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Unique Binding Characteristics of Antipsychotic Agents Interacting with Human Dopamine D_{2A}, D_{2B}, and D_3 Receptors

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SUMMARY

In the present study we have compared the pharmacological properties of human dopamine (DA) D_{2A}, D_{2B}, and D_3 receptors expressed in mammalian cell lines, using [^{3}H]raclopride as a radioligand. Most of the compounds tested had about equal affinity for D_{2A} and D_3 receptors, with the exception of remoxipride, which displayed a 10-fold D_2 selectivity, and the aminotetralin (+)-UH 232, which displayed a 5-fold D_3 selectivity. Several antipsychotic agents, including clozapine and substituted benzamides, bound with 2–3-fold higher affinity to the D_{2B} (short) than to the D_{2A} (long) isoform, whereas others failed to differentiate between the two isoforms. The atypical antipsychotic agent clozapine bound in a biphasic manner and with unexpectedly high affinity (35 nM) to the D_{2B} receptor, suggesting that clozapine may not be as D_4 selective as reported previously. In addition, remoxipride, a new antipsychotic agent with low potential to produce extrapyramidal side effects, displayed 2–3-fold higher affinity for the D_{2B} receptor than for the D_{2A} receptor. Furthermore, sodium differently regulated clozapine and benzamide binding to the various DA receptor subtypes. Thus, sodium decreased the affinity of clozapine for D_{2A} and D_{2B} receptors about 3-fold, whereas the affinity for D_3 receptors was unaltered. In contrast, the affinity of raclopride for the three DA receptor subtypes was increased by sodium. Whether the unique characteristics of the binding of clozapine and benzamides to cloned DA receptors demonstrated in the present study are related to the favorable clinical properties of these compounds remains to be elucidated.

DA receptors, which belong to the family of guanine nucleotide-binding protein-coupled receptors, have historically been divided on the basis of both functional and pharmacological characteristics into two subgroups, D_1 and D_2 (1). D_1 receptors are coupled positively whereas D_2 receptors are coupled negatively to adenylyl cyclase. In addition, linkage of D_2 receptors to other transduction mechanisms, including K^+ and Ca^{2+} channels, phosphatidylinositol turnover, and Na^+-H^+ exchange, has been reported (2, 3).

In the last few years molecular biological techniques have revealed additional subtypes of DA receptors (for reviews, see Refs. 4 and 5). D_1 and D_5 receptors have been cloned and found to be similar in their amino acid sequences as well as in their pharmacological profiles, whereas D_2, D_3, and D_4 receptors are structurally and pharmacologically related. Furthermore, two alternatively spliced D_2 receptor isoforms exist, differing in length by 29 amino acids in the putative third cytoplasmic loop (6–9). The human long isoform, designated D_{2A}, contains 443 amino acids and the short isoform, D_{2B}, contains 414 amino acids.

The various D_2-like receptors reveal different distributions in the central nervous system. Both D_2 receptor isoforms, as localized by mRNA analysis, are represented in all areas where D_2 receptor expression has been demonstrated. However, D_{2A} mRNA seems to predominate in areas where the D_2 receptor density is high, such as basal ganglia, olfactory bulb, and anterior pituitary (4, 5, 8–10). The D_3 and D_4 receptor mRNAs are less abundant than D_2 mRNA and are found in more restricted areas (11–13). The highest levels of D_3 receptor mRNA are found in the olfactory tubercle-islands of Calleja complex and the nucleus accumbens, i.e., regions in limbic brain areas (11, 12), whereas D_4 receptor mRNA is found at relatively high levels in the frontal cortex, midbrain regions, amygdala, and medulla (13).

DA receptors are major targets for drugs used in the treatment of many neuropsychiatric diseases, including schizophrenia. The differential anatomical distribution of various DA receptor subtypes is of great interest, because the efficacy of antipsychotic agents is believed to depend upon the blockade of DA receptors in the mesolimbocortical regions (14), i.e., areas associated with cognitive and emotional functions. On the other hand, the EPS may depend upon the blockade of DA receptors in the basal ganglia (14). Due to the restricted distribution of D_3 receptor mRNA primarily in limbic brain regions, it could be suggested that D_3-selective drugs may produce fewer EPS than drugs that antagonize both D_2 and D_3 receptors.

ABBREVIATIONS: DA, dopamine; Gpp(NH)p, 5′-guanylylimidodiphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; EPS, extrapyramidal side effects.

Whether such drugs will have any antipsychotic or other clinical effects remains, however, to be elucidated.

In this study we have compared the pharmacological properties of human D_{2A}, D_{2B}, and D_3 receptors expressed in mammalian cell lines. In contrast to previous reports, we found differences in the receptor binding characteristics not only between D_2 and D_3 receptors but also between the two isoforms of the D_2 receptor.

