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Differentiating Dopamine D_2 Ligands by Their Sensitivities to Modification of the Cysteine Exposed in the Binding-Site Crevice

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SUMMARY

Cys^{118}, in the third membrane-spanning segment of the dopamine D_2 receptor, is exposed in the binding-site crevice. Cys^{118} reacts with the highly polar, sulfhydryl-specific reagents methanethiosulfonate ethylammonium (MTSEA) and methanethiosulfonate ethyltrimethylammonium (MTSET), and this reaction is retarded by the presence of antagonists and agonists. The reaction of MTSEA covalently attaches $—SCH_2CH_2NH_3^+$ to the cysteine sulfhydryl, producing a lysine-like side chain. The reaction of MTSEA with Cys^{118} decreased the affinity of substituted-benzamide antagonists, such as YM-09151–2, by 50–2800-fold, whereas the affinities of other antagonists, such as *N*-methyl-spiperone, were decreased ≤6-fold. Agonist affinities were decreased 3–12,000-fold. Mutation of Cys^{118} to Lys had effects similar to that of the reaction of Cys^{118} with MTSEA. In contrast, mutation to the uncharged Met, the side-chain volume of which is similar to that of Lys, had much lesser effects on binding. All of the agonists and antagonists contain a positively charged nitrogen that is thought to interact with the side chain of Asp^{114}, located one α-helical turn above Cys^{118}. If this nitrogen is close to Asp^{114}, then in the substituted-benzamides, the group on the nitrogen or the pyrrolidine ring itself could extend toward Cys^{118}. Modification of Cys^{118} would then interfere with binding. The reaction of MTSET with Cys^{118} covalently attaches $—SCH_2CH_2N(CH_3)_3^+$, which is bulkier and ~2 Å longer than the $—SCH_2CH_2NH_3^+$ added by MTSEA. In contrast to MTSEA, MTSET had equally large effects on the binding of YM-09151–2 and *N*-methyl-spiperone. Therefore, the effect on binding depends on both the size and the charge of the side chain substituted for that of Cys^{118}.

The dopamine receptors, like the homologous receptors for the biogenic amines and for acetylcholine, bind neurotransmitters present in the extracellular medium and couple this binding to the activation of intracellular G proteins (1–3). The binding sites of these receptors are formed among their seven, mostly hydrophobic, membrane-spanning segments (2–4) and are accessible to charged, water-soluble agonists, like dopamine. Thus, for each of these receptors, the binding site is contained within a water-accessible crevice, the binding-site crevice, that extends from the extracellular domain of the receptor into the membrane-spanning domain. Some of the residues forming the surface of this crevice are in contact with bound agonists or antagonists, whereas others may have a structural role.

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To identify and characterize the binding-site crevice residues, we are mutating each of the residues in the membrane-spanning segments of the dopamine D_2 receptor to cysteine. If a substituted-cysteine reacts with a polar, sulfhydryl-specific reagent and if this reaction is retarded by the presence of a reversibly binding agonist or antagonist, we conclude that the residue for which the cysteine was substituted is in the binding-site crevice. We detect the reaction by its irreversible block of the subsequent binding of radiolabeled ligand.

The reagents we have used are derivatives of MTS: the positively charged MTSEA and MTSET and the negatively charged methanethiosulfonate ethylsulfonate (5). These reagents are approximately the same size as dopamine, with maximum dimensions of 10 Å × 6 Å. They form mixed disulfides with cysteine sulfhydryls, covalently attaching $—SCH_2CH_2X$, where X is NH_3^+, $N(CH_3)_3^+$, or SO_3^- (Fig. 1). These highly polar MTS derivatives should react much faster

ABBREVIATIONS: MTS, methanethiosulfonate; MTSEA, methanethiosulfonate ethylammonium; MTSET, methanethiosulfonate ethyltrimethylammonium; MMTS, methylmethanethiosulfonate; YM-09151–2, (±)-*N*-(1-benzyl-2-methylpyrrolidine-3-yl)-5-chloro-2-methoxy-4-(methylamino) benzamide; DO 749, (+)-5-(aminosulfonyl-*N*-[1-benzyl-2-pyrrolidinyl)methyl]-2-methoxybenzamide; DO 748, (+)-5-(aminosulfonyl-*N*-[1-phenylethyl-2-pyrrolidinyl)methyl]-2-methoxybenzamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline.

