Two forms of the rat D_2 dopamine receptor as revealed by the polymerase chain reaction

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Received 12 January 1990

We have used the polymerase chain reaction technique (PCR) to clone the cDNA of the D_2 dopamine receptor from rat striatal mRNA. Two major PCR products were produced; one product was identical to a previously published rat cDNA, while the other, more abundant product differed only by an 87-nucleotide insert located in the region of the putative third cytoplasmic loop of the D_2 receptor. A PCR approach for determining message abundance was used to determine the relative message abundance of the two forms of the D_2 receptor in a variety of tissues. Possible implications of the two forms of the D_2 receptor for dopamine-mediated signal transduction are discussed.

D_2 dopamine receptor subtype; G-protein coupled receptor; Polymerase chain reaction

1. INTRODUCTION

Dopamine receptors have been classified into D_1 and D_2 subtypes [1] and the cDNA of a rat dopamine D_2 receptor has been cloned, sequenced and expressed by Civelli and colleagues and shown to be a member of the family of G-protein-linked receptors [2]. To establish our own cell line expressing this dopamine D_2 receptor, we designed oligonucleotide primers based on the published nucleotide sequence [2] in order to amplify rat striatal cDNA for the D_2 receptor by the polymerase chain reaction (PCR) method [3]. By using PCR, we could circumvent cDNA library construction and screening and perhaps amplify other potentially interesting cDNAs which might share homology for the oligonucleotide primers.

We now report that our PCR amplification of the rat striatal cDNA for the D_2 receptor results in essentially two amplified products. These products differ only by a single 87-nucleotide insert and are otherwise identical to the published sequence of the rat D_2 receptor. Additionally, we detail our PCR strategy for detecting differences in the amplified products and for obtaining full-length sequences for the two forms of the D_2 receptor. Finally, we present data on the relative abundance of the two forms in a variety of tissues.

2. EXPERIMENTAL

2.1. *Cell lines*

COS-1 cells were obtained from ATCC and maintained in Dulbecco's Modified Eagles Medium (Sigma) containing 10% fetal calf serum (Sigma) and 1 × antibiotic/antimycotic solution (Sigma) at 37°C and 95% air/5% CO_2. MMQ cells cloned from the rat 7315a anterior pituitary tumor [4] were obtained from Dr Robert MacLeod of the U.Va. and maintained in RPMI 1640 containing 2.4% fetal calf serum (Hyclone), 7.3% horse serum (Irvine Scientific), 2 mM glutamine, 1 × antibiotic/antimycotic and 18 μg/ml gentamicin at 37°C and 95% air/5% CO_2.

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2.2. *Tissue dissection*

Rats were killed by decapitation and the brains, pituitary glands, and livers were removed and dissected on ice. Striata, substantia nigra and cerebellum were dissected from each brain. The anterior pituitary and intermediate lobe were dissected from each pituitary. All tissues were frozen on dry-ice or liquid nitrogen immediately upon dissection and then stored at −70°C.

2.3. *RNA extraction*

RNA was prepared from cells or tissues by the RNAzol method (Cinna/Biotecx) in which cells or tissues were homogenized in RNAzol solution (2 ml/100 mg tissue or 10^7 cells) containing guanidinium thiocyanate, phenol and 2-mercaptoethanol and the RNA extracted following addition of chloroform and centrifugation. The aqueous phase was removed and the RNA precipitated by isopropanol at −20°C. The RNA yield was approximately 1 mg/g tissue.