Experimental Procedures

Materials. Mouse fibroblast (Ltk^-) cells expressing human D_{2A} and D_{2B} receptors and rat pituitary (GH_4C_1) cells expressing human D_{2B} receptors were obtained from Dr. O. Civelli (Vollum Institute, Portland, OR). CHO cells expressing human or rat D_3 receptors were purchased from INSERM Institute (Paris, France). All tissue culture reagents were obtained from GIBCO Ltd. (Paisley, Scotland, UK) except streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) and benzylpenicillin K (Astra). The following substances were generously donated: (+)-AJ 76 and (+)-UH 232 by Dr. A. Johansson, Department of Organic Pharmaceutical Chemistry (University of Uppsala, Uppsala, Sweden), sertindole by Dr. J. Arnt, Lundbeck A/S (Copenhagen, Denmark), amisulpride by Delagrange (Paris, France), amperozide by Kabi Pharmacia (Lund, Sweden), clozapine by Sandoz Pharma Ltd. (Basel, Switzerland), and pimozide and risperidone by Janssen Pharmaceutica (Beerse, Belgium). Raclopride, remoxipride, and [^{3}H]raclopride (batch OA 654/21; specific activity, 46.0 Ci/mmol) were synthetized at the Department of Chemisty, Astra Arcus AB. (+)-Butaclamol, Schering 23390, D-sulpiride, and SKF 38393 were purchased from Research Biochemicals Inc. (Natick, MA), and chlorpromazine, haloperidol, L-sulpiride, and thioridazine were purchased from Sigma. All other chemicals were of analytical grade.

Cells and membrane preparation. The various cell lines were grown in 225-cm^2 flasks with ventilated caps (Costar), in 5% CO_2 in air at 37°. Ltk^- and GH_4C_1 cells were cultured in DMEM supplemented with 10 mM HEPES, 10% fetal calf serum (heat inactivated), and 70 μg/ml benzylpenicillin K/100 μg/ml streptomycin sulfate. Ltk^- and GH_4C_1 cells were selected with Geneticin (G-418, 0.7 mg/ml). CHO cells (deficient in the dihydrofolate reductase gene) were grown in DMEM supplemented as described above except that the fetal calf serum was dialyzed and minimum essential medium amino acid solution (50×) without L-glutamine was added. The cells were detached with 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline. Upon harvesting, the cells were centrifuged (300 × g for 10 min), washed in DMEM two additional times, and homogenized (Dounce homogenizer) in 10 mM Tris·HCl, 5 mM $MgSO_4$, pH 7.4. The homogenate was washed twice in binding buffer (see below) by centrifugation (43,500 × g for 10 min) and was stored in aliquots at −70°.

[^{3}H]Raclopride binding. The frozen cell membranes were thawed, homogenized with a Branson 450 sonifier, and suspended in binding buffer (in mM: 50 Tris·HCl, 120 NaCl, 5 KCl, 1.5 $CaCl_2$, 4 $MgCl_2$, 1 EDTA, pH 7.4 at 22°). The binding assays were performed in duplicate in a total volume of 0.5 ml with a receptor concentration of 80–100 pM (5–25 μg of protein/tube). The binding reaction was initiated by the addition of membranes and was carried out at 22 ± 1° for 60 min (or at 30° for 30 min where indicated). In saturation experiments eight to 16 concentrations of [^{3}H]raclopride were used. In competition experiments 1–2 nM [^{3}H]raclopride was incubated with 10 to 12 concentrations of the competing ligand. Usually, the substances were dissolved in 0.1% ascorbic acid and dilution series were made in binding buffer, using a BIOMEK 1000 robot (Beckman). In experiments where sodium was omitted, 120 mM N-methyl-D-glucamine was added to maintain the ionic strength and the pH was adjusted to 7.4 at 22°. The additions of various drugs did not alter the pH in the final assays. Nonspecific binding was defined with 1 μM (+)-butaclamol. The incubations were terminated by rapid filtration through Whatman GF/B filters and subsequent washing with cold buffer (50 mM Tris·HCl, pH 7.4), using a cell harvester (Brandel or Skatron). Scintillation cocktail (Packard Ultima Gold, 4 ml) was added and the radioactivity was determined in a Packard 2200CA liquid scintillation analyzer at 50% efficiency. Protein concentration was determined by the method of Markwell *et al.* (15), with bovine serum albumin as standard.

