British Journal of Pharmacology (1998) 125, 705–710
http://www.stockton-press.co.uk/bjp

Contributions of cysteine 114 of the human D3 dopamine receptor to ligand binding and sensitivity to external oxidizing agents

[1]Glen L. Alberts, [1]Jeffrey F. Pregenzer & [1,2]Wha Bin Im

[1]Biology II/Neuroscience Research, Pharmacia & Upjohn, Inc., 301 Henrietta Street, Kalamazoo, Michigan 49001, U.S.A.

1 Cysteine 114 (C114) of the human dopamine D3 receptor is located at the helical face of transmembrane segment III (TMIII) near aspartate 110, a counterion for the amine group of catecholamines. The contributions of C114 to receptor function were investigated here using site-directed mutagenetis of C114 to serine.

2 The C114S mutant, as expressed in Sf-9 cells, bound aminotetralin antagonists (UH-232 and AJ-76) and several agonists ((−)3-PPP, apomorphine, pramipexole and quinpirole) with markedly lower affinities as compared to the wild type D3 receptor, but bound other structurally diverse dopaminergic ligands with only minor changes in affinity. Because an N-propyl substituent is the only common structural feature among most affected ligands, we propose that the mutation alters 'a propyl cleft' on the receptor. The mutation hardly affected quinpirole-dependent [^{35}S]-GTPγS binding, suggesting C114 plays a minimal role in receptor-G-protein coupling.

3 N-Ethylmaleimide(NEM), a sulfhydryl modifying agent, blocked ligand binding to the D3 receptor, but not to the C114S mutant. We infer that C114 is the primary residue on the D3 receptor vulnerable to external oxidizing agents. Dopamine D2long and $D4_2$ receptors contain highly homologous TMIII sequences including an equivalent cysteine residue. However, only the D2long receptor, not the $D4_2$ receptor, displayed NEM sensitivity similar to that of the D3 receptor.

4 We conclude that C114 is critical for high affinity interactions between the D3 receptor and ligands containing an N-propyl substituent, and unlike its counterpart in the $D4_2$ receptor, is highly susceptible to external oxidizing agents.

Keywords: D3 dopamine receptor; D2-like dopamine receptors; cysteine residues of D3 receptor; aminotetralins

Introduction

Five major dopaminergic receptors (D1–D5) and their splice variants have been cloned and classified either the D1-like (D1 and D5) or D2-like dopamine receptors (D2, D3 and D4) Civelli *et al*., 1993; Gingrich & Caron, 1993; Seeman & Van Tol, 1994; Lachowitz & Sibley, 1997). The D2-like receptors, in particular, have been shown to mediate neurotransmission involved in motor, cognition and endocrine activities, and their ligands are of potential therapeutic value for psychotic disorders and Parkinson's disease (O'Dell *et al*., 1990; Sokoloff *et al*., 1990, 1992; Sokoloff & Schwarz, 1995). This has generated considerable interest in understanding of structure-function characteristics of D2-like receptors, particularly the identification of key residues in their ligand binding pockets. Earlier studies have shown that ligand binding pockets of catecholamine receptors are contributed primarily by trans-membrane segments (TM), and TMIII in particular is critically involved in receptor ligand interactions, by contributing an aspartate residue (D110 for D3) for an ionic bond with the amine group of catecholamines (Ostrowski *et al*., 1992; Strader *et al*., 1994). In the middle of the D3 TMIII sequence (also D2 and D4 receptors), a cysteine residue (C114) is located only four residues away from D110. This residue could potentially affect ligand binding, because cysteine residues in the active sites of receptors (or enzymes) often play important structural and functional roles, and as the strongest nucleophile in the protein, may become targets of external neurotoxic oxidizing agents (Levine *et al*., 1996). Previously, the equivalent cysteine residue in the D2 receptor was shown to be exposed in the binding-site crevice (Javitch *et al*, 1996). Here we examined the functional contributions of the C114 of the human dopamine D3 receptor by characterizing ligand binding, G-protein coupling and susceptibility to external oxidizing agents of the C114S mutant in comparison with the wild type receptor.

[2]Author for correspondence.

