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Chlorpromazine-Induced Inhibition of Catecholamine Secretion by a Differential Blockade of Nicotinic Receptors and L-type Ca^{2+} Channels in Rat Pheochromocytoma Cells

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ABSTRACT. We investigated the effect of chlorpromazine (CPZ), a phenothiazine neuroleptic, on catecholamine secretion in rat pheochromocytoma (PC12) cells. CPZ inhibited [^{3}H]norepinephrine ([^{3}H]NE) secretion induced by 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), an agonist of nicotinic acetylcholine receptors (nAChRs) with an IC_{50} value of 1.0 ± 0.2 μM. The DMPP-induced rise in cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$ was inhibited by CPZ with an IC_{50} of 1.9 ± 0.1 μM. The DMPP-induced increase in cytosolic free Na^+ concentration $[Na^+]_i$ was also inhibited by CPZ with a similar potency. Furthermore, the binding of [^{3}H]nicotine to PC12 cells was inhibited by CPZ with an IC_{50} value of 2.7 ± 0.6 μM, suggesting that the nAChRs themselves are inhibited by CPZ. In addition, both 70 mM K^+-induced [^{3}H]NE secretion and $[Ca^{2+}]_i$ increase were inhibited by CPZ with IC_{50} of 7.9 ± 1.1 and 6.2 ± 0.3 μM, respectively. Experiments with Ca^{2+} channel antagonists suggest that L-type Ca^{2+} channels are mainly responsible for the inhibition. We conclude that CPZ inhibits catecholamine secretion by blocking nAChRs and L-type Ca^{2+} channels, with the former being more sensitive to CPZ. BIOCHEM PHARMACOL **58**;6:1017–1024, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. chlorpromazine; catecholamine secretion; nicotinic receptor; L-type Ca^{2+} channels; PC12 cells

CPZ‖, a phenothiazine neuroleptic drug, has been widely used in the treatment of schizophrenia. Various neuroleptic drugs including CPZ are known to be effective in preventing delusion and hallucination in paranoid schizophrenia. The antipsychotic effects of the neuroleptic drugs have been associated with their ability to act as dopamine receptor antagonists [1]. Dopaminergic antagonists are thought to act on the modulation of intracellular calcium concentration and neurotransmitter secretion. However, much criticism of the dopamine hypothesis with regard to schizophrenia began to emerge in the 1980s. Clinical observations indicated that dopamine antagonists do little to alleviate the symptoms of schizophrenia and many schizophrenic patients had normal levels of dopamine in their cerebrospinal fluid [2]. Therefore, it seemed possible that CPZ might have some unknown effect on other systems, which would then explain its therapeutic action. CPZ and structurally related compounds have been reported to affect other cellular components, e.g. calmodulin, a Ca^{2+}-binding protein that modulates various cellular functions [3, 4]. CPZ also blocked voltage-sensitive ion channels, including Ca^{2+}, Na^+, and K^+ channels in mouse neuroblastoma NIE-115 cells [5, 6]. Inhibition of voltage-sensitive K^+ channels by CPZ has also been studied in guinea-pig ventricular cells [4], rat sympathetic neurons [7], rat alveolar epithelial cells [8], and PC12 [9]. In addition, Benoit and Changeux [10] have reported that CPZ acts as a closed and open channel blocker of nAChRs in a mouse muscle cell line.

The inhibitory effects of CPZ on a variety of membrane proteins such as receptors and channels have been intensively studied, but little attention has been given to its differential efficacy in the interaction with target sites and the effect of the drug on neurotransmitter secretion, the latter being one of the important final responses induced by the activation of receptors and ion channels. PC12 cells have been widely used as a model system for the study of catecholamine secretion. We, therefore, studied the effect of CPZ on catecholamine secretion in PC12 cells and found that CPZ specifically inhibits catecholamine secretion in-

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‖ *Abbreviations:* CPZ, chlorpromazine; ω-CgTx, ω-conotoxin GVIA; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; IP_3, inositol 1,4,5-trisphosphate; nAChR, nicotinic acetylcholine receptor; [^{3}H]NE, [^{3}H]norepinephrine; $[Na^+]_i$, cytosolic free Na^+ concentration; PC12, rat pheochromocytoma; SBFI/AM, sodium-binding benzofuran isophthalate tetraacetoxymethylester; TCA, trichloracetic acid; and VSCC, voltage-sensitive calcium channel.

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duced by the activation of nAChRs and VSCCs with different potencies.

MATERIALS AND METHODS

Materials

CPZ, DMPP, bradykinin, nifedipine, sulfinpyrazone, Triton X-100, EGTA, Trizma base, and IP_3 were obtained from Sigma Chemical Co. [^{3}H]NE and [^{3}H]IP_3 were obtained from NEN. Fura-2 pentaacetoxymethylester (fura-2/AM) and SBFI/AM were purchased from Molecular Probes. ω-CgTx was obtained from Alomone Labs.

Cell Culture

PC12 cells were grown in RPMI 1640 (GIBCO) supplemented with 10% (v/v) heat-inactivated bovine calf serum (HyClone), 5% heat-inactivated horse serum (HyClone), and 1% antibiotics (GIBCO) in a humidified atmosphere of 5% CO_2/95% air at 37°. The culture medium was changed every 2 days and the cells subcultured weekly.

