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Clozapine interaction with the M_2 and M_4 subtypes of muscarinic receptors

Pavel Michal [a,1], Michaela Lysíková [a,1], Esam E. El-Fakahany [b], Stanislav Tuček [a,*]

[a] *Institute of Physiology, Academy of Sciences of the Czech Republic, Vídenská 1083, 14220 Prague, Czech Republic*
[b] *Neuroscience Research in Psychiatry, University of Minnesota Medical School, 420 Delaware Street S.E., Minneapolis, MN 55455, USA*

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Abstract

Available evidence indicates that the antipsychotic drug clozapine acts as a partial agonist at the muscarinic M_4 and as an antagonist at the M_2 receptors. We wondered whether there is indeed a fundamental difference between its action on these two receptor subtypes, and whether it interacts with their classical or allosteric binding sites. In experiments on Chinese hamster ovary cells stably expressing the M_2 or M_4 receptors, clozapine inhibited the binding of the specific muscarinic ligand [^{3}H]*N*-methylscopolamine to either receptor subtype. The affinity of the high-affinity sites for clozapine was diminished by GTP in the way expected for agonists on both the M_2 and the M_4 receptor subtypes. Arunlakshana–Schild plots of data obtained in saturation binding experiments with [^{3}H]*N*-methylscopolamine at different concentrations of clozapine were linear with a slope of unity. Clozapine did not alter the time course of [^{3}H]*N*-methylscopolamine dissociation from muscarinic M_2 or M_4 receptors. It inhibited the synthesis of cyclic AMP in cells expressing the M_4 receptor subtype, but did not measurably inhibit the synthesis of cyclic AMP in cells expressing the M_2 receptor subtype. We conclude that clozapine has a high affinity for muscarinic M_2 and M_4 receptor subtypes, that it associates with the classical and not with the allosteric binding site, and that it acts as a partial agonist on both the M_2 and the M_4 receptor subtype.

Keywords: Clozapine; Muscarinic receptor; cAMP

1. Introduction

One of the prominent features of the atypical antipsychotic drug clozapine is its high affinity for several types and subtypes of G protein-coupled neurotransmitter receptors (Baldessarini and Frankenburg, 1991; Bymaster et al., 1996). Simultaneous interaction of the drug with multiple synaptic receptors seems to be responsible for the specific features of its action (Nordström et al., 1995; Ashby and Wang, 1996). Although it has been known for a long time that clozapine has a high affinity for the muscarinic receptors for acetylcholine (Miller and Hiley, 1974; Snyder et al., 1974; Richelson and Nelson, 1984), the understanding of the nature of its interactions with these receptors developed slowly and is incomplete.

After the discovery that five subtypes of muscarinic receptors exist in mammals, Bolden et al. (1991, 1992) found in radioligand binding experiments that clozapine acts as an antagonist on all five receptor subtypes. Zorn et al. (1994) investigated the effects of clozapine on the synthesis of cyclic AMP and the production of inositol phosphates in Chinese hamster ovary (CHO) cells stably transfected with the genes for individual muscarinic receptor subtypes and concluded that clozapine acts as selective agonist at muscarinic M_4 receptor subtype and as an antagonist at muscarinic M_1, M_2, M_3 and M_5 receptor subtypes. Zeng et al. (1997) found that clozapine inhibited the synthesis of cyclic AMP and acted as a partial agonist at the M_4 receptors expressed in CHO cells, but had no effect on the synthesis of cyclic AMP in dispersed cells from the striatum. Olianas et al. (1997) examined the action of clozapine on the muscarinic M_4 receptors in much detail and provided convincing evidence that clozapine is a partial muscarinic M_4 receptor agonist in the CHO cells. They suggested that clozapine is also a partial agonist of the M_4 receptors in the striatum and that the

* Corresponding author. Tel.: +420-2-4752620; fax: +420-2-4752488; E-mail: tucek@biomed.cas.cz

[1] P. Michal and M. Lysíková are postgraduate students of pharmacology and neuroscience, respectively, at the First Medical Faculty of Charles University in Prague.

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absence of measurable agonistic effects of clozapine in the striatum depends on lower muscarinic receptor density in striatal membranes.

