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Effects of clozapine on rat striatal muscarinic receptors coupled to inhibition of adenylyl cyclase activity and on the human cloned m4 receptor

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1 Clozapine has recently been claimed to behave as a selective and full agonist at the cloned m4 muscarinic receptor artificially expressed in Chinese hamster ovary (CHO) cells. In the present study we have investigated whether clozapine could activate the rat striatal muscarinic receptors coupled to the inhibition of adenylyl cyclase activity, considered as pharmacologically equivalent to the m4 gene product. In addition, we have examined the effect of the drug on various functional responses following the activation of the cloned m4 receptor expressed in CHO cells.

2 In rat striatum, clozapine (1 nM – 10 μM) caused a slight inhibition of forskolin-stimulated adenylyl cyclase activity, which was not counteracted by 10 μM atropine. On the other hand, clozapine antagonized the inhibitory effect of acetylcholine with a pA_2 value of 7.51. Moreover, clozapine (1 μM) failed to inhibit dopamine D_1 receptor stimulation of adenylyl cyclase activity, but counteracted the inhibitory effect of carbachol (CCh). Clozapine displaced [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) bound to striatal M_4 receptors with a monophasic inhibitory curve and a pK_i value of 7.69. The clozapine inhibition was not affected by the addition of guanosine-5′-*O*-(thio)triphosphate (GTPγS).

3 In intact CHO cells, clozapine inhibited forskolin-stimulated cyclic AMP accumulation with an EC_{50} of 31 nM. This effect was antagonized by atropine. CCh produced a biphasic effect on cyclic AMP levels, inhibiting at concentrations up to 1 μM (EC_{50} = 50 nM) and stimulating at higher concentrations (EC_{50} = 7 μM). Clozapine (0.3 – 5 μM) antagonized the CCh stimulation of cyclic AMP with a pK_i value of 7.47. Similar results were obtained when the adenylyl cyclase activity was assayed in CHO cell membranes.

4 In CHO cells pretreated with the receptor alkylating agent 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (10 μM), the maximal inhibitory effect of clozapine on cyclic AMP formation was markedly reduced, whereas the CCh inhibitory curve was shifted to the right with no change in the maximum.

5 As in rat striatum, in CHO cell membranes the displacement of [^{3}H]-NMS binding by clozapine yielded a monophasic curve which was not affected by GTPγS.

6 Clozapine (10 nM – 10 μM) had a small stimulant effect ($\sim$20%) on the binding of [^{35}S]-GTPγS to CHO cell membranes, whereas CCh caused a 250% increase of radioligand binding. Moreover, clozapine (50 nM – 5 μM) antagonized the CCh-stimulated [^{35}S]-GTPγS binding with a pA_2 value of 7.48.

7 These results show that at the striatal M_4 receptors clozapine is a potent and competitive antagonist, whereas at the cloned m4 receptor it elicits both agonist and antagonist effects. Thus, clozapine behaves as a partial agonist, rather than as a full agonist, at the m4 receptor subtype, with intrinsic activity changing as a function of the coupling efficiency of the receptor to effector molecules.

Keywords: Clozapine; rat striatal M_4 muscarinic receptors; human cloned m4 muscarinic receptor; cyclic AMP; [^{3}H]-N-methylscopolamine binding; [^{35}S]-guanosine-5′-*O*-(thio)triphosphate binding

Introduction

Unlike classical neuroleptics, clozapine can exert an antipsychotic effect without producing significant extrapyramidal side effects (Baldessarini & Frankenburg, 1991). The reason for this atypical neuroleptic profile is still not completely understood. Clozapine has been shown to bind to a number of neurotransmitter receptors, including M_1-M_5 muscarinic receptors, dopamine D_4 receptors, 5-hydroxytryptamine $(5\text{-HT})_{lc}$ and 5-HT_2 receptors, histamine H_1 receptors and α_1-adrenoceptors (Coward, 1992). As extrapyramidal side effects may result from increased striatal cholinergic activity (Miller & Hiley, 1974), it has been proposed that the low incidence of motor disturbances following clozapine administration might be due to the antimuscarinic activity of the drug (Snyder *et al*., 1974; Miller & Hiley, 1976). However, recent studies have obtained evidence that clozapine may act as a muscarinic agonist in the brain. Thus, Rivest & Marsden (1991) found that the administration of an antimuscarinic drug, scopolamine or atropine, attenuated the increase of dopamine metabolism induced by clozapine in striatum and nucleus accumbens of awake, freely moving rats. Similarly, Meltzer *et al*. (1994) observed that scopolamine completely inhibited the ability of clozapine to increase extracellular dopamine, dihydroxyphenylacetic acid and homovanillic acid concentrations in rat striatum. Recently, Zorn *et al*. (1994), with Chinese hamster ovary (CHO) cells transfected with the human cloned m1 – m5 receptor genes, showed that clozapine behaves as an antagonist at the m1, m2, m3 and m5 muscarinic receptors but as a full agonist (EC_{50} 11 nM) at the muscarinic m4 receptor coupled to inhibition of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation. In rat brain, M_4 muscarinic receptors are particularly expressed in striatum (Buckley *et al*., 1988; Levey *et al*., 1991), where they appear to control cyclic AMP formation in an inhibitory manner (McKinney *et al*., 1989; Ehlert *et al*., 1989; Olianas & Onali, 1991; Olianas *et al*., 1996). Because of

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the significance of the central muscarinic receptor activity to the pharmacological actions of clozapine, the present study was designed to investigate whether clozapine could act as a full agonist also at the striatal M_4 receptors coupled to inhibition of adenylyl cyclase activity. For comparison, the action of clozapine at the human cloned m4 receptor expressed in CHO cells was also examined.

Methods

Cell culture

CHO cells expressing the human cloned m4 receptor (CHO/m4 cells) were kindly provided by Prof. A.D. Strosberg (Institut Cochin de Genetique Moleculaire, Paris). The cells were grown as a monolayer culture in Ham's F-12 medium (GIBCO BRL) supplemented with 10% foetal calf serum in a humidified atmosphere (5% CO_2) at 37°C. Cells were grown to ~80% confluency in plastic Petri dishes (Falcon), the medium was removed and the cells were washed with ice-cold phosphate-buffered saline.