Methods

Mutation

Point mutations of C114S and C119S in the D3 and $D4_2$ receptor, respectively, were produced using the procedure of gene splicing by overlap extension (Horton *et al*., 1989). Briefly, we used sense and antisense primers (20–27 nucleotides long) that are complementary to the cDNA region of interest except for the codon for serine in the place of cysteine in the middle of the sequences. The primers for the C114S mutation are 5′GGATGTCATGTCTACAGCCAG-CATCC3′ (base 327–355) as the sense primer, and its complementary sequence as an antisense primer, and for the C119S mutation, 5′CGTCATGTTGTCTACTGCTTCAA (base 345–367) and its complementary sequence. Also we used another pair of outside primers, containing a unique restriction site at the 3′ and 5′ ends for each mutation. Following polymerase chain reactions (PCR) as described elsewhere (Horton *et al*., 1989), we obtained a D3 or D4 cDNA fragment with a cysteine to serine mutation in the middle and unique restriction sites at the 5′ and 3′ ends. The final PCR fragments were digested with proper restriction enzymes to yield sticky ends, and then were subcloned into the PCR-script™ (Stratagene) vector containing the D3 or D4 dopamine receptor cDNA with the complementary ends. All

mutants were confirmed initially with restriction digestion maps, and subsequently with dideoxy sequencing. The D3 and D4 insert (or their mutants) in the PCR-script™ were transferred to PVL1394 (a shuttle vector for baculovirus), and the vectors were used to prepare the recombinant baculoviruses using a BaculoGold kit from Pharmingen. The recombinant baculovirus was purified *via* plaque purification, and its stock with a titer of approximately 1×10^8 plaque-forming particles ml^{-1} was used to infect cultures of Sf-9 cells (5×10^6 viral particles/1×10^6 cells ml^{-1}). The cells were harvested 60–72 h post infection, and used to prepare membranes as described elsewhere (Pregenzer *et al*., 1993).

In some experiments, cell membranes (approximately 300 μg membrane protein) were treated with N-ethylmaleimide (NEM) or 5,5′-dithiobis-[2-nitrobenzoic acid] (DTNB) at various concentrations in medium that contained (mM) HEPES pH 7.4 25, NaCl 100, EDTA 1 and $MgCl_2$ 3, in a total volume of 250 μl for 30 min at 30°C. The mixtures were then diluted 10 fold with the medium for binding experiments containing dithiothreitol at a concentration sufficient to neutralize residual oxidizing agents. We measured binding of radioactive ligands using filtration techniques as described elsewhere (Pregenzer *et al*., 1993). Briefly, [^{3}H]-spiperone binding ([^{3}H]-haloperidol for $D4_2$) was measured in medium that contained (mM) NaCl 100, $MgCl_2$ 2, EDTA 1, HEPES/Tris (pH 7.4) 20, radioactive ligand at various concentrations (0.1–30 nM for typical binding profiles), and 20–80 μg membrane protein, in a total volume of 500 μl at 4°C for 60 min. For Scatchard analysis, we increased the reaction volume to 3 ml to obtain low ratios of bound/free ligand concentrations. The mixture was filtered over a Whatman GF/B filter under vacuum, and the filters were washed rapidly three times with 4 ml of ice-cold 50 mM Tris/HCl buffer (pH 7.4). Non-specific binding was estimated in the presence of excess unlabeled spiperone (10 μM). All stock solutions for ligands were prepared in 0.1% ascorbic acid. Displacement of [^{3}H]-spiperone (for D3) or [^{3}H]-haloperidol (for $D4_2$) by test compounds (competition assay) was measured using the same assay buffer but with the radioactive ligand at 2 nM. Half-maximal inhibitory concentrations (IC_{50} values) were converted to inhibition constants (K_i) (Cheng & Prusoff, 1973).

[^{35}S]-GTPγS binding was measured following the procedure reported earlier (Pregenzer *et al*., 1997) in medium containing (mM) HEPES 25, pH 8.0, NaCl 100, EDTA 1, $MgCl_2$ 3, dithiothreitol 0.5, 0.003% digitonin, 2 nM [^{35}S]-GTPγS (5–3×10^5 c.p.m./assay), and 20–50 μg membrane protein in a total volume of 120 μl. The mixtures also contained indicated ligands at various concentrations. Each binding reaction was initiated by adding the membranes, which had been preincubated with 10 μM GDP for 10 min on ice, and lasted for 30 min at 30°C. The mixtures were then filtered over a Whatman GF/B filter under vacuum, and the filters were washed three times with 4 ml of an ice-cold buffer containing (mM) NaCl 100, Tris/HCl 20, pH 8.0, $MgCl_2$ 25. Agonist-induced [^{35}S]-GTPγS binding was obtained by subtracting that observed without the test ligand. The binding data were analysed using a non-linear regression method (Sigma Plot), and presented as means ± s.e.mean from three or more experiments.

