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Chlorpromazine inhibits store-operated calcium entry and subsequent noradrenaline secretion in PC12 cells

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1 The effect of chlorpromazine on the store-operated Ca^{2+} entry activated *via* the phospholipase C signalling pathway was investigated in PC12 cells.

2 Chlorpromazine inhibited the sustained increase after the initial peak in the intracellular Ca^{2+} concentration produced by bradykinin while having no effect on the initial transient response. The inhibition was lowered by the removal of extracellular free Ca^{2+}. However, chlorpromazine did not inhibit bradykinin-induced inositol 1,4,5-trisphosphate production.

3 Chlorpromazine inhibited the bradykinin-induced noradrenaline secretion in a concentration-dependent manner (IC_{50}: 24 ± 5 μM, $n = 3$).

4 To test for a direct effect of chlorpromazine on store-operated Ca^{2+} entry, thapsigargin, an inhibitor of microsomal Ca^{2+}-ATPase, was used to induce store-operated Ca^{2+} entry in PC12 cells. Chlorpromazine reduced the thapsigargin-induced sustained Ca^{2+} level (IC_{50}: 24 ± 2 μM, $n = 3$), and the inhibition also occluded the inhibitory action of 1-[-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenyl]-1H-imidazole hydrochloride (SK&F96365).

5 The results suggest that chlorpromazine negatively modulates the store-operated Ca^{2+} entry activated subsequent to PLC activation.

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Keywords: Chlorpromazine; bradykinin; phospholipase C; store-operated Ca^{2+} entry; neurotransmitter secretion

Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} ion concentration; fura-2/AM, fura-2 pentaacetoxymethyl ester; $InsP_3$, inositol 1,4,5-trisphosphate; PLC, phospholipase C; SK&F96365, 1-[-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenyl]-1H-imidazole hydrochloride; SOCE, store-operated Ca^{2+} entry

Introduction

Many kinds of neurotransmitters, hormones, and cytokines accomplish their physiological role by increasing cytosolic Ca^{2+} levels. Activation of phospholipase C (PLC)-linked receptors, one of the major pathways of Ca^{2+} signalling, leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate which results in the production of inositol 1,4,5-trisphosphate ($InsP_3$). $InsP_3$ induces elevation of cytosolic free Ca^{2+} by activating Ca^{2+} entry from the extracellular space after depletion of the Ca^{2+} stores. This process is called store-operated Ca^{2+} entry (SOCE), formerly referred to as capacitative Ca^{2+} entry (Putney & McKay, 1999).

SOCE mediates many kinds of important physiological events such as superoxide production in neutrophils (Geiszt *et al*., 1997), platelet aggregation (Huang & Kwan, 1998; Dobrydneva *et al*., 1999), vascular smooth muscle contraction (Zhang *et al*., 1999), insulin secretion (Liu & Gylfe, 1997), and odorant signalling (Zufall *et al*., 2000). In neuronal cells, SOCE is critical to PLC-linked receptor-mediated catecholamine secretion (Powis *et al*., 1996; Koizumi & Inoue, 1998; Fomina & Nowycky, 1999).

Chlorpromazine, one of the phenothiazine neuroleptic drugs, has been used to treat mental disease including schizophrenia (Snyder *et al*., 1974). It also potentiates the effect of drugs such as analgesics, sedatives, and anaesthetics. Until now, chlorpromazine is known to have pleiotropic inhibitory effects on dopamine receptors (Seeman, 1980; Ellenbroek, 1993), calmodulin (Prozialeck & Weiss, 1982; Klockner & Isenberg, 1987), voltage-sensitive ion channels (Ogata *et al*., 1990; Wooltorton & Mathie, 1993; Nakazawa *et al*., 1995; Lee *et al*., 1999), nicotinic acetylcholine receptors (Benoit & Changeux, 1993; Lee *et al*., 1999), the NMDA receptor (Lidsky *et al*., 1997), ATP-sensitive K^+ channels (Muller *et al*., 1991), and $GABA_A$ receptors (Mozrzymas *et al*., 1999). However, nobody had as of yet studied whether chlorpromazine regulates the PLC-mediated signalling pathway or whether chlorpromazine has a regulatory effect on SOCE. Since the action sites are broad, making chlorpromazine a non-specific drug, the regulation of SOCE is important, because it affects the Ca^{2+} signalling of PLC-linked receptors which mediate various physiological events with many kinds of neurotransmitters. Here we report that chlorpromazine regulates the PLC signalling pathway by inhibiting SOCE without attenuating the PLC activity involved in the generation of $InsP_3$ or the Ca^{2+} release from internal stores.