CHO/m4 cell membranes preparation

When cell membranes were prepared for adenylyl cyclase assay, the cells were scraped into an ice-cold buffer containing 10 mM HEPES/NaOH, 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM dithiothreitol (DTT) (pH 7.4) and lysed with a Dounce tissue grinder. The cell lysate was centrifuged at 1,000 *g* for 2 min at 4°C. The supernatant was collected and centrifuged at 32,500 *g* for 30 min at 4°C. The pellet was resuspended in the homogenization buffer at a protein concentration of ~2.0 mg ml^{-1}. For radioligand binding studies, cells were homogenized in an ice-cold buffer containing 25 mM sodium phosphate buffer (pH 7.4) and 5 mM $MgCl_2$ by use of an Ultra-Turrax homogenizer. The cell lysate was centrifuged at 32,500 *g* for 30 min at 4°C and the pellet was resuspended in the same buffer at a protein concentration of ~3 mg ml^{-1}. The membrane preparations were either used immediately or stored at −70°C.

Preparation of membranes from rat striatum

A crude synaptosomal fraction was prepared from striata of male Sprague-Dawley rats (200–250 g) as previously described (Olianas *et al*., 1996) and used for either adenylyl cyclase or radioligand binding assays.

Assay of adenylyl cyclase activity

The enzyme activity of rat striatum was assayed as previously described (Olianas *et al*., 1996). In CHO/m4 cell membranes, the enzyme activity was assayed in a reaction mixture (final volume 100 μl) containing: HEPES/NaOH 50 mM (pH 7.4), $MgCl_2$ 2.3 mM, EGTA 0.3 mM, [α-^{32}P]-ATP 0.05 mM (150–170 c.p.m. $pmol^{-1}$), [^{3}H]-cyclic AMP 0.5 mM (160 c.p.m. $nmol^{-1}$), GTP 50 μM, 3-isobutyl-1-methylxanthine (IBMX) 1 mM, phosphocreatine 5 mM, creatine kinase 50 u ml^{-1}, BSA 50 μg, bacitracin 10 μg, aprotinin 10 kallikrein inhibitor units (KIU) and forskolin 20 μM. The incubation was started by adding the tissue preparation (30–40 μg of protein) and carried out at 25°C for 20 min. [^{32}P]-cyclic AMP was isolated according to Salomon *et al*. (1974).

Receptor alkylation with 1-ethoxycarbonyl-2-ethoxy-1,2-ethoxy-1,2-dihydroquinoline (EEDQ)

Confluent CHO/m4 cells were harvested by incubation in phosphate buffered saline containing 0.5 mM EDTA for 3–5 min at 37°C and centrifuged at 1,000 *g* for 10 min. The cells were resuspended in Krebs-HEPES buffer at a density of ~2.5×10^6 cells ml^{-1}, divided in 1 ml aliquots and incubated at 37°C for 30 min with either vehicle or EEDQ (from 1 μM to 1 mM) freshly dissolved in dimethylsulphoxide. The incubation was terminated by the addition of Krebs-HEPES buffer (mM: NaCl 125, glucose 10, $MgSO_4$ 1.2, KH_2PO_4 1.2, KCl 3.8, $CaCl_2$ 1.2, HEPES NaOH 25; pH 7.4) and the cells were washed 3 times by resuspension and centrifugation. The extent of receptor alkylation was measured by saturation binding of [^{3}H]-NMS (20 pM–2.0 nM) to membranes prepared from vehicle and EEDQ-treated cells. For cyclic AMP assays the cells were labelled with [^{3}H]-adenine as described below.

Assay of cyclic AMP

CHO/m4 cells grown in 36 mm plastic dishes were incubated in Ham's F-12 medium containing 10 μCi ml^{-1} [^{3}H]-adenine for 1 h at 37°C in an incubator. Thereafter, the medium was removed and the cells were incubated in an oxygenated Krebs-HEPES buffer containing 1 mM 3-isobutyl-1-methylxanthine (IBMX), 25 μM FSK and the various test compounds. After 10 min at 37°C, the incubation was stopped by the aspiration of the medium and the addition of an ice-cold solution containing 6% (w/v) perchloric acid and 0.1 mM [^{14}C]-cyclic AMP (3,000 c.p.m.). After 30 min at ice-bath temperature, the solution was neutralized by the addition of 0.6 M KOH and centrifuged. [^{3}H]-cyclic AMP was isolated according to Salomon *et al*. (1974). The recovery of [^{3}H]-cyclic AMP from each sample was corrected on the basis of the recovery of [^{14}C]-cyclic AMP. The formation of [^{3}H]-cyclic AMP is expressed as percentage of total ^{3}H incorporated into the cells (% conversion).

Assay of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding

The binding of [^{3}H]-NMS to rat striatal muscarinic receptors was performed in a final assay volume of 1.2 ml containing 50 mM sodium phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 0.1% BSA, 30 μg of membrane protein and 10 pM–3.0 nM [^{3}H]-NMS. The incubation was carried out at 25°C for 120 min. Nonspecific binding was determined in the presence of 1 μM atropine. The incubation was stopped by adding 4 ml of ice-cold buffer without BSA to each sample followed by immediate filtration through GF/C glass fibre filters presoaked in 0.1% polyethylenimine for at least 18 h. The filters were washed twice with the same buffer, dried and the bound radioactivity was counted by liquid scintillation. Under these conditions, the dissociation constant (K_D) of [^{3}H]-NMS was 61 ± 16 pM, a value similar to that obtained by Waelbroeck *et al*. (1990). The binding capacity (B_{max}) was 3.2 ± 0.5 pmol mg^{-1} protein (mean ± s.e.mean of three experiments). The binding of [^{3}H]-NMS to striatal M_4 receptors was performed under the same conditions following the method described by Waelbroeck *et al*. (1990), which is based on the slower dissociation rate of [^{3}H]-NMS from M_4 receptors as compared to the other subtypes. The samples were preincubated at 25°C for 120 min in the presence of 0.25 nM [^{3}H]-NMS and the test compounds. Thereafter, 1 μM atropine (final concentration) was added to each sample to induce [^{3}H]-NMS dissociation from the receptors and the incubation was continued for 35 min. The incubation was stopped and the bound radioactivity determined as described above. Nonspecific binding was determined by adding 1 μM atropine at the beginning of the preincubation period.