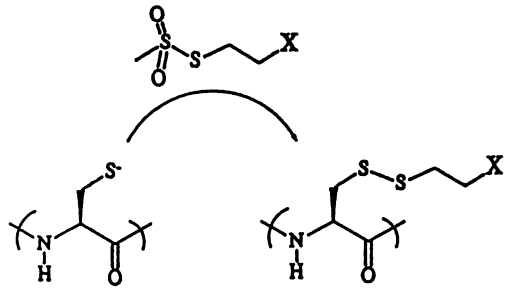
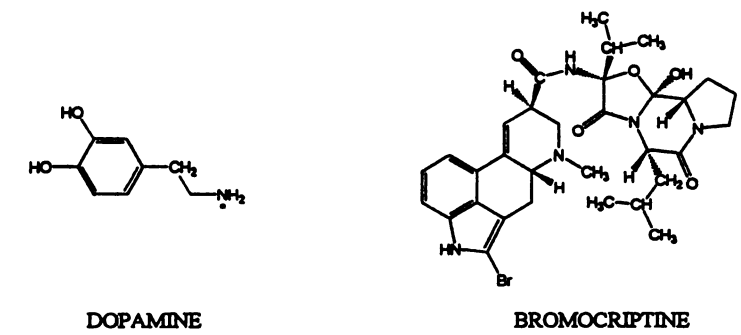


Fig. 1. Reaction of MTS derivatives with a cysteine sulfhydryl. These hydrophilic, lipophobic, sulfhydryl-specific reagents form mixed disulfides with the cysteine sulfhydryl, covalently linking $—SCH_2CH_2X$, where X is NH_3^+, $N(CH_3)_3^+$, or SO_3^-.

with cysteine sulfhydryl groups at the water-accessible surface of the receptor, including the surface of the binding-site crevice, than with sulfhydryls facing the lipid bilayer or buried in the protein interior (6–9). Furthermore, the reaction of the MTS reagents, as exemplified by the reaction of MMTS with 2-mercaptoethanol, is 5×10^9 times faster with the ionized thiolate than with the unionized thiol (10), and only water-exposed cysteine thiols are likely to ionize. Using this approach, we identified 10 residues in the third membrane-spanning segment (8) and 13 residues in the fifth membrane-spanning segment (9) as exposed in the binding-site crevice.

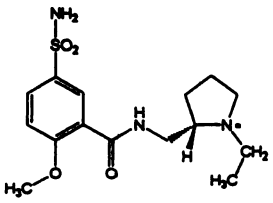
We previously found that binding of the antagonist [^{3}H]YM-09151–2 to wild-type D_2 receptor (before mutagenesis) was irreversibly inhibited by the MTS reagents (7). The positively charged MTSEA and MTSET blocked binding several hundred-fold faster than the negatively charged methanethiosulfonate ethylsulfonate, which is consistent with the affinity of the binding site for positively charged dopamine agonists and antagonists. Furthermore, both agonists and antagonists of the D_2 receptor protected against irreversible inhibition by the MTS reagents. To identify the susceptible cysteine in wild-type receptor, we mutated, one at a time, five transmembrane and two extracellular cysteine residues to serine. Only the mutation of Cys^{118} decreased the susceptibility of antagonist binding to irreversible inhibition by the MTS reagents. Thus, Cys^{118}, a residue in the third membrane-spanning segment, is exposed in the D_2 receptor binding-site crevice (7, 8).

We originally assessed the effects of the MTS reagents on the binding of [^{3}H]YM-09151–2 (Fig. 2), and we were surprised to observe that the effects were far less on the binding of another antagonist, [^{3}H]*N*-methyl-spiperone (Fig. 2). In

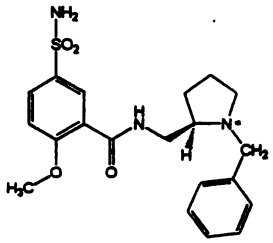


DOPAMINE

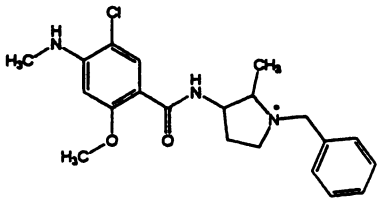
BROMOCRIPTINE



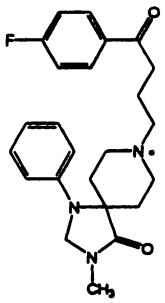
S(-)SULPIRIDE



R(+)DO 749



YM-09151-2



N-METHYL-SPIPERONE

Fig. 2. Structures of the agonists dopamine and bromocriptine; the substituted benzamide antagonists, sulpiride, DO 749, and YM-09151–2; and the nonbenzamide antagonist *N*-methyl-spiperone. +, protonated nitrogen.

the current study, we explored the effects of chemical modification and mutation of Cys^{118} on the affinity of the receptor for various antagonists and agonists.

