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Muscarinic m4 receptor activation by some atypical antipsychotic drugs

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Abstract

To clarify the findings that clozapine is both a muscarinic receptor agonist and antagonist, we examined the effects of neuroleptics on forskolin-stimulated cAMP accumulation in Chinese hamster ovary cells expressing human muscarinic m4 receptors (CHO-hm4) and in rat striatum. With CHO-hm4 cells, clozapine induced a concentration-dependent and atropine-sensitive inhibition on cAMP formation, with $EC_{50} = 60$ nM and $E_{max} = 74\%$ of carbachol maximum. Other atypical neuroleptics, fluperlapine, tenilapine and olanzapine, were similar but less potent, while risperidone, rilapine, quetiapine (ICI 204,636), sertindole, and ziprasidone had almost no effect. Typical neuroleptics, haloperidol, chlorpromazine, fluphenazine, thiothixene, thioridazine, and molindone, showed either no effect or an atropine-resistant inhibition of cAMP formation. However, in rat striatal tissues, clozapine, up to 10 μM, did not show a significant inhibition of cAMP formation, probably due to a relatively low abundance of muscarinic m4 receptors and the presence of multiple types of muscarinic and other receptors, with which clozapine interacts. Nevertheless, muscarinic m4 receptor agonism, to some extent, may be a relevant mechanism for the therapeutic efficacy and side effects of clozapine and some atypical neuroleptics.

Keywords: Clozapine; Muscarinic m4 receptor; Neuroleptic, atypical; Neuroleptics, typical; cAMP; Chinese hamster ovary cell; Striatum, rat

1. Introduction

Clozapine is an atypical neuroleptic with a unique profile in the treatment of schizophrenia compared to other typical antipsychotic agents. It is most effective in decreasing positive and negative signs and symptoms in schizophrenic patients, and it causes very few extrapyramidal side effects (Meltzer, 1995). These adverse effects include Parkinsonian-like signs and symptoms and tardive dyskinesia. Hence, this agent has been used clinically as a representative of a new generation of neuroleptics and an important tool to further our understanding of the etiology, pathophysiology and pharmacotherapy of psychotic illnesses. However, because of the incidence of agranulocytosis induced by clozapine, which limits its clinical use, efforts are being made to develop other atypical neuroleptics with high efficacy and low toxicity.

Clozapine interacts broadly with several neurotransmitter receptors, some of which are implicated in its mechanism of action. Among these receptors, dopamine D_2 and 5-HT_{2A} receptors have been mostly extensively investigated and thought to be most relevant to the atypical mechanism of clozapine (Meltzer, 1994). However, the hypothesis that more potent blockade of 5-HT_{2A} receptors over that of dopamine D_2 receptors is responsible for the atypical features of clozapine (Meltzer et al., 1989), can only partially explain the therapeutic features of this drug.

Muscarinic receptors, which exist in five genetic (m1, m2, m3, m4 and m5) and four pharmacological (M_1, M_2, M_3 and M_4) subtypes (Bonner, 1989), are thought to play a role in many neuropsychiatric disorders. Neuroleptics, antidepressants and antiparkinsonism drugs to varying degrees block muscarinic receptors (Richelson, 1991). In fact, clozapine is a potent and selective muscarinic receptor antagonist (Bolden et al., 1992; Herrling and Misbach-Lesenne, 1982). However, evidence to the contrary exists. Clozapine produces hypersalivation in many patients (Baldessarini and Frankenberg, 1991) and this side effect is blocked by anticholinergic agents (Meltzer, 1992). A partial muscarinic M_1 receptor activation by clozapine in vivo was actually observed (Ögren, 1992). More recently, a report suggested that clozapine acted as an agonist at the cloned muscarinic m4 receptors expressed in transfected cells (Zorn et al., 1994).

We sought to answer whether muscarinic m4 receptor

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activation is a common mechanism of action for currently available atypical neuroleptics. To answer this question, we examined the effects of a group of compounds that have been classified as atypical neuroleptics, in comparison to typical neuroleptics, on forskolin-stimulated cyclic AMP accumulation in Chinese hamster ovary cells expressing human muscarinic m4 receptors (CHO-hm4) and in rat striatum, an area rich in muscarinic m4 receptors (Yasuda et al., 1993). In most cells and neuronal tissues, muscarinic m4 receptors couple to inhibition of adenylyl cyclase activity, so compounds that are agonists at these receptors would inhibit the formation of cyclic AMP in these cells. Our results confirm and extend the previous work of Zorn et al. (1994).

