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The Action of Neuroleptic Drugs on Dopamine-Stimulated Adenosine Cyclic 3′,5′-Monophosphate Production in Rat Neostriatum and Limbic Forebrain

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SUMMARY

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Neuroleptic drugs of various types were more potent inhibitors of dopamine-sensitive production of adenosine cyclic 3′,5′-monophosphate in homogenates of rat brain striatum than non-neuroleptic drugs of similar structures. The most potent drugs were phenothiazines and thioxanthenes with a $-CF_3$ group in position 2 of the tricyclic system and a piperazino side chain. There were also large differences in the effects of *cis* and *trans* isomers of thioxanthenes in which the 2-substituent and the side chain are on the same or opposite side of the double bond connecting the side chain to the ring system. Thus α-flupenthixol, α-clopenthixol, and α-chlorprothixene, which are the *cis* isomers, were considerably more potent than the corresponding β-isomers of the same drugs. α-Flupenthixol was also considerably more potent than the β-isomer in antagonizing the effect of dopamine on cyclic AMP production in the olfactory tubercle and nucleus accumbens, and in antagonizing the potent dopamine agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene in the striatum. These results are discussed in relation to the hypothesis that neuroleptic activity may be related to the blockade of dopamine receptors in the central nervous system.

INTRODUCTION

The most sensitive animal tests of neuroleptic activity used at present are those which utilize the behavioral effects of amphetamine and apomorphine, certain components of which are antagonized by the neuroleptics (1, 2). It is widely believed that the behavioral effects of neuroleptics result from their ability to block dopamine receptors in the central nervous system (3, 4). Many observations on the effects of neuroleptics on dopamine metabolism in brain have been related to their supposed dopamine receptor-blocking activity (3, 5–7).

Recently the second messenger hypothesis of Sutherland has been extended to include the neurotransmitter functions of the catecholamines and other biogenic amines. Support for the involvement of adenosine cyclic 3′,5′-monophosphate in adrenergic neurotransmission has come from electrophysiological and histochemical data on an inhibitory noradrenergic pathway from the locus coeruleus to the Purkinje cells of the cerebellum (8), although this evidence has been disputed (9). It has also been suggested that

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cyclic AMP may be involved in dopamine-mediated transmission, and supportive biochemical and electrophysiological evidence has been obtained from studies on the role of small dopaminergic interneurons in bovine and rabbit superior cervical ganglia (10). Dopamine also stimulated cyclic AMP production in retinal homogenates (11) and in homogenates of brain areas containing a high density of dopamine-containing nerve terminals. These areas include the striatum (12), cortex (13), olfactory tubercle, and nucleus accumbens (14). Homogenates of other brain areas, such as the cerebellum, where little dopamine is found, do not respond in an analogous fashion (12). In addition, rat striatal homogenates do not respond to *beta* adrenergic agonists such as isoprenaline, and respond only to high concentrations of noradrenaline (12). The dopamine-stimulated cyclic AMP production in brain and retinal homogenates could be mimicked by the dopamine receptor-stimulating agent apomorphine and inhibited by neuroleptic drugs such as haloperidol and chlorpromazine (11, 12, 15). Thus, whatever the precise physiological significance of the response of cyclic AMP production to dopamine, these systems seem to represent models of dopamine receptors. We have previously used the rat striatal system to investigate the dopamine-antagonistic action of chlorpromazine and some of its metabolites (16) and the dopamine-like properties of the anti-parkinsonian drug piribedil (ET 495) (17). The present paper describes the effects of several psychotropic agents on dopamine-stimulated cyclic AMP production in rat brain homogenates. These results support the hypothesis that neuroleptic drugs act as antagonists of dopamine at central nervous receptor sites. Some of these results have been presented in a preliminary form elsewhere (18).