Data analysis. The data were analyzed by nonlinear regression using the LIGAND program (16). One- and two-site curve fitting was tested in all experiments. The two-site model was accepted when it significantly improved the curve fit ($p < 0.05$; F test) and when each site accounted for >20% of the receptors. To normalize the data before statistical analysis, the individual K_d and K_i values were converted to pK_d and pK_i values (the negative logarithm of the K_d or K_i value, in molar units). Statistical comparisons of sodium effects were carried out by using Student's paired (for parallel experiments) or unpaired t tests. For multiple tests, pK_d and pK_i values for the various subtypes were compared by using analysis of variance followed by Bonferroni/Dunn (all means) *post hoc* comparison.

Results and Discussion

[^{3}H]Raclopride binds with high affinity to human D_{2A}, D_{2B}, and D_3 receptors. The substituted benzamide [^{3}H]raclopride has been shown to selectively bind to D_2 receptors in various brain tissues (17). In this study we used [^{3}H]raclopride to label cloned human D_{2A}, D_{2B}, and D_3 receptors expressed in mammalian cell lines. We found that [^{3}H]raclopride bound with high affinity, saturability, and low nonspecific binding (5% at the K_d value) to the three DA receptor subtypes. Fig. 1A shows a representative saturation binding curve for [^{3}H]raclopride binding to D_3 receptors expressed in CHO cells. Scatchard analysis of the specific [^{3}H]raclopride binding (Fig. 1B) resulted in a linear plot, consistent with a noncooperative single class of binding sites. The same applies to all Scatchard plots of [^{3}H]raclopride binding to various DA receptor subtypes in the present study. The K_d (nM) and B_{max} (pmol/mg of protein) values for [^{3}H]raclopride were 1.2 ± 0.09 and 2.5 ± 0.15 for D_{2A} (14 experiments), 0.66 ± 0.04 and 2.0 ± 0.16 for D_{2B} (10 experiments), and 1.3 ± 0.11 and 7.6 ± 1.5 for D_3 (12 experiments) receptors, respectively. [^{3}H]Raclopride displayed about 2 times higher affinity ($p < 0.001$) for the D_{2B} receptor than for D_{2A} and D_3 receptors.

Sodium increases the affinity of raclopride for D_{2A}, D_{2B}, and D_3 receptors. Table 1 shows that the omission of sodium ions from the incubation buffer resulted in a statistically significant 2-fold decrease in the affinity of [^{3}H]raclopride for all three receptor subtypes. The B_{max} values were also slightly but not significantly reduced. This effect of sodium on raclopride binding to D_2 and D_3 receptors is in agreement with previous results (18–21).

Several antipsychotic agents display subtype selectivity. The K_i values derived from competition studies of [^{3}H]raclopride binding with various dopaminergic antagonists are summarized in Table 2. Several drugs distinguished between the various DA receptor subtypes. In agreement with previous results (11, 21), the so-called autoreceptor-selective antagonists (+)-AJ 76 and (+)-UH 232 (22) were the only antagonists that had a higher affinity for the D_3 receptor than for the D_2 receptor isoforms, displaying a rank order of potency of $D_3 > D_{2A} \approx D_{2B}$. In addition, amperozide displayed a slight D_3 receptor preference. The most D_2-selective drugs were remoxipride, haloperidol, and risperidone, with 4–18-fold higher affinity for D_2 receptor isoforms than for the D_3 receptor. Indeed, remoxipride

was the only drug that exhibited significant differences in pK_i values between all three DA receptor subtypes, displaying a rank order of potency of $D_{2B} > D_{2A} > D_3$.

In an earlier paper (11), using rat D_3 and rat D_{2A} receptors, it was suggested that typical antipsychotic agents display 10–20-fold lower affinity for D_3 compared with D_{2A} receptors, whereas atypical antipsychotic agents display only 2–3-fold lower affinity. We found no such correlation. In terms of D_3 and D_{2A} receptor affinity, the atypical antipsychotic agent remoxipride was in fact the most selective D_2 antagonist, whereas the structurally related sulpiride displayed no such trend. Haloperidol, as an example of a typical antipsychotic agent, displayed some selectivity for the D_{2A} receptor, whereas chlorpromazine did not distinguish between the two subtypes. Although it seems clear from these data that antagonists can exhibit a certain selectivity for the D_2 or D_3 receptors, this selectivity seems to be unrelated to so-called atypicality.