2. Materials and methods

2.1. Assay of cyclic AMP formation in CHO cells

CHO-hm4 cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 5% FetalClone II bovine serum product (Hyclone Laboratories) and 1% MEM non-essential amino acids, in an atmosphere of 10% CO_2 at 37°C. The cAMP assay was performed with low passage number of confluent cells according to the method described (Pfenning and Richelson, 1990). Briefly, cells were removed by modified Puck's D1 solution containing 2 mM EGTA and collected by low speed centrifugation (500 rpm for 2 min). The cells were then resuspended in a phosphate buffered saline solution containing glucose and sucrose (PBS-GS). To prelabel ATP stores, cells were preincubated with [^{3}H]adenine (20 μCi/10^7 cells in 2 ml suspension) at 37°C in a shaking water bath for 45 min. Cells were then further incubated for 30 min with PBS-GS containing 1.5 mM 3-isobutyl-1-methyl-xanthine, followed by their distribution into 24-well culture plates, to give 10^5 cells in a final volume of 400 μl per well. The responses, in triplicate, were initiated by addition of 1 μM forskolin alone or in combination with a concentration of an agonist or test drug, following a preincubation of the cells with or without atropine for 30 min. The reactions were stopped with the addition of 30 μl of 50% (w/v) trichloroacetic acid and cyclic [^{14}C]AMP (1 nCi) was added as an internal standard. The cyclic [^{3}H]AMP formed in the cells was isolated using ion exchange chromatography with Dowex AG 50 W-X8 column (100–200 mesh, H^+ form) in combination with an alumina column (neutral, type WN-3). The washing buffers (5 ml of 1 mM phosphate buffer, 6 ml of H_2O and 4 ml of 0.1 M imidazole-HCl buffer) were subsequently used. The final 3 ml of eluate was collected in a vial containing 16 ml of Redi-Safe (Beckman Instruments, Fullerton, CA, USA) and counted in a liquid scintillation counter using a dual-isotope program.

2.2. Assay of cyclic AMP formation in rat striatum

Male Wistar rats (about 200 g) were decapitated and quickly dissected. The striata (about 100 mg from each rat, 200 mg for 100 cAMP assays) were removed and put into ice cold D1 buffer. The tissue was minced in D1 solution, passed through a 210 μm Nitex mesh and then a 130 μm Nitex nylon mesh and centrifuged at 2000 rpm for 3 min. The pellet was resuspended in PBS-GS and centrifuged twice at 2000 rpm for 3 min. The pellet was finally resuspended in 2 ml of PBS-GS with 40 μCi [^{3}H]adenine and incubated at 37°C for 45 min. The reaction was centrifuged at 2000 rpm for 3 min to remove the excess of free [^{3}H]adenine before distribution into 24-well plates. The subsequent procedures were the same as those used on cells as described above.

2.3. Materials

The CHO-hm4 cell line was a kind gift from Dr T. Bonner (N.I.M.H., Bethesda, MD, USA). Dulbecco's modified Eagle's medium, MEM non-essential amino acids solution were from Gibco-BRL. FetalClone II bovine serum product was from Hyclone Laboratories (Logan, UT, USA). The radiochemicals [^{3}H]adenine (25 Ci/mmol) and [^{14}C]cAMP (306 mCi/mmol) were purchased from Amersham Life Sciences (Arlington Heights, IL, USA). The following neuroleptics were generously provided by the manufacturers: clozapine and thioridazine from Sandoz (East Hanover, NJ, USA); olanzapine from Eli Lilly (Indianapolis, IN, USA); thiothixene and ziprasidone from Pfizer (New York, NY, USA); quetiapine (ICI 204,636 or Seroquel) from Zeneca (Wilmington, DE, USA); fluphenazine from E.R. Squibb&Sons (Princeton, NJ, USA); molindone from Dupont (Wilmington, DE, USA); and sertindole from Abbott Laboratories (North Chicago, IL, USA). Fluperlapine, rilapine, risperidone and tenilapine were gifts from Dr H. Meltzer (Vanderbilt University, Nashville, TN, USA). Chlorpromazine, haloperidol, forskolin and 3-isobutyl-1-methylxanthine were purchased from Sigma (St. Louis, MO, USA). All other reagents were analytical grade.