The binding of [^{3}H]-NMS to CHO/m4 cell membranes was assayed in a buffer containing 25 mM sodium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 0.1% BSA and 12–15 μg of membrane protein. The [^{3}H]-NMS concentrations ranged from 20 pM to 2 nM and the final assay volume was 1 ml. The incubation was carried out at 30°C for 90 min. Nonspecific binding was determined in the presence of 1 μM atropine. The binding data were analysed by the computer programme EBDA, which yielded the initial estimates of equilibrium binding parameters. These estimates were used

in the non linear curve-fitting computer programme LIGAND (Munson & Rodbard, 1980), which provided the final estimates of the K_D, B_{max} and inhibition constant (K_i). Saturation experiments indicated that in CHO/m4 cell membranes [^{3}H]-NMS bound to a single site with a K_D value of 0.17 ± 0.03 nM and B_{max} of 3.5 ± 0.09 pmol mg^{-1} protein.

Assay of guanosine-5′-O-(3-[^{35}S]-thio)triphosphate ([^{35}S]-GTPγS) binding

CHO/m4 cell membranes were diluted ten fold in 10 mM HEPES/NaOH (pH 7.4), 1 mM EDTA and 0.1% BSA, centrifuged and resuspended in the same buffer. The binding of [^{35}S]-GTPγS was assayed in a reaction mixture (final volume 100 μl) containing: HEPES/NaOH (pH 7.4) 25 mM, $MgCl_2$ 10 mM, EDTA 1 mM, GDP 1 μM, NaCl 100 mM and [^{35}S]-GTPγS 1.0 nM. The incubation was started by the addition of the membrane suspension (1.7–2.0 μg of protein) and was carried out at 30°C for 60 min. The incubation was terminated by adding 5 ml of ice-cold buffer containing 10 mM HEPES/NaOH (pH 7.4) and 1 mM $MgCl_2$, immediately followed by rapid filtration through glass fibre filters (Whatman GF/C) presoaked in the same buffer. The filters were washed twice with 5 ml of buffer and the radioactivity trapped was determined by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 100 μM GTPγS. Assays were performed in duplicate.

Protein content was determined by the method of Bradford (1976), with BSA as a standard.

Statistical analysis

Results are given as mean ± s.e.mean. Concentration-response curves were analysed by a least squares curve-fitting computer programme (GraphPad Prism, San Diego, CA, U.S.A.). Antagonist effects of clozapine were examined according to Arunlakshana-Schild analysis (Arunlakshana & Schild, 1959) and the potency was determined from the ratios between the EC_{50} values of the agonist in the absence and in the presence of different concentrations of the antagonist. The pA_2 values were determined from the x intercepts and calculated by least squares regression analysis of the Schild plots, where the log of the dose ratios (DR) − 1 is plotted as a function of the antagonist concentration. In other experiments where a single concentration of clozapine was examined, the K_i was calculated from the equation:

$$EC_{50}b = EC_{50}a\ (1 + I/K_i)$$

where $EC_{50}a$ and $EC_{50}b$ are the concentrations of the agonist producing half-maximal effect in the absence and in the presence of clozapine, respectively, and I is the concentration of clozapine. For comparison with pA_2 values, K_i values were converted to the logarithmic form (pK_i). Statistical significance of the difference between means was determined by Student's *t* test.

Materials

[α-^{32}P]-ATP (30–40 Ci mmol^{-1}),[2,8-^{3}H]-cyclic AMP (25 Ci mmol^{-1}),[8-^{14}C]-cyclic AMP (45.1 mCi mmol^{-1}) and [^{35}S]-GTPγS (1306 Ci mmol^{-1}), were obtained from New England Nuclear-Du Pont (Bad Homburg, Germany). 1-[N-methyl-^{3}H]-scopolamine methyl chloride ([^{3}H]-NMS) was from Amersham (U.K.). Clozapine, (±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide ((±)-chloro APB HBr) and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) were purchased from Research Biochemical International (Natick, MA, U.S.A.). Guanosine-5′-*O*-(thio)triphosphate (GTPγS) was from Boehringer (Mannheim, Germany). Carbachol chloride (CCh), acetylcholine chloride (ACh), bethanechol chloride, atropine sulphate and the other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Effects on adenylyl cyclase activity of rat striatum

Clozapine, tested at concentrations ranging from 1 nM to 10 μM, caused a slight reduction of the forskolin-stimulated enzyme activity which was maximal at 1 μM and corresponded to 5.5 ± 0.5% decrease of control activity (Figure 1a). Although this effect was statistically significant ($P < 0.05$), it was not antagonized by the addition of a high concentration