Results

The binding properties of the D3 receptor and its C114S mutant were examined in Sf-9 cell membranes with [^{3}H]-spiperone, a specific ligand for the D2-like dopamine receptors. The dissociation constant (K_D) for [^{3}H]-spiperone as obtained from Scatchard analysis was 0.40 ± 0.05 and 2.4 ± 0.1 nM for the wild type and the C114S mutant, respectively, while maximal binding (B_{max}) was 23 ± 3 and 36 ± 2 pmol mg^{-1} protein, respectively. Competition binding experiments were carried out with various ligands for the D2-like receptors (Table 1, Figure 1). The C114S mutant receptor showed markedly altered binding affinities for two aminotetralin antagonists, UH-232 and AJ-76, their K_i values increasing 272- and 102 fold, respectively, when compared to values for the wild type receptor. When other structurally diverse antagonists were examined, however, they exhibited much less changes in their K_i values; chlorpromazine (34 fold), raclopride (28 fold), haloperidol (16 fold), and butaclamol (7 fold), spiperone (6 fold), YM-09151-2 (5 fold). For agonists, we observed noticeable affinity changes for apomorphine (77 fold), (−)3-PPP (56 fold), quinpirole (38 fold), pramipexole (14 fold), but no appreciable changes for ergots, such as cabergoline (3 fold) and lisuride (no change).

For G-protein-coupled receptors, an early step following receptor activation is agonist-induced binding of GTP ([^{35}S]-GTPγS, a non-hydrolyzable analog, for the assay) to Gα

Table 1 Comparison of binding affinities of various ligands to D3 and its C114S mutant, and $D4_2$ and its C119S mutant

Compound	*D3* (K_i, nM)	*D3-C114S* (K_i, nM)	*D4* (K_i, nM)	*D4-C119S* (K_i, nM)
Spiperone	0.4 ± 0.1	2.4 ± 0.1	0.86 ± 0.15	0.95 ± 0.13
Butaclamol	3.0 ± 0.8	23 ± 3	381 ± 49	4800 ± 515
Chlorpromazine	1.0 ± 0.2	34 ± 3	43 ± 5	723 ± 59
YM-9151-02	0.3 ± 0.1	1.5 ± 0.3	0.4 ± 0.1	1.2 ± 0.2
UH-232	4.8 ± 0.5	1308 ± 92	37 ± 6	950 ± 90
AJ-76	31.1 ± 4.6	3154 ± 171	362 ± 48	2322 ± 334
Haloperidol	4.8 ± 0.3	79 ± 2	3.0 ± 0.3	8.6 ± 1.2
Raclopride	3.7 ± 1.5	105 ± 9	1819 ± 224	47888 ± 531
(−)3-PPP	84 ± 9	4763 ± 600	2442 ± 665	1681 ± 267
Apomorphine	23 ± 2	1769 ± 71	7.1 ± 1.5	24 ± 4
Quinpirole	14 ± 1	529 ± 28	209 ± 48	227 ± 21
Pramipexole	5.4 ± 0.6	74 ± 11	253 ± 68	298 ± 43
Lisuride	1.1 ± 0.1	0.7 ± 0.2	11 ± 1	9.5 ± 1.3
Cabergoline	1.3 ± 0.1	4.3 ± 1.0	256 ± 47	205 ± 38

Binding of [^{3}H]-spiperone (D3) or [^{3}H]-haloperidol ($D4_2$) was measured in the presence of test drugs at various concentrations in Sf-9 cell membranes expressing either wild type or mutant receptors. Half-maximal inhibitory concentrations were converted to K_i values (nM) using the Cheng-Prusoff equation. Data are means ± s.e.mean from dose-response profiles obtained from three measurements.