3. Results

With CHO-hm4 cells, forskolin (5 nM to 5 μM) induced a concentration-dependent increase of [^{3}H]cAMP levels. A submaximal concentration of forskolin (1 μM), which caused an increase in [^{3}H]cAMP equivalent to 2–5-fold over basal levels, was chosen to stimulate the response in testing the inhibitory effects of neuroleptics. Like carbachol, clozapine (0.1 nM to 1 μM) produced a concentration-dependent inhibition of the forskolin-stimulated accumulation of cyclic [^{3}H]AMP, with $EC_{50} = 60$

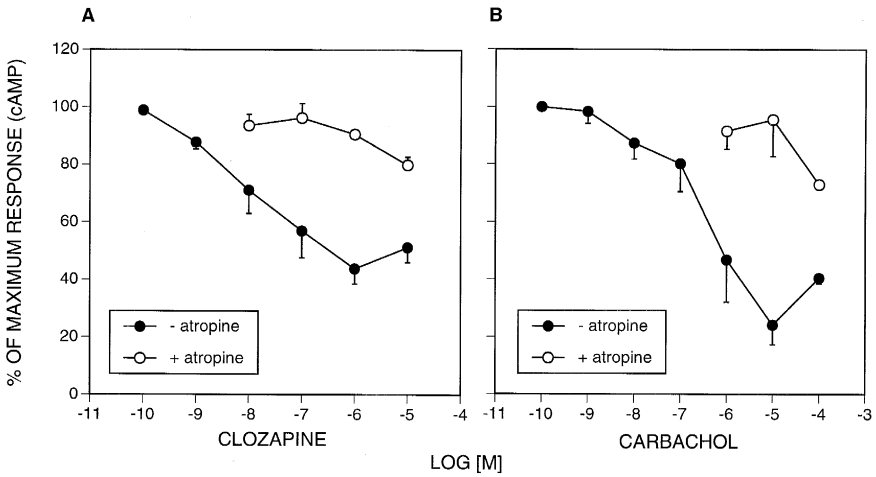

Fig. 1. Concentration dependence of the inhibition by clozapine and carbachol on forskolin-stimulated [^{3}H]cAMP accumulation in CHO cells expressing human muscarinic m4 receptors. The cells were prelabelled with [^{3}H]adenine and stimulated with 1 μM forskolin in the absence (control) and in the presence of the indicated concentrations of drug. (A) Clozapine without and with atropine (10 μM) atropine; (B) carbachol without and with atropine (10 μM). The data are means ± S.E.M. from 3–5 experiments, each point in triplicate, and expressed as a percentage of the maximum response to 1 μM forskolin (2100 dpm/10^5 cells as 100%).

nM and the maximum inhibition of 44%, which was equivalent to 74% of the maximum effect of carbachol (Fig. 1). A reversal of the inhibitory response was seen at higher concentrations of clozapine (10 μM) and carbachol (100 μM) (Fig. 1). In addition to clozapine, atypical neuroleptics fluperlapine, olanzapine and tenilapine also showed a similar but weaker inhibition on forskolin-induced accumulation of [^{3}H]cAMP (Fig. 2). Only at very high concentrations did rilapine (10 μM) have a weak inhibitory effect (Fig. 2). The potency of these compounds is shown in Table 1. The inhibition produced by these agents could be competitively reversed by 10 μM atropine, confirming that the response was via muscarinic receptors (Figs. 1 and 3). However, other atypical neuroleptic compounds tested, including quetiapine, risperidone, sertindole and ziprasidone all at 10 μM, had no effect on muscarinic m4 receptors in this assay. In addition, none of these agents had an effect on the basal concentration of [^{3}H]cAMP in the cells.

All typical neuroleptics, at 1 μM, an effective concentration as m4 agonist for clozapine and others, either caused no significant change (for haloperidol, fluphenazine, thiothixene and molindone), or produced an atropine-insensitive reduction (for chlorpromazine and thioridazine) of the forskolin-induced accumulation of [^{3}H]cAMP (Fig. 3).