2.4. *Oligonucleotides*

Oligonucleotides were synthesized by the Corporate Molecular Biology Group of Abbott Laboratories using the β-cyanoethyl phosphoramidite method on Applied Biosystems models 380A and 380B DNA synthesizers. The sequences of the individual primers were as follows:

Primer A: CCGTGCGTGGATGCGGCGGGAGCTGGC
Primer B: GCATCTCCATTTCCAGCTCCTGAGCTCGG
Primer C: CCGAGCTCAGGAGCTGGAAATGGAGATGC
Primer D: CCTGCAGGGTCGAGAGAAGGCCAAG
Primer E: AATCTACATCGTCCTCCGGAAGCGCC
Primer F: TGGGATGGATCAGGGAGAGTGAGCTG
Probe 1: same as Primer C above
Probe 2: GGCAACTGTACCCACCCTGAG

2.5. *cDNA synthesis*

1–10 μg of total cellular RNA were co-precipitated with 10 μg of

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00145793/90/$3.50

yeast tRNA as carrier and resuspended in ddH_2O. The RNA was primed with D_2 receptor-specific antisense primer and the synthetic reaction was done in a final volume of 25 μl using the components and conditions specified by the manufacturer (BRL) with the exception that Seikagaku avian myeloblastosis virus reverse transcriptase was used. After incubation at 37°C for 1 h, an equal volume of 3 M Na-acetate was added, followed by 4 vols of EtOH for precipitation.

2.6. *DNA amplification*

Oligonucleotide primer sets A–B and C–D were designed to amplify two contiguous stretches of the cDNA which, together, encompassed the entire coding region as well as some non-coding region at the 5′- and 3′-ends (see Fig. 1A). Primer set A–D was used to amplify the full-length cDNA of the D_2 receptor. All reagents and the Taq DNA polymerase for the amplification reaction were purchased from Perkin Elmer Cetus. The reaction was performed for 30 cycles; each cycle consisting of 2 min at 94°C and 6 min at 72°C, using a DNA thermal cycler (Perkin Elmer Cetus). Aliquots of amplified product were either run on agarose gels or subcloned into plasmid vectors.

2.7. *Cloning and sequencing of the full-length cDNA*

Amplified product from the primer set C–D was digested at *Sma*I and *Pst*I sites (see Fig. 1A) and subcloned into a similarly designed pUC18 vector. *E. coli* strain JM109 were transformed by the pUC18 constructs for plasmid amplification and clear plaques were picked and grown as separate clones. Plasmid DNA from each bacterial clone was purified by the CsCl technique [5]. Clones bearing the long form of the D_2 receptor cDNA were identified by restriction digest analysis. The cDNA of the long form of the D_2 receptor was then sequenced from the identified CsCl-purified plasmids using the Sequenase method (United States Biochemicals). Sequencing oligonucleotides were designed to be 150 bp apart. The sequencing reaction and gel were run according to the supplier's protocol.

2.8. *Identification and quantitation of PCR products*

Starting with rat striatal RNA, cDNA synthesis and amplification were performed as described above using the primer set E–F. The PCR reaction was allowed to run for either 10 or 30 amplification cycles and the products run on a 1.5% agarose gel. The resolved products were transferred by capillary action to Zeta-probe membranes (Bio-Rad) for hybridization to oligonucleotide probes labelled at the 5′-end with ^{32}P using bacteriophage T4 polynucleotide kinase. The transfer, hybridization and washing of the membranes were done according to the Bio-Rad protocol. The hybridization probes were Probes 1 and 2 of section 2.4 above. The washed membranes were dried, and autoradiographed. The bands on the autoradiograms were first quantified using an LKB Ultroscan XL laser densitometer and then used to locate the corresponding bands on the membranes which were cut out and placed in vials containing liquid scintillation fluid for quantification by liquid scintillation spectroscopy.