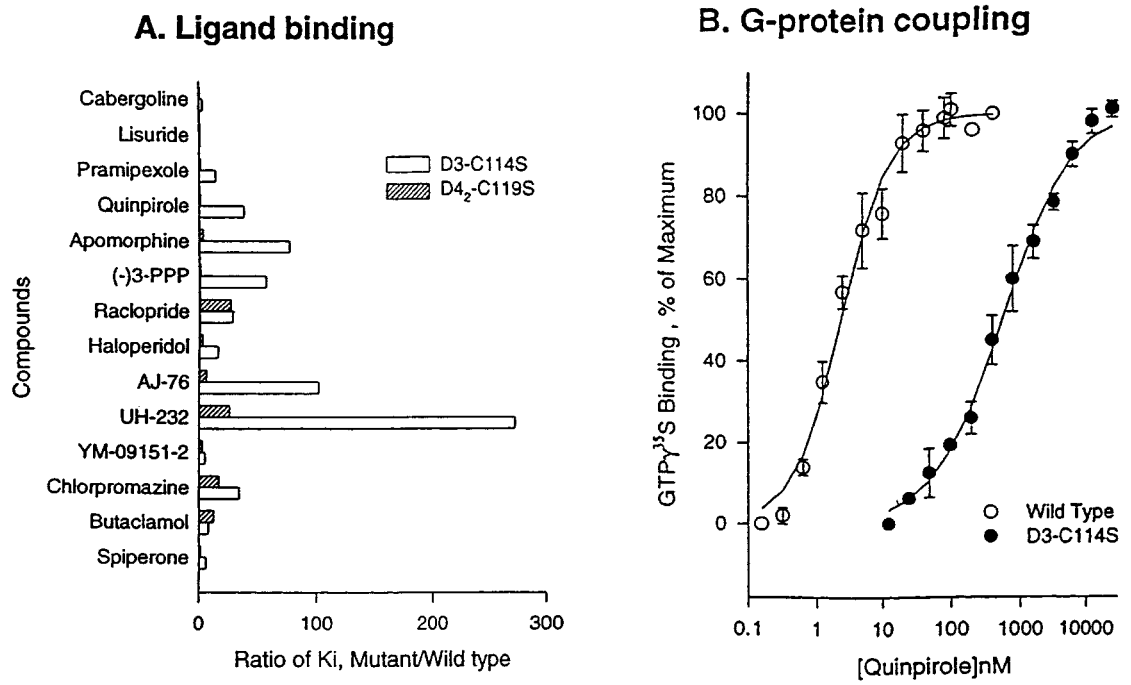

Figure 1 Comparison of ligand binding and G-protein coupling properties of the C114S mutant with those of the wild type human D3 dopamine receptor. (A) Competition binding experiments with [^{3}H]-spiperone were carried out in the presence of test ligands at various concentrations in Sf-9 cell membranes expressing mutant or wild type receptors. The plot shows the K_i ratio of the mutant to the wild type. (B) Dose-response profiles for quinpirole-induced [^{35}S]-GTPγS binding in the Sf-9 cell membrane expressing the wild type (Pregenzer *et al*., 1997) or the C114S mutant. The solid lines show data fitting to a binding isotherm for a single class of binding sites for quinpirole. The maximal binding level for [^{35}S]-GTPγS was 114 ± 6 fmoles mg^{-1} protein and 103 ± 9 fmoles mg^{-1} protein for the wild type and the mutant, respectively, and the EC_{50} ranging from 3–8 nM for the wild type and 543 ± 53 nM for the mutant.

subunits. We have shown previously (Pregenzer *et al*., 1997) that agonists for the D3 receptor, but not antagonists, dose-dependently enhance [^{35}S]-GTPγS binding to Sf-9 cell membranes expressing human D3 dopamine receptors. Quinpirole, in particular, enhanced [^{35}S]-GTPγS binding to a maximal level (E_{max}) of 114 ± 16 fmoles mg^{-1} protein with a half maximal concentration (EC_{50}) ranging from 3–8 nM. In the C114S mutant, quinpirole also dose-dependently enhanced [^{35}S]-GTPγS binding to a maximal level of 103 ± 9 fmoles mg^{-1} protein (Figure 2) with an EC_{50} value of 543 ± 53 nM. The E_{max} was similar to that observed with the wild type receptor in parallel assays, but the EC_{50} was greater than that observed with wild type receptors (6 nM). The latter could be largely attributed to a lower affinity of the mutant receptor for quinpirole (nearly 40 fold lower as compared to that to the wild type). We next examined the susceptibility of C114 to external oxidizing agents, such as DTNB, a bulky hydrophilic agent, and NEM with a moderate ability to penetrate both hydrophilic and hydrophobic phases. NEM treatment dose-dependently reduced binding of [^{3}H]-spiperone or [^{3}H]-YM-09151-2 to the wild type receptor (Figure 2). Scatchard analysis of the data shows that NEM (60 μM) treatment reduced the B_{max} for [^{3}H]-spiperone from 23 ± 3 to 1.6 ± 0.2 pmol mg^{-1} protein, with little effect on its K_D, 0.4 ± 0.1 and 0.32 ± 0.05 nM for the control and the treated, respectively (data not shown). This indicates that the NEM-mediated inhibition is non-competitive. More experiments were carried out with [^{3}H]-YM-09151-2, which displayed the highest affinity to the wild type (K_i of 0.3 nM) as well as to the C114S mutant (1.5 ± 0.3 nM) among the available radioactive ligands. NEM treatment reduced [^{3}H]-YM-09151-2 binding with an IC_{50} of 28.3 ± 1.5 μM while DTNB was much less potent than NEM, showing an IC_{50} value of 4890 ± 754 μM (Figure 2). With the C114S mutant, the IC_{50} value for NEM increased 260 fold, from 28.3 ± 1.5 to 7327 ± 1597 μM, but that for DTNB was not appreciably affected (Figure 2). This indicates that the sulphydryl group of C114 is sensitive to NEM, but is largely inaccessible to DTNB.