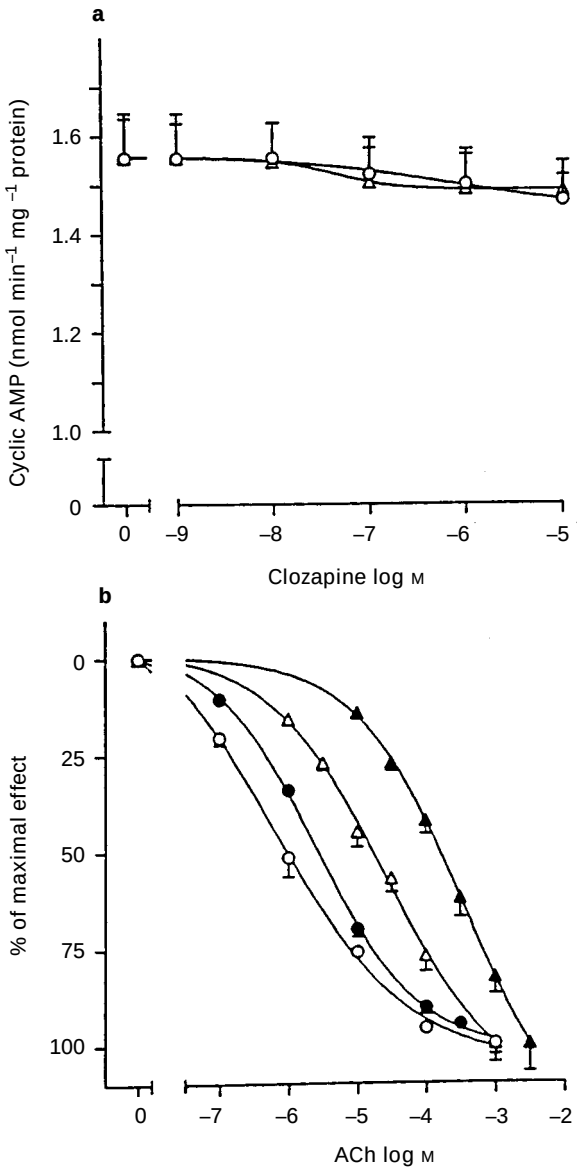

Figure 1 (a) Effect of clozapine on forskolin-stimulated adenylyl cyclase activity of rat striatum. The enzyme activity was assayed at the indicated concentrations of clozapine in the absence (○) and in the presence of 10 μM atropine (△). The concentration of forskolin was 10 μM. (b) Antagonism of acetylcholine (ACh) inhibition of forskolin-stimulated adenylyl cyclase activity of rat striatum by clozapine. The enzyme activity was assayed in the absence (○) and in the presence of 50 nM (●), 500 nM (△) and 5 μM (▲) clozapine. In (a) and (b) data are the mean, and vertical lines show s.e.mean, of three experiments. Enzyme activities (expressed as nmol cyclic AMP min^{-1} mg^{-1} protein ± s.e.mean) were: control, 1.60 ± 0.02; clozapine 50 nM, 1.58 ± 0.02, clozapine 500 nM, 1.54 ± 0.01, clozapine 5 μM, 1.51 ± 0.02 ($n = 3$).

(10 μM) of atropine. Under the same experimental conditions, acetylcholine (ACh) and carbachol (CCh) inhibited the enzyme activity by $25.1 \pm 1.2\%$ and $23.8 \pm 1.9\%$ ($P < 0.001$), respectively. When clozapine was coincubated with ACh, the drug shifted to the right the ACh concentration-response curve without affecting the maximal inhibitory effect (Figure 1b). Analysis of the clozapine antagonism by the method of Arunlakshana & Schild (1959) yielded a pA_2 value of 7.51 ± 0.15 with a slope value of 0.998 ± 0.09.

CCh (50 μM) reduced the adenylyl cyclase stimulation elicited by the dopamine D_1 receptor agonist ($\pm$)-chloro APB HBr (1 μM) by 46.3% ($P < 0.001$). Clozapine (1 μM) failed to inhibit the dopamine D_1 receptor-stimulated cyclic AMP formation. When combined with CCh, clozapine (1 μM) counteracted the inhibitory effect of the cholinoceptor agonist (dopamine D_1 receptor-stimulated enzyme activites, expressed as pmol cyclic AMP min^{-1} mg^{-1} protein $\pm$ s.e.mean, were: control 61.5 ± 2.1, CCh 36.0 ± 1.4, clozapine 63.1 ± 3.2, clozapine + CCh 50.0 ± 2.8, $n = 3$).

Effects on [^{3}H]-NMS binding to striatal M_4 receptors

Clozapine antagonism of [^{3}H]-NMS binding to striatal M_4 receptors yielded a monophasic inhibition curve (Hill coefficient = 1.0) with an IC_{50} of 102 ± 15 nM (Figure 2a). This value corresponded to a pK_i of 7.69 ($pK_i = -\log K_i$). The addition of GTPγS (10 μM) did not affect the clozapine inhibition curve. Conversely, CCh generated a shallow competition curve (Hill coefficient = 0.30), recognizing at least two populations of binding sites, one with high affinity ($pK_i = 7.19$; 44.3%) and the other with low affinity ($pK_i = 5.05$; 55.7%) (Figure 2b). In the presence of GTPγS, the proportion of the high-affinity site was decreased to 23.4% ($P < 0.05$).

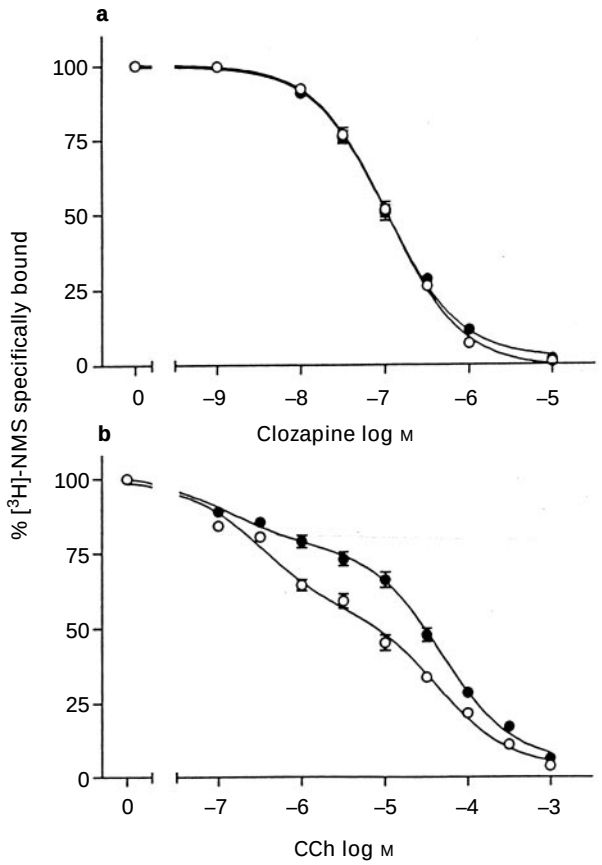

Figure 2 Displacement of [^{3}H]-NMS binding to striatal M_4 sites by clozapine (a) and CCh (b) in the absence (○) and in the presence (●) of 10 μM GTPγS. The [^{3}H]-NMS binding to striatal M_4 sites was assayed as described in Methods. Data are the mean, and vertical lines show, s.e.mean of three experiments.