METHODS

Dopamine-sensitive cyclic AMP production was assayed as described by Kebabian et al. (12). Adult male Sprague-Dawley albino rats were decapitated, and their brains were removed and placed on ice. The neostriatum, nucleus accumbens, and olfactory tubercle were dissected as previously described (14, 19). The neostriatum or other brain area was homogenized in approximately 25 volumes of ice-cold 2 mM Tris-maleate buffer, pH 7.4, containing 2 mM EGTA.[2] Fifty-microliter aliquots of this homogenate were added to assay tubes containing 250 μl of buffer consisting of 80 mM Tris-maleate (pH 7.4), 2 mM $MgSO_4$, 10 mM theophylline, and 0.2 mM EGTA plus drugs as indicated. The tubes were kept in an ice-salt bath, and ATP was added to a final concentration of 0.5 mM. The tubes were incubated with shaking at 30° for 2.5 min and then transferred to a boiling water bath for 2.5 min, followed by centrifugation to sediment the denatured protein. Ten-microliter aliquots of the supernatant solution were taken for analysis of cyclic AMP content by the method of Brown *et al.* (20). The assay was linear for standards of cyclic AMP in the range of 0.5–8.0 pmole.

RESULTS

In accordance with previous reports (12, 14, 17, 21), we found that addition of low concentrations of dopamine to homogenates of rat striatum or limbic nuclei caused approximately a 2-fold increase in cyclic AMP production. In drug antagonism studies a concentration of 100 μM dopamine was used to ensure maximal stimulation. We examined the effects of a series of neuroleptics and other drugs on the stimulation of cyclic AMP production in striatal homogenates produced by this concentration of dopamine (Table 1).

The two most potent compounds, flupenthixol and fluphenazine, have the same 2-substituent (—CF_3) in the tricyclic ring system and the same basic side chain (β-hydroxyethylpiperazinyl). Substitution of a methyl group for the β-hydroxyethyl function in fluphenazine yielded trifluoperazine, which was about 4 times less potent than fluphenazine (Fig. 1). Alterations of the 2-substituent and the basic side chain, as in chlorpromazine, reduced potency still further. The tricyclic antidepressant drug chlorimipramine, a structural analogue of chlorpromazine in which the sulfur atom is

[2] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

TABLE 1

Drug concentrations causing 50% inhibition of stimulation of cyclic AMP production (IC_{50}) in striatal homogenates by 100 μM dopamine, and calculated K_i values

K_i values were calculated from the relationship $IC_{50} = K_i(1 + S/K_m)$, where S is the concentration of dopamine (100 μM) and K_m is the concentration of dopamine required for half-maximal stimulation of adenylate cyclase activity (5 μM). Competitive inhibition has been assumed for all compounds tested (see Fig. 3 and ref. 22).

Drug	IC_{50}	K_i
	M	*M*
1. α-Flupenthixol	2.2×10^{-8}	1.0×10^{-9}
2. (α,β)-Flupenthixol	7.5×10^{-8}	3.5×10^{-9}
3. Fluphenazine	9.2×10^{-8}	4.3×10^{-9}
4. Trifluoperazine	4.0×10^{-7}	1.9×10^{-8}
5. α-Clopenthixol	3.3×10^{-7}	1.6×10^{-8}
6. α-Chlorprothixene	7.8×10^{-7}	3.7×10^{-8}
7. Chlorpromazine	1.0×10^{-6}	4.8×10^{-8}
8. Prochlorperazine	2.2×10^{-6}	1.0×10^{-7}
9. Spiroperidol	2.0×10^{-6}	9.5×10^{-8}
10. Thioridazine	2.8×10^{-6}	1.3×10^{-7}
11. Clozapine	3.5×10^{-6}	1.7×10^{-7}
12. Pimozide	3.0×10^{-6}	1.4×10^{-7}
13. Chlorimipramine	9.0×10^{-6}	4.2×10^{-7}
14. Promazine	6.0×10^{-5}	2.8×10^{-6}
15. Morphine	1.0×10^{-4}	4.8×10^{-6}
16. β-Chlorprothixene	2.0×10^{-5}	9.5×10^{-7}
17. β-Clopenthixol	6×10^{-5}	2.8×10^{-6}
18. β-Flupenthixol	$>10^{-4}$	$>5 \times 10^{-6}$
19. Promethazine	$>10^{-4}$	$>5 \times 10^{-6}$
20. Benztropine	$>10^{-4}$	$>5 \times 10^{-6}$

replaced by a dimethylene bridge, was only 1/10 as active as chlorpromazine. Promazine lacks the 2-chloro substituent of chlorpromazine, and was 60 times less potent than the latter compound. The butyrophenone spiroperidol and diphenylbutylamine pimozide were both somewhat less potent than chlorpromazine. This was also true of thioridazine and clozapine (Figs. 2 and 3). The opiate drug morphine, the anticholinergic benztropine, and the antihistamine phenothiazine promethazine were all very weak antagonists. When a lower stimulating concentration of dopamine was used (10 μM) it was found that lower concentrations of thiorida-

