2S and D2L caused increases in intracellular calcium characteristic of IP_3 production (Hayes et al., 1992; Liu et al. 1992). These results were surprising because in most studies in which coupling to calcium had been examined, D2 receptor activation was correlated with the attenuation of intracellular calcium levels. The coupling of the D2S isoform to calcium influx, and to the activation of K^+ currents has been examined in several cell lines however the coupling of D2L was not reported in these studies (Vallar et al., 1990; Einhorn et al., 1991; Castellano et al., 1993). Vallar et al., (1990) reported that D2S activation decreased intracellular calcium levels in the GH4-C1 cell line. This was the first and only study in which the inhibition of calcium influx by a transfected D2 receptor subtype has been reported. (Unfortunately, the coupling of D2L was not examined in this study.) It is significant that the D2 mediated inhibition of calcium influx, which is typically seen in lactotrophs, was observed in the GH4-C1 cell line. This cell line was originally derived from a somato-lactotroph precursor cell.

To investigate subtype specific modulation of intracellular calcium, AtT20 and GH3 cell lines were stably transfected with either D2L or D2S. These two cell lines, both of

pituitary origin, were chosen with the expectation that the coupling of D2L and D2S would be more likely to reflect the coupling of lactotroph D2 receptors in these particular cell lines than in cell lines derived from other tissue types. This was of particular concern because of observation that D2L and D2S activation resulted in the elevation of intracellular calcium levels by stimulating IP_3 production in fibroblast cell lines (see above). Two different cell lines were chosen for transfection with the anticipation that phenotypic differences in the components of signal transduction (i.e. receptors, G-proteins, and intracellular effectors) could potentially aid in the identification of subtype-specific differences in signalling. AtT20 and GH3 cells have distinct characteristics which probably reflect differences in the origin of the two cell lines: the AtT20 cell line was derived from a corticotroph precursor, while the GH3 cell line was derived from a somato-lactotroph precursor.

Intracellular calcium was studied via a spectrofluorimetric assay using the calcium sensitive dye fluo-3. The inhibition of calcium influx by D2L or D2S activation was examined in AtT20 and GH3 cells stably expressing either isoform. K^+ depolarization, and the L-type calcium channel agonist (BayK 8644) were used stimulate calcium influx in fluo-3 loaded cells. The inhibition of TRH stimulated calcium influx and of calcium release from intracellular stores was also examined in fluo-3 loaded GH3 cells stably expressing D2L or D2S. The coupling of either D2L or D2S to cAMP was not examined since this has been previously studied in several different cell lines (see above).

Some of the findings presented here have appeared in a preliminary report (Society for Neuroscience abstract 281.18, 1992).

Methods

Materials

Reagents and drugs were obtained from the following sources: fluo-3- acetoxymethyl ester (AM) and plueronic acid, were purchased from Molecular Probes (Eugene OR, USA); BSA (fraction V), mianserin hydrochloride, and probenecid were from Sigma; (+)butaclamol hydrochloride, S(-)BayK 8644 (BK), and (-)quinpirole (QP) were from Research Biochemicals International (Natick, MA); thyrotropin releasing hormone (TRH) and somatostatin - cyclic (SS) was purchased from Bachem California (Torrance, CA); pertussis toxin (PTX) was from List Biological Laboratories (Wayland, MA); ^{3}H-spiperone- 21 Ci/mM was from Du Pont - New England Nuclear (Boston, MA). Lipofectin, Dulbecco's modified essential medium (DMEM-cat# 12-604B) with L-glutamine and 4g glucose/l, fetal calf serum, horse serum, and G418, were from GIBCO-BRL (Grand Island, NY). Fluorescence was monitored with a Perkin-Elmer LS-5 fluorimeter equipped with a Perkin-Elmer GP-100 graphics plotter, and a micro-stirrer from Helma (Jamaica, NY). Disposable methacrylate cuvets were purchased from Fisher Scientific (Pittsburgh, PA). A Brandell cell harvester was used with Brandell glass-fiber filter strips for receptor binding assays.

Selection of cell lines.

AtT20 and GH3 cells were grown to approximately 60% confluence in 100 mm tissue culture dishes. For each dish of cells, 10 ug Lipofectin was used to transfect 5ug of an expression vector construct containing the neomycin resistance gene and 5ug of an expression vector construct containing either the D2L or the D2S coding region (for construct details see McChesney et al. 1991). Cells were incubated with the Lipofectin and DNA plasmids for 8 hours at 37 C, 10% CO_2, followed by incubation in fresh media overnight. For a period of 2-4 weeks, the transfected cells were cultured in medium containing 800 ug/ml G418, after which individual colonies were picked and subcultured.

D2 receptor expressing cell lines were identified by receptor binding assays (and calcium fluorescence assays) as described below.

Cell culture and harvest

Cells were routinely grown to ~80-90% confluence in 60 mm or 100 mm tissue culture plates at 37 C with 10% CO_2 in DMEM supplemented with 7% fetal calf serum and 7% horse serum . For binding and fluorescence assays, cells were harvested by treatment for 2 minutes with PBS/1mM EDTA, followed by aspiration, the addition of 10 ml of fresh media, and tituration of the cells from the dishes. The cells were then transferred to petri dishes to prevent reattachment, and were incubated for approximately two hours at 37 C with 10% CO_2. Following incubation, cells were transferred to disposable 15 ml tubes and collected by centrifugation at 300g for 2 min. The pelleted cells were then either frozen at -20 C for subsequent receptor binding assays, or resuspended in buffer appropriate for loading cells with fluo-3 AM (described below).

Receptor binding assays

Single point saturation binding assays were performed for the purpose of screening subclones for the expression of either D2L or D2S. Scatchard analysis was used to more precisely determine the receptor levels of cell lines chosen for further study. Membranes were prepared from previously frozen cells as follows. Frozen cell pellets were thawed on ice and transferred to a dounce homogenizer. The pellets were homogenized 20 strokes in 4 ml of lysis buffer (5 mM Tris pH 7.4, 2 mM EDTA). The homogenates were spun at 16,000 g for 25 minutes at 4 C), the resulting pellets were resuspended in 4 ml lysis buffer, and spun again (15,000 x g, 25 min., 4 C). The pellets were resuspended in binding buffer (50 mM Tris pH 7.4, 1 mM EDTA, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM, $MgCl_2$, 120 mM NaCl) to a concentration of 1 mg/ml. For single point assays, 100 ug of membranes (duplicate or triplicate samples) were incubated with 1 nM ^{3}H-spiperone in

the presence or absence of 1 uM (+)butaclamol (to determine non-specific binding) for 1h at room temperature, in a total volume of 1 ml. For Scatchard analysis, membranes (quadruplicate samples) were incubated with ^{3}H-spiperperone (0.03 - 2 nM). Incubations were terminated by rapid vacuum filtration through Whatman GF-C filter strips using a Brandell cell harvester, followed by 5 rinses with 4 ml of ice cold buffer (50 mM Tris pH 7, 1 mM EDTA). ^{3}H-spiperone bound to filters was counted in a Beckman liquid scintillation counter. Protein concentrations were determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as a standard. Note: 1 uM mianserin was included in initial assays to block potential ^{3}H-spiperone binding to serotonin receptors, however, since no difference in binding was seen in the presence or absence of mianserin, it was concluded that AtT20 and GH3 cell lines did not have these serotonin receptor subtypes, and was not included in subsequent assays.

Solution hybridization/nuclease protection (SHNP) assays

Total RNA was prepared from cells and SHNP assays were performed using methods described previously (see chapter 2, Snyder et al. 1991).

Fluorescence assay of Ca^{+2}

For loading with fluo-3 AM, cells were harvested (as described above) and resuspended in buffer (26 mM HEPES, 117 mM NaCl, 5.3 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2,PO_4 5.6 mM d-glucose, 2mM $CaCl_2$, 0.1% BSA, 2.5 mM probenecid) to a density of ~1 x 10^6 cells /ml. 2.5 mM probenecid was added to the buffer to prevent leakage of the dye from the cells (Merritt et al, 1990). Cells suspensions were incubated for 1 hour at 37 C in buffer containing 2uM fluo-3 AM and 0.02% plueronic acid, followed by centrifugation at 300 g for 2 minutes. The pelleted cells were resuspended in fresh buffer and were incubated for 15 - 30 minutes at room temperature to remove extracellular traces of fluo-3. The cells were again centrifuged at 300 g for 2 minutes,

followed by resuspension in fresh buffer to a density of 0.5 - 1 x 10^6 cells /ml. Each 60 mM dish of cultured cells harvested generally provided 4 - 6 ml of fluo-3 loaded cells, while 100 mM plates provided 10-12 ml of fluo-3 loaded cells. The cells remained at room temperature until use (up to 1 hour), at which time 2 ml of cells was transferred to a cuvet and a miniature stir bar was added. Calcium fluorescence was monitored at 500 nm excitation and 530 nm emission. The spectrofluorimetric output was recorded by chart recorder. For all samples, baseline fluorescence was measured for 1- 3 minutes before the addition of BK, K^+, or TRH (experiments in which calcium influx was stimulated by K^+ or TRH are discussed below). BK (1 uM final) was added to stimulate calcium influx through voltage gated calcium channels. 2 - 3 minutes after the addition of BK, the D2 receptor agonist, QP was added (1 uM final). 100X stocks of BK and QP were added directly to the cell suspensions. Fluorescence was recorded for 1 - 3 minutes following the addition of QP. Maximum inhibition of BK stimulated fluorescence was generally attained within 0.5 minute of QP addition. For experiments examining the effects of SS on BK stimulated fluorescence, a 100X stock of SS was added to cell suspensions to achieve a final concentration of 1 uM.