The M_2 and M_4 subtypes of muscarinic receptors are very similar in their preferential coupling to the G_i and G_o proteins and in their inhibitory effects on adenylyl cyclase (Caulfield, 1993). Experimental evidence listed above suggests that clozapine acts as a partial agonist at the muscarinic M_4 but as an antagonist at the M_2 receptor subtype. Selectivity of this kind would be important both theoretically and practically. Before accepting it as the definitive explanation, it seemed apposite to explore the alternative possibility that clozapine acts as an allosteric modulator (rather than as a classical orthosteric ligand), and to perform additional direct comparisons of the effects of clozapine on the muscarinic M_2 and M_4 receptors.

2. Methods

2.1. Cells and membranes

Experiments were performed on Chinese hamster ovary (CHO) cells stably transfected with genes for the muscarinic M_2 or M_4 receptor subtype (Buckley et al., 1989). Cells were grown in Dulbecco's modified Eagle's medium containing 10% bovine fetal serum and 0.005% geneticin and treated as described (Jakubík et al., 1995b). They were harvested by mild trypsinization after 7 days in culture. Whole cells were used in experiments with the synthesis of cyclic AMP, whereas radioligand binding experiments were performed on membranes of disrupted cells. To obtain the membranes, cells suspended in the cell culture medium without serum were homogenized with an Ultra-Turrax homogenizer, crude fragments were removed by 3 min centrifugation at $300 \times g$ (repeated twice, with washing), and the sediment obtained after 30 min centrifugation at $60\,000 \times g$ was used for subsequent work.

2.2. Experiments with [^{3}H]N-methylscopolamine binding

Three types of experiments were performed: (a) experiments with radioligand displacement, (b) saturation binding experiments, and (c) experiments with radioligand dissociation. Each incubation tube contained membranes corresponding to 1 million cells, suspended in a final volume of 0.8 ml. The incubation medium consisted of (mM) NaCl (136), KCl (5), $MgSO_4$ (1), Na_2HPO_4 (1), and Na-HEPES (10; Na salt of 4-[2-hydroxyethyl]piperazine-1-ethanesulfonic acid, pH 7.4), and the incubations were at 25°C.

In experiments with radioligand displacement, membranes were incubated with 400 pM [^{3}H]*N*-methylscopolamine, with or without 0.5 mM GTP. After 1 h, clozapine was added to final concentrations of 10^{-11}–10^{-5} M and the incubation was continued for 5 h. It was arrested by rapid filtration in a Brandel cell harvester, using Whatman GF/B glass fibre filters pretreated with 0.3% polyethyleneimine. Nonspecific binding was determined in the presence of 5 μM atropine.

In saturation binding experiments, membranes were incubated with 6 different concentrations of [^{3}H]*N*-methylscopolamine. Where indicated, clozapine was added after 1 h and the incubation was continued for 5 h before being arrested by rapid filtration. The concentrations of clozapine used in individual experiments varied between 50 nM and 5000 nM for the M_2 and between 15 nM and 1500 nM for the M_4 receptor subtype. The concentrations of [^{3}H]*N*-methylscopolamine used in experiments without clozapine were in the range of 30 pM–1000 pM for the muscarinic M_2 receptor subtype and of 15–500 pM for the M_4 subtype. In experiments with clozapine, the concentrations of [^{3}H]*N*-methylscopolamine were increased up to 60-fold. Non-specific binding was determined in the presence of 5 μM–600 μM atropine.

In experiments with radioligand dissociation, membranes were incubated with 400 pM [^{3}H]*N*-methylscopolamine for 1 h, after which atropine was added to a final concentration of 5 μM either alone or together with clozapine (10^{-5} M final concentration), and subsequent decline of [^{3}H]*N*-methylscopolamine binding was followed.

2.3. Experiments with the synthesis of cyclic AMP

Cells were grown for 5 days, harvested, and washed in Krebs–Henseleit buffer (KHB) consisting of (mM) NaCl (118), KCl (4.7), $NaHCO_3$ (25), $MgSO_4$ (1.2), $CaCl_2$ (1.3), and glucose (1.2), which had been preequilibrated with 95% O_2/5% CO_2. They were suspended at a concentration of 30 million cells per 1 ml KHB and preincubated at 37°C in the presence of 10 μCi [^{3}H]adenine per 1 ml KHB. After 90 min, they were sedimented by mild centrifugation and washed twice with KHB. They were then resuspended in KHB containing isobutylmethylxanthine (1 mM final concentration) so as to obtain 2 million cells per 1.0 ml of KHB, and incubated 15 min at 37°C. Aliquots (0.5 ml) of suspended cells were added to incubation tubes containing forskolin (25 μM final concentration) and clozapine or other ligands, and the incubation was continued for 10 min.