The human D2long receptor contains a cysteine residue (C118) equivalent to the C114 of D3, and its interaction with [^{3}H]-YM-09151-2 was blocked, upon either NEM or DTNB treatment. Their IC_{50} values are similar to those observed with the D3 receptor, 93 ± 8 and 5260 ± 1148 μM for NEM and DTNB, respectively (data not shown). The $D4_2$ receptor also contains an equivalent cysteine residue (C119), but this was not sensitive to NEM: [^{3}H]-YM-09151-2 binding was hardly affected by NEM treatment even at 2000 μM, (less than 10% inhibition) while DTNB treatment blocked the ligand binding with an IC_{50} of 1502 ± 128 μM (Figure 2). Mutation of C119 to serine in $D4_2$ produced no appreciable changes in its sensitivity to sulfhydryl agents; again no detectable inhibition by NEM at 2000 μM and a low potency block by DTNB with an IC_{50} of 1080 ± 174 μM (Figure 2). These results indicate that C119 of $D4_2$ is located in an environment quite different from the corresponding cysteine residue of D3 and D2long (see Figure 2).

We also examined ligand binding properties of the C119S mutant of $D4_2$ (Table 1). Scatchard analysis of [^{3}H]-haloperidol binding data yielded K_D values of 3.0 ± 0.3 and 8.6 ± 1.2 nM for the wild type and the mutant receptors, respectively, and B_{max} values of 26 ± 1.2 and 4.2 ± 0.4 pmoles mg^{-1} protein, respectively. Most of the test ligands used

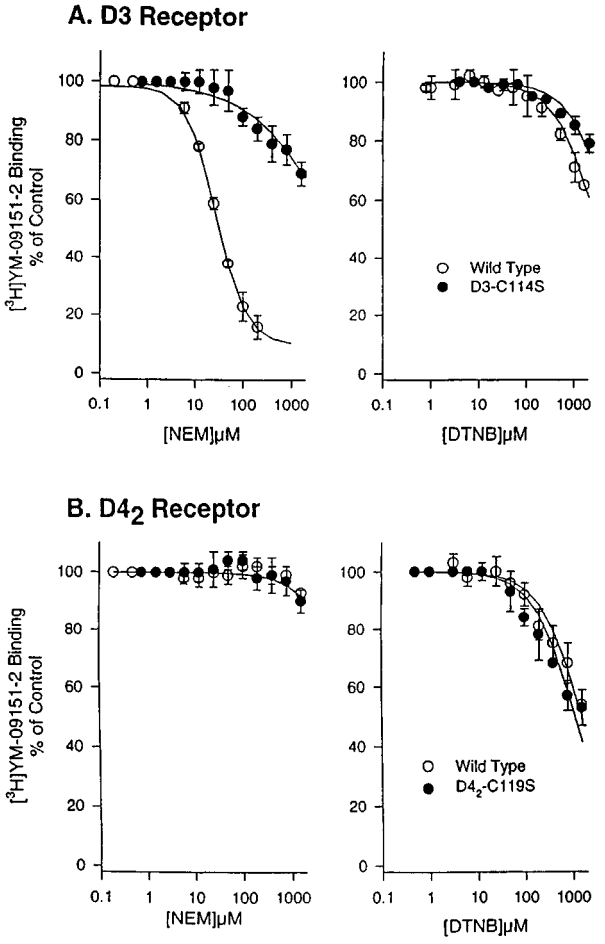

Figure 2 Inhibition of [^{3}H]-YM-09151-2 binding to D3, D4$_2$ and their mutants after pretreatment with either NEM or DTNB at the indicated concentrations. Sf-9 membranes expressing D3, its C114S mutant, D4$_2$ or its C119S mutant were treated with NEM or DTNB at the indicated concentrations for 30 min at 30°C, and the membranes were examined for their ability to bind [^{3}H]-YM-19151-2. The level of binding is expressed as a percentage of that observed with mock treated membranes.

here showed considerably lower affinities to the D4$_2$ than to the D3 receptor; K_i values being 11–491 fold greater than the D3 values except for spiperone, YM-09151-2 and haloperidol (Table 1). The C119S mutation only moderately decreased affinities of a few selective ligands, including UH-232 (26 fold), raclopride (26 fold) and chlorpromazine (17 fold) (Table 1 and Figure 1). These results further support the view that the binding pocket of D4$_2$ is quite distinct from that of the D3 receptor.