The % inhibition by QP of BK stimulated fluorescence was determined as follows: [(fluorescence 0.5 min after the addition of QP - (extrapolated) baseline fluorescence 0.5 min after the addition of QP) / (fluorescence at the time of QP addition - (extrapolated) baseline fluorescence at time of QP addition)] x 100. The % inhibition by SS of BK stimulated fluorescence was determined in the same manner.

PTX sensitivity

Cells grown in 60 mm plates to ~80% confluency were incubated (10% CO_2,, 37 C) for 16 h with PTX (0.01 - 100 ng/ml). Following PTX pretreatment, cells were harvested and loaded with fluo-3 as described above. The inhibitory effect of 1 uM QP on BK stimulated fluorescence was determined as described above. For each concentration of

PTX tested, 2 60 mm plates of cells were treated, and 2-3 measurements (of QP inhibition of BK stimulated fluorescence) were obtained from each plate of cells; measurements obtained from the two plates of cells were averaged. The inhibition of BK stimulated fluorescence by QP of PTX treated cells was expressed as the percentage of the maximum inhibition of BK stimulated fluorescence. Maximum inhibition of BK stimulated fluorescence was generally attained in untreated control cells and in cell treated with concentrations of PTX < or = to 0.1 ng/ml.

Dopaminergic modulation of calcium influx in the presence of elevated K^+

The inhibition of depolarization induced calcium influx by 1 uM QP was examined in AtT20 and GH3 cell lines expressing D2L or D2S. Cells were harvested and loaded with fluo-3 as described above. In one type of experiment, the baseline fluorescence of cells was established, then a stock solution of 2.5 M KCl was added (directly to cuvets containing cell suspensions) to achieve one of the following concentrations: 15, 30, 55, or 105 mM. Following KCl addition, fluorescence was monitored 2-4 minutes until it stabilized (at a higher level - due to the opening of voltage gated calcium channels resulting from depolarization). The ability of D2L and D2S to modulate the K^+ stimulated calcium influx was then tested by adding QP to the cell suspensions and monitoring fluorescence for several minutes. In a variation of the experiment described above, 1 uM BK was added after KCL, followed by 1 uM QP 2-3 minutes later. In a second type of experiment, aliquots of fluo-3 loaded cells were divided, with half resuspended in their usual (5 mM K^+) buffer, and half resuspended in the usual buffer with extra KCl added (55 - 125 mM), and with a compensatory decrease in NaCl - to maintain the normal osmolarity. These pairs of samples were monitored in the standard way (as described earlier: baseline fluorescence was recorded 1-3 minutes, 1 uM BK was added, followed 2-3 minutes later by the addition of 1 uM QP.

Dopaminergic modulation of TRH stimulated calcium fluxes

In experiments with GH3 cell lines examining the ability of D2L and D2S to modulate TRH stimulated calcium fluxes, suspensions of fluo-3 loaded cells were monitored 1-3 minutes to establish baseline fluorescence. QP (or nothing for controls) was added to cell suspensions 1 -2 minutes prior to the addition of 1 uM TRH. The cells were monitored several minutes after the addition of TRH to record the initial "spike" in fluorescence due to the mobilization of calcium from intracellular stores, and to record the subsequent "plateau" of fluorescence due to the entry of extracellular calcium. The heights of calcium "spikes" and "plateaus" obtained in the presence or absence of 1 uM QP were compared. In some cases QP was added several minutes after TRH stimulation in order to further examine its effect on calcium fluorescence during the "plateau" phase.

A two tailed Student's T test was used to determine whether there was a statistically significant difference between TRH stimulated calcium spikes in the presence or absence of QP. The ability of D2L or D2S to modulate TRH stimulated calcium influx was assessed by the qualitative comparison of fluorescence recorded in the presence or absence of 1 uM QP during the plateau phase.

Results

Cell line selection and initial characterization of D2 agonist and antagonist sensitivity.

AtT20 cell lines selected for G418 resistance were initially screened for the presence of D2L or D2S receptors by fluorescence assays. 1 uM QP was found to inhibit BK stimulated fluorescence in approximately half of the G418 selected cell lines. The inhibition of BK stimulated fluorescence by QP was blocked by the prior addition of the D2 receptor antagonist +butaclamol (2 uM - data not shown). ^{3}H-spiperone binding assays were subsequently performed to confirm the presence of transfected receptors.

The lack of D2 receptors in wild type AtT20 and GH3 cells was supported by the lack of QP sensitivity in fluorescence assays, and by the absence of ^{3}H-spiperone binding sites. 10 different transfected cell lines expressing D2L or D2S were identified. There were no pairs of cell lines identified which expressed identical levels of D2L and D2S receptors. Four cell lines were chosen for further study, AL13 and AL16, expressing the D2L receptor, and AS1 and AS5, expressing the D2S receptor (see Table 1)

The selection of GH3 cell lines expressing transfected D2 receptors proved to be more difficult. Non-transfected GH3 cells displayed greater resistance to G418 toxicity than did non-transfected AtT20 cells. G418 resistant GH3 cells (from non-transfected control plates) displayed characteristics not seen in wild type GH3 cells, such as more rapid growth rates, lack of calcium mobilization in response to thyrotropin releasing hormone (TRH) stimulation, and altered morphology - the cells were more rounded and appeared less differentiated. The cells also tended to grow in piles rather than spread out on the plates. Thus for the selection of D2 expressing cell lines, most of the colonies picked for expansion were chosen because they displayed growth and morphological characteristics similar to wild-type GH3 cells. The expanded clones were screened by fluorescence assays for sensitivity to QP (inhibition of BK stimulated fluorescence) and for sensitivity to TRH. The results of these assays indicated that both D2L and D2S were coupled to the inhibition of BK stimulated calcium influx in transfected GH3 cells. In the presence of 2 mM +butaclamol, QP had no effect on BK stimulated fluorescence. Selected GH3 cell lines were also screened for ^{3}H-spiperone binding (data not shown). Of 26 selected lines, 11 displayed TRH responses similar to wild-type GH3 cells; most of these lines also displayed growth rates and morphological characteristics similar to wild-type GH3 cells. Of the GH3 clones with TRH responses, four had high QP responses and relatively high ^{3}H-spiperone binding; these were chosen for the comparison of D2L and D2S mediated signal transduction (see Table 1).

Since AtT20 and GH3 cells possessed endogenous somatostatin (SS) receptors which (similar to D2 receptors) had been reported to inhibit calcium influx (Israel & Vincent, 1990), the inhibition of BK stimulated fluorescence by SS was tested in non-transfected and transfected cell lines. The initial reason for testing the SS responsiveness was to determine whether unintended variability in cell signalling might have occurred during the process of cell line selection (with G418) - variability other than the loss of TRH responsiveness observed in some GH3 cell lines. In wild-type AtT20 and GH3 cells, stimulation by SS led to complete attenuation of BK stimulated fluorescence. The SS response (like the QP response) was PTX sensitive, indicating coupling via G-proteins of the G_i/G_o family. AtT20 lines selected for D2 expression displayed wild-type SS responses. In contrast, GH3 lines selected for D2 expression displayed a wide range of SS responsiveness, ranging from ~20 - 80% inhibition of BK stimulated fluorescence. Interestingly, lines displaying low SS responses also displayed low or no QP response, while lines displaying high SS responses displayed a full range of QP responses. This result suggested that lines with low SS and QP responses may have had lower levels of a G-protein to which these receptors are coupled. This hypothesis was supported by the finding that in experiments with several GH3 cell lines expressing similar levels of D2L (as measured by ^{3}H-spiperone binding), large differences in the ability of QP to inhibit BK stimulated fluorescence was observed. The same was found for several GH3 lines expressing D2S.

Because some lines have been reported to develop D2R expression with growth factor treatment (Missale et al, 1991) solution hybridization /nuclease protection assay was performed with several transfected cell lines (Fig. 1). As anticipated, each cell line examined expressed either D2L or D2S mRNA which were represented on the resulting autoradiograms by protected bands at 76 and 143, or 219, respectively.