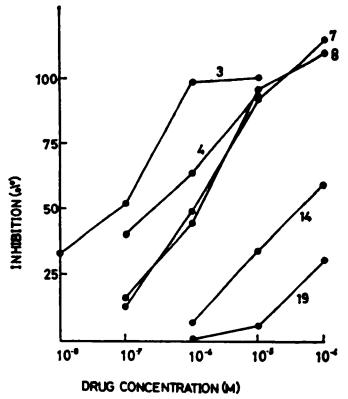

FIG. 1. *Effect of phenothiazines on dopamine-stimulated cyclic AMP production in striatal homogenates*

Basal level of cyclic AMP production was 45.5 ± 3.6 pmoles/sample (2 mg, wet weight), and stimulated level (100 μM dopamine), 88.7 ± 9.1 pmoles/sample (means and standard errors for six experiments). Each point is the mean of at least five separate incubations; standard errors were less than ±10% of means. Numbers refer to the compounds in Table 1. At 0.1 mM some drugs caused more than 100% inhibition, probably by inhibiting basal cyclic AMP production.

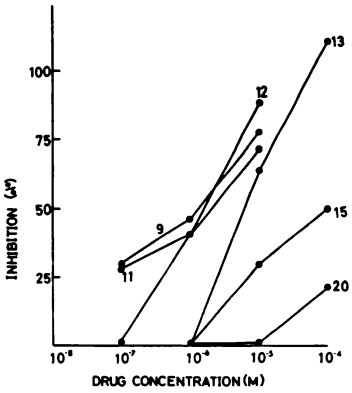

FIG. 2. *Effects of various drugs on dopamine-stimulated cyclic AMP production in striatal homogenates*

Basal level of cyclic AMP production was 43.4 ± 4.7 pmoles/sample (2 mg, wet weight), and stimulated level (100 μM dopamine), 88.1 ± 10.7 pmoles/sample (means and standard errors for six experiments). Each point is the mean of at least five separate incubations; standard errors were less than ±10% of means. Numbers refer to those in Table 1. At 0.1 mM chlorimipramine caused more than 100% inhibition.

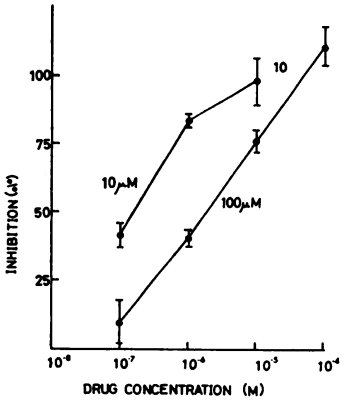

FIG. 3. *Effect of thioridazine on stimulation of cyclic AMP production in striatal homogenates by 100 μM and 10 μM dopamine*

Basal levels of cyclic AMP production in these two experiments were 37.2 and 39.0 pmoles/sample (2 mg, wet weight). Stimulated levels were 69.0 pmoles with 10 μM dopamine and 76.5 pmoles with 100 μM dopamine per sample, respectively.

zine were required to antagonize the effect (Fig. 3), indicating the competitive nature of the inhibition. The structures of the drugs used are shown in Fig. 4.

The thioxanthenes have a double bond connecting the side chain to the heterocyclic nucleus, and it is therefore possible for them to exhibit geometric *cis-trans* isomerism, in which the 2-substituent and the amine side chain are on the same or opposite side of the double bond. The relative effects of the different isomeric forms of the thioxanthenes flupenthixol, clopenthixol, and chlorprothixene are shown in Fig. 5.