$[Ca^{2+}]_i$ Measurement

$[Ca^{2+}]_i$ was determined with the help of the fluorescent Ca^{2+} indicator fura-2 as described previously [11]. In brief, PC12 cell suspensions were incubated in fresh serum-free RPMI 1640 medium containing fura-2/AM (3 μM) for 40 min at 37° under continuous stirring. After loading, the cells were washed twice with Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose, and 5 mM Hepes buffer adjusted to pH 7.4) to remove any extracellular dye. Sulfinpyrazone (250 μM) was added to all solutions to prevent dye leakage [12]. For the fluorometric measurement of $[Ca^{2+}]_i$, the cell suspension was placed into a quartz cuvette in a thermostatically controlled cell holder at 37° and continuously stirred. Fluorescence ratios were measured by an alternative wavelength time-scanning method (dual excitation at 340 and 380 nm; emission at 500 nm) using a Shimadzu RF-5000 spectrofluorometer. Calibration of the fluorescent signal in terms of $[Ca^{2+}]_i$ was performed as described by Grynkiewicz *et al.* [13].

Measurement of [^{3}H]NE Secretion

The release of catecholamine from PC12 cells was measured using a previously reported method [14]. In brief, PC12 cells were transferred to 24-well plates that had been coated with rat tail collagen [15] at a density of 5×10^5 cells per well. After the cells were allowed to stabilize for one day, they were loaded with [^{3}H]NE (1 μCi/mL; 68 pmol/mL) by incubation in serum-free RPMI containing 0.1 mM ascorbic acid for 1 hr at 37° in 5% CO_2/95% air. The cells were then twice washed with Locke's solution and incubated in Locke's solution for 15 min for stabilization. Then, the cells were again incubated with fresh Locke's solution for 10 min to measure basal secretion of [^{3}H]NE. After two measurements of basal secretion, the cells were incubated for 10 min in Locke's solution containing the drug under test. The medium was then removed from each well and centrifuged at 2000 *g* for 30 sec to exclude detached PC12 cells from the supernatant. Residual catecholamines in each well were extracted from the cells by addition of 0.1 N HCl. Scintillation cocktail was added to the medium and the cell extract. Radioactivity was measured in a scintillation counter. The amount of [^{3}H]NE secreted was expressed as percentage of total [^{3}H]NE content.

Measurement of Inositol 1,4,5-trisphosphate

IP_3 concentration in the cells was determined by competition assay using [^{3}H]IP_3 as reported previously [16]. To determine bradykinin-evoked IP_3 production, the PC12 cells were stimulated with bradykinin for 15 sec. The reaction was terminated by aspirating the medium off the cells and adding 15% (w/v) ice-cold TCA containing 10 mM EGTA. The cells were left on ice for 30 min to extract the water-soluble inositol phosphates. TCA was removed with diethyl ether. The final extract was neutralized with 200 mM Trizma base and its pH adjusted to approximately 7.4. Assay buffer (0.1 M Tris buffer containing 4 mM EDTA and 4 mg/mL BSA), [^{3}H]IP_3 (0.1 μCi/mL), and IP_3-binding protein were added to the cell extract. The mixture was incubated for 15 min on ice and then centrifuged at 2000 *g* for 10 min. Water and scintillation cocktail were added to the pellet to measure radioactivity. IP_3 concentration in the sample was determined based on a standard curve and expressed as picomoles per microgram of protein in the soluble cell extract obtained with TCA. The IP_3-binding protein was prepared from bovine adrenal cortex according to the method of Challiss *et al.* [17].

$[Na^+]_i$ Measurement

$[Na^+]_i$ was measured using the fluorescent Na^+ indicator SBFI as previously described in a report by Park *et al.* [18]. In brief, the PC12 cell suspension was incubated in fresh RPMI 1640 medium containing 15 μM SBFI/AM, 10% bovine calf serum, and 0.2% Pluronic F-127 for 2 hr at 37° with continuous stirring. The cells were then washed twice with fresh RPMI 1640 medium and left at room temperature until use. Sulfinpyrazone (250 μM) was added to all solutions to prevent dye leakage [12]. Fluorescence ratios were taken with alternate excitations at 340 and 380 nm and emission at 530 nm. Changes in $[Na^+]_i$ are presented as fluorescence ratios.

Inhibition of [^{3}H]Nicotine Binding

Binding of [^{3}H]nicotine to intact cells was measured as previously described by Higgins and Berg [19]. Intact PC12 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 indicated concentrations of CPZ 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 and scraped into 0.5 mL of 5% TCA, and the radioactivity was measured by liquid scintillation counting. Non-specific binding, determined by co-incubation 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 % of specific binding.

Analysis of Data

All experiments were performed independently more than twice, and all quantitative data are expressed as the means ± SEM. IC_{50} values were calculated with the AllFit program [20]. Unpaired Student's *t*-test was performed for the comparison between two experimental groups. Differences were considered significant when probability (P) values were <0.05.