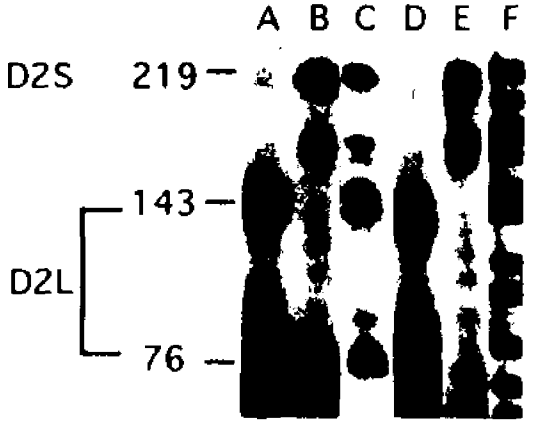


Figure 1. Autoradiogram of a SHNP assay of D2L and D2S mRNA. Lanes A-C represent SHNP assays of total RNA samples were derived from the following sources: A, a cell lines stably expressing the D2L receptor; B, a cell line stably expressing the D2S receptor; C, rat striatum. Lanes D and E show the SHNP assay of synthetic RNA standards representing D2L and D2S, respectively. Size markers (Msp1 digested PBR322) are in lane F.

AtT20	fmol/mg	GH3	fmol/mg
AS1	-866 ± 90	GS23	80 ± 8
AS5	452 ± 58	GS103	92 ± 8
AL13	1678 ± 173	GL13	-269 ± 32
AL16	57 ± 4	GL14	-314 ± 29

Table 1. Receptor levels of cell lines selected for studies comparing the PTX sensitivity of D2L and D2S. Receptor levels are expressed as fmol/mg protein. AtT20 receptor levels represent the averages obtained from 2 - 3 different Scatchard experiments, while GH3 receptor levels represent the averages obtained from 3 -5 different Scatchard experiments. In each experiment quadruplicate determinations of specific and nonspecific binding were carried out for each 3H-spiperone concentration.

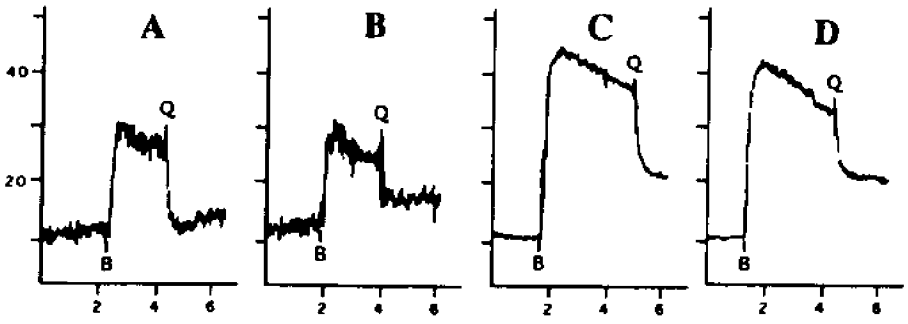


Figure 2. The inhibition of BK stimulated fluorescence by the activation of D2L or D2S in selected AtT20 and GH3 cell lines. Fluorescence is recorded in arbitrary units on the y axis; time is recorded in minutes on the x axis. 1 uM BK, which was used to stimulate fluorescence, was added to fluo-3 loaded cell suspensions as indicated. The fluorescence recordings shown are of two AtT20 cell lines expressing A) D2L (AL16), and B) D2S (AS5) and of two GH3 cell lines expressing D) D2L (GL13) and E) D2S (GS103).

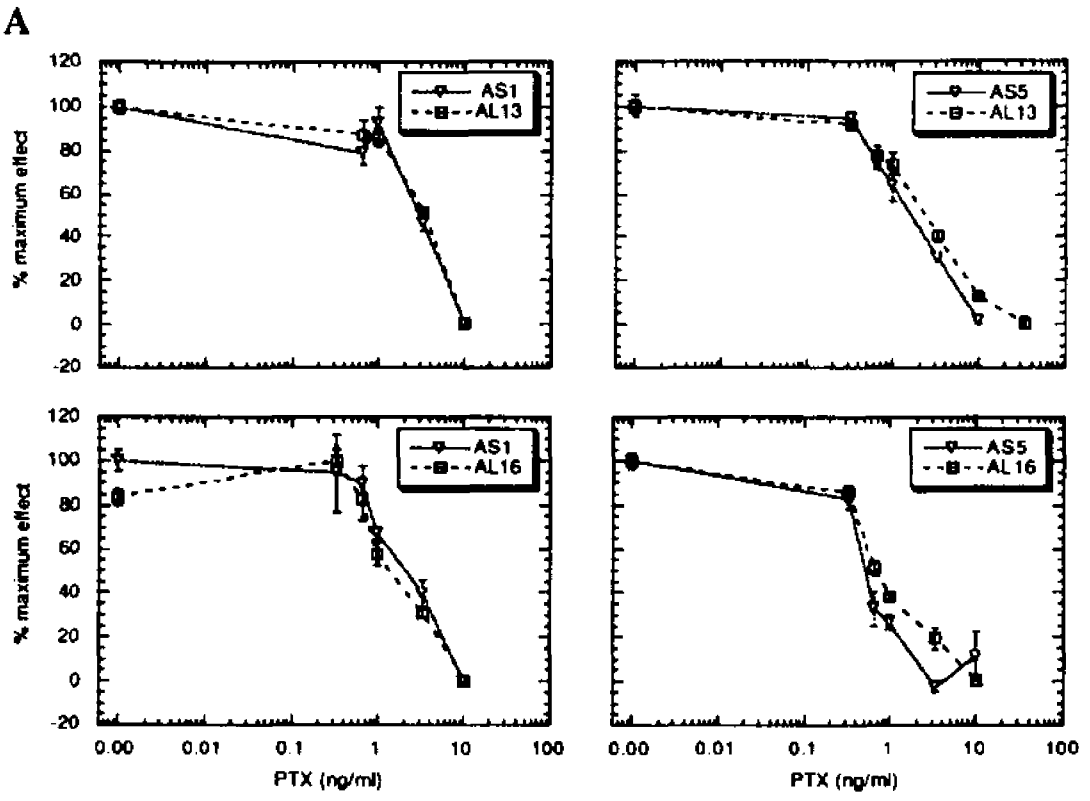
The inhibition of BK stimulated fluorescence by D2L or D2S activation is sensitive to PTX pretreatment

D2L and D2S were coupled to the inhibition of BK stimulated fluorescence in all of the stably transfected AtT20 and GH3 cell lines (Fig 2). The PTX sensitivity of D2L and D2S inhibition of BK stimulated calcium influx was examined. PTX provides an indirect means of comparing the G_o/G_i family G-proteins mediating the response obtained by activation of each receptor. PTX, which irreversibly inactivates G-proteins of the G_i/G_o) family via ADP-ribosylation, has been used as to examine differences in the G-protein coupling of the two receptors. In JEG-3 cells, differences in the PTX sensitivity of D2L and D2S were reported by Montmayeur and Borrelli (1991). These investigators indirectly studied the coupling of the two isoforms to the inhibition of cAMP accumulation by examining the ability of D2L or D2S to modulate transcription of a gene coupled to a cAMP sensitive promoter. Interestingly, D2S was nearly 100X less sensitive to PTX treatment, however, it was only slightly more effective (~20%) than D2L at inhibiting gene transcription from the cAMP sensitive promoter.

To investigate possible differences in G-protein coupling of the D2L and D2S in AtT20 and GH3 cells, we examined the ability of the two isoforms to inhibit BK stimulated calcium influx after PTX pretreatment. Cells incubated for 16 h with increasing concentrations of PTX (0.01-100 ng/ml) were harvested, loaded with fluo-3, and assayed for the ability of 1 uM QP to inhibit BK stimulated Ca^{+2} fluorescence. Because the maximum inhibition of BK stimulated fluorescence by QP varied in the different cell lines, the results (see Fig. 3) were expressed as % maximum effect (effect = inhibition of BK stimulated fluorescence by 1 uM QP). A maximum effect was seen in untreated control cells and in cells treated with concentrations of PTX $\leq$ 0.1 ng/ml. There was no evidence for an effect of PTX at concentrations $\leq$ 0.1 ng/ml. Therefore, in order to provide a more accurate estimate of the maximum effect, values (for the inhibition of BK stimulated fluorescence by QP) obtained at PTX concentrations $\leq$ 0.1 ng/ml were

averaged. The average value was used for normalization of the curves (see Fig. 3). In general, the attenuation of QP responses by PTX was evident at concentrations > 0.1 ng/ml, while the maximal attenuation of QP responses was achieved at PTX concentrations ≥ 10 ng/ml. For each experiment a single pair of cell lines expressing D2L and D2S was compared. In stably transfected AtT20 cell lines, no difference was observed in the PTX sensitivity of D2L and D2S mediated signaling (Fig. 3A). In GH3 cell lines, differences in PTX senstivity were minimal. The slight (~ 3-fold) increase in the sensitivity of D2S observed in one experiment most likely represents experimental variation as no differences in sensitivity were observed in the other three experiments performed. The similar PTX sensitivity of D2L and D2S coupling to calcium channels in GH3 and AtT20 cells contrasts with the 100-fold difference in PTX sensitivity of D2L and D2S coupling to cAMP in JEG-3 cells (Montmayeur and Borrelli, 1991). The results of the present study are consistent with the hypothesis that D2L and D2S are coupled to the inhibition of calcium influx by the same G-protein in AtT20 and GH3 cells.