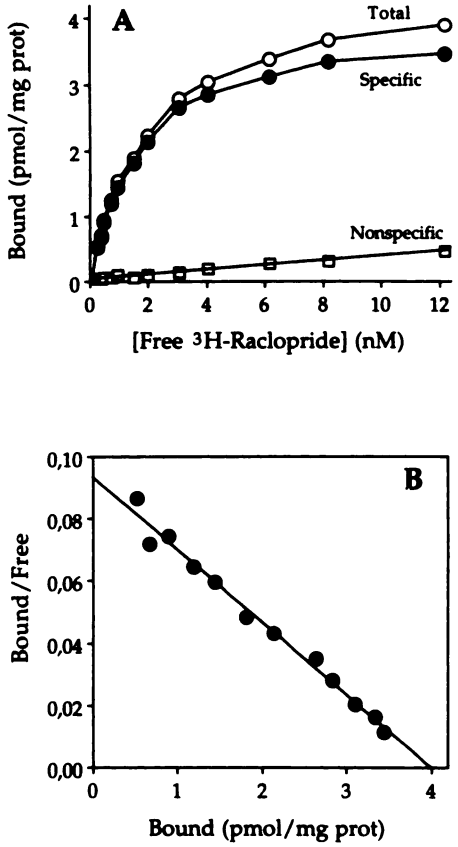

Fig. 1. [^{3}H]Raclopride binding to human dopamine D_3 receptors. A, Representative saturation binding curve for [^{3}H]raclopride binding to D_3 receptors expressed in CHO cells. The saturation study was performed as described in Experimental Procedures. B, Scatchard plot of the specific [^{3}H]raclopride binding. K_d (1.7 nM) and B_{max} (4.0 pmol/mg of protein) values determined from the Scatchard analysis were in good agreement with the values obtained with the LIGAND program.

TABLE 1

Effect of sodium on [^{3}H]raclopride binding to cloned human DA D_{2A}, D_{2B}, and D_3 receptors

The saturation studies were performed in parallel, and dissociation constants (K_d) and receptor densities (B_{max}) were calculated as described in Experimental Procedures. The results are means ± standard errors of four or five experiments. For statistical analysis, using Student's paired t test, the K_d values were converted to pK_d values. The comparisons given in the table are comparisons between the different pK_d and B_{max} values with and without sodium.

Receptor Subtype	K_d	B_{max}
	nM	pmol/mg of protein
D_{2A}		
+NaCl	1.15 ± 0.22	2.69 ± 0.095
−NaCl	2.52 ± 0.43[a]	2.09 ± 0.25
D_{2B}		
+NaCl	0.60 ± 0.063	2.28 ± 0.37
−NaCl	1.22 ± 0.11[b]	1.76 ± 0.35
D_3		
+NaCl	1.20 ± 0.21	6.03 ± 1.6
−NaCl	1.97 ± 0.33[c]	4.28 ± 1.1

[a] $p < 0.001$.
[b] $p < 0.01$.
[c] $p < 0.05$.

Substituted benzamides show higher affinity for D_{2B} receptors than for D_{2A} receptors. In guanine nucleotide-binding protein-coupled nonpeptide receptors, important amino acids for ligand binding are believed to be located in the transmembrane regions of the receptor protein (23). Because the two splice variants of the D_2 receptor differ in their amino acid sequences only in the putative third cytoplasmic loop (6–9), they are expected to possess similar ligand-binding properties. In agreement with this, no differences in binding affinities of various dopaminergic antagonists between D_{2A} and D_{2B} receptors were reported previously (6, 8). However, in the present study we demonstrate that several antagonists display 2–3-fold higher affinity for the D_{2B} than the D_{2A} receptor; these include raclopride, remoxipride, and both isomers of sulpiride, as well as chlorpromazine, clozapine, and thioridazine (Table 2). These substances are not structurally related, ranging from substituted benzamides to tricyclic compounds. With the exception of remoxipride, these compounds displayed similar affinities for D_{2A} and D_3 receptors (Table 2), with the following rank order of potency: $D_{2B} > D_{2A} \approx D_3$. Other antipsychotic agents, including haloperidol, amisulpride, amperozide, and risperidone, as well as the selective D_1 antagonist SCH23390 and the autoreceptor-selective antagonists (+)-AJ 76 and (+)-UH 232, failed to differentiate between the two isoforms.

The molecular mechanism underlying the different binding affinities of some antagonists for D_{2A} and D_{2B} receptors is unclear but is probably related to altered conformations of the receptor proteins due to the different lengths of the third intracellular loop. Consistent with this, we also found that the affinities of [^{3}H]raclopride for rat and human D_3 receptors were different (K_d = 3.7 ± 0.6 nM, four experiments, and K_d = 1.3 ± 0.1 nM, 12 experiments, respectively; $p < 0.001$), with that for the human D_3 receptor being 2–3 times higher. The homology between rat and human D_3 receptors in their amino acid sequences in the region of the transmembrane domains is 97% (21). However, the human D_3 receptor has a deletion of 46 amino acids in its putative third intracellular loop (21) and thus resembles the D_{2B} receptor with a 29-amino acid deletion in its third cytoplasmic loop (6–9). These data suggest that the length of the third cytoplasmic loop has an impact on the binding affinity of at least some antagonist ligands.