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Methods

Cell Culture

PC12 cells were grown in RPMI 1640 supplemented with 10% (v v^{-1} heat-inactivated bovine calf serum, 5% (v v^{-1} heat-inactivated horse serum, and 1% v v^{-1} penicillin (5,000 U ml^{-1})/streptomycin (5,000 μg ml^{-1} solution. The cells were cultured in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed every 2 days, and the cells were subcultured weekly.

Measurement of noradrenaline secretion

Catecholamine secretion by PC12 cells was measured following the method reported by Suh & Kim (1994). In brief, cells were loaded with [^{3}H]-noradrenaline (1 μCi ml^{-1}) during an incubation in RPMI 1640 for 1 h at 37°C in 5% CO_2/95% air. The cells were washed twice and then incubated in Locke's solution (NaCl 154 mM, KCl 5.6 mM, glucose 5.6 mM, $CaCl_2$ 1 mM, $MgCl_2$ 500 μM, and 5 mM HEPES buffer adjusted to pH 7.4) for 15 min to let them stabilize. Then the cells were incubated again in fresh Locke's solution for 15 min to measure basal secretion. The cells were subsequently stimulated with the drugs under test for 15 min. The medium was removed from the wells and residual catecholamines were extracted from the cells by addition of 10% trichloroacetic acid. The radioactivity was measured with a scintillation counter. The amount of [^{3}H]-noradrenaline secreted was calculated and expressed as percentage of total [^{3}H]-noradrenaline content.

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

$[Ca^{2+}]_i$ was determined using the fluorescent Ca^{2+} indicator fura-2 as described previously (Suh *et al*., 1995). Briefly, the cell suspension in Locke's solution was incubated with 3 μM fura-2 pentaacetoxymethyl ester (fura-2/AM) for 50 min at 37°C under continuous stirring. The fura-2-loaded cells were then washed twice with fresh Locke's solution. Sulfinpyrazone (250 μM) was added to all solutions to prevent dye leakage. For the fluorimetric measurement of $[Ca^{2+}]_i$, 1×10^6 cells ml^{-1} were placed into a quartz cuvette in a thermostatically controlled cell holder at 37°C and continuously stirred. Fluorescence ratios were monitored with dual excitation at 340 and 380 nm and emission at 500 nm. Calibration of the fluorescent signal in terms of $[Ca^{2+}]_i$ was performed as described by Grynkiewicz *et al*. (1985) using the following equation:

$$[Ca^{2+}]_i = K_D(R - R_{min})(R_{max} - R)^{-1}S_{f2}S_{b2}{}^{-1}$$

where R is the ratio of fluorescence emitted by excitation at 340 and 380 nm. S_{f2} and S_{b2} are the proportionality coefficients at 380 nm excitation of Ca^{2+}-free fura-2 and Ca^{2+}-saturated fura-2, respectively. In order to obtain R_{min}, the fluorescence ratios were measured after adding 4 mM EGTA, 30 mM Trizma base, and 0.1% Triton X-100 to the cell suspension. To obtain R_{max}, the cell suspension was then treated with $CaCl_2$ at a final concentration of 4 mM, and the fluorescence ratio measured.

Mn^{2+} quenching of fura-2 fluorescence

The Mn^{2+} quenching assay was performed as described by Song *et al*. (1998) to measure the influx of Ca^{2+} from the extracellular space. Briefly, fura-2-loaded cells (5×10^6 cells ml^{-1}) were placed into a quartz cuvette in a thermostatically controlled cell holder at 37°C and continuously stirred. Fluorescence was excited at 360 nm, i.e., the isosbestic wavelength at which Ca^{2+} does not affect fura-2 fluorescence and at which, therefore, any changes are caused by Mn^{2+} quenching. Emission was recorded at 500 nm. In order to quantify the amount of Mn^{2+} influx, we monitored the slope of Mn^{2+}-induced changes in fluorescence as well as total changes after applying 1 mM $MnCl_2$ and the drugs to be tested. We quantified this data by measuring the period of time over which the fluorescence changes from 1.6 to 1.4 relative fluorescence units.