Figure 3. The effects of varying concentrations of PTX on the inhibition of BK stimulated fluorescence by 1 uM QP. Each graph represents a single experiment comparing two cell lines; one expressing D2L and one expressing D2S. The concentration of PTX used to pretreat cells (for 16 hours) is indicated by the x axis. The results were expressed as the % maximum effect (y axis) with effect = inhibition of BK stimulated fluorescence by 1 uM QP. A) Two AtT20 lines expressing D2L (AL13 & AL16) were alternatively compared with two different AtT20 lines expressing D2S (AS1 & AS5). B) Two GH3 lines expressing D2L (GL13 & GL14) were alternatively compared with two different GH3 lines expressing D2S (GS23 & GS103).


A
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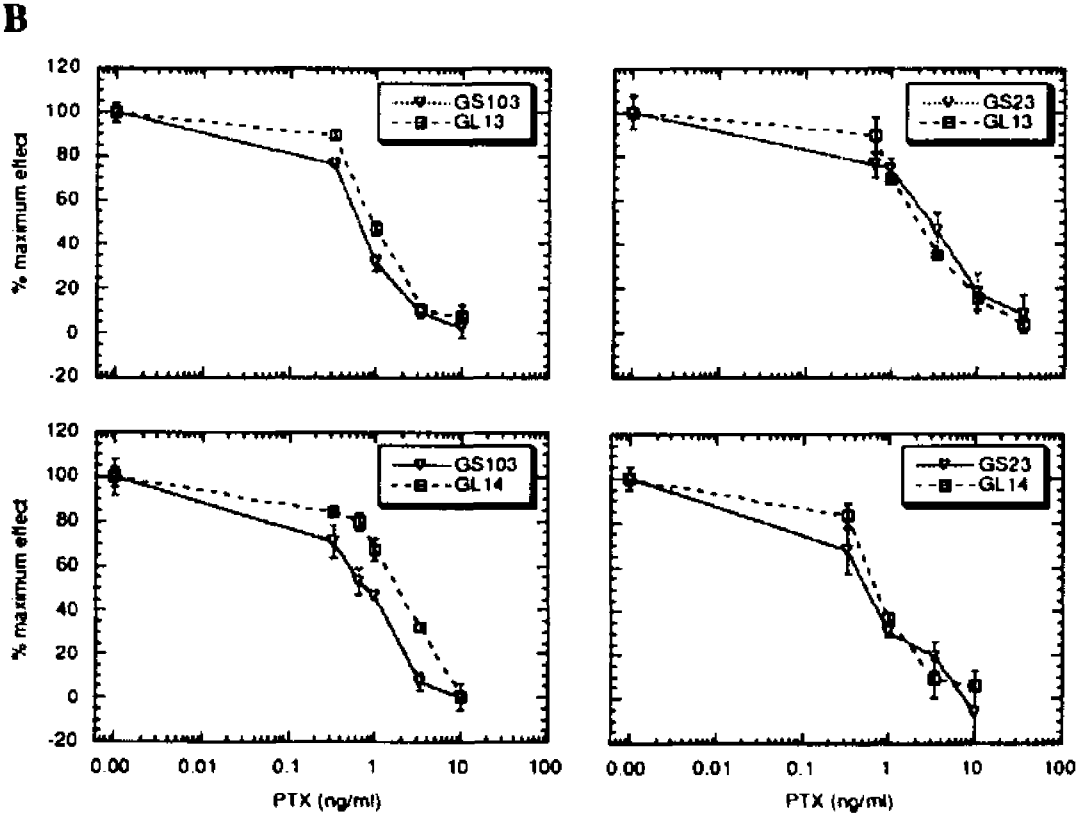
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D2L or D2S activation attenuated calcium fluorescence stimulated by [K^+] < 55 mM; K^+ or BK stimulated calcium fluoresecence were not attenuated at [K^+]≥ 55mM.

Cell depolarization results in calcium influx in cells possessing voltage gated calcium channels. Because depolarization is a normal physiological modulator of calcium channels (unlike BK), it was of interest to examine the effects of D2L or D2S receptor activation on depolarization induced calcium influx. In these experiments varying concentrations of extracellular KCl were used to depolarize cells. The addition of KCl directly to fluo-3 loaded cell suspensions, caused a rapid increase in fluorescence, which generally decreased (10-50%) over a period of several minutes before stabilizing. In a series of experiments, suspensions of fluo-3 loaded AtT20 or GH3 cells (expressing either D2L or D2S) were depolarized by the addition of KCl to one of the following concentrations: 15, 30, 55, or 105 mM. KCl was added directly to cell suspension in cuvets. After fluorescence had stabilized (typically 2-4 minutes after KCl depolarization), 1 uM QP was added to the cell suspensions. At KCl concentrations of 15 and 30 mM, the addition of QP led to a rapid decrease in fluorescence by ~25 - 75% (Fig 4A). The decrease in fluorescence by QP was greater in cells stimulated with 15 mM KCl than with 30 mM KCl.. When cells were depolarized by the addition of 55 or 105 mM KCl, no decrease in fluorescence was observed upon the addition of QP (Fig 4 B&C). In a second set of experiments, cells were resuspended in a high-K^+ buffer (55 or 105 mM) in which the concentration of Na^+ was reduced to maintain osmolarity. No attenuation of K^+ stimulated fluorescence was observed upon the addition of 1 uM QP. This result was observed for AtT20 and GH3 cells expressing either D2L or D2S. In a variation of this experiment, aliquots of fluo-3 loaded cells were divided into two samples, each pair of sample of cells was spun and resuspended in new buffer. For each pair of samples, one sample was resuspended in buffer containing 5 mM K^+, and one sample was resuspended in buffer containing either 55 or 105 mM K^+,. 1 uM BK was used to stimulate calcium influx. 2-3 minutes after the addition of BK, the inhibition of

fluorescence by 1 uM QP was tested. QP caused a decrease in fluorescence only in cells resuspended in the 5 mM K^+ buffer, but not in cells resuspended in the high K^+ buffer (Fig. 4 D-G). It was observed that 1 uM SS was also ineffective in lowering fluorescence in the presence of 105 mM KCl (Fig. 4F).

The inability of QP to attenuate fluorescence in the presence of KCl > 55 mM suggested that both D2L and D2S were uncoupled to the inhibition of calcium influx in the presence of high K^+. This may indicate that the inhibition of calcium influx by D2L and D2S occurs secondarily to the activation of K^+ efflux. K^+ efflux would be expected to hyperpolarize the cell, leading to the closure of voltage gated calcium channels. Alternatively, the coupling of D2L and D2S to calcium channels may be effected at elevated membrane potentials as has been observed for other GPCRs. These possibilities are discussed in greater detail below in the Discussion.