Effects on cyclic AMP accumulation in CHO/m4 cells

Clozapine produced a concentration-dependent inhibition of cyclic AMP accumulation in CHO/m4 cells (Figure 3a). The half-maximal effect occurred at 30.8 ± 3.8 nM whereas the maximal inhibition was reached at 1 μM and corresponded to $72 \pm 4.0\%$ decrease of basal value ($P < 0.001$, $n = 6$). The addition of atropine (1 μM) markedly antagonized the clozapine inhibitory effect. CCh had a biphasic effect on cyclic AMP accumulation, eliciting inhibition at concentrations ranging from 10 nM to ~ 1 μM ($EC_{50} = 50.2 \pm 5.7$ nM) and stimulation at higher concentrations ($EC_{50} = 7.0 \pm 0.5$ μM) (Figure 3b). The extent of maximal inhibition elicited by CCh was similar to that produced by clozapine and the combination of the two compounds did not produce a further decrease of cyclic AMP accumulation (results not shown). The cholinoceptor agonist bethanechol caused inhibition of cyclic AMP accumulation ($EC_{50} = 3.2 \pm 1.0$ μM) followed by a slight stimulation with

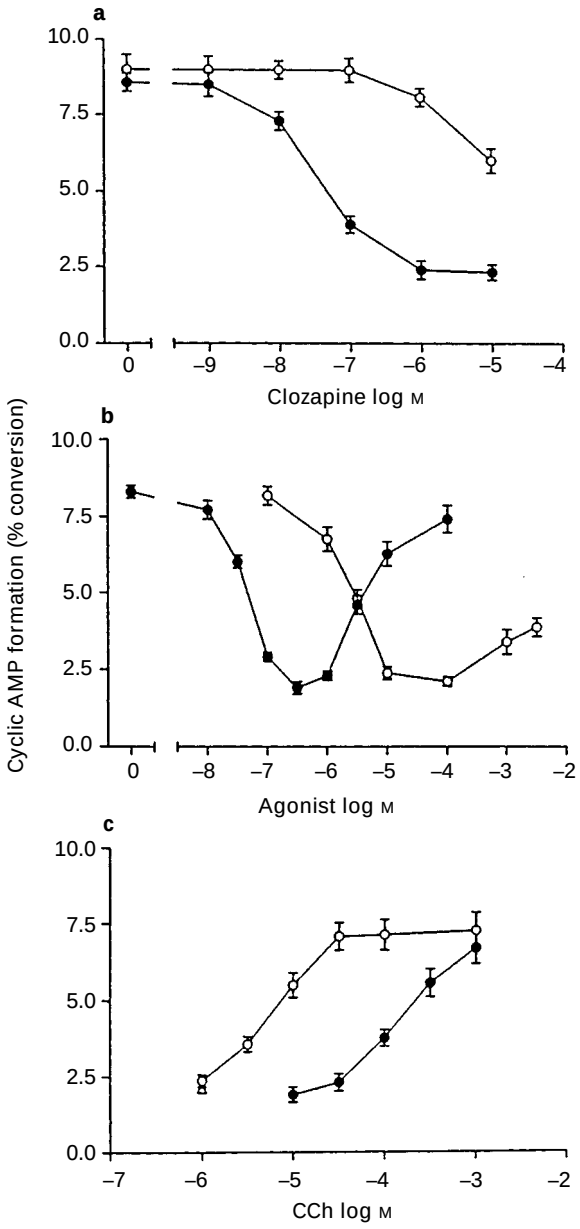

Figure 3 Cyclic AMP accumulation in CHO/m4 cells. Cells prelabelled with [^{3}H]-adenine were incubated with 25 μM forskolin in the presence of the indicated concentrations of the following drugs: clozapine without (●) and with (○) 1 μM atropine (a); carbachol (●) and bethanechol (○) (b); carbachol (CCh) without (○) and with 1 μM clozapine (●) (c). Data are the mean and vertical lines show s.e.mean of three experiments.

concentrations > 100 μM. When combined with CCh, clozapine (1 μM) caused a 30 fold rightward shift of the stimulating part of the CCh curve with an apparent pK_i of 7.47 ± 0.1 (Figure 3c).

Effects on adenylyl cyclase activity of CHO/m4 membranes

In membranes of CHO/m4 cells, clozapine caused a concentration-dependent inhibition of forskolin-stimulated adenylyl cyclase activity with an EC_{50} value of 45 ± 5.2 nM and a maximal effect was reached at 1 μM (Figure 4a). Atropine (1 μM) completely antagonized the inhibition seen with 1 μM clozapine (results not shown). On the other hand, as observed in intact cells, CCh produced a biphasic effect with inhibition of the enzyme activity at concentrations from 10 nM to 1 μM (EC_{50} = 30 ± 4.1 nM) and stimulation at higher concentrations (EC_{50} = 12.5 ± 1.7 μM). The maximal inhibitory effect of clozapine corresponded to 88 ± 7.5% of that of CCh. When clozapine was combined with CCh, the curve for the stimulant effect of the latter agonist was shifted to the right with increasing clozapine concentration (Figure 4b). The estimated pA_2 value of clozapine was 7.43 ± 0.09 with a slope of 1.05 ± 0.08 (n = 3).

Effects of EEDQ treatment of CHO/m4 cells

Preincubation of CHO/m4 cells with the receptor alkylating agent EEDQ at concentrations ranging from 1 μM to 1 mM resulted in a progressive reduction in the binding of [^{3}H]-NMS (results not shown). In CHO/m4 cells pretreated with 10 μM EEDQ, which reduced the B_{max} of [^{3}H]-NMS binding by 65.1 ± 3.8% (n = 3), clozapine failed to inhibit cyclic AMP accumulation significantly (Figure 5). On the other hand, the EEDQ treatment shifted to the right the inhibitory curve for CCh by ~10 fold, without significantly reducing the maximal inhibitory effect of the agonist. Moreover, the stimulant effect of CCh was not present in EEDQ-treated cells.

Effects on [^{3}H]-NMS binding to CHO/m4 cell membranes

In CHO/m4 cell membranes, CCh displaced the binding of [^{3}H]-NMS with a shallow curve (Hill coefficient 0.61 ± 0.04), indicating the presence of at least two components: a high-affinity site (K_i = 0.45 μM; 49%) and a low-affinity site (K_i = 18 μM; 51%) (Figure 6a). In the presence of 100 μM GTPγS, the CCh displacement curve was steeper (Hill coefficient 0.80 ± 0.03) with a decrease in the fraction of the high-affinity site to 28% (P > 0.05). On the other hand, clozapine displayed a monophasic displacement curve (Hill coefficient = 0.91 ± 0.07) with a K_i value of 16.7 ± 1.2 nM (pK_i = 7.77) (Figure 6b). The addition of GTPγS failed to affect the clozapine competition curve.