Measurement of $InsP_3$ production

$InsP_3$ generation was determined by competition assay of [^{3}H]-$InsP_3$ for binding protein as described previously (Park *et al*., 1997). Confluent cells on a 6-well plate were stimulated with the drugs under test. The reaction was terminated by addition of ice-cold 5% trichloroacetic acid containing 10 mM EGTA. The supernatant of the lysate was saved and treated with diethylether to remove the trichloroacetic acid. The aqueous fraction after a final extraction was neutralized with 200 mM Trizma base and adjusted to pH 7.4. Twenty μl of the extract was added to 20 μl of assay buffer (0.1 M Tris buffer containing 4 mM EDTA) and 20 μl of [^{3}H]-$InsP_3$ (100 nCi ml^{-1}). Then 20 μl of a solution containing the binding protein was added and the mixture incubated for 15 min on ice and centrifuged at $2000 \times g$ for 5 min. One hundred μl of water and 1 ml of liquid scintillation cocktail were added to the pellet to measure the radioactivity. The $InsP_3$ concentration of the sample was determined by comparison to a standard curve and expressed as pmole (mg of protein)$^{-1}$. The total cellular protein concentration was measured by the Bradford method after lysis of the cells with trichloroacetic acid.

Analysis of data

All quantitative data were expressed as means ± s.e.mean. The results were analysed using the analysis of variance test. We calculated the IC_{50} values using the Microcal Origin for Windows program. Differences were considered significant only for $P < 0.05$.

Materials

Chlorpromazine, bradykinin, thapsigargin, SK&F96365, and sulfinpyrazone were purchased from Sigma (St. Louis, MO, U.S.A.). Fura-2/AM was obtained from Molecular Probes (Eugene, OR, U.S.A.). [^{3}H]-Noradrenaline and [^{3}H]$InsP_3$ were purchased from NEN (Boston, MA, U.S.A.). RPMI 1640 and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY, U.S.A.). Bovine calf serum and horse serum were obtained from HyClone (UT, U.S.A.).

Results

We studied the effect of chlorpromazine on the PLC-mediated $[Ca^{2+}]_i$ increase and noradrenaline secretion in PC12 cells, cells that have been widely used as a model system for the study of catecholamine secretion. Bradykinin stimulates B_2 bradykinin receptors and activates PLC in this cell line (Nardone *et al*., 1994; Suh *et al*., 1995; Kim *et al*., 1999). Bradykinin-induced Ca^{2+} increase happens in a transient fashion. It reaches peak level within a few seconds and then falls back to the basal level in 200 s. The transient Ca^{2+} increase is partly due to the fast desensitization of the bradykinin receptors and is often detected in PLC-mediated signalling. Chlorpromazine inhibited the $[Ca^{2+}]_i$ rise evoked by bradykinin (Figure 1A). The inhibition was reversible, a full response to bradykinin having been obtained after a 7-min wash with drug free solution (data not shown). The inhibition was more obvious in the 'Ca^{2+}-decreasing state', during which the Ca^{2+} level returns from the peak level to the normal level, rather than in the 'Ca^{2+}-increasing state' represented by the initial peak (Figure 1A). In order to determine the chlorpromazine-induced inhibition of the Ca^{2+} rise comparing the Ca^{2+} levels during the 'Ca^{2+}-increasing state' and at the 'Ca^{2+}-decreasing state', we calculated the cytosolic Ca^{2+} level at the initial point (depicted as 2 s) and the Ca^{2+} level at 90 s from the data of Figure 1A and depicted the quantified data in Figure 1B. The chlorpromazine-induced inhibition of the Ca^{2+} increase was more dramatic during the 'Ca^{2+}-decreasing state' rather than 'Ca^{2+}-increasing state' (Figure 1B). In the absence of extracellular Ca^{2+}, a chlorpromazine-induced inhibition was not observed (Figure 1C). But when the extracellular Ca^{2+} was reintroduced to a normal level (2.2 mM), inhibition became again apparent (Figure 1C). We quantified the Ca^{2+} level of the data in Figure 1C at the point of bradykinin-treatment (which shows only bradykinin-induced Ca^{2+} release from the intracellular Ca^{2+} pools) and subsequent 4 mM $CaCl_2$ treatment (which induced bradykinin-induced Ca^{2+} influx from the extracellular space) and depicted them in Figure 1D). The chlorpromazine-induced inhibition of the Ca^{2+} increase was more obvious in the Ca^{2+} influx from the extracellular medium than in the Ca^{2+} release from the intracellular stores (Figure 1D). These results suggest that the chlorpromazine-induced inhibition only affected the Ca^{2+} influx caused by bradykinin. In Figure 1B,D, the sustained rise in $[Ca^{2+}]_i$ produced by re-introducing Ca^{2+} into the Ca^{2+}-free medium after bradykinin stimulation was greater than the sustained rise evoked by bradykinin stimulation in the presence of extracellular Ca^{2+}. We speculate that this is caused by a difference in the filling state of the intracellular