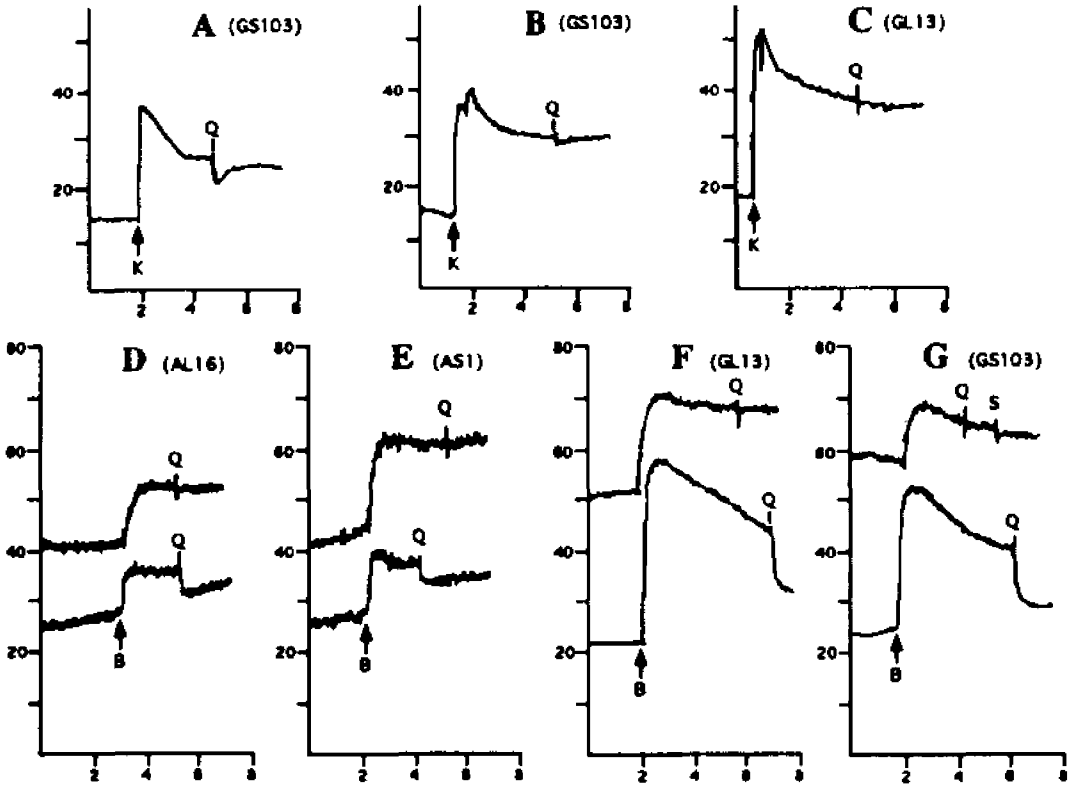

Figure 4. The inhibition of calcium influx by D2L and D2S in K^+ depolarized cells. Fluorescence is recorded in arbitrary units on the y axis. Time is recorded in minutes on the x axis. Calcium influx in fluo-3 loaded cells was stimulated by the addition of KCl (A-C) or 1 uM BK (D-G). 1 uM QP and 1 uM BK were added as indicated by Q or B, respectively. Cells in (A-C) were resuspended in buffer containing 5 mM K^+. They were depolarized by the addition KCl (K). A) GS103 cells were depolarized by the addition of KCl to a final concentration of 15 mM. B) GS103 cells were depolarized by the addition of KCl to a final concentration of 55 mM. C) GL13 cells were depolarized by the addition of KCl to a final concentration of 55 mM. Note; the addition of QP attenuated fluorescence when the extracellular K^+ concentration was 15 mM (A), but not when the extracellular K^+ concentration was 55 mM (B&C). Aliquots of cells in (D-G) were divided and resuspended in either buffer containing 5 mM K^+ (lower traces), or in buffer containing 55 mM K^+ (upper traces). The following cell lines were used: (D) AL16; (E) AS1; (F) GL13; (G) GS103. Note that no attenuation of BK stimulated fluorescence was observed in cells resuspended in buffer containing 55 mM K^+.

TRH induced calcium release from intracellular stores was not affected by the activation of D2L or D2S. TRH induced calcium influx was inhibited by both isoforms.

The inhibition of TRH induced calcium fluxes was examined in fluo-3 loaded GH3 cells expressing either D2L or D2S. TRH stimulation of GH3 cells has been shown to cause the rapid mobilization of calcium from intracellular stores via the generation of IP_3, followed by the influx of extracellular calcium (Rebecchi & Gershengorn, 1983; Albert & Tashjian, 1984). In fluorescence recordings of fluo-3 loaded GH3 cell suspensions, the initial release of calcium appeared as a sharp peak or "spike", lasting approximately 1 minute, while the entry of extracellular calcium, which has been termed the "plateau phase", appeared as a bell-shaped curve of lower magnitude which generally lasted several minutes (Fig.5). The ability of D2L and D2S to modulate each of these stages of calcium fluorescence was tested. The magnitude of fluorescence during TRH induced calcium spikes was unaffected by the prior addition of 1 uM QP to suspensions of cells expressing either D2L (results not shown) or D2S (Fig. 5 A&B). However, the magnitude of fluorescence during the plateau phase was significantly attenuated (Fig. 5, A&B). In addition, when QP was added during the plateau phase, calcium fluorescence was rapidly reduced (Fig. 5 C&D).

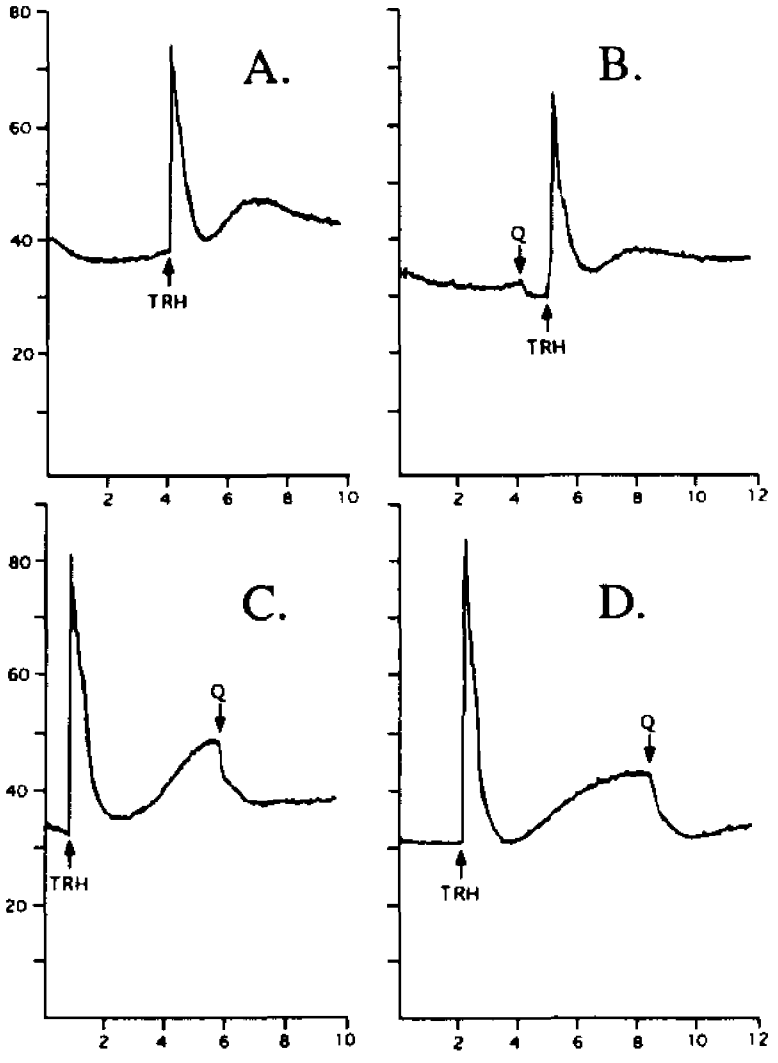

Figure 5. Stimulation with TRH and QP of GH3 cell lines expressing either D2S (A-C) or D2L (D). Fluorescence is recorded in arbitrary units on the y axis. Time is recorded in minutes on the x axis. The addition of 1 uM TRH or of 1 uM QP (Q) is indicated.

The inhibition of BK stimulated fluorescence by SS and QP are not additive in GH3 cell lines expressing either D2L or D2S.

In GH3 cells, the activation of native SS receptors results in the inhibition of calcium influx (Kleuss et al., 1991). This response is specifically mediated by the coupling of SS receptors to the G-protein Go (Kleuss et al., 1991; Kleuss et al., 1992; Kleuss et al., 1993). By examining the additivity (or lack of additivity) of calcium influx inhibition by SS and QP in transfected GH3 cell lines, we hoped to gain insight into the G-protein coupling of D2L and D2S. If the responses mediated by SS and QP were mediated by different G-proteins, then it would be predicted that stimulation of cells with SS and QP might increase the total inhibition of BK stimulated fluorescence in an additive fashion. A lack of additivity might indicate the coupling of both receptors to the same G-protein. This result would not be completely unexpected since it has been observed that the inhibition of calcium influx by many GPCRs is mediated by coupling to the G_o G-protein (Hesheler et al., 1987; Kleuss et al., 1991; Lledo et al., 1992; Taussig et al., 1992).

The inhibition of BK stimulated fluorescence by SS was observed in non-transfected AtT20 and GH3 cells, and in cell lines selected for the expression of D2L or D2S. The activation of SS receptors in AtT20 cells lines led to the complete inhibition of BK stimulated fluorescence (data not shown) making these cell lines unsuitable for the study of SS and QP additivity. In contrast, SS stimulation of stably transfected GH3 cells led to varying degrees of inhibition of BK stimulated calcium fluorescence, depending on the particular cell line tested (as discussed above in *cell line selection*). One possible explanation for the variation in SS responsiveness is that during cell line selection and subsequent passage, the growth of cells expressing lower levels of G-proteins of the G_i/G_o family was favored. The selection of GH3 cell lines with less than maximal inhibition of BK stimulated calcium fluorescence was fortuitous since it permitted us to examine the additivity of QP and SS responses. In several cell lines, the activation of SS receptors, or

transfected D2 receptors (either D2L or D2S) resulted in a 60-80% decrease in BK stimulated fluorescence (Fig. 6). From the magnitude of these responses it would be predicted that the co-stimulation of D2 and SS receptors would result in the complete inhibition of BK stimulated fluorescence, if the responses mediated by each receptor were independent of each other. However, when cells were stimulated by both QP and SS, the complete inhibition of BK stimulated fluorescence was not observed (Fig. 6). In some cases, the stimulation with QP and SS resulted in a slight increase in the total inhibition (of BK stimulated fluorescence) above the inhibition (of BK stimulated fluorescence) mediated by either agonist alone (Fig. 6 C&D), however, there were no cases in which the total inhibition (of BK stimulated fluorescence) was fully additive.

The inhibition of BK stimulated fluorescence by SS was completely blocked by pretreatment of cells for 16 h with 10 ng/ml PTX (data not shown).