Experimental Procedures

Materials. [^{3}H]YM-09151–2 (87 Ci/mmol) and [^{3}H]*N*-methylspiperone (84 Ci/mmol) were obtained from DuPont-NEN. Raclopride was a gift from ASTRA (Södertälje, Sweden); piquindone was a gift from Dr. M. Teeter (Boston College, Chestnut Hill, MA); DO 748 and DO 749 were gifts from Dr. P. Sokoloff (Cenre Paul Broca Inserm, Paris, France); and unlabeled YM-09151–2 was a gift from the Yamanouchi Pharmaceutical Co. (Tokyo, Japan). Fluoro-benzylspiperone was obtained from Tocris Cookson; all other drugs were obtained from Research Biochemicals. The MTS reagents were synthesized according to the method of Stauffer and Karlin (5).

Stable and transient transfection. Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium/F-12 (1:1) containing 3.15 g/liter glucose (Specialty Media) in 10% bovine calf serum (Hyclone) at 37° and 5% CO_2. A cell line stably expressing human D_2 receptor was developed as described previously (7). Cells were transiently transfected with lipofectamine (GIBCO) as described previously (8).

Preparation of cells. At 48 hr after transient transfection or when nearly confluent for stably transfected cells, cells were washed with PBS (8.1 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 138 mM NaCl, 2.7 mM KCl, pH 7.2), briefly treated with PBS containing 5 mM EDTA, and then dissociated in PBS. Cells were pelleted at 1000 × *g* for 5 min at 4° and resuspended for binding or treatment with MTS reagents.

[^{3}H]YM-09151–2 and [^{3}H]*N*-methyl-spiperone binding. Whole cells from a 35-mm plate were resuspended in 450 μl of buffer A (25 mM HEPES, 140 mM NaCl, 5.4 mM KCl, 1 mM EDTA, 0.006% bovine serum albumin, pH 7.4). Cells were then diluted 20-fold with buffer A. [^{3}H]YM-09151–2 and [^{3}H]*N*-methyl-spiperone binding was determined according to a modification of reported procedures (8). For saturation binding, duplicate borosilicate tubes contained six different concentrations of ligand in buffer A with 300 μl of cell suspension in a final volume of 0.5 ml. Competition assays had a final volume of 2 ml and 8–10 concentrations of the indicated drugs. The mixture was incubated at room temperature for 60 min and then filtered with a Brandel cell harvester through Whatman 934AH glass fiber filters (Brandel). The filter was washed twice with 5 ml of 10 mM Tris·HCl, 120 mM NaCl, pH 7.4, at room temperature. Specific [^{3}H]YM-09151–2 binding and specific [^{3}H]*N*-methyl-spiperone binding were defined as total binding minus nonspecific binding in the presence of 1 μM (+)-butaclamol.

Reactions with MTS reagents. Aliquots of cell suspension in buffer A were incubated with freshly dissolved MTS reagents at the stated concentrations at room temperature for 2 min. Cell suspensions were then diluted and assayed for binding as described above.

Site-directed mutagenesis. Mutations were generated as previously described (8) and were confirmed by DNA sequencing. Mutants are presented as wild-type residue/residue number/mutant residue; the residues are given in the single-letter code.

Results

A 2-min application of 2.5 mM MTSEA to intact cells stably expressing wild-type D_2 receptor lowered specific [^{3}H]YM-09151–2 binding, assayed near its K_D, by ~95% (Fig. 3A). This decrease in binding resulted from an ~45-fold decrease in the affinity of the receptor for [^{3}H]YM-09151–2 (Fig. 3A). The inhibition could not be reversed by removal of the MTS reagents but could be reversed partially with 100 mM 2-mercaptoethanol (data not shown), which could reduce the disulfide bond formed between $—SCH_2CH_2NH_3^+$ and cysteine. In contrast to its effect on [^{3}H]YM-09151–2, the reaction with