RESULTS

Inhibitory Effect of CPZ on DMPP-Induced [^{3}H]NE Secretion

The effect of CPZ, a typical antipsychotic drug, on catecholamine secretion was investigated in PC12 cells. CPZ by itself did not affect the basal [^{3}H]NE secretion (data not shown). Stimulation of the cells with 100 μM DMPP, an activator of the nAChR, increased [^{3}H]NE secretion by 4.5 ± 0.3% of the total endogenous content (open circle in Fig. 1A). Incubation of cells with CPZ decreased the DMPP-induced [^{3}H]NE secretion in a concentration-dependent manner, with an IC_{50} of 1.0 ± 0.2 μM (Fig. 1A). Submicromolar concentrations of CPZ already reduced the DMPP-induced secretion significantly (19% inhibition with 300 nM CPZ), while the maximal inhibitory concentration was 10 μM. To further characterize the CPZ-induced inhibition of catecholamine secretion, we tested various concentrations of DMPP in the presence of CPZ at the IC_{50} level (Fig. 1B). DMPP at a concentration above 1 μM significantly evoked [^{3}H]NE secretion, and the amount of secreted [^{3}H]NE increased as the concentration of DMPP increased (open circles in Fig. 1B). CPZ inhibited DMPP-induced [^{3}H]NE secretion at all DMPP concentrations tested with similar potency (closed circles in Fig. 1B). The result suggests that CPZ acted in a non-competitive manner, in good agreement with previous reports [21, 22].

Inhibitory Effect of CPZ on the DMPP-Induced $[Ca^{2+}]_i$ Increase

Since an increase in $[Ca^{2+}]_i$ is an essential step in catecholamine secretion, we tested the effect of CPZ on the rise in $[Ca^{2+}]_i$. CPZ by itself had no effect on $[Ca^{2+}]_i$ (data not shown), but when the cells were incubated with various

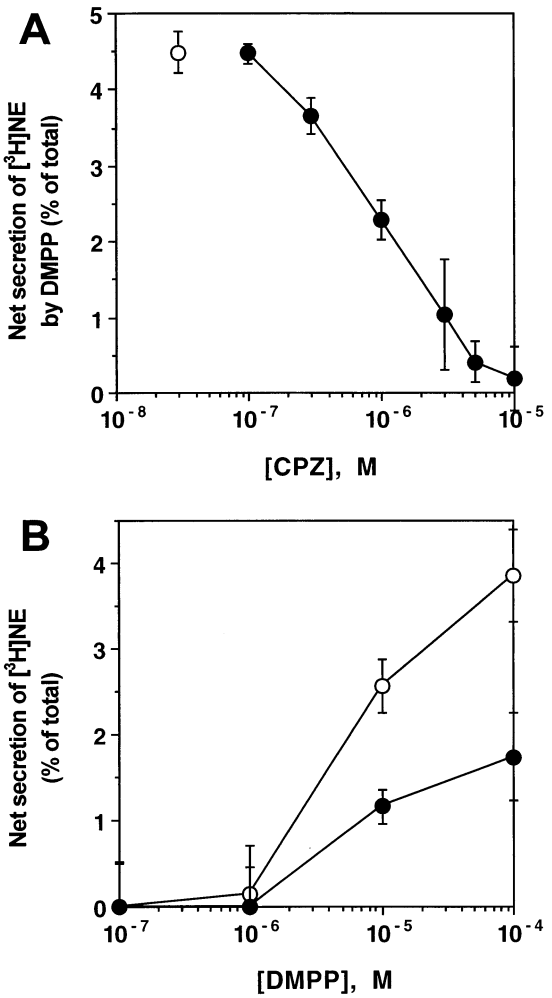

FIG. 1. Inhibitory effect of CPZ on DMPP-induced [^{3}H]NE secretion by PC12 cells. (A) [^{3}H]NE secretion induced by 100 μM DMPP in the presence of the indicated concentrations of CPZ (●) was determined. Secretion of [^{3}H]NE induced by DMPP alone (○) in the absence of CPZ is presented. Four separate experiments were done, and the results were reproducible. Data are the means ± SEM (bars) values of triplicate measurements. (B) [^{3}H]NE-loaded PC12 cells were treated with the indicated concentrations of DMPP in the absence (○) or presence (●) of CPZ at the IC_{50} (1 μM). The secreted [^{3}H]NE was measured as described under Materials and Methods and is expressed as % of total [^{3}H]NE. Three separate experiments were done, and the results were reproducible. Data are the means ± SEM (bars) values of triplicate measurements.

concentrations of CPZ, a subsequent DMPP-induced $[Ca^{2+}]_i$ rise was inhibited, with an IC_{50} of 1.9 ± 0.1 μM (Fig. 2B). The inhibition by 1 μM CPZ was 30.4 ± 4.0% (trace b in Fig. 2A). CPZ at 10 μM completely abolished the DMPP-induced $[Ca^{2+}]_i$ rise (trace c in Fig. 2A). In addition, as the concentrations of DMPP were increased, greater $[Ca^{2+}]_i$ rises were achieved, attaining a maximum at 100 μM DMPP (open circles in Fig. 2C). When PC12 cells were exposed to various concentrations of DMPP in the presence of 1 μM CPZ all $[Ca^{2+}]_i$ rises were inhibited with similar potency (closed circles in Fig. 2C). These