3. RESULTS AND DISCUSSION

The PCR product from the primer set C–D (Fig. 1A) ran on an agarose gel as a single band of predicted size whereas the product from primer set A–B (Fig. 1A) ran as two distinct bands. The lower band was of the predicted size; the upper band was more abundant (Fig. 1B). The D_2 receptor cDNA has a unique *Bam*HI site adjacent to the initiation ATG codon. When cut with *Bam*HI, both bands from this primer set were reduced to the same extent (Fig. 1B), suggesting sequence homology between the products beyond that for the common priming regions. The sequence for the long form (see Fig. 2) was found to be identical to that

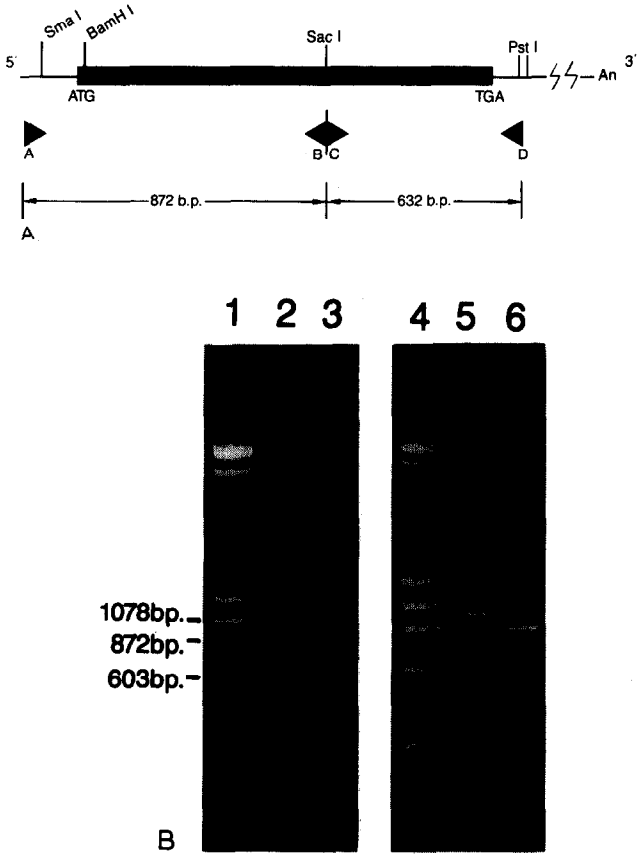

Fig. 1. PCR amplification of the rat D_2 receptor cDNA. (A) Schematic for the PCR amplification showing locations of the primer sets A–B, C–D and A–D. (B) Ethidium bromide stained agarose gels: lanes 1 and 4, size markers; lane 2, tRNA control; lane 3, PCR product of the C–D primer set; lane 5, PCR products of the A–B primer set; lane 6, PCR products of the A–B primer set cut with *Bam*HI (note the non-specific bands are not cut by *Bam*HI).

of the previously published sequence for the cDNA of the rat D_2 receptor [2] except for the presence of an 87-bp insert. The D_2 receptor, like other G-protein-coupled receptors, is thought to be an integral membrane protein of 7 transmembrane spanning regions which connect an N-terminal extracellular head to a COOH-terminal cytoplasmic tail and the 87-nucleotide insert is within the region coding for the putative third cytoplasmic loop of the receptor [6]. Within the insert there are 2 consensus sites for N-glycosylation and 2 cysteines which could form a disulfide bridge or serve as sites for lipid modification.

To determine the relative abundance of the two forms of the D_2 receptor, the primer set E–F was used to amplify, by PCR, the cDNAs reverse transcribed from mRNA for the short or long forms of the receptor (Fig. 3A). Total RNA from a variety of tissues was used for the cDNA synthesis. The primer set E–F was designed so that amplified product originating from mRNA for the short form would be 215 bp while product from the message encoding the 87-bp insert would