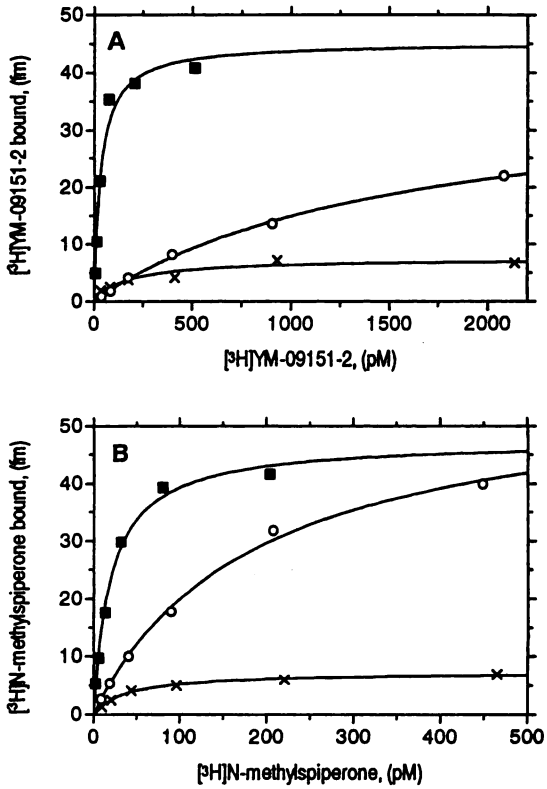


Fig. 3. Effect of MTSEA and MTSET on the binding of [^{3}H]YM-09151–2 (A) and [^{3}H]*N*-methyl-spiperone (B). Cells stably transfected with dopamine D_2 receptor (■) were reacted with 2.5 mM MTSEA (○) or 10 mM MTSET (×) for 2 min as described in Experimental Procedures. Cells were then diluted and assayed for [^{3}H]YM-09151–2 or [^{3}H]*N*-methylspiperone binding as described. Data are from a representative experiment with duplicate determinations and were fit to a saturation binding isotherm by nonlinear regression. This experiment was performed three times with similar results.

MTSEA lowered the affinity of the receptor for [^{3}H]*N*-methylspiperone by ~6-fold (Fig. 3B). Reaction with MTSET, however, which attaches the longer and bulkier $—SCH_2CH_2N(CH_3)_3^+$ to the cysteine sulfhydryl, equally reduced the apparent number of binding sites for both antagonists (Fig. 3).[1] MMTS, which attaches the smaller, uncharged $—SCH_3$ to the cysteine sulfhydryl, had no significant effect on the binding of either antagonist (data not shown).

Because [^{3}H]*N*-methyl-spiperone binding to D_2 receptor modified by MTSEA was still readily measurable, we determined the affinities of a number of specific antagonists and agonists competing with [^{3}H]*N*-methyl-spiperone binding before and after reaction of the D_2 receptor with 2.5 mM MTSEA. Reaction of MTSEA with Cys^{118} reduced the affinities of the substituted-benzamide antagonists YM-09151–2, eticlopride, DO 749, raclopride (Fig. 4A), DO 748, sulpiride, and metoclopramide and of piquindone by 50–2800-fold (Table 1). In marked contrast, the affinities of antagonists from other structural classes, including fluoro-benzyl-spiperone, pimozide, haloperidol (Fig. 4B), fluphenazine, butaclamol, and

[1] The normalized B_{max} values for MTSEA- and MTSET-treated receptors were 82 ± 4% and 24 ± 4% for [^{3}H]YM-09151–2 and 116 ± 5% and 20 ± 4% for [^{3}H]*N*-methyl-spiperone, respectively (mean ± standard error, three experiments). K_D values for control, MTSEA-treated, and MTSET-treated receptors were 30 ± 7, 1300 ± 290, and 240 ± 100 pM for [^{3}H]YM-09151–2 and 22 ± 5, 140 ± 25, and 140 ± 61 pM for [^{3}H]*N*-methyl-spiperone, respectively (mean ± standard error, three experiments).