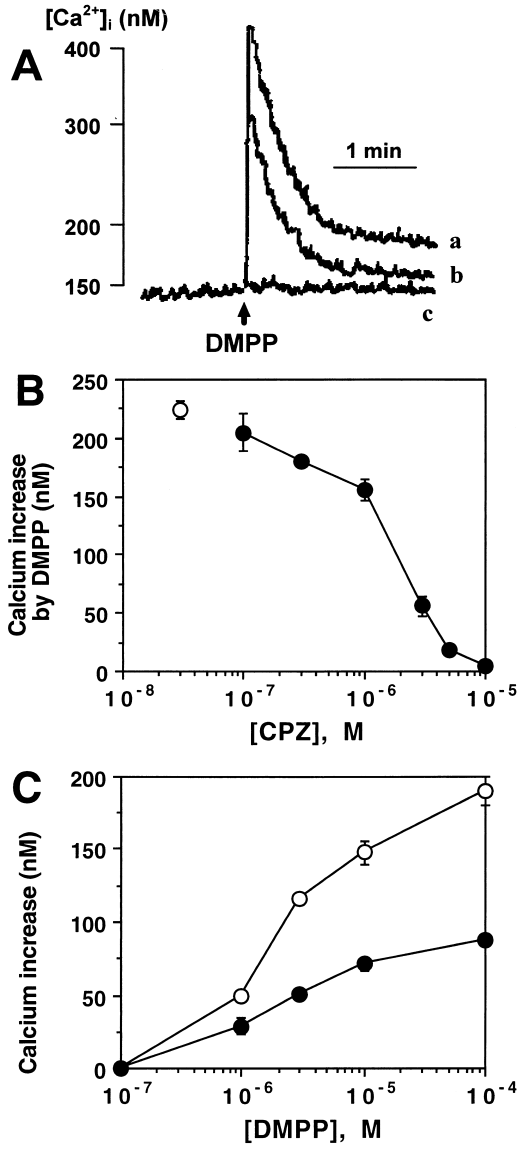

FIG. 2. Inhibitory effect of CPZ on DMPP-induced $[Ca^{2+}]_i$ elevation in PC12 cells. (A) The intracellular $[Ca^{2+}]_i$ rise induced by 100 μM DMPP was measured in the absence (trace a) or presence (1 μM, trace b; 10 μM, trace c) of CPZ. The experiments were performed 4 times independently and typical Ca^{2+} transients are presented. (B) The $[Ca^{2+}]_i$ rise was induced with 100 μM DMPP after a 3-min preincubation of the cells with the indicated concentrations of CPZ (●). The peak height of each stimulation was compared to that of the control $[Ca^{2+}]_i$ rise caused by 100 μM DMPP alone (○). Three separate experiments were done, and the results were reproducible. Data are the means ± SEM (bars) values of triplicate measurements. (C) The $[Ca^{2+}]_i$ rise induced by the indicated concentrations of DMPP in the absence (○) or presence (●) of CPZ at the IC_{50} (1 μM) was measured. Two separate experiments were done, and the results were reproducible. Data are means ± SEM (bars) values of triplicate measurements.

results are in good agreement with those obtained for the [^{3}H]NE secretion, suggesting that CPZ inhibits DMPP-induced [^{3}H]NE secretion by decreasing the rise in $[Ca^{2+}]_i$.

Inhibition of nAChR Activity by CPZ

Since the activation of nAChRs has been known to induce membrane depolarization, leading to a subsequent activation of VSCCs, a DMPP-induced $[Ca^{2+}]_i$ increase can occur through both nAChRs and VSCCs. In contrast, voltage-sensitive Na^+ channels are not activated upon nicotinic or high K^+ stimulation and Na^+ entry into chromaffin cells following DMPP treatment occurs entirely through nAChRs [23, 24]. In our PC12 cells, the Na^+ increase induced by DMPP was not affected by tetrodotoxin, an inhibitor of voltage-sensitive Na^+ channels (data not shown). Therefore, the inhibition of the rise in DMPP-induced $[Ca^{2+}]_i$ cannot simply be attributed to the inhibition of nAChRs. However, Na^+ entry can occur through nAChRs, but not through VSCCs. We, therefore, measured the effect of CPZ on the DMPP-induced $[Na^+]_i$ rise to test whether nAChRs themselves were inhibited by CPZ. As shown in Fig. 3, A and B, pretreatment of cells with 1 μM CPZ decreased the DMPP-induced $[Na^+]_i$ rise by 57.1 ± 0.1%, while CPZ at 10 μM caused near complete inhibition of the DMPP-induced response. These results suggest that CPZ inhibits the activity of the nAChRs. In order to confirm that CPZ blocks nAChR directly, we tested the effect of CPZ on [^{3}H]nicotine binding. Competition between [^{3}H]nicotine and CPZ for nAChR binding was indeed observed. Figure 3C shows a competition curve for CPZ using labeled nicotine. CPZ effectively competed for binding with [^{3}H]nicotine with an IC_{50} of 2.7 ± 0.6 μM, a value similar to that obtained for the DMPP-induced [^{3}H]NE secretion and the $[Ca^{2+}]_i$ rise. The results suggest that blockage of nAChRs is responsible for the inhibition of DMPP-induced catecholamine secretion by CPZ.

