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Inhibition of nicotinic acetylcholine receptors and calcium channels by clozapine in bovine adrenal chromaffin cells

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Abstract

The effects of clozapine on the activities of nicotinic acetylcholine receptors (nAChRs) and voltage-sensitive calcium channels (VSCCs) were investigated and compared with those of chlorpromazine (CPZ) in bovine adrenal chromaffin cells. [^{3}H]Norepinephrine ([^{3}H]NE) secretion induced by activation of nAChRs was inhibited by clozapine and CPZ with half-maximal inhibitory concentrations (IC_{50}) of 10.4 ± 1.1 and 3.9 ± 0.2 μM, respectively. Both cytosolic calcium increase and inward current in the absence of extracellular calcium induced by nicotinic stimulation were also inhibited by clozapine and CPZ, but the greater inhibition was achieved by CPZ. In addition, [^{3}H]nicotine binding to chromaffin cells was inhibited by clozapine and CPZ with IC_{50} values of approximately 19 and 2 μM, respectively. On the other hand, [^{3}H]NE secretion induced by high K^+ was inhibited by clozapine and CPZ with similar IC_{50} values of 15.5 ± 3.8 and 17.1 ± 3.9 μM, respectively. Our results suggest that clozapine, as well as CPZ, inhibits nAChRs and VSCCs, thereby causing inhibition of catecholamine secretion, and that clozapine is much less potent than CPZ in inhibiting nAChRs.

Keywords: Clozapine; Chlorpromazine; Nicotinic acetylcholine receptors; Catecholamine secretion; Chromaffin cells

1. Introduction

The dopamine hypothesis of schizophrenia proposes that schizophrenia results from too much dopaminergic activity and that the antipsychotic drugs used act by blocking dopamine receptors in the nervous system. The strongest evidence of the dopamine hypothesis is that classical antipsychotic drugs such as CPZ and haloperidol bind and block dopamine D_2 receptors in a way that is directly related to their clinical antipsychotic potencies [1]. The report that the number of D_2 or D_2-like receptors is elevated in postmortem schizophrenia brain tissue [2] supports the dopamine hypothesis. However, the dopamine hypothesis of schizophrenia is profoundly challenged by the atypical antipsychotic drug clozapine.

Clozapine is a neuroleptic agent that is structurally different from CPZ, a phenothiazine with two benzene rings linked by a sulfur and a nitrogen atom. Clozapine consists of a dibenzodiazepine derivative with a piperazinyl side chain. It has been classified as an atypical neuroleptic drug due to its unique neuropharmacological profile (for review see [3]). Clozapine has been useful in treatment-resistant patients and in treating the negative symptoms of schizophrenia, while it has a high affinity for D_4 receptors rather than D_2 receptors [4]. In addition, in some patients treated with typical antipsychotics, the therapeutic response was still poor despite D_2 receptor blockage. On the other hand, all patients treated with clozapine showed significant clinical improvement with a lower level of D_2 receptor blockage [5].

In addition to dopamine receptors, many membrane proteins, such as voltage-sensitive Ca^{2+}, Na^+, and K^+ channels, have been reported to be inhibited by rather high concentrations of many classical antipsychotics [6–10]. We recently reported that CPZ inhibited nAChRs and L-type VSCCs, thereby causing inhibition of catecholamine secretion in rat pheochromocytoma cells [11]. In contrast to the

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Abbreviations: CPZ, chlorpromazine; nAChR, nicotinic acetylcholine receptor; VSCC, voltage-sensitive calcium channel; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; SBFI, sodium-binding benzofuran isophthalate; [^{3}H]NE, [^{3}H]norepinephrine; DMEM/F-12, Dulbecco's modified Eagle's medium/F-12; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; $[Na^+]_i$, cytosolic free Na^+ concentration; and EPS, extrapyramidal side effects.

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extensive studies of classical antipsychotics with regard to their inhibitory effects on neurotransmitter release and the activity of ion channels and nAChRs, little attention has been given to the action of clozapine. Indeed, comparative studies of classical and atypical antipsychotics might provide a clue toward an understanding of the clinical efficacy of clozapine and the side effects caused by classical antipsychotics. Furthermore, investigation of the effect of clozapine on catecholamine secretion and ion fluxes and comparison with the inhibitory effects induced by CPZ are necessary to understand the clinical efficacy and physiological effects of clozapine. Adrenal chromaffin cells have been widely used as a model system by which to investigate the mechanism of neurosecretion [12–14]. Both nicotinic cholinergic agonists and high K^+ have been extensively used to investigate the mechanisms of calcium increase and catecholamine secretion induced by activation of nAChRs and VSCCs [14,15]. By using the well-established model system, we investigated the effect of clozapine on catecholamine secretion and ion fluxes induced by the activation of nAChRs and VSCCs. Our results suggest that clozapine inhibits nAChRs and VSCCs, leading to the subsequent inhibition of calcium increase and catecholamine secretion. Furthermore, clozapine was much less potent than CPZ in the inhibition of nAChRs.

2. Materials and methods

2.1. Materials

Clozapine, CPZ, and DMPP were purchased from Sigma. Fura-2/acetoxymethylester, SBFI/acetoxymethylester, and Pluronic F-127 were obtained from Molecular Probes, Inc. [^{3}H]NE and [^{3}H]nicotine were purchased from NEN Life Science Products.

2.2. Chromaffin cell preparation

Chromaffin cells were isolated from bovine adrenal medulla by two-step collagenase digestion as previously described [12]. For measurement of [^{3}H]NE secretion and the [^{3}H]nicotine binding assay, cells were plated in 24-well plates at a density of 5×10^5 cells per well. Chromaffin cells transferred to 100-mm culture dishes (1×10^7 cells per dish) were used to measure cytosolic free calcium and sodium concentrations. The cells were maintained in DMEM/F-12 (Life Technologies, Inc.) containing 10% bovine calf serum (HyClone) and 1% antibiotics (Life Technologies, Inc.). Chromaffin cells were incubated in a humidified atmosphere of 5% CO_2/95% air at 37° for 3–7 days before use.