Discussion

Utilizing site-directed mutagenesis and sulfhydryl group oxidizing agents, we investigated the functional role of the sulfhydryl group of C114 in TM III, a domain critical for D3 receptor-ligand interactions (Ostrowski *et al*., 1992), and the susceptibility of C114 to oxidation. C114 is about one helical turn away from D110, a residue involved in an ionic bond with the amine group of catecholamines, and is thus likely exposed to, or at least proximal to, the ligand binding pocket. In the present investigation, we used the C114S mutant of D3 to demonstrate that the sulphydryl group at position 114 is critically important for receptor-ligand interactions. Particularly, the C114S mutant exhibited markedly lower affinities for aminotetralin analogs (AJ-76 and UH-232), by two orders of magnitude, as compared to wild type D3 receptors. Furthermore, several agonists ((−)3-PPP, apomorphine, quinpirole and pramipexole but not ergots) exhibited considerably lower affinities to C114S receptors (Table 1 and Figure 1), while other structurally diverse ligands showed lesser affinity changes. Interestingly, an N-propyl substituent (N,N-dipropyl for UH-232) is the only common structural feature among the affected ligands except for apomorphine. Earlier binding studies with 2-aminotetralins (Malmberg *et al*, 1994) has shown that at least one N-propyl substituent is essential for optional binding of this ligand class to D3 receptors, and it has been proposed that there is a propyl cleft on the D3 receptor. According to the model, the cleft extends from D110 and M113 of TMIII towards TMII and TMVII (Malmberg *et al*., 1994). We propose that C114, although not noted expressively in the model, may contribute to the propyl cleft not only because of its proximity to D110 and M113, but also because we found that the C114S mutation selectively affects the affinities of those ligands with at least one N-propyl group.

Previously (Alberts *et al*., 1998), we prepared a D3/D1 chimeric receptor in which the TMIII region from residues 104–114 (TMIII (104–114, DVFVTLDVMMC)) was replaced with the corresponding D1 sequence (NIWVAFDIMCS, including seven divergent residues which are underlined). This chimera, which has C113 and S114 instead of the M113 and C114 of D3, showed markedly lower affinities for UH-232 and AJ-76, (−)3-PPP, quinpirole, and pramipexole as compared to the wild type receptor, but displayed minimal changes in affinity for ergots (Alberts *et al*., 1998). These characteristics of the chimera are similar to those of the D3 C114S mutant examined here, and support the view that C114 critically contributes to D3 receptor-ligand interactions.

With respect to G-protein activation in the C114S mutant, quinpirole enhanced [^{35}S]-GTPγS binding to the same level as in the wild type receptor, except for a right shift in the dose-response profile, which probably reflects a lower affinity of the mutant receptor for quinpirole than the wild type receptor. Another factor potentially affecting [^{35}S]-GTPγS binding is receptor expression which was somewhat greater for the mutant receptor than for the wild type receptor (36 ± 2 vs 23 ± 2 pmol mg^{-1} protein, respectively). Previous studies with G-protein-coupled receptors have shown that a predominant population of receptors, when expressed at high levels in SF-9 cells, display phenotypes of G-protein-uncoupled receptors, primarily due to limiting endogenous G-proteins (Butkerait *et al*., 1995). This is consistent with our data that maximal [^{35}S]-GTPγS binding was only 100 fmol mg^{-1} protein while receptor expression was well above 20 pmol mg^{-1} protein. Apparently, at receptor densities attained with high expression systems like Sf-9 cells, [^{35}S]-GTPγS binding is primarily limited by endogenous G-proteins, and is not directly proportional to receptor density.