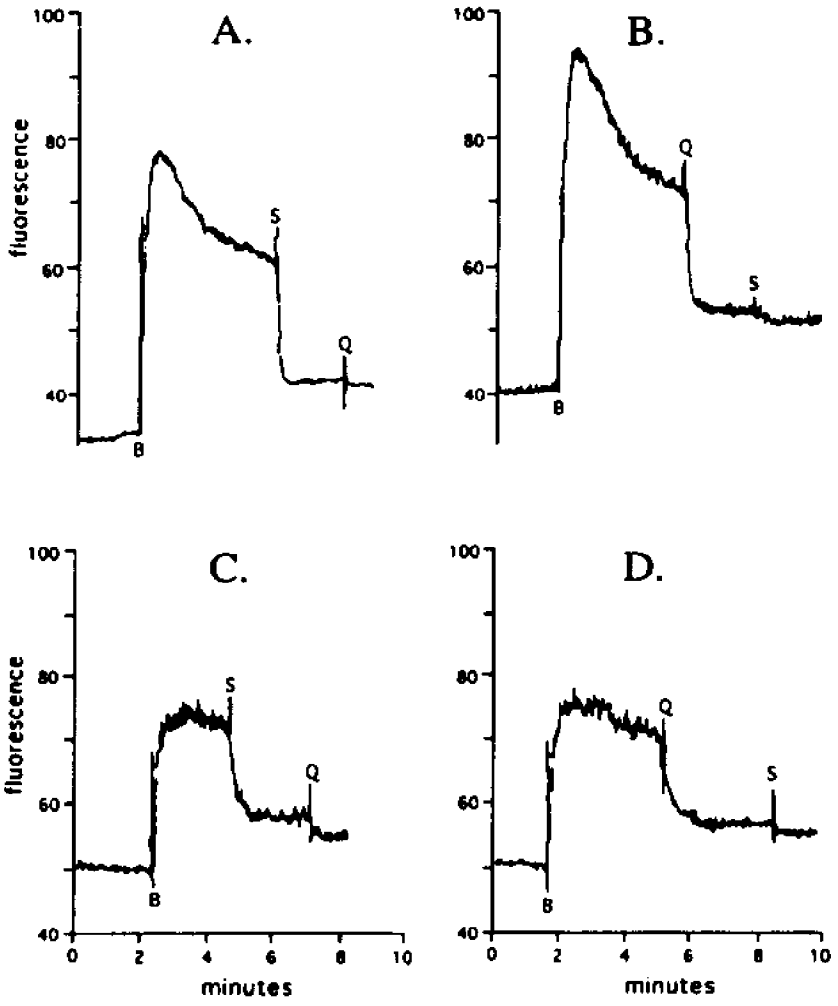

Figure 6. Fluorescence recordings of stably transfected GH3 cell lines demonstrating the lack of additivity of QP and SS responses. Fluorescence is recorded in arbitrary units on the y axis. Time is recorded in minutes on the x axis. Cells were loaded with fluo-3 as described in the methods section. Each sample of loaded cells was divided into two portions to generate a pair of fluorescence recordings from cells expressing D2L (A &B) or from cells expressing D2S (C &D). Baseline fluorescence was recorded for approximately two minutes at which time BayK 8644 (BK) was added to a final concentration of 1 uM. SS(S) and QP (Q) were added to final concentrations of 1 uM at the indicated times. The order in which SS and QP were added was exchanged within each pair of recordings.

Discussion

To examine the subtype specific regulation of calcium fluxes, we stably transfected D2L and D2S into two different cell lines of pituitary origin, AtT20 and GH3. Our studies have shown that in both cell lines, the activation of either D2L or D2S can mediate the suppression of intracellular calcium levels. Both isoforms attenuated calcium influx stimulated by BayK 8644 (BK), and by K^+ depolarization. In addition, both isoforms attenuated calcium influx stimulated by TRH in transfected GH3 cell lines.

In isolated lactotrophs, the activation of D2 receptors has been shown to specifically inhibit voltage gated calcium currents through L- and T-type channels (Lledo et al., 1990). To investigate the specific coupling of D2L and D2S to L-type calcium channels, we utilized the dihydropyridine compound BK, which is an agonist of L-type calcium channels. In both cell lines, BK stimulation caused an immediate increase in calcium influx, which was rapidly attenuated by the activation of either isoform. L-type calcium channels are distinguished from other voltage gated currents (N, T, and P) by their activation at relatively high membrane potentials (-10 mV- 0 mV) and by their extremely slow rates of inactivation (for review see Tsien et al., 1988-TINS 11:431-438). In many endocrine cell types, the activation of L-channels is associated with prolonged enhancement of hormone secretion (Cota and Hiriart, 1989).

The G-protein coupling of D2L and D2S to the inhibition of BK stimulated calcium influx was studied in AtT20 and GH3 cell lines using PTX, and by comparison to the inhibition of calcium influx by SS receptors in GH3 cells. PTX pretreatment of transfected AtT20 or GH3 cells blocked (or diminished) the effects of D2L or D2S activation on BK stimulated calcium influx. These results indicated that in both cell lines, both isoforms were coupled (to the inhibition of BK stimulated calcium influx) via G-proteins of the G_o/G_i family. The responses mediated by D2L and D2S appeared to be similarly sensitive to pretreatment with different concentrations of PTX. In contrast, the

PTX sensitivity of cAMP modulation by D2L and D2S in JEG-3 cells differed by nearly 100-fold (Montmayeur & Borelli, 1991). In JEG-3 cells, D2S appeared to be slightly more effective than D2L in mediating the inhibition of cAMP accumulation (Montmayeur & Borelli, 1991). Interestingly, the transfection of the JEG-3 cells with $G_{i\alpha 2}$, which JEG-3 cells lack, was found to enhance the ability of D2L to inhibit cAMP accumulation , suggesting that the differences in responsiveness and PTX sensitivity were due to the absence of the G_{i2} through which D2L was more efficiently coupled to adenylate cyclase (Montmayeur et al., 1993). These studies indicate that the examination of PTX sensitivity may be useful in establishing differences in the G-protein coupling of two receptors in cases where no difference (or a small difference) in signaling is observed. In our cell lines, the similarity of the PTX sensitivity of D2L and D2S mediated responses suggests that both isoforms are coupled with similar efficiency to the inhibition of BK stimulated calcium influx. It cannot be determined, however, whether each isoform is coupled via the same G-protein, or via different G-proteins. This question was addressed in the comparison of D2L, D2S and SS mediated responses in GH3 cells.

Comparison of the effects of QP and SS, separately, and together, demonstrated that there was essentially no additivity of their effects on BK stimulated fluorescence. The lack of additivity suggests that D2L, D2S, and the SS receptors may be coupled via the same G-protein to calcium influx. In GH3 cells, it has been demonstrated that the SS receptor is coupled to the inhibition of calcium currents by the G-protein, G_o, which has the specific subunit composition $\alpha_{o2}\beta_3\gamma_4$ (Kleuss et al., 1991; Kleuss et al., 1992; Kleuss et al., 1993). It is tempting to speculate that the levels of this particular G-protein limited the maximum inhibition by SS and QP of BK stimulated calcium fluorescence in our transfected GH3 cell lines. An alternative explanation for the observed lack of additivity is that SS and D2 receptors are coupled via different G-proteins to a common second messenger or intracellular effector, which in turn terminates the opening of calcium

channels by BK, and that levels of this second messenger or intracellular effector limit the magnitude of the total response. A third explanation for the non-additivity of the QP and SS mediated responses is that the activation of one receptor led to the (rapid) desensitization of the other receptor. This is unlikely because little or no desensitization of the receptor initially stimulated was observed at the time in which the second agonist was added (the second agonist was added 2-4 minutes after the addition of the first agonist). Desensitization of a response generally occured during a 10 minute period following agonist addition; it was apparent by gradual rise in fluorescence (up to the level observed before agonist addition) as the stimulated receptor became less effective at inhibiting calcium influx. Furthermore, in experiments in which significant desensitization of the first response was observed (generally at later time points), the subsequent addition of the second agonist led to a greater response than in experiments in which no desensitization of the first response was observed. This result indicates homologous desensitization of the receptor initially stimulated, and a lack of heteologous desensitization of the second receptor stimulated.