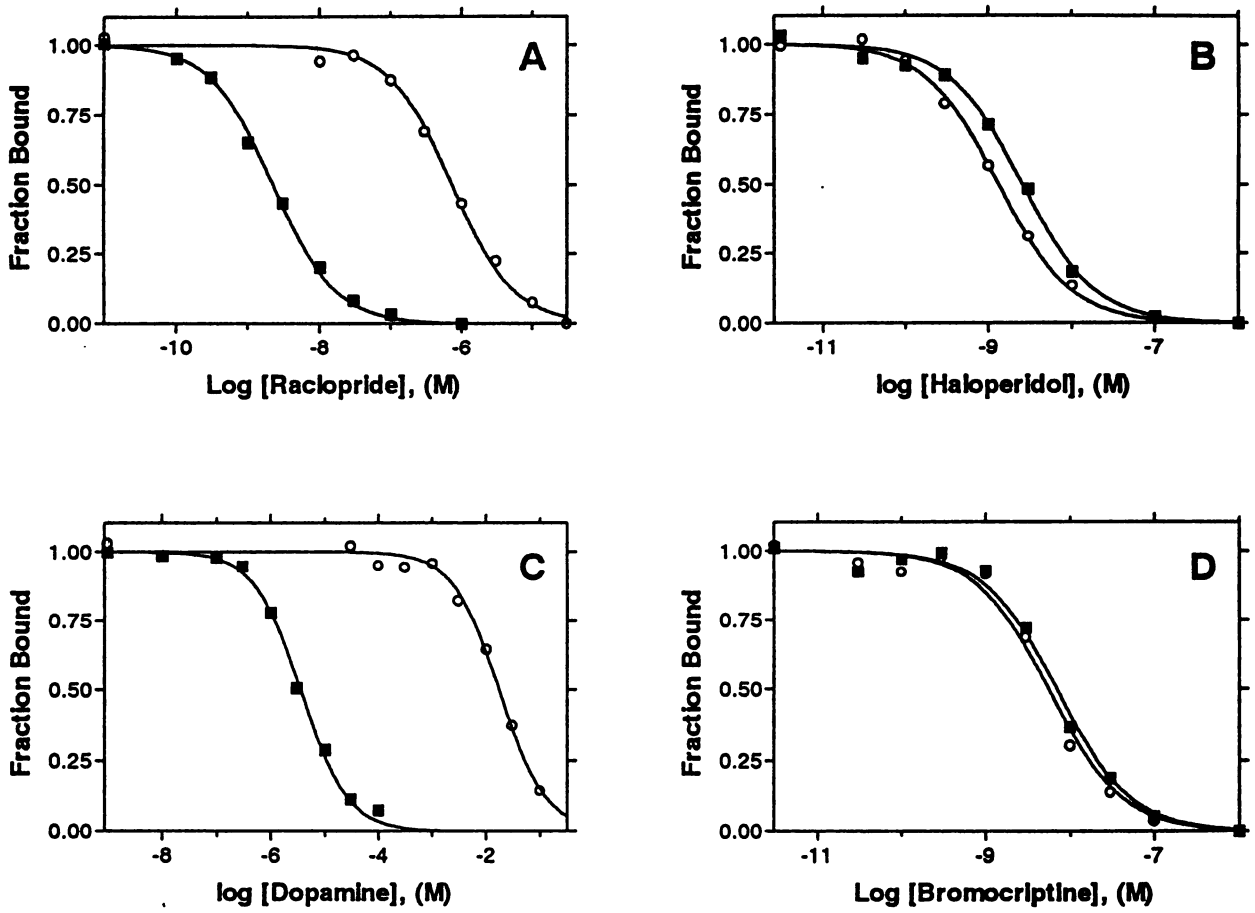


Fig. 4. Effect of reaction with MTSEA on the affinity of D_2 receptor for antagonists and agonists. Cells stably transfected with dopamine D_2 receptor were treated with (○) or without (■) 2.5 mM MTSEA for 2 min and assayed with [^{3}H]*N*-methyl-spiperone (80 pM) in the presence of nine concentrations of the indicated drug as described in Experimental Procedures. Data from representative experiments are shown and are fit to a one-site competition model by nonlinear regression.

clozapine, were reduced by ≤5-fold (Table 1). Large differences were also seen in the effects of MTSEA on agonists: although the affinity of dopamine was reduced 12,000-fold (Fig. 4C), the affinity of bromocriptine was reduced only 3-fold (Fig. 4D). Intermediate effects were seen with other agonists (Table 1).

Reaction of cysteine with MTSEA produces a lysine-like side chain. We directly mutated Cys^{118} to lysine. The affinity of $C^{118}K$ for sulpiride and for dopamine was less than that of wild-type receptor by ~10,000-fold (Fig. 5), whereas the affinity for [^{3}H]*N*-methyl-spiperone was 10-fold less than that of wild-type receptor (data not shown).[2] Mutation of Cys^{118} to serine or alanine decreased the affinity for [^{3}H]*N*-methyl-spiperone by <2-fold, and mutation to Met decreased the affinity for [^{3}H]*N*-methyl-spiperone by ~5-fold (data not shown). The affinities of $C^{118}S$, $C^{118}A$, and $C^{118}M$ for sulpiride and for dopamine were only slightly different than those for wild-type (Fig. 5).