2.3. Measurement of [^{3}H]NE secretion

Catecholamine secretion from chromaffin cells was measured in 24-well plates following the method reported by Park *et al*. [14]. In brief, cells were loaded with [^{3}H]NE (1 μCi/mL; 68 pmol/mL) by incubation in DMEM/F-12 containing 0.01% ascorbic acid for 1 hr at 37° in 5% CO_2/95% air. The cells were washed twice with Ca^{2+}-free Locke's solution and then incubated in Locke's solution (154 mM NaCl, 5.6 mM KCl, 5.6 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES buffer adjusted to pH 7.4) for 15 min to stabilize them. Then, the cells were incubated with Locke's solution for 10 min to measure basal secretion. The cells were subsequently stimulated with the drugs under test for 10 min. After the incubation, the medium was removed from each well and transferred to a scintillation vial. Finally, residual catecholamine in the cells was extracted by addition of 10% trichloroacetic acid, and the extract was transferred to a scintillation vial. The radioactivity in each vial was measured with a scintillation counter. The amount of [^{3}H]NE secreted was calculated as a percentage of total [^{3}H]NE content. Net stimulated secretion was obtained by subtracting the basal from the stimulated secretion. In order to measure the effect of the antipsychotic drug treatment, the drug was added to the medium before measurement of both basal and stimulated secretion.

2.4. $[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was determined with the help of the fluorescent Ca^{2+} indicator fura-2 as reported previously [16]. Briefly, the chromaffin cell suspension was incubated with fresh serum-free DMEM/F-12 medium containing fura-2/AM (3 μM) for 40 min at 37° with continuous stirring. The cells were then washed with Locke's solution and left at room temperature until use. Sulfinpyrazone (250 μM) was added to all solutions to prevent dye leakage [17]. Addition of sulfinpyrazone caused a small (less than 10%) potentiation in the peak level of cytosolic calcium elevation induced by DMPP or high K^+ treatment (data not shown). Nevertheless, the inhibitory effect of clozapine and chlorpromazine was similar in the absence or presence of sulfinpyrazone in experimental solutions (data not shown). Fluorescence ratios were measured by an alternative wavelength time scanning method (dual excitation at 340 and 380 nm; emission at 500 nm). Calibration of the fluorescent signal in terms of $[Ca^{2+}]_i$ was performed at the end of each experiment as described by Grynkiewicz *et al*. [18]. $[Ca^{2+}]_i$ was calculated using the equation

$$[Ca^{2+}]_i = K_d[R - R_{min})/(R_{max} - R)](S_{f2}/S_{b2})$$

where R_{min} is the fluorescence ratio obtained when Ca^{2+} is chelated by successive addition of 4 mM EGTA, 30 mM Trizma base, and 0.1% Triton X-100. Then, R_{max} was obtained by adding 4 mM $CaCl_2$ to saturate fura-2. S_{f2} and S_{b2} are the proportionality coefficients of free fura-2 and Ca^{2+}-saturated fura-2, respectively.

2.5. Electrophysiological recording

Whole-cell patch-clamp recordings were performed to measure inward current through nAChRs at room temperature with an Axopatch 200B amplifier (Axon Instruments) and Digidata 1200 interface as described previously [19], with some modifications. Isolated chromaffin cells were plated on glass coverslips and incubated for 1–2 days at 37° under a 5% CO_2-containing atmosphere. The pipettes were fire-polished and had a final resistance of 3–4 MΩ. The bath solution for whole-cell recordings had the following composition (in mM): 156.2 NaCl, 5.6 KCl, 10 glucose, 5 HEPES, pH 7.4 with NaOH. An internal solution of the following composition was used (in mM): 140 CsCl, 1 $MgCl_2$, 3 EGTA, 10 HEPES, pH 7.4 with CsOH. Currents were filtered on line at 2–10 kHz, sampled at 2–5 kHz, and collected on an IBM-compatible computer using the pClamp6 program. Unless specified, the holding potential was −100 mV. All values are given as means ± SEM.

2.6. Inhibition of [^{3}H]nicotine binding

Binding of [^{3}H]nicotine to intact cells was measured as previously described by Park *et al.* [14]. Intact chromaffin cells in 24-well plates (5×10^5 cells/well) were washed twice with Locke's solution and incubated with 20 nM [^{3}H]nicotine and the indicated concentrations of antipsychotic drugs for 40 min at 25°. Then, the cells were washed three times with 1 mL ice-cold Ca^{2+}-free Locke's solution containing 100 μM EGTA. Finally, the cells were lysed by being scraped into 0.5 mL 5% trichloroacetic acid, and the radioactivity was measured by liquid scintillation counting. Non-specific binding, determined by coincubation with 1 mM nicotine, amounted to less than 20% of total binding, and was routinely subtracted from the total binding. The binding data were analyzed and expressed as percentage of specific binding.

2.7. Statistical analysis

All quantitative data were expressed as means ± SEM. IC_{50} values were calculated with the Microcal Origin for Windows program.

3. Results

3.1. Inhibitory effects of clozapine and CPZ on DMPP-induced [^{3}H]NE secretion

In order to study the effect of clozapine on catecholamine secretion, [^{3}H]NE-loaded chromaffin cells were treated with clozapine. Clozapine (up to 100 μM) by itself did not induce significant [^{3}H]NE secretion (data not shown). DMPP, a well-known activator of nAChRs [14,15, 20–22], induced [^{3}H]NE secretion by 17.2 ± 2.4% of the total endogenous content. Clozapine decreased DMPP-induced [^{3}H]NE secretion in a concentration-dependent manner, with a half-maximal inhibitory concentration (IC_{50}) of 10.4 ± 1.1 μM (Fig. 1). Clozapine at a 100-μM concentration completely blocked DMPP-induced [^{3}H]NE secretion. CPZ also inhibited DMPP-induced [^{3}H]NE secretion in a concentration-dependent manner with an IC_{50} of 3.9 ± 0.2 μM, which is lower than that of clozapine. In addition, CPZ completely inhibited the DMPP-induced response at a concentration of 30 μM. These results suggest that although clozapine and CPZ both inhibit DMPP-induced [^{3}H]NE secretion, clozapine is less potent in this inhibition.