Clozapine binds with high affinity to the D_{2B} receptor. An important finding in the present study involved the unique characteristics of the binding of clozapine to the DA receptor subtypes. Table 3 shows that the apparent affinities ($K_{0.5}$ values) of clozapine for D_{2A}, D_{2B}, and D_3 receptors were 60 nM, 35 nM, and 86 nM, respectively. Furthermore, biphasic displacement curves for clozapine at D_{2B} receptors and in some experiments even at D_{2A} and D_3 receptors were observed. About 60%

TABLE 2

Potencies of various dopaminergic antagonists to inhibit [^{3}H]raclopride binding to cloned human D_{2A}, D_{2B}, and D_3 receptors

The competition studies were performed and the K_i values were calculated as described in Experimental Procedures. The results are means ± standard errors of n experiments. For statistical analysis, the K_i values were converted to pK_i values and subjected to analysis of variance followed by *post hoc* Bonferroni/Dunn comparisons.

Substance	D_{2A} (Ltk$^-$ cells)		D_{2B} (Ltk$^-$ cells)		D_3 (CHO cells)	
	K_i	n	K_i	n	K_i	n
	nM		nM		nM	
(+)-AJ 76	80.3 ± 20	3	78.8 ± 4.1	3	35.0 ± 4.9[a–c]	4
Amisulpride	0.973 ± 0.34	3	0.898 ± 0.27	3	3.01 ± 1.1	4
Amperozide	420 ± 94	4	322 ± 26	4	235 ± 19	5
Chlorpromazine[d]	1.14 ± 0.12	3	0.550 ± 0.077[a,c]	3	1.16 ± 0.14[b,c]	3
Clozapine[e]	59.8 ± 7.8	8	35.0 ± 4.7[a,c]	7	83.3 ± 9.9[b,f]	7
Haloperidol	0.665 ± 0.11	4	0.532 ± 0.19	4	2.74 ± 0.55[a,b,g]	5
Pimozide[d]	0.720 ± 0.22	3	0.350 ± 0.050	3	0.530 ± 0.20	3
Raclopride	2.30 ± 0.21	5	0.999 ± 0.10[a,f]	7	1.80 ± 0.21[b,g]	8
Remoxipride	125 ± 15	7	54.3 ± 5.0[a,f]	7	969 ± 40[a,b,f]	5
Risperidone	1.71 ± 0.32	4	1.29 ± 0.053	4	6.70 ± 1.5[h]	5
SCH 23390	267 ± 37	3	284 ± 52	3	314 ± 55	3
Sertindole[d]	0.622 ± 0.045	3	0.381 ± 0.014	3	1.63 ± 0.30[h]	3
L-Sulpiride	7.44 ± 1.3	4	2.50 ± 0.18[a,g]	5	7.99 ± 0.97[b,f]	6
D-Sulpiride	1040 ± 160	4	393 ± 72[a,g]	4	1550 ± 190[b,f]	4
Tiaspirone[d]	0.453 ± 0.13	3	0.352 ± 0.14	3	0.622 ± 0.27	3
Thioridazine[d]	2.34 ± 0.097	3	1.19 ± 0.11[a,c]	3	2.30 ± 0.35[b,c]	2
(+)-UH 232	14.4 ± 2.4	6	12.5 ± 1.1	4	2.89 ± 0.62[a,b,f]	4

[a] Comparison of pK_i value for D_{2A} versus D_{2B} or D_3.
[b] Comparison of pK_i values for D_{2B} versus D_3.
[c] $p < 0.05$.
[d] Incubated at 30° for 30 min.
[e] $K_{0.5}$ values; for further details, see Table 3.
[f] $p < 0.001$.
[g] $p < 0.01$.
[h] D_{2A} versus D_3, $p < 0.01$; D_{2B} versus D_3, $p < 0.001$.

TABLE 3

Effect of sodium on clozapine binding to cloned human D_{2A}, D_{2B}, and D_3 receptors

The competition studies were performed and the K_i values were calculated as described in Experimental Procedures. The results are means ± standard errors of n experiments. The term $K_{0.5}$ is used instead of K_i because the Hill coefficient is substantially less than 1. K_h and K_l represent affinity values of high- and low affinity sites, respectively. R_h indicates the percentage of receptors in the high affinity state. For statistical analysis, the $K_{0.5}$ values were converted to p$K_{0.5}$ values. The effect of sodium on p$K_{0.5}$ values given in the table was tested with Student's unpaired t test.