Discussion

Cys^{118}, a residue in the middle of the third membrane-spanning segment, is exposed in the D_2-receptor binding-site crevice. Reaction of Cys^{118} with MTSEA, which attaches $—SCH_2CH_2NH_3^+$ to the cysteine sulfhydryl, dramatically reduced the binding of the substituted-benzamide [^{3}H]YM-09151–2. Surprisingly, reaction with MTSEA only modestly reduced the affinity of the nonbenzamide antagonist [^{3}H]*N*-methyl-spiperone. Consistent with these results, the affinities of the substituted-benzamide derivatives tested and of piquindone were reduced 50–2800-fold after reaction with MTSEA, whereas the affinities of other nonbenzamide antagonists tested were reduced <6-fold. Because the binding of the nonbenzamide antagonists is only slightly affected, reaction of MTSEA with Cys^{118} does not disrupt the structure of the binding site. Instead, the irreversible inhibition of binding by MTSEA is likely due to steric and/or electrostatic repulsion of the antagonists by the new lysine-like side chain generated by the reaction of MTSEA with Cys^{118}.

All D_2 ligands contain a positively charged nitrogen that is thought to interact with the side chain of Asp^{114} (3, 12–14), located one α-helical turn above Cys^{118} (8). If this nitrogen is close to Asp^{114}, then in the substituted-benzamides, the group on the nitrogen or the pyrrolidine ring itself could extend toward Cys^{118}. The mutation of Cys^{118} to lysine affected binding identically to the reaction of Cys^{118} with MTSEA, i.e., the affinity of the $C^{118}K$ mutant for [^{3}H]*N*-methyl-spiperone was reduced ~10-fold, whereas its affinity for sulpiride and for dopamine was reduced ~10,000-fold

[2] The levels of expression of $C^{118}M$ and $C^{118}K$ were ~10–20% of those observed for wild-type receptor and for the $C^{118}S$ and $C^{118}A$ mutants (data not shown).

TABLE 1

Effect of reaction with MTSEA on the affinity of the dopamine D_2 receptor for antagonists and agonists

Human embryonic kidney 293 cells stably transfected with dopamine D_2 receptor were treated with or without 2.5 mM MTSEA for 2 min as described in Experimental Procedures. Cells were then diluted and assayed with [^{3}H]*N*-methyl-spiperone (80 pM) in the presence of nine concentrations of the indicated drug as described. The apparent K_i value was determined according to the method of Goldstein and Barrett (11) using the IC_{50} value obtained by fitting the data to a one-site competition model by nonlinear regression. Piquindone, a pyrroloisoquinoline, is grouped with the substituted benzamides because of the shared sodium regulation of binding and effects of MTSEA on binding. The mean ± standard deviation values are shown for varying numbers of independent experiments (*n*) each with duplicate determinations.

	K_i	$K_{i(MTSEA)}$	*n*	$K_{i(MTSEA)}/K_i$
	nM	nM		
Substituted-benzamides				
YM-09151–1	0.023 ± 0.005	1.2 ± 0.14	5	51
Eticlopride	0.034 ± 0.006	4.5 ± 0.59	2	130
DO 749	0.20 ± 0.009	190 ± 0.001	3	950
Piquindone	0.30 ± 0.009	70 ± 33	2	240
Raclopride	0.49 ± 0.023	430 ± 28	2	900
DO 748	0.91 ± 0.12	620 ± 11	3	680
(−)-Sulpiride	1.7 ± 0.019	4600 ± 970	2	2800
Metoclopramide	5.0 ± 1.4	6400 ± 470	2	1300
Other antagonists				
Fluoro-benzyl-spiperone	0.099 ± 0.006	0.32 ± 0.01	2	3
Pimozide	0.32 ± 0.14	0.62 ± 0.39	2	2
Haloperidol	0.35 ± 0.12	0.72 ± 0.053	2	2
Fluphenazine	0.40 ± 0.22	0.24 ± 0.012	2	1
(+)-Butaclamol	0.48 ± 0.052	0.87 ± 0.16	2	2
Clozapine	43 ± 14	210 ± 74	2	5
Agonists				
Terguride	0.44 ± 0.005	3.5 ± 0.12	2	8
Bromocriptine	1.3 ± 0.012	3.7 ± 0.29	2	3
(±)-2-(*N*-phenylethyl-*N*-propyl)amino-5-hydroxytetralin hydrochloride	5.8 ± 1.7	1300 ± 300	2	220
Dopamine	970 ± 160	$11 \times 10^6 \pm 1.3 \times 10^6$	3	12,000
Quinpirole	1400 ± 300	37,000 ± 5,300	2	27

(Fig. 5). Thus, MTSEA produces its effects by modifying Cys^{118}, and the resulting lysine-like side chain (or the slightly smaller lysine side chain generated by mutagenesis) is responsible for the observed effects on ligand binding.