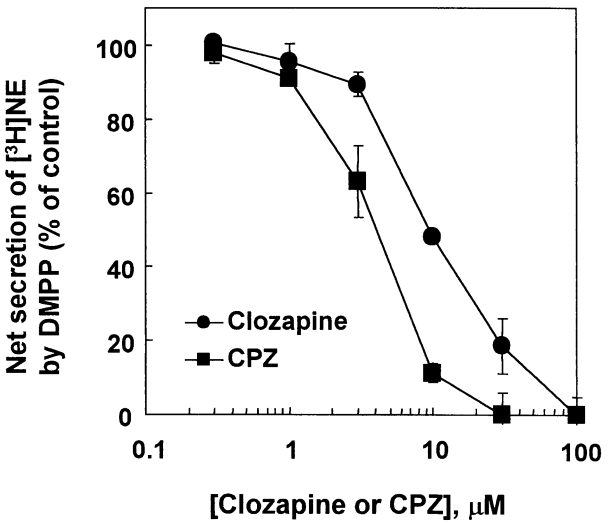

Fig. 1. Inhibitory effects of clozapine and CPZ on DMPP-induced catecholamine secretion by chromaffin cells. [^{3}H]NE-loaded chromaffin cells were treated with 10 μM DMPP in the presence of the indicated concentrations of the antipsychotics. The secreted [^{3}H]NE was measured in triplicate as described under Materials and Methods and is expressed as a percentage of the averaged DMPP-induced [^{3}H]NE secretion in the absence of antipsychotics. Such triplicate unpaired experiments were done independently five times from more than two different cell preparations. Data are means ± SEM (bars) values.

3.2. Inhibitory effects of clozapine and CPZ on DMPP-induced [Ca^{2+}]$_i$ elevation

Since an increase in [Ca^{2+}]$_i$ is an essential step in catecholamine secretion, we tested the effect of the two antipsychotics on the DMPP-induced [Ca^{2+}]$_i$ rise. Clozapine and CPZ (up to 100 μM) had no effect on the basal [Ca^{2+}]$_i$ (data not shown). DMPP induced a rapid increase in [Ca^{2+}]$_i$ (Fig. 2A), and this increase could be inhibited by clozapine in a concentration-dependent manner with an IC_{50} of 9.0 ± 0.9 μM (Fig. 2B). CPZ also inhibited the DMPP-induced [Ca^{2+}]$_i$ rise with an IC_{50} of 3.0 ± 1.7 μM, suggesting that CPZ is the more potent inhibitor. As seen in Fig. 2A, the inhibitory effect of 3 μM clozapine was much less potent than that induced by 3 μM CPZ.