Binding Parameter	+NaCl			−NaCl		
	Affinity	R_h	n	Affinity	R_h	n
	nM	%		nM	%	
D_{2A}						
$K_{0.5}$	59.8 ± 7.8		8	18.4 ± 4.0[a]		4
K_h	24.5 ± 7.0	63	4[b]	4.76 ± 0.17	77	2[b]
K_l	302 ± 150		4	148 ± 68		2
D_{2B}						
$K_{0.5}$	35.0 ± 4.7		7	11.3 ± 0.99[a]		5
K_h	9.41 ± 1.8	61	7[b]	2.07 ± 0.58	61	5[b]
K_l	126 ± 25		7	80.5 ± 31		5
D_3						
$K_{0.5}$	86.3 ± 9.9		7	87.8 ± 10[c]		4
K_h	25.1 ± 14	46	2[b]	13.1	40	1[b]
K_l	171 ± 63		2	187		1

[a] $p < 0.001$.
[b] Number of experiments with a significantly better fit to a two-site model.
[c] Not significant, $p > 0.05$.

of the D_{2B} receptors displayed a remarkably high affinity for clozapine, with a K_{high} value of 9 nM, whereas 40% had a K_{low} of 126 nM. The mean Hill coefficient of clozapine for D_{2B} receptors was 0.70 ± 0.07 (seven experiments). The corresponding Hill values for D_{2A} and D_3 receptors were 0.81 ± 0.04 (eight experiments) and 0.81 ± 0.05 (seven experiments), respectively. All of the Hill coefficients were significantly different from 1 ($p < 0.01$), but there was no statistically significant difference between the various subtypes ($p > 0.05$). In comparison, Hill coefficients for raclopride were 0.98 ± 0.02, 0.94 ± 0.04, and 0.92 ± 0.02 for D_{2A}, D_{2B}, and D_3 receptors, respectively. Due to the variation in the number of experiments showing biphasic competition curves with clozapine, control experiments using 23 concentrations (four points per log unit) were carried out with D_{2A} and D_{2B} receptors. However, these studies gave results similar to those obtained with fewer data points (data not shown). Thus, two experiments of four with D_{2A} receptors, and three of five with D_{2B} receptors, gave a statistically better curve fit with the two-site model ($p < 0.05$). The Hill coefficients for clozapine were 0.88 ± 0.05 for D_{2A} receptors (four experiments) and 0.77 ± 0.06 for D_{2B} receptors (five experiments). The addition of the nonhydrolyzable GTP analogue Gpp(NH)p (100 μM) to the incubation buffer did not have any effect on clozapine binding. We also tested the characteristics of the binding of clozapine to the D_{2B} receptor expressed in GH_4C_1 (rat pituitary) cells, and we found that even in these cells clozapine displaced [^{3}H]raclopride at the D_{2B} receptor with high affinity and in a biphasic manner (data not shown).

Sodium decreases the affinity of clozapine for the D_{2A} and D_{2B} receptors but not the D_3 receptor. Fig. 2 and Table 3 demonstrate that the binding of clozapine to the various DA receptor subtypes was differently regulated by sodium ions. The omission of sodium from the incubation buffer led to a 3-fold increase in the affinity of clozapine for D_{2A} and D_{2B} receptors, whereas the affinity of clozapine for D_3 receptors was not changed. The displacement by clozapine of [^{3}H]raclopride binding to the D_{2B} receptor in the absence of sodium was still best described with a two-site model (Table 3). It is noteworthy that the binding affinity of clozapine for D_4 receptors has also been