Inhibitory Effect of CPZ on High K^+-Evoked Responses

Since VSCCs can become activated after stimulation of nAChRs, we investigated the effect of CPZ on catecholamine secretion induced by the activation of VSCCs. Stimulation of cells with 70 mM K^+ raised [^{3}H]NE secretion by 13.2 ± 0.7% (open square in Fig. 4A). CPZ produced a concentration-dependent inhibition of the 70 mM K^+-evoked [^{3}H]NE secretion, with an IC_{50} of 7.9 ± 1.1 μM (Fig. 4A). At 50 μM, CPZ completely inhibited the high K^+-evoked response. We then tested whether the inhibition of 70 mM K^+-evoked [^{3}H]NE secretion was due to the inhibition of VSCCs. When cells were incubated with various concentrations of CPZ, the subsequent $[Ca^{2+}]_i$ rise induced by 70 mM K^+ became decreased with an IC_{50} of 6.2 ± 0.3 μM (Fig. 4B), in good agreement with the results for [^{3}H]NE secretion. This suggests that VSCCs are also inhibited by CPZ treatment. The IC_{50} values for the inhibition of 70 mM K^+-induced responses are significantly greater than those for the inhibition of DMPP-induced responses ($P < 0.05$ by unpaired Student's *t*-test).

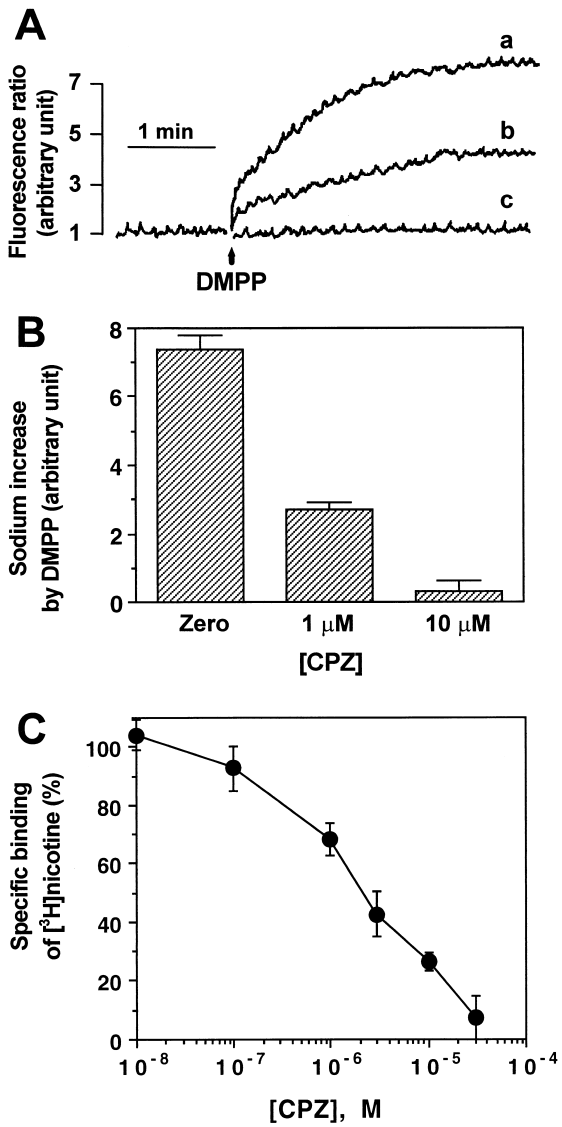

FIG. 3. Inhibitory effect of CPZ on $[Na^+]_i$ elevation and [^{3}H]nicotine binding. (A) The $[Na^+]_i$ rise induced by 100 μM DMPP was measured in the absence (trace a) or presence (1 μM, trace b; 10 μM, trace c) of CPZ. The experiments were performed 3 times independently and typical Ca^{2+} transients are presented. (B) Statistical analysis of the $[Na^+]_i$ rise induced by 100 μM DMPP in the presence of the indicated concentrations of CPZ. Three separate experiments were done, and the results were reproducible. Data are means ± SEM (bars) values of triplicate measurements. (C) PC12 cells were incubated with 20 nM [^{3}H]nicotine and various concentrations of CPZ for 40 min at 25°. Displacement of [^{3}H]nicotine by unlabeled CPZ (●) is presented. Non-specific binding was determined in the presence of 1 mM unlabeled nicotine. The data represent the means of two experiments, each carried out in triplicate.