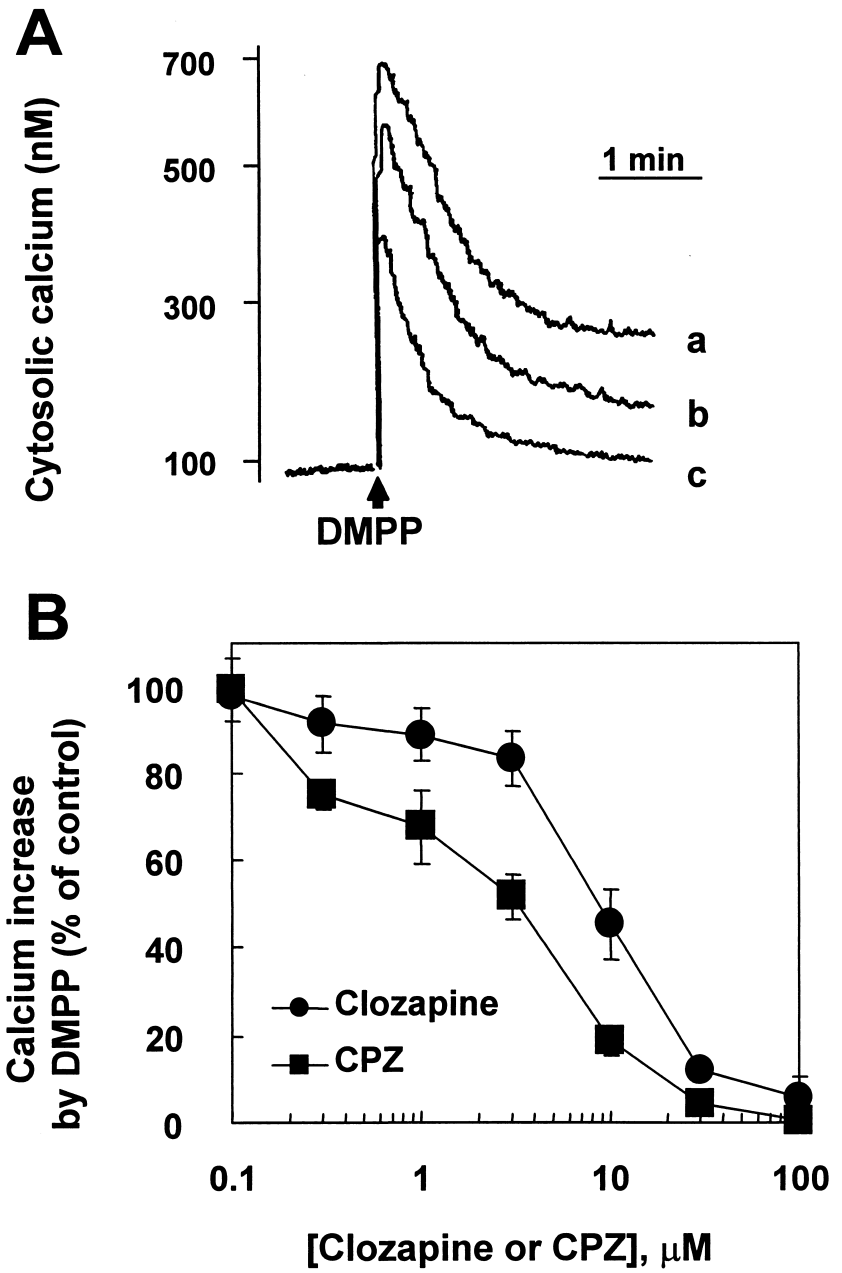

Fig. 2. Inhibitory effects of clozapine and CPZ on DMPP-induced $[Ca^{2+}]_i$ elevation in chromaffin cells. (A) Fura-2-loaded chromaffin cells were treated with 10 μM DMPP in the absence (trace a) or presence of 3 μM clozapine (trace b) and CPZ (trace c). The experiments were performed independently 5 times and typical Ca^{2+} transients are presented. (B) Fura-2-loaded chromaffin cells were treated with 10 μM DMPP in the presence of the indicated concentrations of antipsychotics. The peak height of each stimulation was measured and is expressed as a percentage of the control peak height achieved by DMPP alone. The unpaired experiments were done separately six times from more than two different cell preparations. Data are means ± SEM (bars) values.

3.3. Inhibitory effects of clozapine and CPZ on inward current through nAChRs

Because it is well known that DMPP-induced catecholamine secretion and calcium increase require a prior increase in $[Na^+]_i$ through nAChR [14,15], we first tested the effect of clozapine and CPZ on the DMPP-induced $[Na^+]_i$ rise by using the Na^+-sensitive fluorescent dye SBFI. Clozapine and CPZ inhibited the DMPP-induced $[Na^+]_i$ increase with an IC_{50} of 1.8 ± 0.1 and 0.5 ± 0.1 μM, respectively. The IC_{50} values obtained from SBFI-loaded cells were much lower than those from measurements of $[^3H]$NE secretion and calcium increase. Since the basal level of the fluorescence ratio in SBFI-loaded cells were decreased immediately by the addition of high concentrations of clozapine and CPZ (data not shown), the shift in IC_{50} may have been caused by quenching of SBFI fluorescence by clozapine and CPZ. In order to assess more clearly the effect of the antipsychotics on sodium influx through nAChRs, we performed whole-cell patch clamp experi-