Effects on [^{35}S]-GTPγS binding to CHO/m4 cell membranes

CCh maximally stimulated [^{35}S]-GTPγS binding to CHO/m4 cell membranes by 3.5 fold with an EC_{50} value of 3.2 ± 0.5 μM (Figure 7a). Clozapine, at concentrations ranging from 10 nM to 10 μM, produced only a slight increase in [^{35}S]-GTPγS

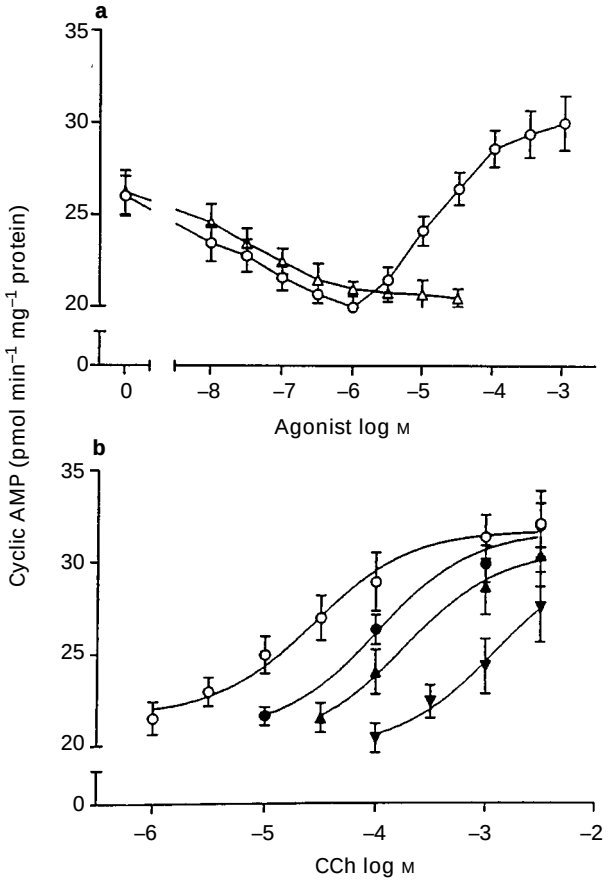

Figure 4 (a) Effects of carbachol and clozapine on adenylyl cyclase activity of CHO/m4 cell membranes. The enzyme activity was assayed in the presence of 20 μM forskolin at the indicated concentrations of carbachol (○) and clozapine (△). (b) Antagonism of carbachol (CCh) stimulation of adenylyl cyclase by clozapine. The enzyme activity was assayed at the indicated concentrations of CCh without (○) and with 0.3 (●), 1.0 (▲) and 5.0 (▼) μM clozapine. Data are the mean, and vertical lines show s.e.mean, of three experiments.

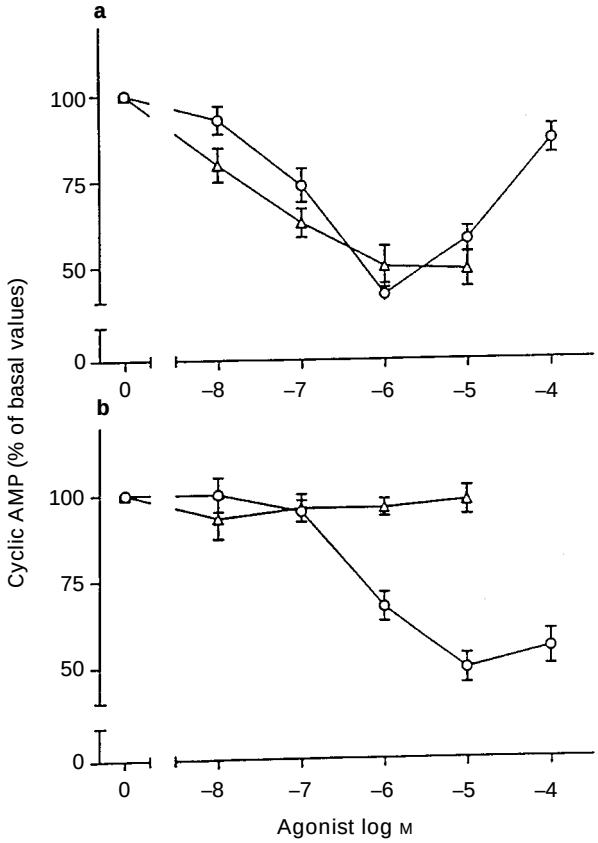

Figure 5 Effects of EEDQ treatment on cyclic AMP accumulation in CHO/m4 cells. Cells were pretreated with either vehicle (a) or 10 μM EEDQ (b) and then labelled with [^{3}H]-adenine. Cyclic AMP formation was determined in the presence of the indicated concentrations of carbachol (○) and clozapine (△). Data are the mean, and vertical lines show s.e.mean, of three experiments.

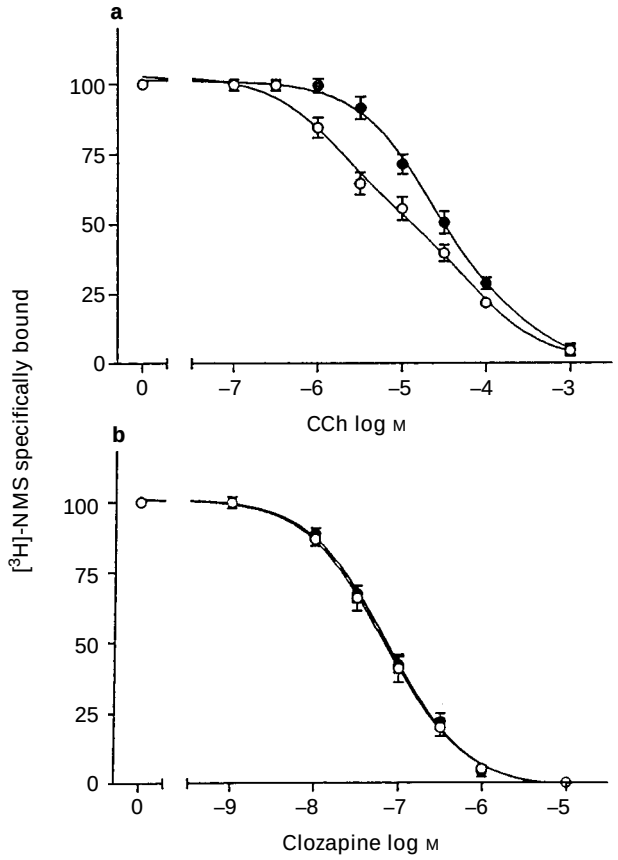

Figure 6 Displacement of [^{3}H]-NMS binding to CHO/m4 cell membranes by carbachol (CCh) (a) and clozapine (b) in the absence (open symbols) and in the presence (solid symbols) of 100 μM GTPγS. Data are the mean, and vertical lines show s.e.mean, of three experiments.