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       10                  30                  50
GCTGCCGGAGGGGCGGCCGTGCGTGGATGCGGCGGGAGCTGGAAGCCTCCAGCAGCCGGC
       70                  90                 110
GCCTTCTCTGGCCCCGGGCGCCATATGGCTTGAAGAGCCGTGCCACCCAGTGGCCCCACT
      130                 150                 170
GCCCCAATGGATCCACTGAACCTGTCCTGGTACGATGACGATCTGGAGAGGCAGAACTGG
          MetAspProLeuAsnLeuSerTrpTyrAspAspAspLeuGluArgGlnAsnTrp
      190                 210                 230
AGCCGGCCCTTCAATGGGTCAGAAGGGAAGGCAGACAGGCCCCACTACAACTACTATGCC
SerArgProPheAsnGlySerGluGlyLysAlaAspArgProHisTyrAsnTyrTyrAla
      250                 270                 290
ATGCTGCTCACCCTCCTCATCTTTATCATCGTCTTTGGCAATGTGCTGGTGTGCATGGCT
MetLeuLeuThrLeuLeuIlePheIleIleValPheGlyAsnValLeuValCysMetAla
      310                 330                 350
GTATCCCGAGAGAAGGCTTTGCAGACCACCACCAACTACTTGATAGTCAGCCTTGCTGTG
ValSerArgGluLysAlaLeuGlnThrThrThrAsnTyrLeuIleValSerLeuAlaVal
      370                 390                 410
GCTGATCTTCTGGTGGCCACACTGGTAATGCCGTGGGTTGTCTACCTGGAGGTGGTGGGT
AlaAspLeuLeuValAlaThrLeuValMetProTrpValValTyrLeuGluValValGly
      430                 450                 470
GATTGGAAATTCAGCAGGATTCACTGTGACATCTTTGTCACTCTGGATGTCATGATGTGC
AspTrpLysPheSerArgIleHisCysAspIlePheValThrLeuAspValMetMetCys
      490                 510                 530
ACAGCAAGCATCCTGAACCTGTGTGCCATCAGCATTGACAGGTACACAGCTGTGGCAATG
ThrAlaSerIleLeuAsnLeuCysAlaIleSerIleAspArgTyrThrAlaValAlaMet
      550                 570                 590
CCCATGCTGTATAACACACGCTACAGCTCCAAGCGCCGAGTTACTGTCATGATTGCCATT
ProMetLeuTyrAsnThrArgTyrSerSerLysArgArgValThrValMetIleAlaIle
      610                 630                 650
GTCTGGGTCCTGTCCTTCACCATCTCCTGCCCACTGCTCTTCCGACTCAACAATACAGAC
ValTrpValLeuSerPheThrIleSerCysProLeuLeuPheArgLeuAsnAsnThrAsp
      670                 690                 710
CAGGGTGAGTGTATCATTGCCAACCCTGCCTTTGTGGTCTACTCCTCCATTGTCTCATTC
GlnGlyGluCysIleIleAlaAsnProAlaPheValValTyrSerSerIleValSerPhe
      730                 750                 770
TACGTGCCCTTCATCGTCACTCTGCTGGTCTATATCAAAATCTACATCGTCCTCCGGAAG
TyrValProPheIleValThrLeuLeuValTyrIleLysIleTyrIleValLeuArgLys
      790                 810                 830
CGCCGGAAGCGGGTCAACACCAAGCGCAGCAGTCGAGCTTTCAGAGCCAACCTGAAGACA
ArgArgLysArgValAsnThrLysArgSerSerArgAlaPheArgAlaAsnLeuLysThr