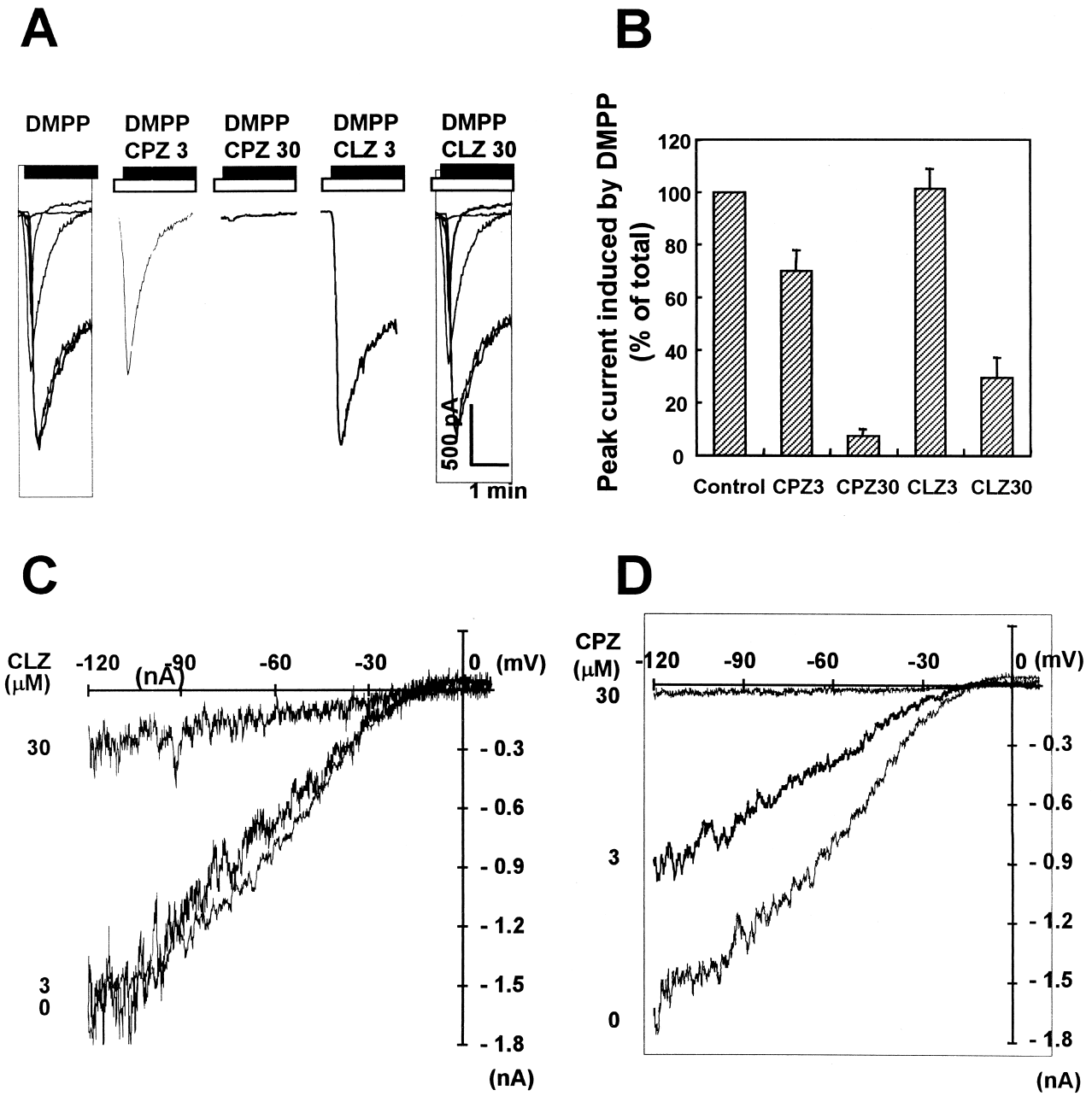

Fig. 3. Inhibitory effects of clozapine and CPZ on DMPP-induced inward current in chromaffin cells. The whole-cell configuration of the patch clamp technique was established in single isolated chromaffin cells after 2–3 days of culture. The membrane potential was held at −100 mV. The superfused cells were challenged with 10 μM DMPP for 2 min. After washing for 10 min, the cells were first treated with 3 or 30 μM clozapine (CLZ) or CPZ for 3 min and then stimulated with 10 μM DMPP in the presence of the indicated concentrations of the antipsychotic drug. The cells were washed again for 20 min and stimulated with DMPP to confirm the recovery. The experiments were performed 4–5 times independently, and typical current traces (A) and statistical analyses (B) of the inhibition of peak current by clozapine and CPZ are presented. (C) and (D) Effects of clozapine and CPZ on current–voltage relationship of nAChRs. The cells were treated with 10 μM DMPP in the presence of the indicated concentration of clozapine or CPZ, then ramp pulse protocol was applied from −120 to 10 mV for 360 msec at the time of peak. The experiments were performed 4–5 times independently, and typical current traces are presented.

ments and investigated the effect of clozapine and CPZ on the nAChR-mediated sodium current in the absence of extracellular calcium. As shown in Fig. 3, A and B, DMPP elicited an inward current, with this current declining after reaching a peak level in the continued presence of DMPP. The nicotinic current was inhibited by 30 μM clozapine and CPZ to 29.2 ± 8.1% (N = 4) and 7.5 ± 2.7% (N = 5) of the control, respectively. Whereas clozapine had little inhibitory effect at 3 μM (100.0 ± 8.2%, N = 5), CPZ at 3 μM significantly inhibited the DMPP-induced current (70.3 ± 7.7%, N = 5). The nAChR-mediated current–voltage relationship in the absence or presence of antipsy-

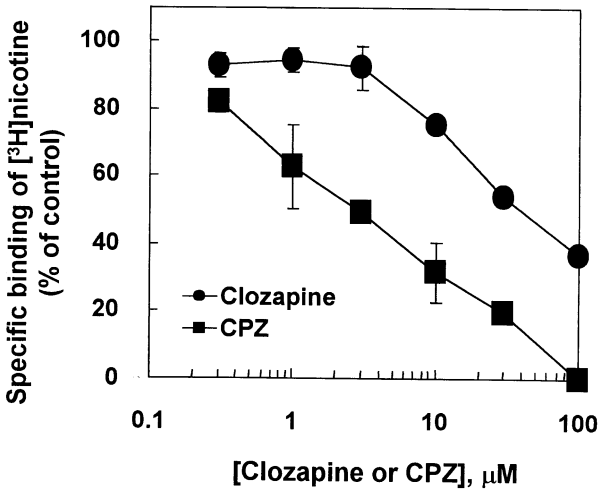

Fig. 4. Competition between antipsychotics and [^{3}H]nicotine for binding to the nAChR. Chromaffin cells were incubated with 20 nM [^{3}H]nicotine and various concentrations of clozapine or CPZ for 40 min at 25°. Specific binding of [^{3}H]nicotine is presented. Non-specific binding was determined in the presence of 1 mM unlabeled nicotine. Triplicate unpaired experiments were done independently three times from more than two different cell preparations. Data are means ± SEM (bars) values.

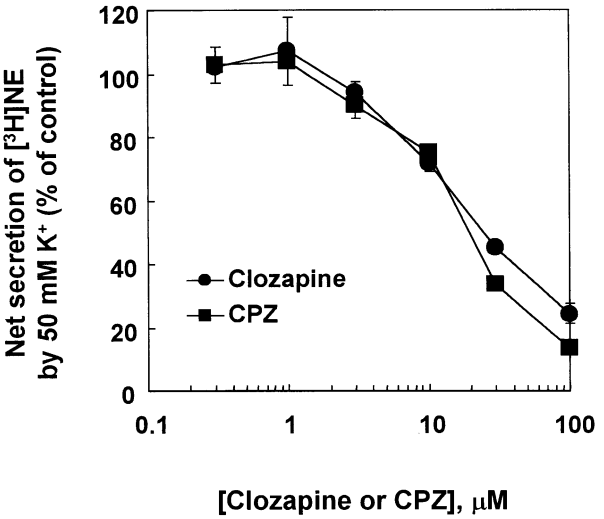

Fig. 5. Inhibitory effect of clozapine and CPZ on DMPP-induced catecholamine secretion by chromaffin cells. [^{3}H]NE-loaded chromaffin cells were treated with 50 mM K^+ in the presence of the indicated concentrations of antipsychotics. The secreted [^{3}H]NE was measured in triplicate as described under Materials and Methods and is expressed as a percentage of the averaged DMPP-induced [^{3}H]NE secretion in the absence of antipsychotics. Such triplicate unpaired experiments were done independently three times from more than two different cell preparations. Data are means ± SEM (bars) values.

chotics was accomplished by applying a ramp pulse from −120 to 10 mV for 360 msec at the time of peak. As can be seen in Fig. 3, C and D, there was no apparent shift in the voltage dependence of the current in response to clozapine and CPZ. Furthermore, the inhibitory effects of the two antipsychotics were often reversible, although it took as much as 20 min to wash clozapine completely (data not shown).

3.4. Inhibitory effects of clozapine and CPZ on [^{3}H]nicotine binding

Because both calcium increase and inward current induced by nicotinic stimulation could be inhibited by clozapine and CPZ, it was possible that the two antipsychotics directly inhibited nAChRs. We therefore performed an nAChR binding competition analysis between [^{3}H]nicotine and the antipsychotics. As shown in Fig. 4, both clozapine and CPZ inhibited [^{3}H]nicotine binding, and the inhibition by CPZ was more potent. The IC_{50} values for clozapine and CPZ were 19.1 ± 4.8 and 2.0 ± 2.0 μM, respectively. The results suggest that inhibition of [^{3}H]nicotine binding by clozapine and CPZ indeed causes the inhibition of DMPP-induced $[Na^+]_i$ and $[Ca^{2+}]_i$ increases and catecholamine secretion. In addition, the more potent CPZ inhibition of [^{3}H]nicotine binding contributes to a stronger inhibition of the DMPP-induced response.