The incubation was arrested by adding 50 μl HCl (2.5 M) to each incubation tube, together with [^{14}C]cyclic AMP as a tracer for checking the recovery. The samples were put on acid alumina columns (1.5 g of Sigma acid alumina type WA-1 per column), and the tubes were washed with 1 ml water, which was also put on the columns. The elution was with 7 ml water, followed by 2 ml of 0.2 M ammonium acetate, and then additional 3 ml of 0.2 M ammonium acetate. The last three ml of the eluate contained cyclic AMP and were collected in scintillation vials and mixed with 14 ml of Ecolite + (ICN Pharmaceuticals)

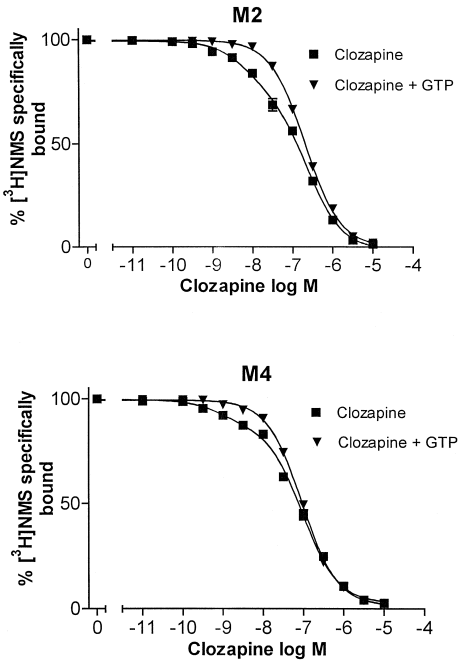

Fig. 1. Inhibition of the specific binding of [^{3}H]*N*-methylscopolamine to the membranes of CHO cells expressing muscarinic receptors of the M_2 (top) or M_4 (bottom) subtype. Incubations were in the presence of 400 pM [^{3}H]*N*-methylscopolamine, either without (■) or with 0.5 mM GTP (▼), and of clozapine at the concentrations indicated on the abscissa. Abscissa: log of the concentration (M) of clozapine. Ordinate: Per cent of [^{3}H]*N*-methylscopolamine binding in the absence of clozapine. Each point represents the mean (±S.E.M) of three experiments with incubations performed in triplicates. See Table 1 for parameters computed from these experiments.

scintillation cocktail. The radioactivity of the collected eluate was counted with the use of a double-labelling program in a Beckmann scintillation counter.

2.4. Data treatment

Values of IC_{50}, K_d, K_i, B_{max}, nH (Hill slope coefficient) and f_{high} (fraction of receptors displaying high affinity for the ligand) were computed with the GraphPad Prism (GraphPad Software, San Diego, CA) program.

3. Results

3.1. Inhibition of [^{3}H]N-methylscopolamine binding to the muscarinic M_2 and M_4 binding sites by clozapine

As shown in Fig. 1, the binding of [^{3}H]*N*-methylscopolamine to membranes of CHO cells expressing the muscarinic M_2 or M_4 receptor subtype became diminished and finally fully abolished in the presence of increasing concentrations of clozapine. In the absence of GTP, the [^{3}H]*N*-methylscopolamine displacement curves were shallow (Table 1; nH values of 0.68 and 0.73 for the M_2 and M_4 binding sites, respectively) and the fit was significantly better when based on the assumption of two binding sites for clozapine rather than of one site. In the presence of 0.5 mM GTP, the displacement curves were steeper (nH values of 1.00 and 1.01 for the M_2 and M_4 binding sites, respectively) and the fit was better on the assumption of one site rather than of two sites. The affinity of the muscarinic M_4 receptors for clozapine was higher than that of the M_2 receptors; this was true both for the high- and the low-affinity sites.