In each case the α-isomer was considerably more potent than the β-isomer. This was particularly marked with α- and β-flupenthixol. The activity of flupenthixol in the α,β-mixture, which is used clinically (2), was entirely due to the α-isomer. We also examined the relative effects of α- and β-flupenthixol on the stimulation of cyclic AMP formation produced by 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene. This compound is an analogue of dopamine with the side chain fixed in an extended conformation. We have shown it to be a potent dopamine-like stimulant of the striatal cyclic AMP production system (21). Here again there was a large difference in antagonist

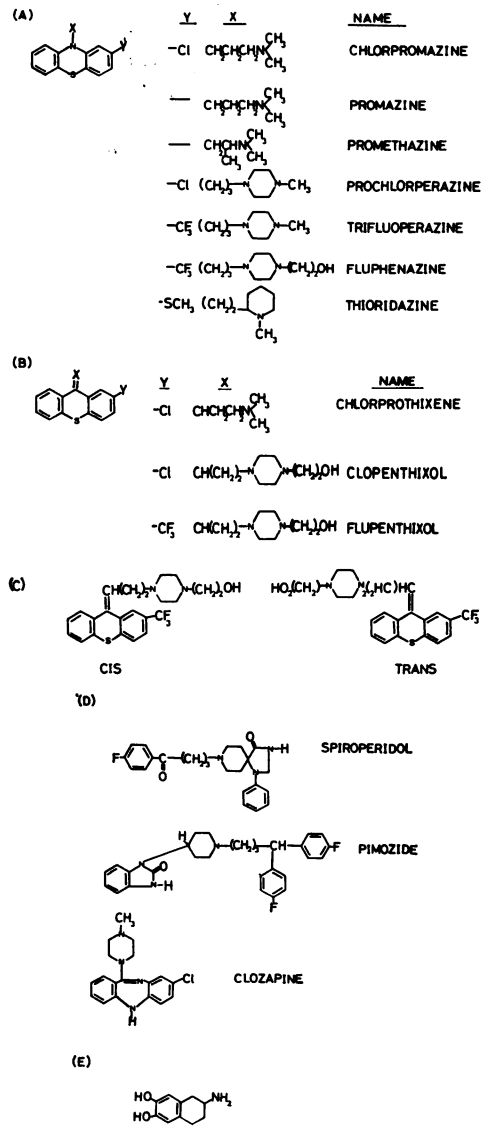

FIG. 4. *Structures of drugs used*

A. Phenothiazine neuroleptic agents. B. Thioxanthene neuroleptic agents. C. *cis/trans* isomers of flupenthixol. D. Non-phenothiazine neuroleptic agents. E. 2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene.

potency between α- and β-flupenthixol (Table 2).

There is some evidence that neuroleptic activity may be related to blockade of dopamine receptors in the limbic system rather than in the basal ganglia, blockade of the latter receptors being most probably

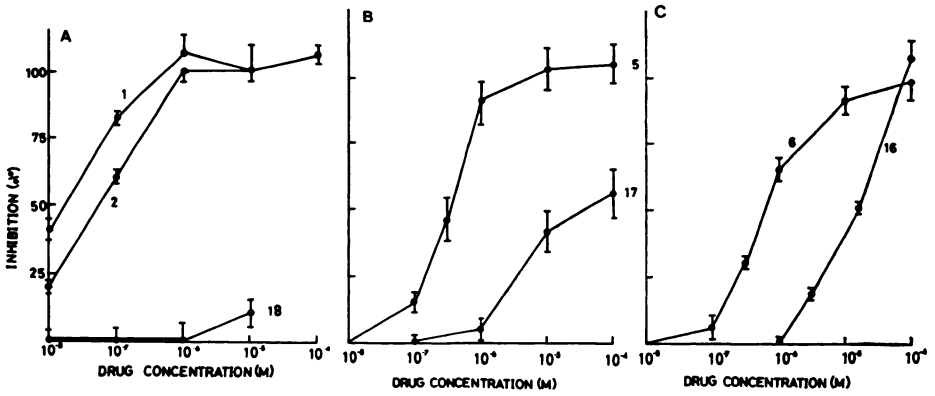

FIG. 5. *Effect of α- and β-thioxanthene isomers on dopamine-stimulated cyclic AMP production in striatal homogenates: flupenthixol (A), clopenthixol (B), and chlorprothixene (C)*

Basal activity was 33.0 (A), 37.5 (B), and 28.5 (C) pmoles/tube (2 mg, wet weight). Activity in the presence of 100 μM dopamine was 69.0 (A), 75.0 (B), and 57.0 (C) pmoles/tube (2 mg, wet weight). Each point is the mean and standard error for at least five separate incubations. Numbers refer to those in Table 1.