In isolated lactotrophs, D2 receptor stimulation has been shown to inhibit calcium influx and prolactin secretion induced by K^+ depolarization (Malgaroli et al., 1987). Because depolarization is a physiological modulator of voltage gated calcium channels (unlike BK), it was of interest to examine the inhibition of K^+ stimulated calcium influx by D2L and D2S. K^+ depolarization would be expected to stimulate calcium influx through a variety of voltage gated calcium channels, depending on the level of depolarization achieved. In our experiments, QP was added several minutes after KCl (induced depolarization), at which time only L- and (some) N-type channels, which inactivate very slowly, would be expected to be open. In AtT20 and GH3 cell lines the activation of either isoform attenuated calcium influx stimulated by 15 or 30 mM (extracellular) KCl, but not calcium influx stimulated by $[K^+] \geq 55$ mM. Surprisingly, the inhibition of BK stimulated calcium influx by D2L and D2S was blocked by extracellular

$[K^+] \geq 55$ mM. One possible explanation for this result is that the attenuation of calcium influx by D2L and D2S was an indirect effect of the activation of K^+ efflux, and that in the presence of extracellular $[K^+] \geq 55$ mM, efflux was prevented. K^+ efflux would be expected to hyperpolarize the cells, leading to a decrease in cell excitability and to the closure of voltage gated calcium channels. The coupling of D2 receptors to K^+ currents has been demonstrated in several cell types (Einhorn et al., 1991; Lacey et al., 1987; Freedman and Weight, 1988). In lactotrophs D2 receptor are specifically coupled to A- and M-type calcium currents (Lledo et al., 1990b). In GH3-C1 cells -which are related to GH3 cells, D2S activation caused cell hyperpolarization, probably through the activation of K^+ channels (Vallar et al. 1990). It is important to note however, that calcium current inhibition can also occur in the absence of K^+ efflux. In lactotrophs, Ca^{+2} and K^+ currents were independently modulated by the coupling of D2 receptors to two different G-proteins, G_o and G_{i3} respectively (Lledo et al., 1992).

An alternative explanation for the effect of high extracellular K^+ concentrations on the modulation of calcium influx by D2L and D2S is that the coupling of these receptors to calcium channels was effected at elevated membrane potentials. It has been reported that cell depolarization (or a single depolarizing prepulse) can block the inhibition of voltage activated (L-type or N-type) calcium channels by D2 and adrenergic receptors in chick peripheral sensory neurons (Marchetti et al., 1986). Elevated membrane potential similarly effects the inhibition of calcium currents by many other GPCRs, including receptors for somatostatin, adenosine, acetylcholine, $GABA_B$, dynorphin, and leu-enkephalin (see Carbone and Swandulla, 1990 and refs. therein). In our experiments, the attenuation of BK or K^+ stimulated fluorescence by SS was also blocked in the presence of $[K^+] \geq 55$ mM. The voltage sensitivity of the inhibition of calcium influx suggests that either the G-proteins are conformationally altered in some way such that they can no longer modulate calcium channels, as has been proposed by Carbone & Swandulla (1990), or that the calcium channels are altered conformationally by positive membrane

potentials such that they can no longer interact with G-proteins. If the G-proteins coupled to calcium channels are conformationally altered by voltage, then it might be expected that G-proteins which modulate other intracellular effectors (adenylate cyclase, phospholipase C, K^+ channels, etc.) may also be voltage sensitive. The effect of membrane potential on the ability (or inability) of particular G-proteins to interact with particular intracellular effectors may provide a means by which certain patterns of signaling are selected for under different physiological conditions. In neurons, it has been proposed that the elevation of membrane potential by a train of action potentials may play a role in overcoming presynaptic inhibition (or neurotransmitter release) mediated by GPCRs that inhibit calcium influx (Elmslie et al., 1990). Similarly, the depolarization of endocrine cells might promote secretion by alleviating the inhibitory influence of dopamine and/or other neuromodulators on calcium influx. In cells with inexcitable cell membranes (due to the absence of voltage gated Na^+ channels) the inhibition of calcium influx by D2 and other GPCRs may play a role in suppressing calcium oscillations or in decreasing the frequency of calcium oscillations. It has been observed that calcium oscillations occur in the absence of changes in membrane potential in some cells (Rink and Jacob, 1989).

The simulation of pituitary lactotrophs by TRH promotes prolactin secretion, which can be blocked by dopaminergic activity (for review see Ben Jonathan, 1985). In lactotrophs and GH3 cells, TRH has a biphasic effect on calcium levels, characterized by a brief initial rise in calcium levels (spike phase), followed by a more prolonged period (plateau phase) in which calcium levels are elevated above baseline, but are lower than the levels achieved during the initial calcium spike (Margaroli et al., 1987; Albert and Tashjian, 1984). These two stages of calcium elevation are characteristically induced by hormones and neurotransmitters that activate phospholipase C. The initial calcium spike is due to IP^3 mediated release of calcium from intracellular stores (Vallar et al, 1988; Rebecchi et al, 1983), while the plateau phase involves the influx of extracellular calcium

(Vallar et al, 1988; Albert and Tashjian, 1984). These biphasic changes in intracellular calcium levels have been directly correlated with the biphasic release of prolactin from GH3 cells (Albert and Tashjian, 1984).

In lactotrophs, D2 receptor activation inhibits the elevation of intracellular calcium level during the spike and plateau phases (Malgaroli et al., 1987). In our GH3 cell lines, the activation of D2L or D2S did not inhibit the initial rapid rise or "spike" in intracellular calcium induced by TRH. In contrast, the activation of either isoform caused the attenuation of calcium influx observed during the plateau phase.

The mechanism by which lactotroph D2 receptors inhibit spike phase calcium levels has not been established. Malgaroli et al., (1987) found that the dopaminergic inhibition of calcium levels during the spike phase was relieved by treatments that elevated cAMP (forskolin, cholera toxin), however, other investigators have reported no change in the dopaminergic inhibition of calcium levels or of inositol phosphate production in the presence of forskolin (Journot et al., 1987). It has been proposed that the D2 mediated suppression of inositol phosphate production may involve the blockade of non-dyhydropyridine sensitive calcium channels (Enjalbert et al., 1990). The lack of effect of D2L and D2S on the TRH induced calcium spike in our studies may be due to a phenotypic difference in the signal transduction components present in GH3 cells and lactotrophs. Alternatively, a different D2 receptor subtype present in the lactotroph which mediates the inhibition of calcium levels and IP_3 metabolism during the spike phase.

The mechanisms responsible for calcium entry during the plateau phase are not completely understood. In GH3 cells, it has been shown that the overall rise in calcium during the plateau can be separated into two components, a dyhydropyridine sensitive component, and a dyhydropyridine insensitive component. This indicates the involvement of at least two different classes of calcium channels (Albert & Tashjian, 1984). It has recently been reported that during an IP_3 mediated calcium spike, a soluble

factor is released which induces calcium influx through a non-voltage-dependent calcium channel, during the plateau phase (Randriamampita and Tsien, 1993; Parekh et al., 1993). In our experiments, the activation of D2L or D2S only partially attenuated calcium influx during the plateau phase. This was evident when QP was added to cell suspensions before TRH. This result indicates that some calcium influx, possibly through non-voltage gated calcium channels, is not inhibited by D2 receptor activation.

In summary, our studies have shown that the D2L and D2S receptor isoforms can effectively inhibit the influx of calcium through voltage gated calcium channels in two different cell lines. Although differences in the ability of each isoform to inhibit calcium influx could not be resolved, the responses mediated by both receptors were similar. Studies with PTX and SS suggest that a similar, if not identical, signal transduction pathway is used by both D2L and D2S in the inhibition of calcium influx.

CHAPTER 7

General Discussion

Genetic diversity of dopamine receptors

The cloning of the first D2 receptor (Bunzow et al., 1988) quickly led to the identification by us (Snyder et al., 1989), and several other investigators (DalToso et al., 1989; Giros et al., 1989; Grandy et al., 1989; Monsma et al., 1989; Selbie et al., 1989) of a second D2 receptor (discussed in chapter 1). Except for the inclusion of a stretch of 29 novel amino acids in the putative third cytoplasmic loop, this new D2 receptor isoform was identical to the first D2 receptor reported. This receptor, called D2L (L, because it was longer) and the original D2 receptor (renamed D2S) represented alternatively spliced mRNA variants of a single gene. Since the cloning of the D2(L&S) isoforms, the related D3 and D4 receptors have been identified. These receptors are encoded by separate genes. In addition, two receptors of the pharmacological D1 class, the D1 and the D5 (also called D1B) have been isolated (Zhou et al. 1990; Monsma et al. 1990; Weinshank et al. 1991; Grandy et al. 1991; Sunahara et al. 1991).

The D2(L&S) receptors are encoded by a single gene, as are the receptor variants of the D3 receptor subfamily. In contrast, the D4 receptor subfamily is encoded by multiple polymorphic genes (VanTol et al., 1992). The "D2" receptor genes are distinguished from the "D1" receptor genes (and those of the other catecholamine receptors) by the presence of introns. The rat and human D2 receptor genes contain at least six introns within the coding region (Araki et al., 1992; O'Malley et al., 1990). The rat D3 receptor gene is reported to have five introns within the coding region (Giros et al., 1991). The human D4 receptor gene receptor genes each contain three introns within the coding region (VanTol et al., 1992).