Another phenotype of G-protein-coupled receptors is the GTP-induced agonist affinity changes that are sensitive to receptor density. For example, the D2 receptor, when expressed at a density greater than 1 pmol mg^{-1} protein, showed no detectable high affinity states for agonists, again because only a small fraction of receptors is coupled to endogenous target G-proteins (Boundy *et al*., 1996). Generally, GTP-induced agonist affinity changes were reported in the literature at a receptor density less than 0.5 pmol mg^{-1} protein (Parker *et al*., 1994; Butkerait *et al*., 1995; Boundy *et al*., 1996). In addition, the degree of GTP-induced agonist affinity changes is quite

variable from one receptor to another. For example, with the D2 receptor, more than 100 fold differences were observed in agonist affinities between the G-protein-coupled and -uncoupled receptors, while only small affinity differences (less than 10 fold) have been reported for the D3 receptors (Sokoloff & Schwatz, 1995). Indeed, we were not able to detect GTP-sensitivity of agonist binding to D3 receptors, even at a receptor density less than 1 pmol mg^{-1} protein. Our current binding data for agonists thus represent low affinity states. From all these considerations, the agonist-mediated [^{35}S]-GTPγS binding seems to be the appropriate way to evaluate G-protein coupling processes for D3, especially with the Sf-9 high expression system, and our current data are consistent with the view that the C114 appears to play a minimal role in receptor coupling to cellular G-proteins. Also, we would like to point out that in the Sf-9 cell system, receptor density is variable, even from batch to batch, because of a number of factors, such as culture conditions, the age of Sf-9 cells, cell harvest time, titers of recombinant baculoviruses, and unpredictable expression efficacy of virions carrying different cDNA, even if only slightly modified cDNA. In our hands, receptor density varied from 1–36 pmol mg^{-1} protein despite our reasonable efforts to control these factors in a uniform manner. It has been demonstrated, however, that ligand binding property is not affected by receptor density (Alberts *et al*., 1988).

The current data also demonstrate that C114 is the primary residue sensitive to an externally applied oxidizing agent, NEM (medium-sized amphiphilic), but not to DTNB (bulky and hydrophilic). This indicates that C114 is located in a binding region probably contiguous with the extracellular aqueous phase, but spatially restricted. Interestingly, the two residues adjacent to C114 toward the extracellular side are methionine (M112 and M113), which is known as an endogenous antioxidant in proteins (Levine *et al*., 1996). These methionines, therefore, could partially protect the C114 sulphydryl group in the D3 receptor from external oxidizing agents. It is also noteworthy that the human D_3 receptor has five additional cysteine residues in the transmembrane segments, C37 (TMI), C51 (TMI), C122 (TMIII), C166 (TMIV) and C341 (TMVI). Their apparent NEM-insensitivity could arise either from their presence in non-critical binding regions or from their limited exposure to the extracellular side.

The D2long and $D4_2$ receptors also contain a cysteine residue equivalent to C114 of D3. The NEM-sensitivity we observed here (see Figure 2) indicates that the cysteine residue of D3 may share similar environments with that of D2, but not with that of $D4_2$. In fact, the NEM-insensitivity of $D4_2$ is surprising, because the TMIII region is highly conserved among the D2-like receptors; the only divergent residue near the cysteine is leucine ($D4_2$) in the place of M113 (D3). It appears that despite their high homology in TMIII, the overall conformation of $D4_2$ differs appreciably from those for D2 and D3, and could lead to its insensitivity to NEM. This view is supported by our current observations that binding profiles of the $D4_2$ observed with test ligands were considerably different from those of D3.

Of some interest is whether this differential sensitivity to external oxidizing agents among the D2-like receptors is related to pathophysiological processes. One of the currently popular themes is that oxidative stresses arising from both intracellular and extracellular sources may compromise functional integrity of the receptors, which in turn could lead to pathological processes. With respect to extracellular oxidizing stresses, D2long and D3 could be more vulnerable than $D4_2$. So, it will be of some future interest to examine the extent to which C114 of D3 is oxidized *in situ* (or C118 in D2long), and how such oxidized receptors contribute to pathophysiological processes.

We thank Dr John E. Bleasdale for his helpful comments on the manuscript.

References

ALBERTS, G.L., PREGENZER, J.F. & IM, W.B. (1998). Identification of transmembrane regions critical for ligand binding to the human D3 dopamine receptor using various D3/D1 transmembrane chimeras. *Mol. Pharmacol.,* in press.

BOUNDY, V.A., LU, L. & MOLINOFF, P.B. (1996). Differential coupling of rat D2 dopamine receptor isoforms expressed in *Spodoptera frugiperda* insect cells. *J. Pharmacol. Exp. Therap.,* **276,** 784–794.

BUTKERAIT, P., ZHENG, Y., HALLAK, H., GRAHAM, T.E., MILLER, H.A., BURRIS, K.D., MOLINOFF, P.B. & MANNING, D.R. (1995). Expression of the human 5-hydroxytryptamine$_{1A}$ receptor in Sf9 cells. *J. Biol. Chem.,* **270,** 18691–18699.