3.2. Apparent K_d for the binding of [^{3}H]N-methylscopolamine in the presence of clozapine

We performed saturation binding experiments with [^{3}H]*N*-methylscopolamine in the absence and in the presence of clozapine. In the absence of clozapine, apparent K_d values for the specific binding of [^{3}H]*N*-methylscopolamine to the M_2- and M_4-CHO cell membranes were 190 pM and 120 pM, respectively, and the B_{max} values were 12.6 fmol and 11.9 fmol [^{3}H]*N*-methylscopolamine binding sites per million M_2- and M_4-CHO cells, respectively. The apparent K_d values were substan-

Table 1
Inhibition by clozapine of [^{3}H]*N*-methylscopolamine binding to the membranes of CHO cells expressing muscarinic receptors of the M_2 or M_4 subtype
Data have been computed from experiments shown in Fig. 1. Expression f_{high} denotes the fraction of receptors with the high affinity for clozapine.

Receptor subtype	GTP	K_i (95% confidence interval)	K_{i-high} (95% confidence interval)	K_{i-low} (95% confidence interval)	f_{high} (±S.E.M.)	nH (±S.E.M.)
M_2	0		1.81×10^{-9} M (0.67×10^{-9}–4.87×10^{-9})	5.98×10^{-8} M (4.22×10^{-8}–8.48×10^{-8})	0.24 ± 0.04	0.68 ± 0.05
M_2	0.5 mM	5.13×10^{-8} M (4.77×10^{-8}–5.53×10^{-8})				1.00 ± 0.03
M_4	0		1.54×10^{-10} M (0.22×10^{-10}–11.10×10^{-10})	1.63×10^{-8} M (1.21×10^{-8}–2.20×10^{-8})	0.13 ± 0.03	0.73 ± 0.05
M_4	0.5 mM	1.57×10^{-8} M (1.46×10^{-8}–1.69×10^{-8})				1.01 ± 0.03

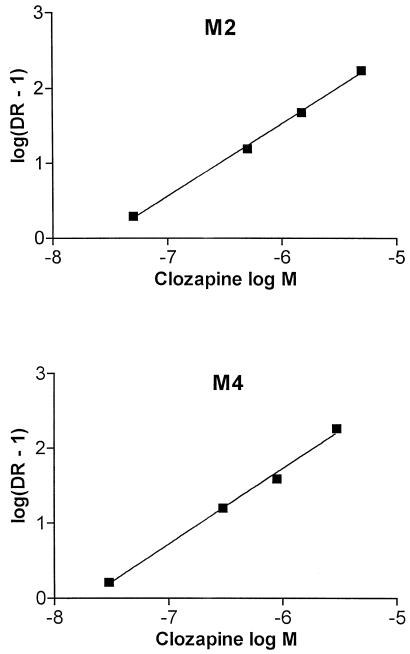

Fig. 2. Arunlakshana–Schild plot of data on the inhibition of specific [^{3}H]*N*-methylscopolamine binding to membranes of CHO cells expressing muscarinic receptors of the M_2 (top) or M_4 (bottom) subtype. The plot is based on data obtained in saturation binding (Scatchard-type) experiments performed with [^{3}H]*N*-methylscopolamine in the presence of clozapine at four different concentrations and in its absence. Abscissa: log of the concentration (M) of clozapine. Ordinate: log(DR-1), where DR = (apparent K_d for the binding of [^{3}H]*N*-methylscopolamine in the presence of clozapine)/(apparent K_d for the binding of [^{3}H]*N*-methylscopolamine in the absence of clozapine).

tially higher when determined in the presence of clozapine, which was applied at concentrations corresponding to 1- to 100-fold the K_i values for clozapine discovered in the displacement experiments shown in Fig. 1. The Arunlakshana and Schild (1959) plot of the data (Fig. 2) yielded straight lines within a hundred-fold range of clozapine concentrations, both for the M_2 and the M_4 receptor subtype. The slope of the straight lines was 0.97 for the muscarinic M_2 and 1.00 for the M_4 receptors. The K_i values for the inhibition of [^{3}H]*N*-methylscopolamine binding by clozapine computed from data in Fig. 2 were 45 nM on the M_2 and 18 nM on the M_4 receptor subtype.

3.3. Clozapine and [^{3}H]N-methylscopolamine dissociation

In experiments following the rate of [^{3}H]*N*-methylscopolamine dissociation as revealed after the addition of 5 μM atropine, the addition of 10 μM clozapine had no effect on the speed with which the binding of [^{3}H]*N*-methylscopolamine to the M_2 or M_4 receptors became diminished (data not shown). The k_{off} values obtained with and without clozapine were 0.33 min^{-1} and 0.04 min^{-1} on the M_2 and M_4 receptor subtypes, respectively.