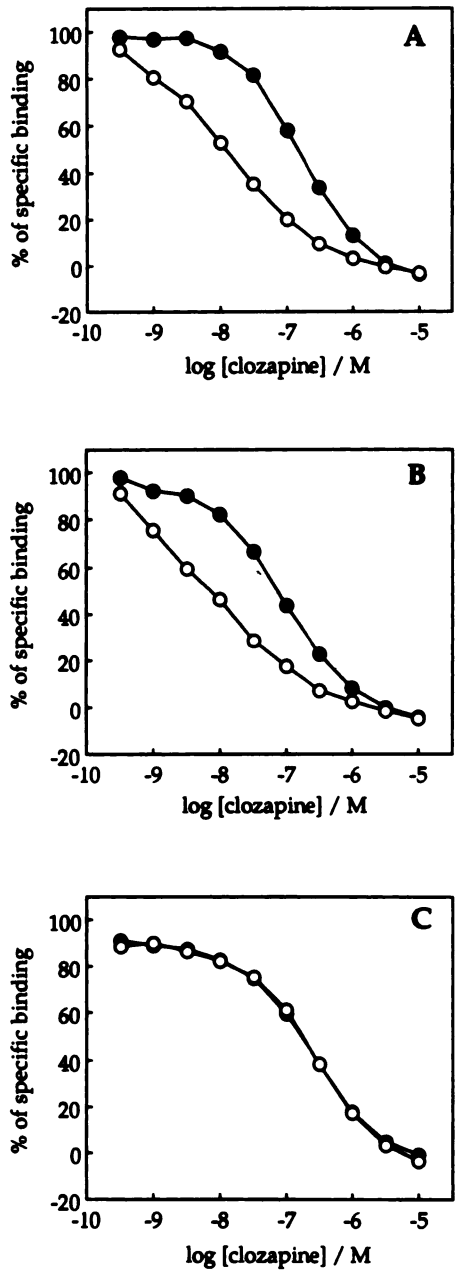

Fig. 2. Effect of sodium on clozapine binding to D_{2A} (A), D_{2B} (B), and D_3 (C) receptors. [^{3}H]Raclopride and various concentrations of clozapine were incubated in the absence (○) or presence (●) of 120 mM NaCl. Representative clozapine displacement curves from parallel experiments are shown. For additional details, see Table 3.

reported to decrease in the presence of sodium (25). In addition, Seeman (10) recently reported that the binding of clozapine to anterior pituitary D_2 receptors is also sodium sensitive, with sodium reducing the affinity of clozapine by about 2-fold.

The biphasic binding, as well as the decreased affinity of clozapine seen after the addition of sodium ions, is consistent with an agonist-like receptor-ligand interaction (24). However, the insensitivity to Gpp(NH)p is in disagreement with this. To our knowledge, clozapine has not been reported to have agonist properties in any functional studies. Whether the biphasic binding of clozapine found in the present study is due to partial agonist activity or is related to experimental conditions (such as ion composition or pH of the buffer) is presently under investigation in our laboratory.

Conclusions

The DA hypothesis of schizophrenia is strongly supported by the observation that all antipsychotic agents block DA receptors both *in vivo* and *in vitro* (10, 26). Furthermore, there is an excellent correlation between clinically effective plasma water levels of a large number of antipsychotic agents and their affinities for D_2 receptors (10, 26). However, clozapine, an antipsychotic agent with very low EPS potential, has been shown to have about 10 times lower free plasma concentration than would be predicted from its D_2 receptor binding affinity (10, 13). Therefore, the cloning of the D_4 receptor, with a relatively high affinity for clozapine (9–25 nM in the presence of sodium) (13, 25), led to the suggestion that clozapine exerts its antipsychotic action via the D_4 receptor. Furthermore, the reported low concentration of D_4 receptor mRNA in motor areas (13) was put forward to explain the low EPS potential of this antipsychotic agent.

The present results show that clozapine binds to D_{2B} receptors with an affinity comparable to that for D_4 receptors. Thus, at therapeutically effective plasma levels clozapine probably occupies not only D_4 receptors (10) but also the D_{2B} receptors. Thus, the short isoform of the D_2 receptor may be of interest as a target receptor for clozapine. Interestingly, remoxipride also shows a higher affinity for this D_2 receptor isoform. Furthermore, we found that sodium differently regulates the binding of clozapine and raclopride to various DA receptor subtypes. Whether these unique characteristics of the binding of antipsychotic agents to cloned receptors are similar to those *in vivo* remains to be demonstrated.