On rat striatal tissue, 1 μM forskolin caused an increase

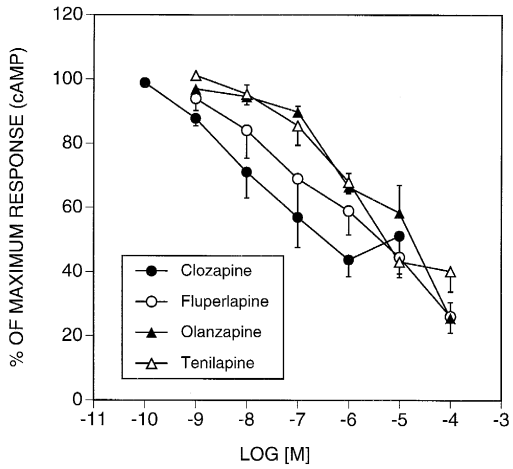

Fig. 2. Concentration dependence of the inhibition by several atypical neuroleptics on forskolin-stimulated [^{3}H]cAMP accumulation in CHO cells expressing human muscarinic m4 receptors. The cells were prelabelled with [^{3}H]adenine and stimulated with 1 μM forskolin in the absence (control) and presence of the indicated concentrations of clozapine, fluperlapine, olanzapine and tenilapine. The data are means ± S.E.M. from 4–5 experiments, each point in triplicate, and expressed as a percentage of the maximum response to 1 μM forskolin (2100 dpm/10^5 cells as 100%).

Table 1
Potency of atypical neuroleptics as agonists in inhibition of forskolin (1 μM)-induced accumulation of cyclic [^{3}H]AMP on the human muscarinic m4 receptors expressed in CHO cells

Atypical neuroleptics	EC_{50} (nM) [a]	*n*
Clozapine	60 ± 10	7
Tenilapine	600 ± 100	3
Fluperlapine	600 ± 100	4
Olanzapine	1900 ± 200	6
Rilapine	N.E. [b]	3
ICI 204,636	N.E.	3
Risperidone	N.E.	3
Sertindole	N.E.	3
Ziprasidone	N.E.	3
Carbachol [c]	290 ± 70	4

[a] Data (EC_{50} or concentration at 50% of maximal response) are expressed as geometric means ± S.E.M. (see Bolden et al., 1992, for references for this type of analysis). [b] N.E. = no effect. [c] Carbachol is not a neuroleptic.

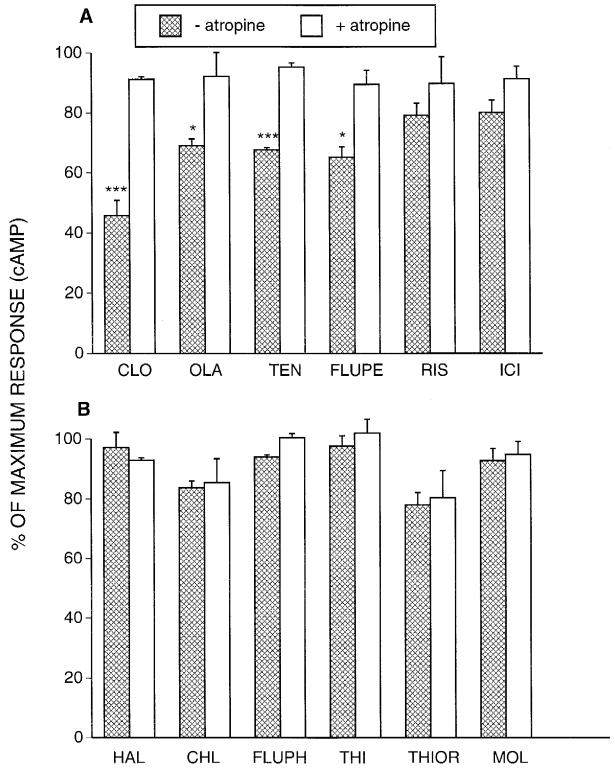

Fig. 3. Effects of atypical (A) and typical (B) neuroleptics on forskolin-stimulated [^{3}H]cAMP accumulation on CHO cells expressing human muscarinic m4 receptors. The cells were prelabelled with [^{3}H]adenine and then stimulated with 1 μM forskolin following the pretreatment with 1 μM of clozapine (CLO), tenilapine (TEL), fluperlapine (FLUPE) and olanzapine (OLA); 10 μM of risperidone (RIS); 100 μM quetiapine (QUI); or 1 μM of haloperidol (HAL), chlorpromazine (CHL), fluphenazine (FLUPH), thiothixene (THI), thioridazine (THIOR) or molindone (MOL). In each case, experiments were done in the absence (hatched bars) and in the presence (open bars) of 10 μM atropine. The data are means ± S.E.M. from 3–6 experiments, each point determined in triplicate, and expressed as a percentage of the maximum response to 1 μM forskolin (control, 2100 dpm/10^5 cells as 100%). ** $P < 0.01$, *** $P < 0.001$, compared to the control (Student's *t*-test).

of cAMP of 1–3-fold above basal levels. Clozapine, up to 10 μM, had no significant effect on either basal or forskolin-induced cAMP accumulation even though carbachol, at high concentrations (10 μM and higher), showed a modest inhibition (percentage of control response ± S.E.M., 71 ± 1%, $P < 0.001$ by Student's *t*-test vs. control) of this response that was partially reversed by atropine (82 ± 5%, $P > 0.05$ vs. control).