3.4. Inhibition of cyclic AMP synthesis by carbachol and clozapine

Carbachol inhibited the forskolin-stimulated synthesis of cyclic AMP in intact cells in a biphasic manner (Fig. 3). The inhibition progressively increased as the concentrations of carbachol rose from 10^{-8} to 10^{-5} M but became smaller at higher carbachol concentrations. The maximum inhibition induced by carbachol was 53% in the M_2-CHO cells and 60% in the M_4-CHO cells. When used at concentrations of 10^{-8} M–10^{-5} M, clozapine inhibited the synthesis of cyclic AMP in the M_4-CHO cells with a maximum inhibitory effect of 39% but had no effect on the M_2-CHO cells (Figs. 3 and 4). The EC_{50} values computed from experiments in Fig. 3 were 488 nM and 1875 nM for

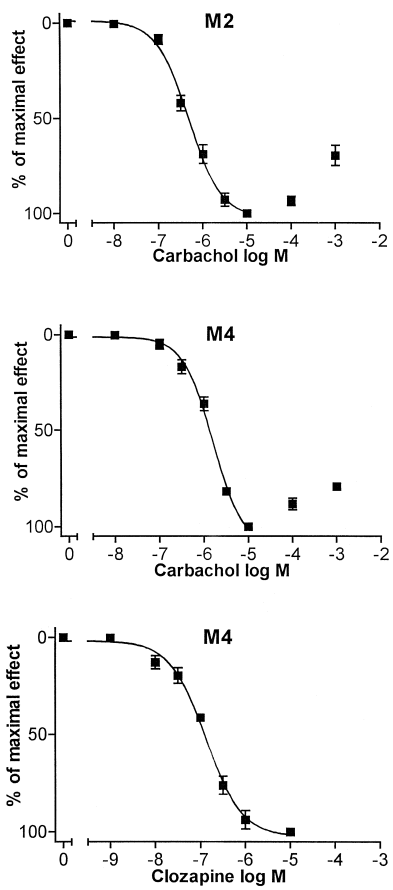

Fig. 3. Effects of carbachol and clozapine on the forskolin-stimulated synthesis of cyclic AMP in CHO cells expressing muscarinic receptors of the M_2 (top) or the M_4 (middle and bottom) subtype. Abscissa: log of the concentration (M) of carbachol (top and middle) or clozapine (bottom). Ordinate: Percent of maximal inhibition of the synthesis of cyclic AMP induced by carbachol or clozapine. The maximal inhibition induced by carbachol was 53% in the cells expressing the muscarinic M_2 and 60% in those expressing the M_4 receptor subtype. Clozapine produced no measurable inhibition in cells expressing the M_2 receptor subtype (not shown here) and maximally 39% inhibition in those expressing the M_4 receptor subtype. Data are means (±S.E.M) of three experiments with incubations performed in triplicates.

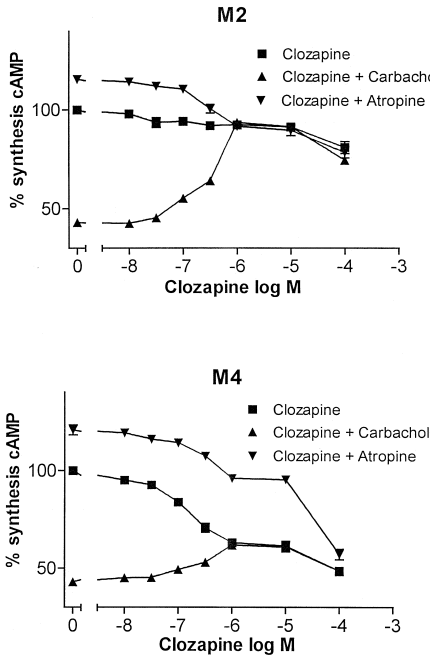

Fig. 4. Effect of clozapine on the forskolin-stimulated synthesis of cyclic AMP in CHO cells expressing muscarinic receptors of the M_2 (top) or the M_4 (bottom) subtype. Clozapine was present either alone (■), or simultaneously with 10 μM carbachol (▲), or with 5 μM atropine (▼). Abscissa: log of the concentration (M) of clozapine. Ordinate: Synthesis of cyclic AMP in the presence of the indicated concentrations of clozapine, carbachol and atropine, expressed as per cent of the synthesis in the absence of any ligand. Note that the points most to the left in the graphs indicate the synthesis of cyclic AMP in the absence of clozapine and in the absence of any modulator (■), or in the presence of 10 μM carbachol alone (▲), or in the presence of 5 μM atropine alone (▼). Points represent means (±S.E.M) of three experiments, with incubations performed in triplicates.

the effect of carbachol on the M_2- and M_4-CHO cells, respectively, and 135 nM for the effect of clozapine on the M_4-CHO cells.