      850                 870                 890
CCACTCAAGGGCAACTGTACCCACCCTGAGGACATGAAACTCTGCACCGTTATCATGAAG
ProLeuLysGlyAsnCysThrHisProGluAspMetLysLeuCysThrValIleMetLys
      910                 930                 950
TCTAATGGGAGTTTCCCAGTGAACAGGCGGAGAATGGATGCTGCCCGCCGAGCTCAGGAG
SerAsnGlySerPheProValAsnArgArgArgMetAspAlaAlaArgArgAlaGlnGlu
      970                 990                1010
CTGGAAATGGAGATGCTGTCAAGCACCAGCCCCCCAGAGAGGACCCGGTATAGCCCCATC
LeuGluMetGluMetLeuSerSerThrSerProProGluArgThrArgTyrSerProIle
     1030                1050                1070
CCTCCCAGTCACCACCAGCTCACTCTCCCTGATCCATCCCACCACGGCCTACATAGCAAC
ProProSerHisHisGlnLeuThrLeuProAspProSerHisHisGlyLeuHisSerAsn
     1090                1110                1130
CCTGACAGTCCTGCCAAACCAGAGAAGAATGGGCACGCCAAGATTGTCAATCCCAGGATT
ProAspSerProAlaLysProGluLysAsnGlyHisAlaLysIleValAsnProArgIle
     1150                1170                1190
GCCAAGTTCTTTGAGATCCAGACCATGCCCAATGGCAAAACCCGGACCTCCCTTAAGACG
AlaLysPhePheGluIleGlnThrMetProAsnGlyLysThrArgThrSerLeuLysThr
     1210                1230                1250
ATGAGCCGCAGAAAGCTCTCCCAGCAGAAGGAGAAGAAAGCCACTCAGATGCTTGCCATT
MetSerArgArgLysLeuSerGlnGlnLysGluLysLysAlaThrGlnMetLeuAlaIle
     1270                1290                1310
GTTCTCGGTGTGTTCATCATCTGCTGGCTGCCCTTCTTCATCACGCACATCCTGAATATA
ValLeuGlyValPheIleIleCysTrpLeuProPhePheIleThrHisIleLeuAsnIle
     1330                1350                1370
CACTGTGATTGCAACATCCCACCAGTCCTCTACAGCGCCTTCACATGGCTGGGCTATGTC
HisCysAspCysAsnIleProProValLeuTyrSerAlaPheThrTrpLeuGlyTyrVal
     1390                1410                1430
AACAGTGCCGTCAACCCCATCATCTACACCACCTTCAACATCGAGTTCCGCAAGGCCTTC
AsnSerAlaValAsnProIleIleTyrThrThrPheAsnIleGluPheArgLysAlaPhe
     1450                1470                1490
ATGAAGATCTTGCACTGCTGAGTCTGCCCCTTGCCTGCACAGCAGCTGCTTCCCACCTCC
MetLysIleLeuHisCys
     1510                1530                1550
CTGCCTATGCAGGCCAGACCTCATCCCTGCAAGCTGTGGGCAGAAAGGCCCAGATGAACT
     1570                1590
TGGCCTTCTCTCGACCCTGCAGGCCCTGCAGTGTTA
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Fig. 2. Nucleotide and amino acid sequences of the cDNA of the rat D_2 dopamine receptor containing the 87-bp insert (boxed). Possible *N*-glycosylation sites and cysteines within the insert are underlined.