Inhibition of L-type VSCCs by CPZ

PC12 cells express several types of VSCCs, among which the L- and N-types are major components [11, 25]. To determine which type of Ca^{2+} channel is inhibited by CPZ, we carried out measurements of [^{3}H]NE secretion and $[Ca^{2+}]_i$ using nifedipine and ω-CgTx, L- and N-type VSCC blockers, respectively. As shown in Fig. 5A, the inhibition of 70 mM K^+-evoked [^{3}H]NE secretion observed with nifedipine and ω-CgTx was 90.9 ± 1.7% and 12.6 ± 2.6%, respectively. There was no more inhibition when 5 μM CPZ and nifedipine were added together (91.0 ± 1.9%). However, simultaneous treatment with 5 μM CPZ and ω-CgTx resulted in a greater inhibition (66.4 ± 1.2%) than treatment with ω-CgTx (12.6 ± 2.6%) or CPZ (37.5 ± 13.1%) alone. The 70 mM K^+-evoked $[Ca^{2+}]_i$ rise was further inhibited by CPZ in the presence of ω-CgTx (inhibition by 53.9 ± 3.9%) compared to CPZ treatment alone (inhibition by 43.1 ± 3.4%) (Fig. 5B). In addition,

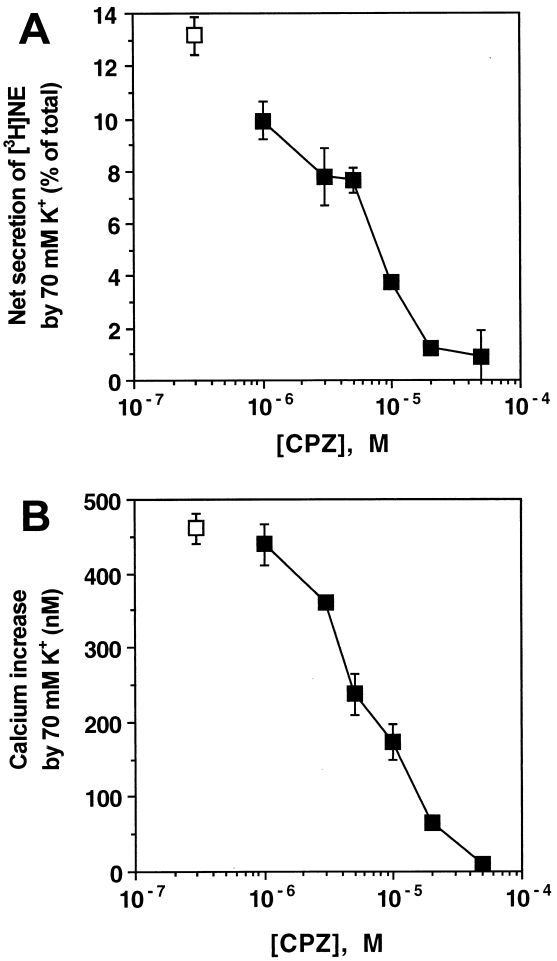

FIG. 4. Inhibitory effect of CPZ on 70 mM K^+-induced responses. (A) [^{3}H]NE secretion induced by 70 mM K^+ in the presence of the indicated concentrations of CPZ (■) was determined. Secretion of [^{3}H]NE induced by 70 mM K^+ alone (□) in the absence of CPZ is also presented. Four separate experiments were done, and the results were reproducible. Data are the means ± SEM (bars) values of triplicate measurements. (B) The $[Ca^{2+}]_i$ rise induced by 70 mM K^+ was measured after a 3-min preincubation with the indicated concentrations of CPZ (■). The $[Ca^{2+}]_i$ rise induced by 70 mM K^+ alone (□) in the absence of CPZ is also presented. The peak height of each stimulation was compared to that of the control $[Ca^{2+}]_i$ increase caused by 70 mM K^+ alone. Four separate experiments were done, and the results were reproducible. Data are the means ± SEM (bars) values of triplicate measurements.