4. Discussion

In the present study, we found that at the cloned human muscarinic m4 receptors expressed in CHO cells, atypical neuroleptic agents fall into two groups. The first group showed muscarinic m4 receptor activation and included clozapine, tenilapine, fluperlapine and olanzapine. The second group showed no or weak receptor activation and included risperidone, rilapine, quetiapine, sertindole and ziprasidone. These data confirm and extend the findings of Zorn et al. (1994). Since all the typical neuroleptic compounds tested, including haloperidol, did not share the m4 stimulatory effect, this mechanism may partially explain the atypical profile of clozapine and a few related compounds.

Clozapine has a range of activities at many other receptors, notably 5-HT_{2A} and 5-HT_{1C} receptors, α_1-adrenoceptors and dopamine D_4 receptors (Coward, 1992; Van Tol et al., 1991). Several hypotheses have been put forth to account for its higher efficacy and lower extrapyramidal side effects over typical antipsychotic drugs. These include a much higher affinity for 5-HT_{2A} over dopamine D_2 receptors (Meltzer, 1991), a higher affinity for D_4 over dopamine D_2 receptors (Van Tol et al., 1991) and high affinity for muscarinic m1 receptors (Bolden et al., 1992). However, it is more likely that multiple biological mechanisms including muscarinic m4 receptor activation is responsible for the diverse differences between clozapine and typical neuroleptics.

Clozapine has long been recognized as a muscarinic receptor antagonist, which was proposed as the basis for its relative lack of extrapyramidal side effects. Early experiments showed that clozapine functioned as a potent antagonist of muscarinic receptors in some central neurons and in peripheral tissues from animals (Stille et al., 1971; Snyder et al., 1974; Fosbraey et al., 1980; Herrling and Misbach-Lesenne, 1982). With the discovery of five cloned subtypes of the muscarinic receptor (m1, m2, m3, m4 and m5) (Bonner, 1989), we found clozapine had high affinity and selectivity for the muscarinic m1 receptor (Bolden et al., 1992).

However, clozapine also appeared to cause some effects that were unexpected for a muscarinic receptor antagonist. Clozapine behaved like a muscarinic agonist at inhibiting apomorphine-induced climbing in mice (Lassen, 1979). The phenomena that clozapine antagonized the postjunctional acetylcholine response, while apparently potentiating the prejunctional inhibition by acetylcholine (Fosbraey et al., 1980) might also imply its dual effects as a muscarinic agonist and antagonist. More strikingly, the muscarinic receptor antagonist scopolamine blocked the ability of clozapine, but not that of haloperidol and thioridazine, to increase extracellular dopamine release in the striatum (Meltzer et al., 1994; Rivest and Marsden, 1991). In fact, clozapine behaved as a partial agonist at the M1 site in vivo (Ögren, 1992).

Of all the atypical compounds tested in this study, clozapine was the most potent (EC_{50} = 60 nM), although less so than that (11 nM) reported by Zorn et al. (1994), probably due to the difference in methods for assay. Fluperlapine and olanzapine are chemically similar to clozapine (a dibenzazepine tricyclic) and also are similar pharmacologically (Moore et al., 1992). Both drugs had a similar but lower agonist potency on muscarinic m4 recep-

tors than did clozapine. However, surprisingly, another analogue of clozapine, quetiapine (ICI 204,636), which has a similar antipsychotic profile as does clozapine with experimental animals (Swerdlow et al., 1994; Migler et al., 1993), was extremely weak in this study. This agreed with the previous report that quetiapine had no affinity on muscarinic receptors (Saller and Salama, 1993). The benzisoxazole derivative risperidone, another promising atypical neuroleptic, also lacked an m4 receptor agonist effect. Risperidone has a low extrapyramidal side effect profile only at lower doses (e.g., 6–8 mg/day). At higher doses, risperidone may be indistinguishable from typical neuroleptics such as haloperidol with regard to extrapyramidal side effects (Meltzer, 1995). Like clozapine, this compound is also a potent antagonist of the 5-HT_{2A} receptor, the α_1-adrenoceptor, the histamine H_1 receptor and the dopamine D_2 receptor. However, recently, in a randomized, double-blind, controlled clinical trial of risperidone versus clozapine in patients with chronic schizophrenia, clozapine, but not risperidone, caused an increase in salivation and a mean reduction in heart rate (Klieser et al., 1995). These data suggest that activation of muscarinic receptors may account for differences between clozapine and risperidone.