be 302 bp (Fig. 3A). As an additional check that the PCR products represented cDNAs from the D_2 receptor, products from one tissue, the rat striatum, were Southern blotted and hybridized to

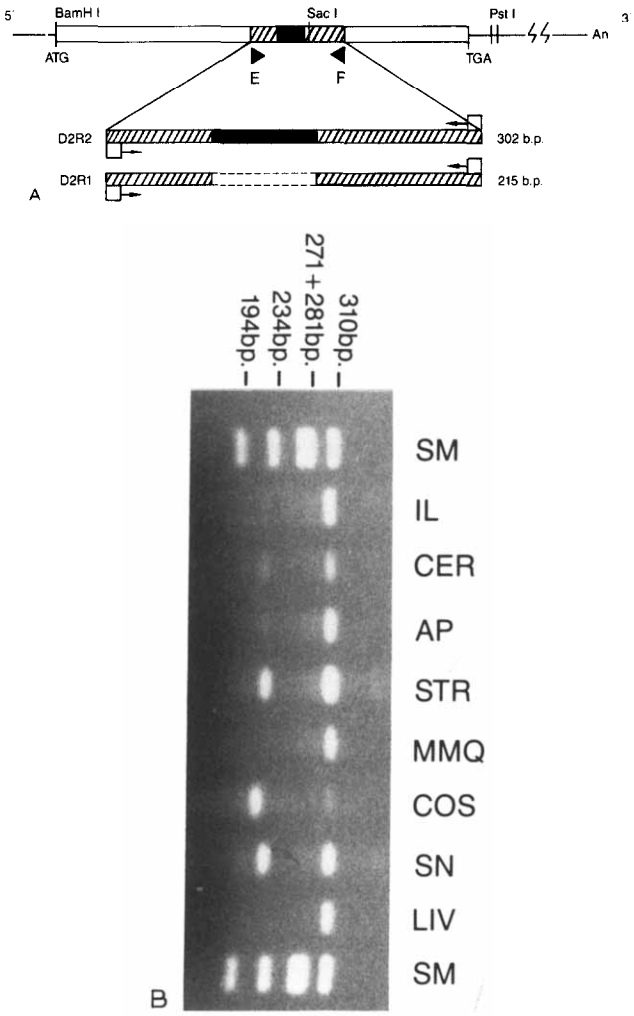

Fig. 3. Tissue distribution of the short and long forms of the D_2 receptor. (A) Schematic for PCR amplification of two discriminable cDNA products for the short and long forms of the D_2 receptor. (B) Left panel: ethidium bromide stained agarose gel of size markers and PCR products; IL = rat intermediate lobe; CER = rat cerebellum; AP = rat anterior pituitary; STR = rat striatum; MMQ = MMQ cell line (rat-derived); COS = COS-1 cell line (monkey-derived); SN = rat substantia nigra; LIV = rat liver.

[^{32}P]oligonucleotide probes. Probe 1 was a 29-mer oligonucleotide starting 12 nucleotides 3′ to the insert and therefore common to the two forms of the receptor while Probe 2 was a 26-mer unique to the insert. As seen in Fig. 4, Probe 1 hybridized to the long and short PCR products while Probe 2 selectively hybridized to the long form. Moreover, the relative amounts of the two forms were the same following 10 or 30 cycles of PCR amplification. This demonstrates that the relative amounts of the PCR products most likely reflect the relative amounts of the original transcripts and not the preferential amplification of one of the cDNAs since the latter would alter the ratio of PCR products as amplification proceeded from 10 to 30 cycles.

The main result is that the longer PCR product was clearly present and more abundant than the short form

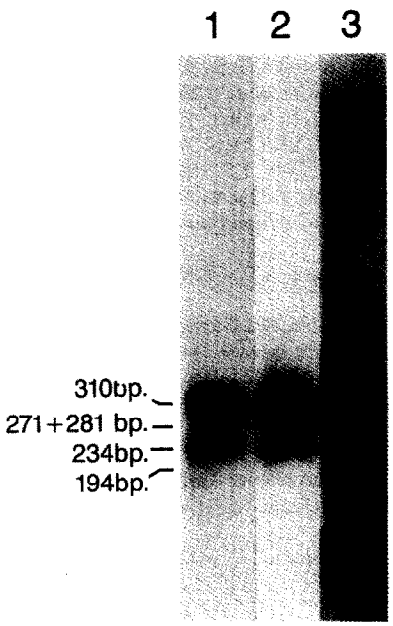

Fig. 4. Autoradiograms of Southern blots of rat striatal cDNA amplified for 10 or 30 PCR cycles using primer set E–F and hybridized to oligonucleotide probes 1 or 2. Lane 1: 10 cycles, Probe 1. Film exposed for 24 h. Long form represents 67% of D_2 receptor cDNA as determined by laser densitometry. Lane 2: 30 cycles, Probe 1. Film exposed for 30 min. Long form represents 71% of D_2 receptor cDNA as determined by both laser densitometry and liquid scintillation spectroscopy. Lane 3: 30 cycles, Probe 2. Film exposed for 30 min.

in every rat tissue studied (Fig. 3B). In striatum and substantia nigra, approximately 70% of D_2 transcripts coded for the longer, insert-plus form (Fig. 4). In the liver and in pituitary tissues of anterior pituitary and intermediate lobe, as well as in the pituitary-derived MMQ cells, the short form was either not detectable or barely present. In the cerebellum, which is not generally recognized as a D_2 receptor-expressing tissue, both forms were faintly detected, and again, the PCR product for the long form predominated. The only exception to the general pattern of relative abundance was found in the monkey-derived COS-1 cell in which the lower band product predominated and was a slightly smaller size.