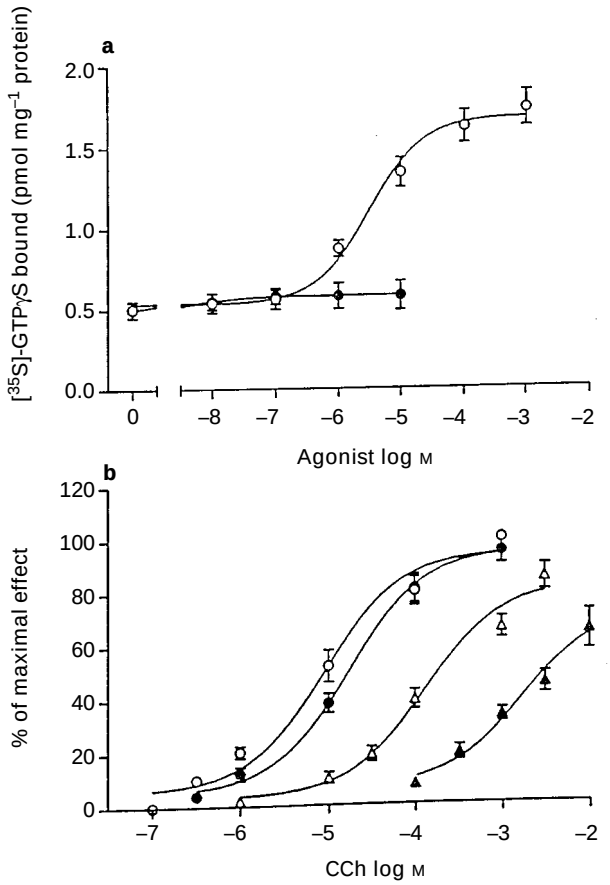

Figure 7 (a) Effects of carbachol (○) and clozapine (●) on [^{35}S]-GTPγS binding to CHO/m4 cell membranes. (b) Stimulation of [^{35}S]-GTPγS binding by carbachol (CCh) in the absence (○) and presence of 50 nM (●), 500 nM (△) and 5.0 μM (▲) clozapine. Data are the mean, and vertical lines show s.e.mean, of three experiments.

binding which corresponded to a ~20% increase of basal value. When combined with CCh, clozapine antagonized the CCh stimulation of [^{35}S]-GTPγS binding with a pA_2 value of 7.48 ± 0.10 and a Schild slope of 1.08 ± 0.05 ($n = 3$).

Discussion

Clozapine has been shown by Zorn *et al.* (1994) to act as a selective and full agonist at the cloned m_4 receptor expressed in CHO cells. However, the present study showed that clozapine does not behave as a full agonist at the muscarinic receptors coupled to inhibition of adenylyl cyclase of rat striatum, which can be considered as a valid example of native M_4 receptors (McKinney *et al.*, 1989; Elhert *et al.*, 1989, Olianas & Onali, 1991; Olianas *et al.*, 1996). Clozapine produced only a slight reduction of forskolin-stimulated enzyme activity when compared to the full inhibitory responses elicited by either ACh or CCh. Moreover, the clozapine inhibition was not antagonized by atropine, indicating that the effect is not due to an action on muscarinic receptors. On the other hand, when added together with ACh, clozapine antagonized the ACh-induced inhibition of adenylyl cyclase activity. The slope value of the Schild plot was not different from unity, suggesting competitive antagonism. Besides inhibiting basal and forskolin-stimulated cyclic AMP formation, muscarinic receptor agonists effectively depress dopamine D_1 receptor-stimulated adenylyl cyclase activity of rat striatum (Olianas *et al.*, 1983; Kelly & Nahorski, 1986). This effect also appears to be mediated through the stimulation of M_4 receptors (Olianas *et al.*, 1996). However, clozapine failed to inhibit the striatal dopamine D_1 receptor activity, a result that contrasts with the putative M_4 agonist activity of the drug. On the other hand, clozapine significantly counteracted the CCh inhibition of dopamine D_1 receptor-stimulated adenylyl cyclase, indicating that the drug exerts an antagonist effect on this response.

A possible agonist activity of clozapine at the striatal M_4 receptors was also investigated in radioligand binding assays, by use of [^{3}H]-NMS as a radioligand and following the methodology for selective labelling of M_4 sites described by Waelbroeck *et al.* (1990). In these assays, clozapine displaced the radioligand binding with a monophasic curve, indicating the recognition of a single class of binding sites. The estimated pK_i value of clozapine (7.69) correlated with the pA_2 value displayed for the antagonism of the ACh inhibition of adenylyl cyclase activity (7.51) and is close to the affinity of the drug for the cloned human muscarinic m4 receptor subtype observed in the present study ($pK_i = 7.77$) and in that by Bolden *et al.* (1992) (7.95). Moreover, the clozapine displacement curve was not shifted to the right by GTPγS, a property more typical of antagonist rather than agonist binding to G protein-coupled receptors (Nathanson, 1987). However, CCh clearly recognized two affinity states of the M_4 receptor, one with high and the other with low affinity. As expected for agonist binding, the addition of GTPγS reduced the proportion of sites with high affinity for CCh. Thus, radioligand binding assays also fail to demonstrate characteristics consistent with an agonist action of clozapine at striatal M_4 receptors.

In agreement with the previous observation by Zorn *et al.* (1994), clozapine inhibited the forskolin-stimulated cyclic AMP accumulation in CHO/m4 cells. This effect was concentration-dependent, saturable and antagonized by atropine, as expected for an action mediated by the m4 receptor. The clozapine inhibition is quantitatively similar to and not additive with that produced by CCh. Moreover, the clozapine in-

hibitory effect appears to result from the activation of an m4 receptor negatively coupled to adenylyl cyclase, as it could be observed also when the enzyme activity was directly assayed in CHO/m4 cell lysates.