3.5. Inhibitory effects of clozapine and CPZ on high K^+-induced catecholamine secretion

In addition to their effects on the nAChR-mediated signaling pathway, the effects of clozapine and CPZ on high K^+-induced responses were also investigated. Clozapine inhibited 50 mM K^+-induced [^{3}H]NE secretion in a concentration-dependent manner, with an IC_{50} of 15.5 ± 3.8 μM (Fig. 5). In contrast to its effects on the DMPP-induced responses, clozapine at 100 μM did not completely block high K^+-induced [^{3}H]NE secretion. Interestingly, CPZ inhibited high K^+-induced [^{3}H]NE secretion with a similar IC_{50} of 17.1 ± 3.9 μM. These results suggest that both clozapine and CPZ inhibit high K^+-induced catecholamine secretion with similar potency.

3.6. Inhibitory effects of clozapine and CPZ on high K^+-induced $[Ca^{2+}]_i$ rise

We also tested the effect of the two antipsychotics on high K^+-induced $[Ca^{2+}]_i$ rise. K^+ at 50 mM induced a rapid increase in $[Ca^{2+}]_i$ (Fig. 6A), and this increase was nearly completely blocked by omitting extracellular Ca^{2+} (data not shown), suggesting that high K^+-induced Ca^{2+} elevation is caused by VSCC activation. The result is in good agreement with previous reports in which VSCCs played a major role in high K^+-induced Ca^{2+} elevation [23,24]. The calcium increase induced by 50 mM K^+ was inhibited by clozapine in a concentration-dependent manner, with an IC_{50} of 54.8 ± 6.0 μM (Fig. 6B). CPZ also inhibited DMPP-induced $[Ca^{2+}]_i$ rise, but with an IC_{50} of 23.8 ± 2.4 μM, suggesting that CPZ is a slightly more potent inhibitor. The inhibitory effects induced by treatment with 3 μM of clozapine and CPZ were almost negligible (Fig. 6A), suggesting that these

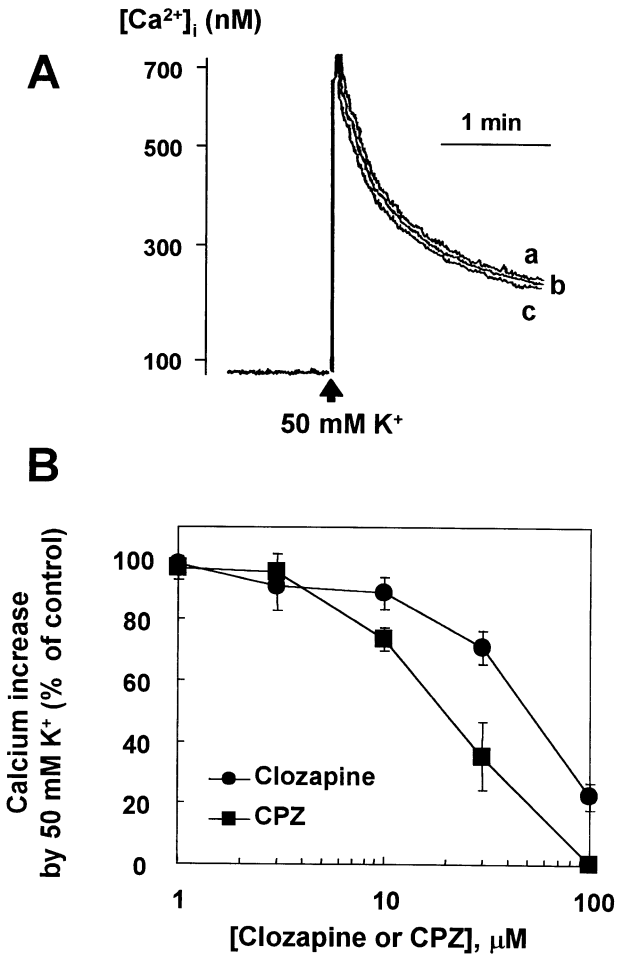

Fig. 6. Inhibitory effect of clozapine or CPZ on 50 mM K^+-induced $[Ca^{2+}]_i$ elevation in chromaffin cells. (A) Fura-2-loaded chromaffin cells were treated with 50 mM K^+ in the absence (trace a) or presence of 3 μM clozapine (trace b) or CPZ (trace c). The experiments were performed independently 3 times and typical Ca^{2+} transients are presented. (B) Fura-2-loaded chromaffin cells were treated with 50 mM K^+ in the presence of the indicated concentrations of antipsychotics. The peak height of each stimulation was measured and is expressed as a percentage of the control peak height caused by 50 mM K^+ alone. The unpaired experiments were done separately six times from more than two different cell preparations. Data are means ± SEM (bars) values.

antipsychotics have a relatively lesser effect on VSCCs than on nAChRs.

4. Discussion

Our results clearly show that, like CPZ, clozapine inhibits both nAChRs and VSCCs. The inhibitory effects lead to the subsequent inhibition of calcium increase and catecholamine secretion. We previously reported that CPZ inhibited the nAChR- and VSCC-mediated catecholamine secretion of rat pheochromocytoma PC12 cells by blocking nAChRs and VSCCs [10]. Therefore, clozapine is similar to CPZ in that both antipsychotics inhibit catecholamine secretion induced by the activation of nAChRs and VSCCs. Their common inhibitory effects might thus explain their common therapeutic effects as antipsychotics. Another possible hypothesis is that the common side effects caused by long-term antipsychotic treatment may be attributed to these inhibitory effects.

Expression of D_4 and D_5 dopamine receptors in bovine adrenal chromaffin cells has been demonstrated by radioligand-binding assay [25], fluorescence microscopy [26], and polymerase chain reaction [27]. It has been reported that agonists selective for either D_1- or D_2-like dopamine receptor subfamilies inhibited nAChR- and VSCC-mediated calcium increase and catecholamine secretion in bovine chromaffin cells [28,29]. Since neuroleptic drugs such as CPZ and clozapine have been well known to antagonize the activity of dopamine receptors, the inhibitory effects on nAChRs and VSCCs reported here are not likely to be related to the activity of dopamine receptors.