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References

1. Kebabian, J. W., and D. B. Calne. Multiple receptors for dopamine. *Nature (Lond.)* **277**:93–96 ((1979).
2. Vallar, L., and J. Meldolesi. Mechanisms of signal transduction at the dopamine D_2 receptor. *Trends Pharmacol. Sci.* **10**:74–77 (1989).
3. Ganz, M. B., J. A. Patcher, and D. L. Barber. Multiple receptors coupled to adenylate cyclase regulate Na-H exchange independent of cAMP. *J. Biol. Chem.* **265**:8989–8992 (1990).
4. Sibley, D. R., and F. J. Monsma. Molecular biology of dopamine receptors. *Trends Pharmacol. Sci.* **13**:61–69 (1992).
5. Civelli, O., J. R. Bunzow, D. K. Grandy, Q. Y. Zhou, and H. H. M. Van Tol. Molecular biology of the dopamine receptors. *Eur. J. Pharmacol.* **207**:277–286 (1991).
6. Grandy, D. K., M. A. Marchionni, H. Makam, R. E. Stofko, M. Alfano, L. Frothingham, J. B. Fischer, K. J. Burke-Howie, J. R. Bunzow, A. C. Server, and O. Civelli. Cloning of the cDNA and gene for a human D_2 dopamine receptor. *Proc. Natl. Acad. Sci. USA* **86**:9762–9766 (1989).
7. Monsma, F. J., L. D. McVittie, C. R. Gerfen, L. C. Mahan, and D. R. Sibley. Multiple D_2 dopamine receptors produced by alternative RNA splicing. *Nature (Lond.)* **342**:926–929 (1989).
8. Giros, B., P. Sokoloff, M. P. Martres, J. F. Riou, L. J. Emorine, and J. C. Schwartz. Alternative splicing directs the expression of two D_2 dopamine receptor isoforms. *Nature (Lond.)* **342**:923–926 (1989).
9. Dal Toso, R., B. Sommer, M. Ewert, A. Herb, D. B. Pritchett, A. Bach, B. D. Shivers, and P. H. Seeburg. The dopamine D_2 receptor: two molecular forms generated by alternative splicing. *EMBO J.* **8**:4025–4034 (1989).
10. Seeman, P. Dopamine receptor sequences: therapeutic levels of neuroleptics occupy D_2 receptors, clozapine occupies D_4. *Neuropsychopharmacology* **7**:261–284 (1992).
11. Sokoloff, P., B. Giros, M. P. Martres, M. L. Bouthenet, and J. C. Schwartz. Molecular cloning and characterization of a novel dopamine receptor (D_3) as a target for neuroleptics. *Nature (Lond.)* **347**:146–151 (1990).
12. Bouthenet, M. L., E. Souil, M. P. Martres, P. Sokoloff, B. Giros, and J. C. Schwartz. Localization of dopamine D_3 receptor mRNA in the rat brain using *in situ* hybridization histochemistry: comparison with dopamine D_2 receptor mRNA. *Brain Res.* **564**:203–219 (1991).
13. Van Tol, H. H. M., J. R. Bunzow, H. C. Guan, R. K. Sunahara, P. Seeman,

H. B. Niznik, and O. Civelli. Cloning of the gene for a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. *Nature (Lond.)* **350**:610–614 (1991).
14. Creese, I. Classical and atypical antipsychotic drugs: new insights. *Trends Pharmacol. Sci.* **6**:479–483 (1983).
15. Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87**:206–210 (1978).
16. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
17. Köhler, C., H. Hall, S. O. Ögren, and L. Gawell. Specific *in vitro* and *in vivo* binding of ^{3}H-raclopride. *Biochem. Pharmacol.* **34**:2251–2259 (1985).
18. Stefanini, E., A. M. Marchisio, P. Devoto, F. Vernaleone, R. Collu, and P. F. Spano. Sodium-dependent interaction of benzamides with dopamine receptors. *Brain Res.* **198**:229–233 (1980).
19. Reader, T. A., S. Boulianne, E. Molina-Holgado, and K. M. Dewar. Effects of monovalent cations on neostriatal dopamine D_2 receptors labeled with [^{3}H]raclopride. *Biochem. Pharmacol.* **40**:1739–1746 (1990).
20. Neve, K. A. Regulation of dopamine D2 receptors by sodium and pH. *Mol. Pharmacol.* **39**:570–578 (1991).
21. Sokoloff, P., M. Andrieux, R. Besançon, C. Pilon, M. P. Martres, B. Giros, and J. C. Schwartz. Pharmacology of human dopamine D_3 receptor expressed in a mammalian cell line: comparison with D_2 receptor. *Eur. J. Pharmacol.* **225**:331–337 (1992).
22. Johansson, A. M., L. E. Arvidsson, U. Hacksell, J. L. G. Nilsson, K. Svensson, and A. Carlsson. Resolved *cis*- and *trans*-2-amino-5-methoxy-1-methyltetralins: central dopamine receptor agonists and antagonists. *J. Med. Chem.* **30**:602–611 (1987).
23. Strader, C. D., I. S. Sigal, and R. A. F. Dixon. Structural basis of β-adrenergic receptor function. *FASEB J.* **3**:1825–1832 (1989).
24. Grigoriadis, D., and P. Seeman. Complete conversion of brain D_2 dopamine receptors from the high- to the low-affinity state for dopamine agonists, using sodium ions and guanine nucleotide. *J. Neurochem.* **44**:1925–1935 (1985).
25. Van Tol, H. H. M., C. M. Wu, H. C. Guan, K. Ohara, J. R. Bunzow, O. Civelli, J. Kennedy, P. Seeman, H. B. Niznik, and V. Jovanovic. Multiple dopamine D_4 receptor variants in the human population. *Nature (Lond.)* **358**:149–152 (1992).
26. Seeman, P. Dopamine receptors and the dopamine hypothesis of schizophrenia. *Synapse* **1**:133–152 (1987).

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