We included cerebellum, liver and COS-1 cells in our tissue distribution analysis to serve as negative controls, thinking these tissues to be negative for D_2 receptor expression. However, we have since learned that others have found evidence for D_2 receptor transcripts in cerebellum using the slot-blot method and have verified these results by radioligand binding experiments using the new high-affinity D_2 radioligand, [^{125}I]epidepride (personal communication, K. Neve). We expect our results in liver to be similarly confirmed. The result from the COS-1 cells is more perplexing, since it was the only tissue where the shorter form predominated. However, the size of the shorter form was slightly smaller than predicted (see Fig. 3B) and we do not know whether this reflects a species difference in the D_2 receptor (the COS-1 cell is derived from African green monkey kidney epithelium) or a product that is not a D_2 receptor.

The results of the present study indicate that two transcripts exist for the rat D_2 dopamine receptor which differ only by the presence of an 87-bp insert. The complete identity of the two forms outside the insert suggest that they are alternatively spliced transcripts. Alternative splicing is consistent with evidence of introns in the rat D_2 receptor gene [2] despite the lack of introns in the majority of G-protein-coupled receptor genes sequenced to date [7].

What is the significance of the insert in the putative third cytoplasmic loop of the D_2 receptor? Assuming that the receptor protein contains the insert, it is natural to focus on possible implications for signal transduction since this loop has been shown to be important for signal transduction of α- and β-adrenergic receptors and muscarinic acetylcholine receptors [8,9]. However, when the analysis has been narrowed to within the loop, the transducing regions appear to be restricted to the 5′- and 3′-ends of the loop which is in keeping with the location of the peripheral transducing G-proteins at the cytoplasmic surface of the membrane [10,11]. Since the insert is outside of these regions how could it influence D_2 receptor signal transduction? One possibility is that the insert provides a site of membrane attachment thereby creating sub-loops within this large cytoplasmic loop. Recent work on the Ras proteins, for example, suggests that lipid modification of cysteines by palmitoylation or polyisoprenylation might play a role in anchoring the peripheral proteins to the plasma membrane [12,13] and a similar role for palmitoylation of adjacent cysteines has been proposed to create a fourth cytoplasmic loop of rhodopsin [14]. In this regard it is interesting to note two adjacent cysteines in the third cytoplasmic loop of the M1 muscarinic acetylcholine receptor. Moreover, in the β_2-adrenergic receptor, the mutation of a carboxyl tail cysteine to prevent its palmitoylation results in the uncoupling of the receptor from activation of adenylate cyclase [15]. Two cysteines (Cys-268 and Cys-277) are present in the 29 amino acid insert of the D_2 receptor and the latter is part of a possible consensus sequence for polyisoprenylation [16].

It is known that D_2 receptor activation by dopamine leads to inhibition of adenylate cyclase, increased membrane potassium conductance, and inhibition of calcium currents [17] and that D_2 receptor purified from bovine anterior pituitary exhibits a selectivity for one of the G-proteins designated G_{i2} [18]. Whether the 87-bp, 29 amino acid insert directs the protein among these transduction pathways and whether the insert might work by altering G-protein affinity via the formation of cytoplasmic sub-loops are important issues to be resolved.

Acknowledgements: We thank Drs Marc Caron and Kim Neve for their kindness in sharing pre-publication results, Dr Donald Frail for many helpful discussions and Dr Peter DiStefano for quantification of the autoradiograms.

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