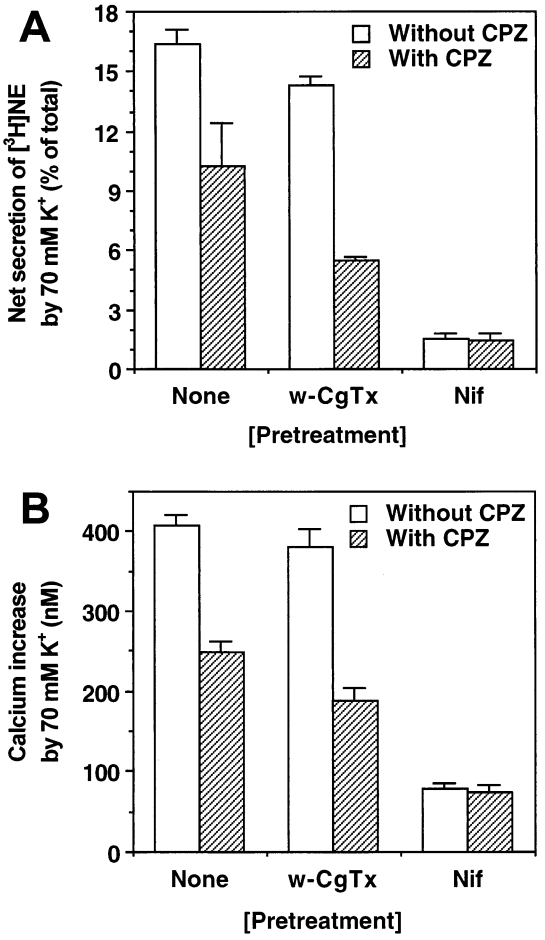

FIG. 5. Selective inhibition of L-type Ca^{2+} channels by CPZ. (A) [^{3}H]NE-loaded PC12 cells were preincubated with CPZ (5 μM), nifedipine (Nif, 5 μM), or ω-CgTx (10 μM) for 10 min. The cells were then treated with 70 mM K^+ in the presence of CPZ and/or Ca^{2+} channel blockers. Secreted [^{3}H]NE was measured as described under Materials and Methods and is expressed as % of total [^{3}H]NE. Two experiments were done, and the results were reproducible. Data are means ± SEM (bars) values of triplicate measurements. (B) PC12 cells were incubated with CPZ (5 μM) for 3 min and 70 mM K^+ was added. In the case of nifedipine (Nif, 5 μM) or ω-CgTx (10 μM), each treatment was for 5 min before the addition of 70 mM K^+. The net increase in $[Ca^{2+}]_i$ was obtained by subtracting the basal level of $[Ca^{2+}]_i$ from the peak level after the 70 mM K^+ stimulation. Three separate experiments were done, and the results were reproducible. Data are means ± SEM (bars) values of triplicate measurements.

when PC12 cells were incubated with ω-CgTx up to 1 hr, the simultaneous treatment with ω-CgTx and CPZ still showed additive inhibition (data not shown), suggesting that ω-CgTx-sensitive N-type VSCCs are not affected by 5 μM CPZ. Among VSCCs, the L-type was preferentially inhibited by CPZ, but the N-type was also blocked by a high concentration (50 μM) of CPZ (Fig. 4B). These results suggest that it is the inhibition of L-type VSCCs that mainly contributes to the inhibition of 70 mM K^+-evoked [^{3}H]NE secretion by CPZ.

Lack of a CPZ Effect on Bradykinin-Induced Responses

We examined whether CPZ affects bradykinin-induced responses resulting from the activation of phospholipase C [26]. Bradykinin induced a concentration-dependent rise in $[Ca^{2+}]_i$, reaching a maximal level at 100 nM (open triangles in Fig. 6A). When cells were pretreated with CPZ at concentrations of up to 10 μM, at which point the activity of nAChRs was completely inhibited, the peak height of the bradykinin-induced $[Ca^{2+}]_i$ increase was not affected (closed triangles in Fig. 6A), and there was only a slight decrease of the sustained Ca^{2+} level. CPZ also had no effect on the bradykinin-induced IP_3 generation (Fig. 6B). In addition, CPZ (from 1 to 10 μM) did not significantly inhibit $[Ca^{2+}]_i$ elevation induced by the calcium ionophore ionomycin (240 ± 5 nM, compared to the control 257 ± 5

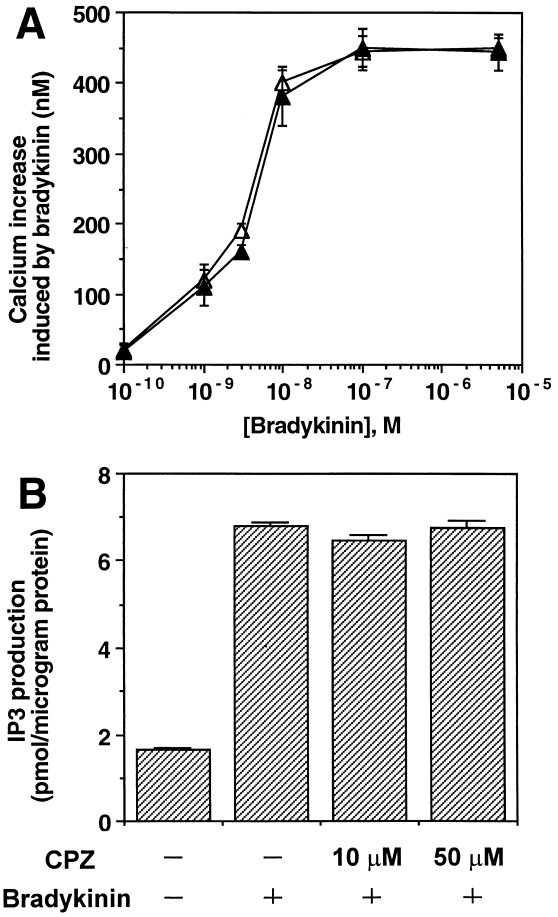

FIG. 6. Lack of a CPZ effect on bradykinin-induced responses. (A) The $[Ca^{2+}]_i$ rise induced by the indicated concentrations of bradykinin in the absence (△) or presence (▲) of 5 μM CPZ was measured. Two separate experiments were done, and the results were reproducible. Data are means ± SEM (bars) values of triplicate measurements. (B) PC12 cells were stimulated with bradykinin in the presence of the indicated concentrations of CPZ, and the cellular concentration of IP_3 was determined as described under Materials and Methods and expressed as picomoles per microgram of protein. Three separate experiments were done, and the results were reproducible. Data are mean values ± SEM (bars) of triplicate measurements.

nM; $P = 0.15$ by Student's t-test, N = 3). These results indicate that the action of CPZ on nAChRs and VSCCs is specific and without any effect on bradykinin receptor-mediated phospholipase C activation and calcium increase by a calcium ionophore.