CHENG, Y-C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099–3108.

CIVELLI, O., BUNZOW, J.R. & GRANDY, D.K. (1993). Molecular diversity of the dopamine receptors. *Annu. Rev. Pharmacol. Toxicol.,* **32,** 281–307.

GINGRICH, J.A. & CARON, M.G. (1993). Recent advances in the molecular biology of dopamine receptors. *Annu. Rev. Neurosci.,* **16,** 299–321.

HORTON, R.M., HUNT, H.D., HO, S.N., PULLEN, J.K. & PEASE, L.R. (1989). Engineering hybrid genes without the use of restriction enzymes: Gene splicing by overlap extension. *Gene,* **77,** 61–68.

JAVITCH, J.A., FU, D. & CHEN, J. (1996). Differentiating dopamine D2 ligands by their sensitivities to modification of the cysteine exposed in the binding-site crevice. *Mol. Pharmacol.,* **49,** 692–698.

LACHOWITZ, J.E. & SIBLEY, D.R. (1997). Molecular characteristics of mammalian dopamine receptors. *Pharmacol. Toxicol.,* **81,** 105–113.

LEVINE, R.L., MOSONI, L., BERLETT, B.S. & STADTMAN, E.R. (1996). Methionine residues as endogenous antioxidants in proteins. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 15026–15040.

MALMBERG, Å., NORDALL, G., JOHANSSON, A.M., MOHELL, N. & HACKSELL, U. (1994). Molecular basis for the binding of 2-aminotetralins to human dopamine D_{2A} and D_3 receptors. *Mol. Pharmacol.,* **46,** 299–312.

O'DELL, S.J., HOSTE, G.J., WIDMARK, C.B., SHAPIRO, R.M., POTKIN, S.G. & MARSHALL, J.F. (1990). Chronic treatment with clozapine or haloperidol differentially regulates dopamine and serotonin receptors in the brain. *Synapse,* **6,** 146–153.

OSTROWSKI, J., KJELSBERG, M.A., CARON, M.G. & LEFKOWITZ, R.J. (1992). Mutagenesis of the β2-adrenergic receptor: How structure elucidates function. *Annu. Rev. Pharmacol. Toxicol.,* **32,** 167–183.

PARKER, E.M., GINSEL, D.A., IBEN, L.G., NOWAK, H.P., MAHLE, C.D., YOCCA, F.D. & GAUGHAN, G.I. (1994). Characterization of human 5HT1 receptors expressed in Sf9 cells. *Europ. J. Pharmacol.,* **268,** 43–53.

PREGENZER, J.F., ALBERTS, G.L. & IM, W.B. (1997). Agonist-induced [^{35}S]-GTPγS binding in the membranes of *Spodoptera frugiperda* insect cells expressing the human D3 dopamine receptor. *Neurosci. Lett.,* **226,** 91–94.

PREGENZER, J.F., IM, W.B., CARTER, D.B. & THOMSEN, D.R. (1993). Comparison of interaction of [^{3}H]-muscimol, t-butylbicyclophosphoro[^{35}S]-thionate, and [^{3}H]-flunitrazepam with cloned $GABA_A$ receptors of the $\alpha1\beta2$ and $\alpha1\beta2\gamma2$ subtypes. *Mol. Pharmacol.,* **43,** 801–806.

SEEMAN, P. & VAN TOL, H.M. (1994). Dopamine receptor pharmacology. *Trends Pharmacol. Sci.,* **15,** 265–270.

SOKOLOFF, P., GIROS, B., MARTRES, M-P., BOUTHENET, M-L. & SCHWARZ, J-C. (1990). Molecular cloning and characterization of a novel dopamine receptor (D3) as a target of neuroleptics. *Nature,* **347,** 146–151.

SOKOLOFF, P., MARTRES, M-P., GIROS, B., BOUTHENET, M-L. & SCHWARZ, J-C. (1992). The third dopamine receptor (D_3) as a novel target for antipsychotics. *Biochem. Pharmacol.,* **43,** 659–666.

SOKOLOFF, P. & SCHWARTZ, J-C. (1995). Novel dopamine receptors half a decade later. *Trends Pharmacol. Sci.,* **16,** 270–275.

STRADER, C.D., FONG, T.M., TOTA, M.R. & UNDERWOOD, D. (1994). Structure and function of G protein-coupled receptors. *Annu. Rev. Biochem.,* **63,** 101–132.

(Received April 15, 1998
Revised June 28, 1998
Accepted July 23, 1998)