Fig. 4 shows the results of experiments in which the synthesis of cyclic AMP was measured either after the addition of various concentrations of clozapine alone, or of various concentrations of clozapine together with a fixed concentration of carbachol (10 μM) or atropine (5 μM). In these experiments, 5 μM atropine alone enhanced the synthesis of cyclic AMP by 15% and 20% in the M_2- and M_4-CHO cells, respectively, apparently by suppressing the constitutive activity of muscarinic receptors (Jakubík et al., 1995a).

In experiments on the M_2-CHO cells (Fig. 4A), 10 μM carbachol alone inhibited the synthesis of cyclic AMP by 57% and its inhibitory effect was nearly completely eliminated by 1 μM clozapine. In experiments on the M_4-CHO cells (Fig. 4B), 10 μM carbachol alone inhibited the synthesis of cyclic AMP by 58% and its inhibitory effect was diminished (but not eliminated) by clozapine. In the presence of 10 μM carbachol plus 1 μM clozapine, cyclic AMP synthesis was inhibited by 38%, which was the same as in the presence of 1 μM clozapine alone.

The inhibition of cyclic AMP synthesis in the M_4-CHO cells by 0.1–10 μM clozapine was prevented by 5 μM atropine. At the same time, 1 μM and 10 μM clozapine eliminated the enhancement of cyclic AMP synthesis induced by atropine both in the M_2- and the M_4-CHO cells.

When the concentration of clozapine was raised from 10 μM to 100 μM, the synthesis of cyclic AMP became diminished in the M_2-CHO cells (Fig. 4A) and the inhibition of cyclic AMP synthesis became deepened in the M_4-CHO cells (Fig. 4B). At this stage, the effects of clozapine were not prevented by atropine.

4. Discussion

Basic characteristics of the effects of allosteric modulators on muscarinic receptors have been summarized in recent reviews (Tuček and Proška, 1995; Christopoulos and Lanzafame, 1998; Holzgrabe and Mohr, 1998). In radioligand binding experiments, two main features help to distinguish an allosteric modulator with a negative effect on the binding of a classical ligand from a compound which acts competitively: (a) The modulator alters radioligand binding by producing a finite change in the affinity of the receptor for the radioligand, and there is a ceiling to its inhibitory effect on radioligand binding (Ehlert, 1988). This is best revealed by the non-linearity of the Arunlakshana and Schild (1959) plots of the binding data (Stockton et al., 1983), and by the inability of the allosteric modulator to completely inhibit the binding of the radioligand at its high concentrations. (b) The allosteric modulator alters (usually slows down, in the case of muscarinic receptors) the dissociation rate of classical ligands.

In our experiments, clozapine did not act as an allosteric modulator by either criterion. The Arunlakshana–Schild plots of its effects on [^{3}H]*N*-methylscopolamine binding were linear and had a slope of unity both on the muscarinic M_2 and the M_4 receptors, and the dissociation rates of [^{3}H]*N*-methylscopolamine were not affected by the presence of clozapine.

The curves describing the inhibition of [^{3}H]*N*-methylscopolamine binding in the presence of increasing concentrations of clozapine were shallow when the experiments were performed in the absence of GTP, both on membranes from cells expressing the muscarinic M_4 ($nH = 0.73$) and the M_2 ($nH = 0.76$) receptors. In the presence of GTP, however, the inhibitory curves became steep, with Hill slopes close to unity. Such behavior depends on the ability of GTP to produce dissociation of receptor–G protein complexes and is typical of receptor agonists (Haga et al., 1986; Florio and Sternweis, 1989; Matesic et al., 1989; Rinken, 1996), while it never occurs with receptor antagonists. It seems necessary to conclude from the data in Fig. 1 and Table 1 that clozapine acts as an agonist both on muscarinic M_4 and M_2 receptor subtypes. We note that

Olianas et al. (1997) did not reveal any effect of 10 μM GTPγS (guanosine-5′-*O*-(thio)triphosphate) on the inhibition of [^{3}H]*N*-methylscopolamine binding to the M_4 receptors by clozapine, and the reason of the difference is not clear; perhaps the use of 500 μM GTP in our experiments was more suitable to reveal the phenomenon than the use of 10 μM GTPγS by Olianas et al. (1997), or the cell membranes used differed in their receptor/G protein ratios.