Although both clozapine and CPZ inhibit nAChRs and VSCCs, clozapine is much less potent in inhibiting nAChRs. In contrast, the inhibition of VSCCs was similar between clozapine and CPZ. The more potent inhibition of nAChRs by CPZ suggests that the additive clinical effects elicited by CPZ treatment, such as EPS, may be due to the more potent inhibition of nAChRs. Until now, the underlying mechanism of EPS has not been clearly elucidated. There have been interesting reports concerning a relationship between nicotinic activity and side effects caused by antipsychotics. This hypothesis is based on the observation that the very high rates of cigarette smoking among schizophrenics, nearly 90%, have been noted in a variety of studies in comparison to the general population's rate of 25% [30,31]. Since nicotine is the principal psychoactive component in tobacco and has a wide variety of pharmacological effects, several lines of evidence suggest the likelihood of nicotine self-medication by schizophrenics [32]. Decina *et al*. [33] found that schizophrenics who smoked had significantly lower rates of neuroleptic-induced parkinsonism. Levin *et al*. [34] reported that nicotine was effective in reversing some of the adverse side effects of haloperidol and improving cognitive performance in schizophrenia. It is interesting that typical and atypical antipsychotics differed with regard to cigarette smoking. Whereas haloperidol caused a dose-related increase in *ad lib*. smoking [35], clozapine caused a significant decrease in such smoking [36]. These results are in good accord with our findings here showing that CPZ, a typical antipsychotic drug, is much more potent in inhibiting nAChRs, suggesting that the adverse side effects of typical antipsychotics are related to the inhibition of nAChRs. Therefore, it will be very interesting to look for a correlation between the extent of nAChR inhibition and adverse side effects such as EPS. Such studies could contribute to the development of new drugs with reduced side effects [4,37].

Many reports have suggested that therapeutic plasma clozapine concentration ranges between approximately 1

and 2 μM [38,39], a concentration at which clozapine did not have any significant inhibitory effect on DMPP- or high K^+-evoked responses in our study. Elevated plasma concentrations of clozapine up to 10 μM in schizophrenic patients seem to cause toxic side effects such as a mild anticholinergic syndrome with sinus tachycardia [39,40]. Therefore, it is likely that side effects rather than therapeutic effects might be caused by the clozapine-induced inhibition of nAChRs. However, other clinical studies have suggested that therapeutic effects of clozapine could be induced by higher plasma concentrations of clozapine than 1 μM in treatment-refractory schizophrenic patients [41,42]. These clinical reports together with the results presented here suggest that the inhibition of nAChRs and VSCCs by clozapine may cause either side effects or therapeutic effects in schizophrenic patients depending upon the clinical situation. More detailed clinical study will be required to define a correlation between the inhibitory effects of clozapine elucidated here and its clinical effects in schizophrenic patients including those resistant to treatment.

Acknowledgments

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References

[1] Seeman P, Chau-Wong M, Tedesco J, Wong K. Brain receptors for antipsychotic drugs and dopamine: direct binding assays. Proc Natl Acad Sci USA 1975;72:4376–80.
[2] Seeman P, Ulpian C, Bergeron C, Riederer P, Jellinger K, Gabriel E, Reynolds GP, Tourtellotte WW. Bimodal distribution of dopamine receptor densities in brains of schizophrenics. Science 1984;225: 728–31.
[3] Seeman P, Van Tol HH. Dopamine receptor pharmacology. Trends Pharmacol Sci 1994;15:264–70.
[4] Owens DG. Adverse effects of antipsychotic agents. Do newer agents offer advantages? Drugs 1996;51:895–930.
[5] Pilowsky LS, Costa DC, Ell PJ, Murray RM, Verhoeff NP, Kerwin RW. Clozapine, single photon emission tomography, and the D_2 dopamine receptor blockade hypothesis of schizophrenia. Lancet 1992;340:199–202.
[6] Klockner U, Isenberg G. Calmodulin antagonists depress calcium and potassium currents in ventricular and vascular myocytes. Am J Physiol 1987;253:H1601–11.
[7] Ogata N, Yoshii M, Narahashi T. Psychotropic drugs block voltage-gated ion channels in neuroblastoma cells. Brain Res 1989;476: 140–4.
[8] Ogata N, Yoshii M, Narahashi T. Differential block of sodium and calcium channels by chlorpromazine in mouse neuroblastoma cells. J Physiol 1990;420:165–83.
[9] Jacobs ER, DeCoursey TE. Mechanisms of potassium channel blocker in rat alveolar epithelial cells. J Pharmacol Exp Ther 1990; 255:459–72.
[10] Nakazawa K, Ito K, Koizumi S, Ohno Y, Inoue K. Characterization of inhibition by haloperidol and chlorpromazine of a voltage-activated K^+ current in rat phaeochromocytoma cells. Br J Pharmacol 1995;116:2603–10.
[11] Lee IS, Park TJ, Suh BC, Kim YS, Rhee IJ, Kim KT. Chlorpromazine-induced inhibition of catecholamine secretion by a differential blockade of nicotinic receptors and L-type Ca^{2+} channels in rat pheochromocytoma cells. Biochem Pharmacol 1999;58:1017–24.
[12] Kilpatrick DL, Ledbetter FH, Carson KA, Kirshner AG, Slepetis R, Kirshner N. Stability of bovine adrenal medulla cells in culture. J Neurochem 1980;35:679–92.
[13] Kim KT, Westhead EW. Cellular responses to Ca^{2+} from extracellular and intracellular sources are different as shown by simultaneous measurements of cytosolic Ca^{2+} and secretion from bovine chromaffin cells. Proc Natl Acad Sci USA 1989;86:9881–5.
[14] Park TJ, Shin SY, Suh BC, Suh EK, Lee IS, Kim YS, Kim KT. Differential inhibition of catecholamine secretion by amitriptyline through blockage of nicotinic receptors, sodium channels, and calcium channels in bovine adrenal chromaffin cells. Synapse 1998;29: 248–56.
[15] Gandia L, Villarroya M, Sala F, Reig JA, Viniegra S, Quintanar JL, Garcia AG, Gutierrez LM. Inhibition of nicotinic receptor-mediated responses in bovine chromaffin cells by diltiazem. Br J Pharmacol 1996;118:1301–7.
[16] Park TJ, Chung S, Han MK, Kim UH, Kim KT. Inhibition of voltage-sensitive calcium channels by the A_{2A} adenosine receptor in PC12 cells. J Neurochem 1998;71:1251–60.
[17] Di Virgilio F, Fasolato C, Steinberg TH. Inhibitors of membrane transport system for organic anions block fura-2 excretion from PC12 and N2A cells. Biochem J 1988;256:959–63.
[18] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[19] Buisson B, Gopalakrishnan M, Arneric SP, Sullivan JP, Bertrand D. Human alpha4beta2 neuronal nicotinic acetylcholine receptor in HEK 293 cells: A patch-clamp study. J Neurosci 1996;16:7880–91.
[20] Liu PS, Lin YJ, Kao LS. Effects of caffeine on Ca^{2+} fluxes and secretion in bovine chromaffin cells. Eur J Pharmacol 1995;291:265–72.
[21] Kim KT, Choi SY, Park TJ. Neomycin inhibits catecholamine secretion by blocking nicotinic acetylcholine receptors in bovine adrenal chromaffin cells. J Pharmacol Exp Ther 1999;288:73–80.
[22] Park TJ, Lee IS, Ha H, Kim KT. Temperature sensitivity of catecholamine secretion and ion fluxes in bovine adrenal chromaffin cells. Mol Cells 1999;9:67–71.
[23] Lopez MG, Villarroya M, Lara B, Sierra RM, Albillos A, Garcia AG, Gandia L. Q- and L-type Ca^{2+} channels dominate the control of secretion in bovine chromaffin cells. FEBS Lett 1994;349:331–7.
[24] Lomax RB, Michelena P, Nunez L, Garcia-Sancho J, Garcia AG, Montiel C. Different contributions of L- and Q-type Ca^{2+} channels to Ca^{2+} signals and secretion in chromaffin cell subtypes. Am J Physiol 1997;272:C476–84.
[25] Lyon RA, Titeler M, Bigornia L, Schneider AS. D_2 dopamine receptors on bovine chromaffin cell membranes: identification and characterization by [^{3}H]*N*-methylspiperone binding. J Neurochem 1987;48: 631–5.
[26] Artalejo CR, Ariano MA, Perlman RL, Fox AP. Activation of facilitation calcium channels in chromaffin cells by D_1 dopamine receptors through a cAMP/protein kinase A-dependent mechanism. Nature 1990;348:239–42.