DISCUSSION

There have been many reports describing many kinds of membrane proteins that can be inhibited by CPZ, such as nicotinic receptors and voltage-sensitive ion channels [4–10]. However, the effect of CPZ on the release of neurotransmitters had received little attention up to now [27–29]. In particular, an intensive study of the effect of neuroleptic drugs on the secretion of catecholamines had not yet been performed. Because the synaptic level of catecholamines such as dopamine [30] and norepinephrine [31] is thought to play a critical role in schizophrenia, both the secretion of catecholamines into the synapse and its modulation by neuroleptic drugs is physiologically important. We used PC12 cells, a model system for the study of catecholamine secretion [25, 26], in order to investigate the effect of a typical antipsychotic drug, chlorpromazine, on catecholamine secretion. Our studies indicate that CPZ inhibits catecholamine secretion evoked by nicotinic stimulation or membrane depolarization. The results imply that, when presynaptic dopaminergic or noradrenergic neurons are exposed to nicotinic stimulation or membrane depolarization in the presence of CPZ, catecholamine secretion is inhibited and the amount of catecholamine at the synaptic cleft is decreased. This may be one of the therapeutic mechanisms of neuroleptics. In addition, Blaha and Lane [28] reported that repeated treatment with classical antipsychotic drugs, but not atypical antipsychotics, reduces the basal dopamine release in the striatum, suggesting that this decrease in nigrostriatal dopamine release is linked to the extrapyramidal side effects. Therefore, it would be of interest to compare the effects of classical and atypical antipsychotics on catecholamine secretion. We are at present investigating a correlation between the adverse effects caused by CPZ treatment, such as the extrapyramidal side effects [32, 33], and the inhibitory action of CPZ on catecholamine secretion.

Our study suggests that CPZ inhibits catecholamine secretion by blocking nicotinic receptors and L-type VSCCs. Since the stimulation of nicotinic receptors leads to membrane depolarization and the subsequent activation of VSCCs, it could be presumed that the inhibition of the DMPP-induced $[Ca^{2+}]_i$ rise by CPZ might be due to the inhibition of VSCCs by CPZ. However, the data showing that both the elevation in $[Na^+]_i$ and the [^{3}H]nicotine binding evoked by DMPP were inhibited by CPZ with similar IC_{50}s strongly suggests that inhibition of the nAChR itself, not the VSCCs, mainly contributes to the inhibition of DMPP-evoked catecholamine secretion. Since CPZ is known to be a non-competitive channel blocker of nAChRs because of its interaction with a unique high-affinity site that is distinct from the agonist-binding site [23, 24, 34], CPZ might not be expected to influence the binding of [^{3}H]nicotine to the nAChRs. However, it is likely that the binding of either agonists or non-competitive blockers to distinct sites may cause a conformational change in nAChRs, leading to the influence of one substance on the binding of the other. It has been reported that CPZ binding can be enhanced by agonist treatment. This would support the notion that the binding of an agonist induces a conformational change in the nAChR, switching it to an active state with an open cation channel and thus making it easier for CPZ to reach its high-affinity site [35]. In reverse, it may be possible that a non-competitive blocker may affect agonist binding. Takayama *et al.* [36] reported that, in the rat brain, non-competitive blockers affected agonist binding to nAChR: local anesthetics (procaine, tetracaine, and cocaine), phencyclidine, and mecamylamine all inhibited [^{3}H]methylcarbamylcholine binding to nAChRs. In PC12 cells, a similar effect to that described for the rat brain may occur, given that we observe the displacement of [^{3}H]nicotine binding to nAChR by CPZ. Structural and pharmacological heterogeneity of the nAChRs [37] may explain this variation in the effects of CPZ on agonist binding.

L-type VSCCs were also inhibited by CPZ albeit with lower sensitivity than the nAChRs, resulting in the inhibition of catecholamine secretion evoked by membrane depolarization. Our data suggest that L-type VSCCs are more susceptible to CPZ than N-type VSCCs. Furthermore, it seems likely that CPZ directly interacts with target membrane proteins without the involvement of a second messenger system, considering its competition with [^{3}H]nicotine binding and the rapid onset of the inhibition. On the other hand, bradykinin-induced activation of PLC was not affected by CPZ treatment, suggesting that nAChRs and VSCCs are selectively inhibited by CPZ with different sensitivities. Wode-Helgodt *et al.* [38] reported that clinical improvement and extrapyramidal symptoms correlate with the concentration of CPZ in plasma and cerebrospinal fluid in psychotic patients. Although further clinical experiments are required, the concentration-dependent inhibitory effects of CPZ could provide the clue towards the understanding of the concentration-dependent therapeutic effects or the side effects of CPZ treatment.

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