Clozapine inhibited the synthesis of cyclic AMP in the M_4-CHO cells but the maximum inhibition achieved was lower than the maximum inhibition produced by carbachol. Clozapine thus acted as a partial agonist at muscarinic M_4 receptors. When added simultaneously with carbachol, it diminished the inhibitory effect of carbachol (Fig. 4B). On the other hand, clozapine did not measurably alter the synthesis of cyclic AMP in the M_2-CHO cells when applied at concentrations of up to 10 μM. It did diminish, however, the inhibition produced by carbachol (Fig. 4A). Effects similar to those of clozapine at the M_2-CHO cells (Fig. 4A), might be produced either by a compound which acts as an antagonist, or by a compound which is a weak partial agonist in an inefficiently coupled receptor–G protein–effector system (see Olianas et al., 1997).

In view of data obtained in radioligand binding experiments with and without GTP, we interpret the action of clozapine on muscarinic M_2 receptors as that of a weak partial agonist. The ineffectiveness of clozapine regarding the inhibition of cyclic AMP synthesis in the M_2- as opposed to the M_4-CHO cells did not depend on a difference in receptor density, since that was nearly the same in both cell types, but it may be that the coupling with G_i proteins and adenylyl cyclase was more tight in the case of the muscarinic M_4 than of the M_2 receptor subtype. Differences in the efficiency of the coupling of different receptor subtypes to their related G proteins have been described previously for muscarinic (McKinney et al., 1991) and very thoroughly for β-adrenergic receptors (Green et al., 1992; Levy et al., 1993).

Atropine alone enhanced the synthesis of cyclic AMP in the M_2 and M_4 CHO cells (Fig. 4A and B), in harmony with earlier observations (Jakubík et al., 1995a). Apparently, atropine acts as a negative antagonist (''inverse agonist'') in the system used, and blocks the constitutive activity of muscarinic receptors in CHO cells. The fact that clozapine did not have the same effect as atropine on the synthesis of cyclic AMP in the M_2-CHO cells is compatible with the assumption that clozapine is a weak partial agonist, but it would not contradict the alternative assumption that it is a neutral (rather than a negative) antagonist. When applied at the high concentration of 100 μM, clozapine induced some inhibition of cyclic AMP synthesis even in the M_2-CHO cells, and deepened the inhibition observed in the M_4-CHO cels, compared to that observed with 10 μM clozapine. These effects were not blocked by 5 μM atropine. It seems possible that 100 μM clozapine has some non-muscarinic action, but we did not pursue investigations in this direction.

In summary, clozapine has a very high affinity for the M_2 and M_4 subtypes of muscarinic receptors. Importantly, the affinity which we have found is several fold higher than the affinities reported for clozapine in relation to dopamine D_1 and D_2 receptors (Ashby and Wang, 1996; Bymaster et al., 1996). Since the therapeutically effective concentrations of clozapine in the blood plasma are close to 1 μM (Perry et al., 1991) and the concentrations of clozapine in the cerebrospinal fluid seem to be only five times lower than those in the plasma (Nordin et al., 1995), one has to assume extensive binding of clozapine to muscarinic receptors in patients during the treatment with the drug. Our data indicate that clozapine binds to the classical (not to the allosteric) binding sites of muscarinic M_2 and M_4 receptors, and that it acts as a weak partial agonist of these receptors. However weak they are, the agonistic properties of clozapine at muscarinic M_2 and M_4 receptors are likely to affect the state and distribution of G proteins in the membranes and to play an important role in the general action of clozapine, which involves the interplay between a number of G protein-coupled receptors in neural cells.

5. Note added in proof

Agonistic properties of clozapine on the M_1, M_2 and M_3 subtypes of muscarinic receptors have recently been described by M.C. Olianas and coll. (Neuropsychopharmacology 20:263–270, 1999).

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