[27] Dahmer MK, Senogles SE. Dopaminergic inhibition of catecholamine secretion from chromaffin cells: evidence that inhibition is mediated by D_4 and D_5 dopamine receptors. J Neurochem 1996;66:222–32.
[28] Bigornia L, Allen CN, Jan CR, Lyon RA, Titeler M, Schneider AS. D_2 dopamine receptors modulate calcium channel currents and catecholamine secretion in bovine adrenal chromaffin cells. J Pharmacol Exp Ther 1990;252:586–92.
[29] Dahmer MK, Senogles SE. Differential inhibition of secretagogue-stimulated sodium uptake in adrenal chromaffin cells by activation of D_4 and D_5 dopamine receptors. J Neurochem 1996;67:1960–4.
[30] Hughes JR, Hatsukami DK, Mitchell JE, Dahlgren LA. Prevalence of smoking among psychiatric outpatients. Am J Psychiatry 1986;143:993–7.
[31] O'Farrell TJ, Connors GJ, Upper D. Addictive behaviors among hospitalized schizophrenic patients. Addict Behav 1983;8:329–33.
[32] Lohr JB, Flynn K. Smoking and schizophrenia. Schizophr Res 1992;8:93–102.
[33] Decina P, Caracci G, Sandik R, Berman W, Mukherjee S, Scapicchio P. Cigarette smoking and neuroleptic-induced parkinsonism. Biol Psychiatry 1990;28:502–8.
[34] Levin ED, Wilson W, Rose JE, McEvoy J. Nicotine–haloperidol interactions and cognitive performance in schizophrenics. Neuropsychopharmacology 1996;15:429–36.
[35] McEvoy J, Freudenreich O, Levin E, Rose J. Haloperidol increases smoking in patients with schizophrenia. Psychopharmacology 1995;119:124–6.
[36] McEvoy J, Freudenreich O, McGee M, VanderZwaag C, Levin E, Rose J. Clozapine decreases smoking in patients with chronic schizophrenia. Biol Psychiatry 1995;37:550–2.
[37] Shen WW. A history of antipsychotic drug development. Compr Psychiatry 1999;40:407–14.
[38] Chang WH, Lin SK, Lane HY, Hu WH, Jann MW, Lin HN. Clozapine dosages and plasma drug concentrations. J Formos Med Assoc 1997;96:599–605.
[39] Broich K, Heinrich S, Marneros A. Acute clozapine overdose: plasma concentration and outcome. Pharmacopsychiatry 1998;31:149–51.
[40] Renwick AC, Renwick AG, Flanagan RJ, Ferner RE. Monitoring of clozapine and norclozapine plasma concentration–time curves in acute overdose. J Toxicol Clin Toxicol 2000;38:325–8.
[41] Perry PJ, Miller DD, Arndt SV, Cadoret RJ. Clozapine and norclozapine plasma concentrations and clinical response of treatment-refractory schizophrenic patients. Am J Psychiatry 1991;148:231–5.
[42] Hasegawa M, Gutierrez-Esteinou R, Way L, Meltzer HY. Relationship between clinical efficacy and clozapine concentrations in plasma in schizophrenia: effect of smoking. J Clin Psychopharmacol 1993;13:383–90.