However, there are a number of aspects that differentiate the action of clozapine from that of CCh and other full muscarinic agonists. Thus, CCh elicits a biphasic effect on cyclic AMP accumulation, with an inhibitory effect at low concentrations followed by a stimulant effect at higher concentrations. By contrast, clozapine caused only inhibition of cyclic AMP accumulation. The same difference was detected in the adenylyl cyclase assay. When added with CCh, clozapine antagonized the CCh stimulation of cyclic AMP accumulation and adenylyl cyclase activity with potencies (pK_i-pA_2 values = 7.47 – 7.43) close to its EC_{50} value in eliciting the inhibition of cyclic AMP accumulation. Thus, clozapine displays agonist and antagonist effects with similar potencies. This property is consistent with the possibility that clozapine acts as a partial agonist at the cloned m4 receptor. A likely explanation for the finding that clozapine elicits an inhibitory response quantitatively similar to that of CCh is that in CHO cells the m4 receptor couples to the inhibition of cyclic AMP formation with high efficiency, possible as a consequence of receptor overexpression and its high affinity for pertussis toxin-sensitive G proteins, which mediate the cyclase inhibition (Jones *et al*., 1991). Under this condition, it is possible that even low-efficacy agonists may induce a full inhibitory response (Kenakin *et al*., 1992). On the other hand, the m4 receptor appears to be less efficiently coupled to stimulation of cyclic AMP formation. Although the molecular mechanisms involved are not completely understood, the stimulant response requires higher agonist concentrations than the cyclase inhibition and has been shown to be mediated by pertussis toxin-insensitive G proteins (Jones *et al*., 1991). The present study showed that the receptor-mediated stimulation of adenylyl cyclase can be detected in CHO cell membranes in the presence of the Ca^{2+} chelator EGTA, adding further support to the idea that this response is not mediated indirectly through an elevation of Ca^{2+} due to phospholipase C stimulation (Jones *et al*., 1991). In HEK 293 cells transfected with the m4 receptor and type I adenylyl cyclase, the muscarinic stimulation of cyclic AMP formation has been proposed to involve a crossover from inhibitory to stimulant G protein coupling that occurs at maximal receptor activation (Dittman *et al*., 1994). It is possible that a similar mechanism might occur in CHO/m4 cells. Regardless of the mechanism, a full stimulant response is observed only with a high-efficiency agonist, such as CCh, but not with clozapine which conversely acts as an antagonist. Thus, clozapine appears to behave as either 'full' agonist or antagonist depending upon the efficiency of the receptor coupling to the signal transduction machinery.

Evidence for the possible action of clozapine as a partial m4 receptor agonist was also provided by the data obtained with the receptor alkylating agent EEDQ. Partial muscarinic receptor inactivation by EEDQ treatment markedly reduced the clozapine inhibition of cyclic AMP accumulation, whereas it shifted to the right the CCh inhibitory curve with no change of the maximal response. This differential sensitivity to receptor alkylation is consistent with the idea that clozapine acts as a partial agonist, while CCh behaves as a full agonist. Two additional sets of data are in line with this possibility. First, clozapine displacement of [^{3}H]-NMS binding to CHO/m4 cell membranes was monophasic and not affected by GTPγS, an antagonist-like behaviour similar to that observed in rat striatal membranes, whereas CCh recognized high- and low-affinity states of the m4 receptor in a guanine nucleotide-sensitive manner. Secondly, clozapine was found to be a poor stimulator of [^{35}S]-GTPγS binding to CHO/m4 cell membranes and antagonized the large stimulant effect produced by CCh. Previous studies have shown that the stimulation of [^{35}S]-GTPγS binding in CHO cell membranes constitutes a valid assay to evaluate agonist and antagonist properties at the muscarinic receptor subtypes (Lazareno & Birdsall, 1993). The EC_{50} of CCh in stimulating guanine nucleotide exchange is several fold lower than that displayed in inhibiting the adenylyl cyclase activity. Previous studies (Olianas & Onali, 1996) have shown that the addition of 100 mM NaCl, required to amplify the muscarinic stimulation of [^{35}S]-GTPγS binding, significantly reduces the agonist potency. Thus, the lower potency of CCh in the [^{35}S]-GTPγS binding assays, can be ascribed, at least in part, to the presence of 100 mM NaCl. The partial agonist property of clozapine may explain why the drug behaved as an agonist in CHO cells and as an antagonist in striatum at M_4 receptors coupled to adenylyl cyclase. In fact, differences in receptor density and coupling efficiency between the two cell systems are likely to be critical for the intrinsic activity of the drug. With regard to the receptor density, under assay conditions similar to those employed for CHO/m4 cells we found that the B_{max} of [^{3}H]-NMS binding to rat striatal membranes was $\sim$3.2 pmol mg^{-1} protein, a value close to that (3.4 pmol mg^{-1} protein) obtained by Elhert & Tran (1990). Studies in which immunoprecipitation techniques with receptor subtype specific antibodies were used (Yasuda *et al*., 1993), or combined analysis of equilibrium and kinetic binding data (Waelbroeck *et al*., 1990), have estimated that in rat striatum M_4 receptors represent 45% of the total muscarinic receptors. This value corresponds to a density of approximately 1.4 pmol mg^{-1} protein, which is 2.5 fold lower than that present in our CHO/m4 cells (3.5 pmol mg^{-1} protein). Thus, the density of striatal M_4 receptors may not be sufficiently high to allow the expression of the clozapine agonist effect.

Because the agonist effect of clozapine is dependent on receptor density, it is possible that the selectivity of the drug for the m4 receptor described by Zorn *et al*. (1994) is secondary to the receptor overexpression in CHO cells. In this case, the drug may act as a partial agonist at any muscarinic receptor subtype if expressed at a sufficiently high density. This possibility remains to be verified.

In conclusion, the present study shows that in rat striatum clozapine is a potent and competitve antagonist of the adenylyl cyclase inhibiting muscarinic receptors, whereas in CHO/m4 cells the drug behaves as a partial agonist, producing either agonist or antagonist effects probably as a function of the efficiency with which the m4 receptor couples to different signal transduction pathways. If a similar variability in the coupling conditions occurs in native tissues, then clozapine and clozapine-like antipsychotic drugs may differentially modulate the cholinergic transmission through the M_4 receptors, thus expanding their spectrum of possible pharmacological effects.

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