TABLE 2

Effect of α- and β-flupenthixol on stimulation of cyclic AMP (cAMP) production in various brain areas by 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) or dopamine

Results are the means and standard errors for at least five separate incubations.

Area	Agonist (100 μM)	Basal activity	Activity + agonist	Inhibition of stimulated activity	
				α-flupenthixol (1 μM)	β-flupenthixol (1 μM)
		pmoles cAMP/tube	*cAMP/tube*	%	%
Striatum	ADTN	31.0	63.0	105 ± 3.6	NS[a]
Olfactory tubercle	Dopamine	20.0	45.0	93 ± 2.7	NS
Nucleus accumbens	Dopamine	22.0	42.0	90 ± 5.2	NS

[a] No significant inhibition ($p > 0.05$).

related to drug-induced parkinsonism (23–25). Dopamine-stimulated formation of cyclic AMP has been demonstrated in homogenates of the olfactory tubercle and nucleus accumbens from rat brain (14), limbic regions which contain a high density of dopaminergic terminals. In these systems, again, the α-isomer of flupenthixol was much more active than the β-isomer in antagonizing the stimulatory effect of added dopamine on cyclic AMP production (Table 2).

DISCUSSION

Previous studies have indicated that the dopamine-stimulated production of cyclic AMP in basal ganglia and other brain areas represents a valid model for studies of drug interactions with dopamine receptors in the central nervous system (11, 12, 21). The results reported here show that neuroleptic drugs of various structures are more effective as antagonists of dopamine than non-neuroleptic drugs of similar structures. It should be noted that a concentration of 100 μM dopamine was used in the drug antagonism studies reported here. Because the inhibition of dopamine-stimulated cyclic AMP production appears to be of a competitive nature (Fig. 3) (see ref. 22), it was possible to calculate K_i values (see Table 1), which more accurately reflect drug binding affinities than do IC_{50} values.

The critical importance of a 2-substituent and the nature of the basic amine side chain for neuroleptic activity in the phenothiazine and thioxanthene classes is well documented (26). In the present study the most potent compounds, α-flupenthixol and fluphenazine, both have a $-CF_3$ group at position 2 and a β-hydroxyethylpiperazinyl amine side chain. Replacement of the β-hydroxyethyl group by a methyl function as in trifluoperazine or replacement of the $-CF_3$ group by a chlorine atom, as in α-clopenthixol, both led to a fall in potency. The influence of the $-CF_3$ group was also shown by the higher potency of fluphenazine in comparison with prochlorperazine.

Promazine, which has only weak clinical neuroleptic activity (27), lacks a 2-substituent, and had only weak dopamine antagonistic effects. It was of interest that chlorimipramine, a tricyclic antidepressant which differs from chlorpromazine only in having a dimethylene bridge instead of a sulfur atom, had only about $\frac{1}{10}$ the potency of the latter compound. A third criterion for potent neuroleptic activity is the presence of a 3-carbon side chain between the phenothiazine nucleus and the basic amine group (26). The fact that promethazine, an antihistamine, containing a substituted 2-carbon side chain, was almost inactive is thus also in accordance with documented structure-activity relationships for neuroleptics. The above results are consistent with data obtained from other neurochemical and pharmacological systems used to assess central nervous system dopamine receptor blockade. The specificity of the present assay was further supported by the finding that benztropine, an anticholinergic drug which is a potent inhibitor of the uptake of dopamine (28), was inactive. Neuroleptics of other structures, such as clozapine, spiroperidol, and pimozide, were all active in the present system. The potencies of the latter two compounds in the system *in vitro* were low, however, in view of their high potencies in animal tests and clinically. In the case of pimozide a selective uptake by the caudate nucleus has been demonstrated (29). This selective distribution of pimozide in the central nervous system after systemic administration may account for its high potency *in vivo*.