The rat striatum was used to study further the activation by clozapine of muscarinic m4 receptors, because these receptors are relatively enriched in this area (Bernard et al., 1992; Hersch et al., 1994; Levey et al., 1991), which is likely involved with the extrapyramidal side effects of neuroleptics. With the striatum and without selective antagonists, we otherwise were not able to focus specifically on the muscarinic m4 (M_4) subtype. However, clozapine caused no apparent muscarinic receptor activation (up to 10 μM). The reason for this lack of an effect by clozapine is uncertain. Likely, it is due to the existence in this tissue of a highly complex network of muscarinic and other receptors interacting with the adenylyl cyclase system. In general, stimulation of muscarinic m4 or m2 receptors causes a decrease of cAMP through a pertussis toxin-sensitive, inhibitory G-protein. However, stimulation of muscarinic m1, m3, or m5 receptors causes, to a lesser extent, an increase in cAMP levels, even though the main effects of these receptors is the stimulation of inositol phosphate metabolism (Bonner, 1989). Moreover, recent observations indicate that in m4 monoclonal cell systems, muscarinic m4 receptor activation causes either inhibition or stimulation of cAMP levels, depending upon receptor density, agonist concentration, type of G-proteins coupled and type of adenylyl cyclases (Jones et al., 1991; Migeon and Nathanson, 1994; Dittman et al., 1994). Therefore, the effect of clozapine on cAMP levels in rat striatum might represent a summation of a complex of effects of this drug on muscarinic as well as other receptors. More likely, our assay was not sensitive enough to measure an effect of clozapine, since the full muscarinic agonist carbachol caused only a small inhibition of the effect of forskolin.

As mentioned above, the cellular response to muscarinic agonists is mediated by receptors coupling with guanine nucleotide regulatory proteins. In the case of the G-protein coupled receptor family, the receptor binding and G-protein coupling are regulated by factors such as GTP, its analogs and mono- or divalent cations (Na^+, Mg^{2+}) (Wong et al., 1994; Mousseau and Butterworth, 1994). However, the extent of the regulation varies from system to system. In preliminary studies (data not shown) involving muscarinic m4 receptor binding in competition with [^{3}H]quinuclidinyl benzilate, the binding affinity of clozapine ($K_d = 47$ nM) was not significantly shifted by GTP-γ-S (100 μM) or NaCl (120 mM). Further experimentation is necessary to learn the significance of this phenomenon.

The multiple clinical benefits of clozapine may be attributed to its direct interactions with different receptors, including its dual (agonist and antagonist) effects on different subtypes of muscarinic receptors and consequent regulation of neurotransmitter release. Dopamine-acetylcholine balance may be relevant to the expression of schizophrenic symptoms and the muscarinic system may serve to dampen the expression of positive schizophrenic symptoms associated with dopaminergic hyperactivity (Tandon and Greden, 1989). In fact, different subtypes of muscarinic receptors are coincidentally or selectively expressed in some neuronal populations of the striatum that express dopamine receptors and hence regulate striatal function (Bernard et al., 1992). Moreover, both clozapine (Meltzer et al., 1994) and other cholinomimetic drugs (Gorell and Czarnecki, 1986) cause in the striatum an increase of dopamine release, which is blocked by antimuscarinic agents.

In summary, in the CHO-hm4 cell system, activation of muscarinic muscarinic m4 receptors appears to be a pharmacological effect of some, but not all, atypical antipsychotic drugs. The muscarinic activation by clozapine could not be detected in rat striatum, probably due to a relatively low abundance of muscarinic m4 receptors and the presence in this tissue of multiple types of muscarinic and other receptors, with which clozapine interacts. Although the clinical relevance of this receptor activation is uncertain, potential new atypical drugs could be identified by screening for this activity at the cloned muscarinic m4 receptor.

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