In contrast, substitution of Cys^{118} by methionine or the creation of a methionine-like residue by reaction of Cys^{118} with MMTS (which adds —SCH_3 to the cysteine sulfhydryl) had only a small effect on the affinity of the receptor for *N*-methyl-spiperone, dopamine, and sulpiride (Fig. 5). The side-chain volume of methionine is nearly the same as that of lysine; therefore, volume alone (i.e., steric repulsion) cannot account for the differences between the lysine and methionine mutants. The positive charge of the lysine side chain must play a significant role. If the mechanism is purely electrostatic repulsion of ligand, then the positively charged nitrogen in substituted-benzamides and in piquindone is closer to the positively charged side chain of Lys^{118}. The positively charged lysine side chain may also interfere sterically with the binding of the substituted-benzamides and piquindone and some agonists but not other ligands.

The difference in the positions of the substituted-benzamides compared with the other classes of antagonists is subtle, because the reaction of MTSET with Cys^{118} disrupts the binding of *N*-methyl-spiperone as well as of YM-09151–2. The reaction of MTSET with Cys^{118} covalently attaches —$SCH_2CH_2N(CH_3)_3^+$, which is bulkier and ~2 Å longer than the —$SCH_2CH_2NH_3^+$ added by MTSEA.

A pharmacophoric model has been built to account for the structure-activity relationships of a series of substituted-benzamides as well as other D_2 antagonists (15). In addition to the main anchoring areas, the existence was proposed of three accessory binding sites for the *N*-substituent: a small hydrophobic pocket and two different aromatic binding sites (Ar_2 and Ar_3). According to this model, most antagonists occupy only one of the accessory binding sites. The *N*-ethyl substituents of sulpiride and of related 2-pyrrolidinyl benzamides are proposed to interact with a small hydrophobic pocket near the site of interaction of the protonated amine; the larger *N*-benzyl substitutents of the 2-pyrrolidinyl benzamides DO 748 and DO 749 are predicted to interact with the Ar_3 accessory site; and the *N*-benzyl substituent of the 3-pyrrolidinyl benzamide YM-09151–2 is proposed to interact with the Ar_2 accessory site. If Cys^{118} were in or near the hydrophobic pocket, sulpiride would bind very poorly after disruption of this subsite by the reaction of MTSEA. YM-09151–2 might be less affected because it interacts with Ar_2 rather than with the hydrophobic pocket. Similarly, the model would predict that DO 748 and DO 749 be less affected by reaction of MTSEA with Cys^{118} because they interact with Ar_3 rather than with the hydrophobic pocket. However, the affinities of DO 748 and DO 749 were decreased nearly 1000-fold by the reaction of MTSEA with Cys^{118}. This suggests that the MTSEA-modified side chain interferes with a common feature of substituted-benzamide binding, not simply by disrupting binding to a hydrophobic subsite used only by sulpiride-like benzamide antagonists.

Other residues have been identified that affect the binding of substituted-benzamides. Mutation to leucine of His^{394} in the sixth membrane-spanning segment decreases the affinity (≤20-fold) of a subgroup of substituted-benzamide drugs, increases the affinity (≤25-fold) of another subgroup of substituted-benzamide drugs, and has little effect on still other substituted-benzamide drugs or several nonbenzamides (16). It is unclear whether mutation of His^{394} affects the binding of different compounds directly or indirectly through a propagated structural perturbation; nevertheless, various substituted-benzamides are affected differently.