It is known that there are large differences in the pharmacological activities of the *cis* and *trans* isomers of the thioxanthenes (2, 30, 31). It was of interest, therefore, that similar differences existed in the ability of these isomers to block the dopamine-stimulated production of cyclic AMP. It is known from the X-ray (32, 33) and NMR analyses (34) of certain thioxanthenes that the pharmacologically active α-isomers of these compounds have the *cis* configuration; i.e., the 2-substituent and the amine side chain are on the same side of the double bond linking the side chain to the ring system (Fig. 4). The double bond detracts from the conformational mobility of the thioxanthenes in comparison with the phenothiazines. A determination of the X-ray structures of a series of the active isomers, therefore, may give some insight into the most probable conformation required for a phenothiazine or thioxanthene neuroleptic to be a potent antagonist of the dopamine receptor. A possible molecular mechanism for this blockade by chlorpromazine, based on the X-ray structure of chlorpromazine and dopamine, has been suggested (35). Recently the three-dimensional structure of α-chlorprothixene has been determined (36). It is similar in over-all conformation to the crystal structure of chlorpromazine, and model building supports the concept that dopamine could be superimposed on this molecule in a way similar to that suggested for chlorpromazine. In a recent publication (21) we showed that the conformation of dopamine at the dopamine-sensitive adenylate cyclase, and hence probably at the dopamine receptor, is similar to the preferred form found in the crystal by X-ray analysis (37) and in solution by NMR studies (38). In confirmation of this, we found that α- and β-flupenthixol also showed different potencies in antagonizing stimulation produced by 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene, a dopamine agonist which has the side chain fixed in an extended conformation by incorporation into a second ring system. It is this extended conformation of dopamine that Horn and Snyder (35) suggested may be superimposed on a portion of the neuroleptic molecule.

It is known that dopaminergic neurons exist in various regions of the central nervous system apart from those in the nigro-striatal

TABLE 3

Comparison of effects of neuroleptics on dopamine-sensitive adenylate cyclase and behavioral parameters

Drug	IC_{50} for dopamine-sensitive aednylate cyclase	ED_{50}	
		Antagonism of apomorphine-induced stereotypy in rats[a]	Antagonism of amphetamine-induced stereotypy in rats[a]
	M	mg/kg	mg/kg
(α,β)-Flupenthixol	7.5×10^{-8}	0.5	0.2
α-Flupenthixol	2.2×10^{-8}	0.3	0.07
β-Flupenthixol	$>10^{-4}$	>80	>160
Clopenthixol	3.3×10^{-7} (α)	30 (α)	0.2 (α)
Chlorprothixene	7.8×10^{-7} (α)	45 (α,β)	0.5 (α,β)
Fluphenazine	9.2×10^{-8}	0.2	0.08
Chlorpromazine	1.0×10^{-6}	59	0.6

[a] Data from Møller-Nielsen *et al.* (2).

pathway (39). However, the role of these pathways in mediating behavioral responses has not been extensively investigated. It has been suggested that blockade of dopamine receptors in an area outside the basal ganglia, such as in the limbic system or cortex, may be responsible for neuroleptic activity (3, 40). We found that α- and β-flupenthixol had differential effects on dopamine-stimulated cyclic AMP production in homogenates from two limbic forebrain areas which contain dopaminergic synapses. Some neuroleptic drugs also have potent antimuscarinic effects (25, 41). These effects may be expected to modify activity of neuroleptic drugs in the basal ganglia but not in the limbic system, owing to the existence of a dopaminergic cholinergic balance influencing the activity of the extrapyramidal system (42). This type of interaction may explain differences in the effects of neuroleptics in the basal ganglia and limbic system seen *in vivo* (24) rather than intrinsic differences in dopamine receptor topography in different brain regions.

In addition, it is clear that the dopamine-stimulated formation of cyclic AMP in brain homogenates should be useful as a screening procedure for neuroleptic activity. Table 3 shows the good agreement found when the relative effects of neuroleptics on dopamine-stimulated cyclic AMP production in rat striatum are compared with the effects of the same drugs on two currently used behavioral tests for neuroleptic activity. The results reported here support the hypothesis that neuroleptic drugs may antagonize the actions of dopamine at its receptors. However, the precise localization in the central nervous system of the dopamine receptors that are critical for neuroleptic activity remains to be elucidated.

After this manuscript was prepared, a publication presenting results in good agreement with those given here has appeared (22). We have presented our findings in the form of IC_{50} values and also as calculated K_i values in order to facilitate comparison of our findings with those recently published elsewhere.

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