For intron containing genes, the process of alternative mRNA splicing can give rise to multiple mRNA transcripts. As mentioned above, alternative splicing of the D2(L&S) receptor gene produces the receptor isoforms, D2L and D2S. No other splice variants of this gene have been identified. One functional D3 receptor has been identified in humans. However, a large number of variant D3 receptor transcripts encoding truncated and full length receptor proteins were reported during, and subsequent to our own work characterizing the D3 receptor (Giros et al, 1990; Giros et al. 1991; Fishburn et al., 1993; Pagliusi et al., 1993; Nagai et al., 1993; Schmauss et al., 1993). These D3 receptor variants were cloned from several different species. In the mouse, functional splice variants which differ in the third cytoplamic loop have been identified which are analogous to the D2 isoforms we have studied (Fishburn et al., 1993). In our studies (Snyder et al., 1991a&b) and in reports from several other laboratories, similar functional variants have not been identified in rat or human brain. In these species a large number of apparent splice variants have been reported which encode truncated receptor proteins whose putative sequence includes the amino terminal transmembrane domains (Snyder et al., 1991a&b; Giros et al, 1990; Giros et al., 1991; Nagai et al., 1993; Schmauss et al., 1993). The function of these truncated proteins has not been elucidated to date. In one report, however, a truncated human D3 variant, distinct from the variant we identified, has shown a high predominance in certain regions of schizophrenic brain (Schmauss et al, 1993).

One possible role of these truncated receptor proteins, not demonstrated *in vivo* to date, is that of either a modulator of the functional receptor or a component of a heteromeric multiple subunit receptor. This hypothetical role *in vivo* is derived from recent *in vitro* studies which demonstrate that functional muscarinic receptors can be assembled from multiple subunits, each contributing different transmembrane (TM) domains (Maggio et al., 1993a; Maggio et al., 1993b). In one study, functional muscarinic receptors were expressed by co-expressing two chimers containing TM 1-5 muscarinic M3

receptor/TM6-7 α2 adrenergic receptor and TM 1-5 adrenergic receptor/TM6-7 muscarinic receptors (Maggio et al., 1993b). While neither construct alone led to the expression of a functional receptor, co-expression of the two constructs reconstituted a functional coupled muscarinic receptor. These experiments indicate that the transmembrane domains themselves may be sufficient for receptor assembly. By extension, these truncated D3 receptor variants may be capable of assembling with other proteins to form functional receptors in vivo.

A family of D4 receptor genes has been identified in humans (VanTol et al, 1992). An analogous family of genes does not appear to be present in the rat (VanTol et al, 1992). Within the human population, there are at least five different polymorphic genes. These genes vary by the presence of a 48 bp sequence that is repeated two, three, four, five or seven times within the region encoding the third cytoplasmic loop. There are indications that variants with six and eight-fold repeats also exist. The expression of this repeated segment imparts different ligand binding characteristics to the different D4 receptors (VanTol et al, 1992). Of the cloned D2 receptor family, the D4 receptor has been reported to have the highest affinity for the atypical neuroleptic, clozapine (VanTol et al., 1991). It has also recently been reported that the level of D4 receptors is elevated in the brains of schizophrenics (Seeman et al., 1993). These observations suggest that the D4 receptor gene polymorphism may provide the genetic basis for individual differences susceptibility to schizophrenia and in responsiveness to neuroleptic therapy. As in the D2(L&S) and D3 receptor genes, the presence of introns in the D4 receptor genes indicates that alternative mRNA splicing is likely to generate further D4 receptor diversity. To date, however, no splice variants have been reported.

Biological significance of the D2L and D2S splice variants

The evolutionary conservation of the D2L and D2S splice variants in all species examined; including mouse (Montmayeur et al, 1991), rat (Giros et al., 1989), cow (Chio

et al., 1990), and man (Selbie et al.,1989), suggests that the presence of both receptors in mammals is important. In contrast, many of the D3 and D4 receptor variants appear to present in rodents or humans, but not both. D2L and D2S vary by the presence absence of a 29 amino acid (aa) segment within the third cytoplasmic loop. The sequence similarity of the 29 aa across species suggests the functional importance of this sequence. Pharmacologically, D2L and D2S appear to be identical. This is not surprising since the two receptors vary structurally in an intracellular region, while the ligand binding site of catecholamines is postulated to reside within the transmembrane domains (Probst et al, 1992). Mutagenesis studies of other GPCRs indicate that the third cytoplasmic loop is important in phosphorylation mediated desensitization, and in G-protein coupling (for review see Probst et al., 1992). Thus, the 29 aa segment may modify the regulation and/or signal transduction of D2L.

To gain insight into the biological roles of D2L and D2S, we quantitatively mapped the distribution of D2L and D2S mRNA in the rat brain by a sensitive, solution hybridization/nuclease protection assay (Snyder et al., 1990, see Chapter 2). Both mRNA species were found in all tissues examined, with D2L comprising 22-30% of the total mRNA in brain areas, and 11-15% of the total mRNA in the pituitary. The relatively constant ratio of D2L:D2S in the brain does not give us any clues as to what might be the distinct biological roles of D2L and D2S. The distribution and relative abundance of D2L and D2S mRNA roughly corresponded to the distribution and relative abundance of D2 receptors as determined in previous ligand binding studies (Gehlert et al., 1984, Lightman et al., 1982; Palacios and Pazos, 1987). An exception to this was seen in the neurointermediate lobe of the pituitary, where the relative abundance of D2L and D2S mRNA in the pituitary was nearly 2-fold greater than the overall abundance of D2L and D2S mRNA in the striatum. Receptor levels in the striatum are reported to be higher than in the neurointermediate lobe (Gehlert et al., 1984, Lightman et al., 1982; Palacios and Pazos, 1987). The excess D2 receptor mRNA in the neurointermediate lobe consisted

entirely of D2L mRNA. This suggested the possibility that D2L mRNA in the neurointermediate lobe is translated less efficiently than in other tissues, or that the turnover of D2L in the neurointermediate lobe is higher than in other tissues. Differences in the translational efficiency or mRNA stability of the two isoforms may represent mechanisms by which the relative abundance of the two receptor isoforms are regulated.

A difference in the regulation of the mRNA of D2L and D2S has been reported by Kukstas et al. (1991). Using the technique of quantitative PCR, it was shown that the level of D2S mRNA was two-fold higher than the level of D2L mRNA in a population of lactotrophs (representing ~1/2 of the total) from lactating rats. Furthermore, treatment of cells in primary cultures with progesterone or testosterone, altered the ratio of D2S:D2L mRNA; with testosterone reversing the ratio to that observed in male rats. Treatment with estradiol decreased the overall abundance of D2L and D2S mRNA, but it did not change the relative ratio. The abundance of D2S mRNA in this report was very surprising since it had previously been observed that D2L mRNA was the more abundant species in the pituitary, and in all other tissues examined (Snyder et al., 1990; Chio et al, 1990; DalToso et al., 1989; Monsma et al., 1989) In light of the important role D2 receptors play in the inhibition of prolactin secretion, it is tempting to speculate that the levels of D2S are higher during lactation, and/or that differences in the G-protein coupling or the regulation of this receptor make it a less effective inhibitor of prolactin secretion than the D2L receptor.

To examine the regulation and signal transduction of D2L and D2S, these receptors have been transfected into cell lines which lack native D2 receptors. The comparison of D2L and D2S signal transduction in cell lines indicates that there are differences in the desensitization and G-protein coupling of the two isoforms. A study by Liu et al. (1992) indicated that the activation of kinase C resulted in greater desensitization of signaling mediated by D2S than by D2L. These results were somewhat surprising. Due to the presence of several potential phosphorylation sites within the 29 aa segment of D2L, it

might be expected that D2L would be preferentially desensitized. It is possible that conformation of the third cytoplasmic loop of D2S made a phosphorylation site (common to both receptors) more accessible to kinase C, while the conformation of this domain of D2L protected this site from phosphorylation. No other studies comparing the desensitization of D2L and D2S mediated signaling have been reported.

Several reports have indicated that D2L and D2S may be differentially coupled to the inhibition of cAMP. In three different cell lines, CHO-K1, 293 cells, and JEG-3 cells, D2S appeared to be more effective than D2L at inhibiting the accumulation of cAMP (Hayes et al., 1992; DalToso et al., 1989, Montmayeur and Borrelli, 1991). The work of Montmayeur and coworkers indicates that in JEG-3 cells, D2L is preferentially coupled via the G_{i2} to the inhibition of cAMP accumulation.(Montmayeur and Borrelli, 1991; Montmayeur et al., 1992). This study also indicates that if two receptors are coupled to specific intracellular signals via different G-proteins, then it may only be possible to detect the coupling difference in the absence of one of the G-proteins to which one of the receptors is preferentially coupled.

Our studies indicate that D2L and D2S are similarly coupled to the inhibition of calcium influx in AtT20 and GH3 cells. At the limit of resolution of the assay system employed, we can detect no difference in D2L and D2S coupling to L-type calcium channels in either cell line studied. Studies with PTX did not support any differences in G-protein coupling in either expression system. Studies with SS suggest that both isoforms are likely to be coupled via the same G-protein in GH3 cells.

Receptors of the D2 receptor mediate a wide range of effects in the nervous and endocrine systems. They are important in cognition, motor coordination, reward, and in prolactin secretion. The identification of receptors within the D2 family by molecular cloning, and their functional characterization these receptors mammalian cell lines are critical first steps towards understanding the biology of this receptor system in the brain and in the pituitary.

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