The binding of the substituted-benzamides and of piquin-

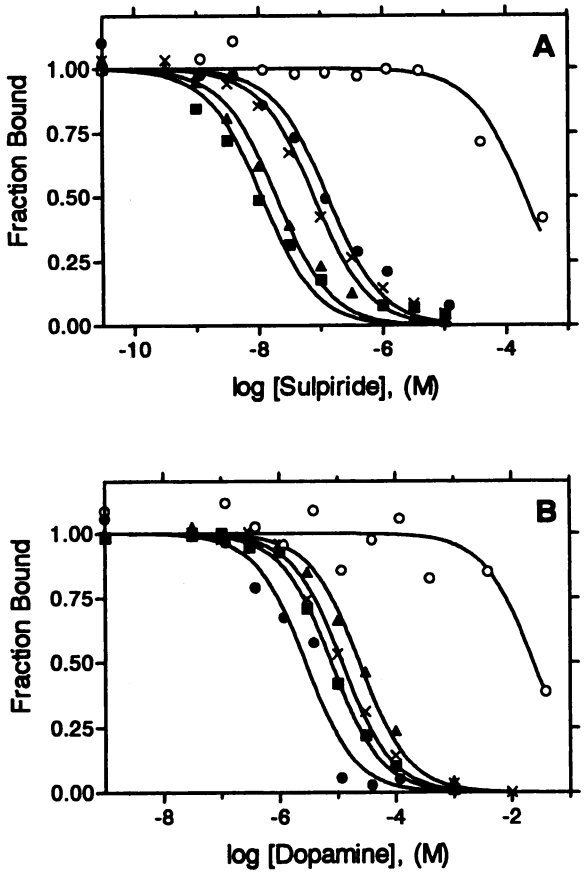


Fig. 5. Effect of mutation of Cys^{118} on the affinity of D_2 receptor for sulpiride (A) and dopamine (B). Cells transiently transfected with wild-type (■) or mutant dopamine D_2 receptor (×, $C^{118}A$; ○, $C^{118}K$; ●, $C^{118}M$; ▲, $C^{118}S$) were assayed with [^{3}H]*N*-methyl-spiperone (320 pM for $C^{118}K$ and $C^{118}M$ and 80 pM for wild-type and all other mutants) in the presence of 9 or 10 concentrations of the indicated drug as described in Experimental Procedures. Data from a representative experiment are shown and are fit to a one-site competition model by nonlinear regression.

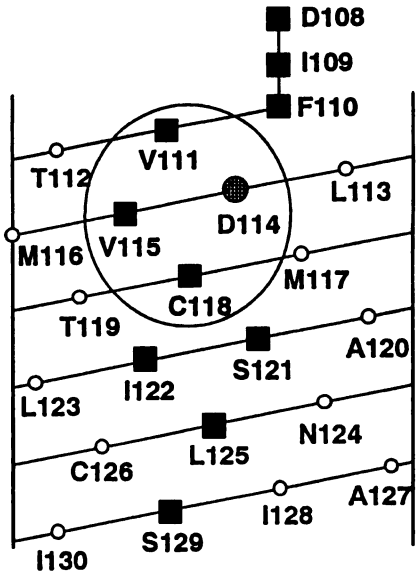


Fig. 6. Helical-net representation of the residues in and flanking the third membrane-spanning segment of the dopamine D_2 receptor. The helical-net representation summarizes the effects of MTSEA on [^{3}H]YM-09151–2 binding to cysteine-substitution mutants (8). ■, Reactive residues. ○, MTSEA had no effect on binding. *Hatched circle*, no binding after cysteine substitution. The most reactive positions, Val^{111}, Val^{115}, and Cys^{118}, are enclosed in an ellipse that includes Asp^{114}, the residue likely to bind the protonated amine of D_2 ligands.

done depends on the presence of sodium (17–19). Mutation to alanine of Asp^{80} in the second membrane-spanning segment decreases the regulation of the affinity of the receptor for substituted-benzamides by sodium (20). The binding of other antagonists, such as the butyrophenone derivative *N*-methyl-spiperone (Fig. 2), is not dependent on the presence of sodium (17, 18). If the modification of Cys^{118} by MTSEA prevents a conformational change that is necessary for the binding of substituted-benzamides and of piquindone but not other antagonists, this would selectively lower the affinity for the benzamides and for piquindone. However, it is unlikely that this explains the effect of MTSEA, since removal of all sodium reduces substituted-benzamide binding by a much smaller factor than does reaction with MTSEA (19, 21).

D_2 ligands are likely to share certain contact residues; other contact residues may be unique to certain ligands or to a structural class of ligands. Although YM-09151–2 and *N*-methyl-spiperone, and indeed all of the antagonists tested, are competitive antagonists (22), their set of contact residues and the space they occupy within the binding-site crevice differ in that only the substituted-benzamides and piquindone are markedly affected by the presence of the modified side chain of Cys^{118}. Furthermore, agonists, which must activate receptor and not simply block activation, also have different sets of contact residues, as the affinity of dopamine was decreased 12,000-fold, whereas that of bromocriptine was decreased only 3-fold.

Using the substituted-cysteine accessibility method, we mapped all of the residues in the third membrane-spanning segment exposed in the binding-site crevice (8). Cysteines substituted for Val^{111}, Val^{115}, and Cys^{118} were the most reactive (Fig. 6), and these residues surround Asp^{114}, which did not tolerate mutation to cysteine. Reaction of MTSEA with $V^{111}C$ and $V^{115}C$ profoundly inhibited the binding of both [^{3}H]YM-09151–2 and [^{3}H]*N*-methyl-spiperone (data not shown). Therefore, these residues are exposed on the surface of the binding-site crevice near Asp^{114} in a region occupied by both ligands.

In summary, our results demonstrate that competitive ligands differ in their exact set of contact residues and in the space they occupy within the binding-site crevice. Similar studies with cysteine-substitution mutants in other positions may provide additional clues to the detailed interaction of ligands within the binding site.

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