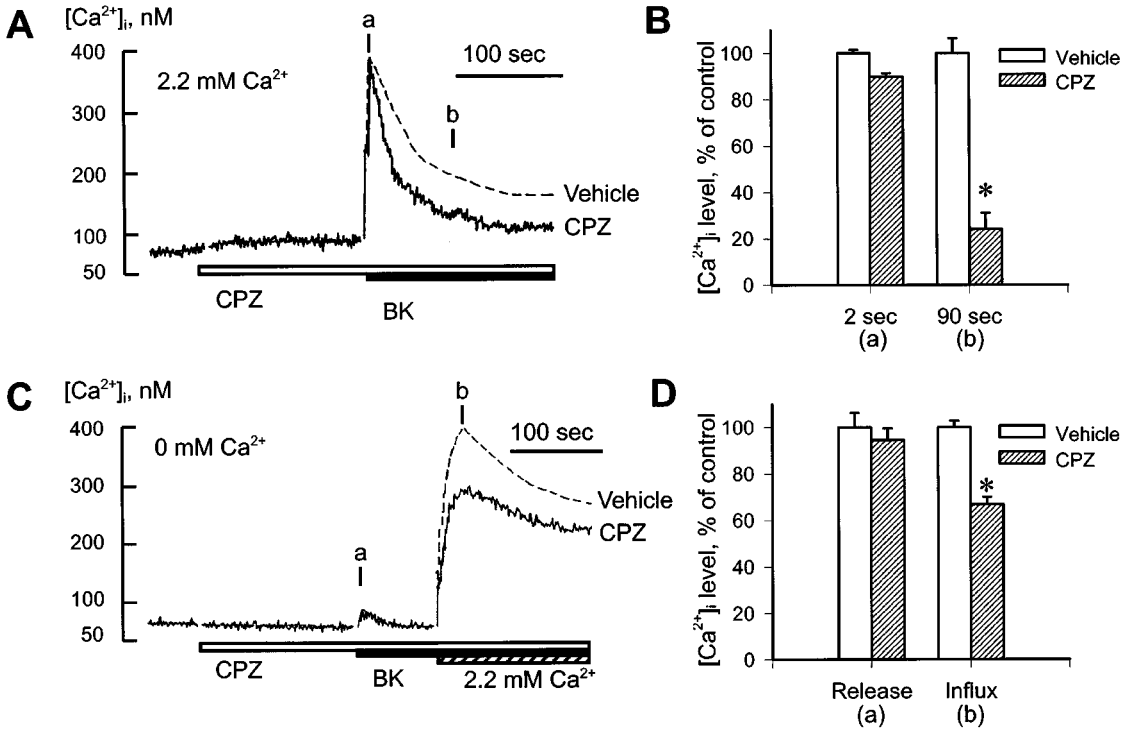

Figure 1 Effect of chlorpromazine on bradykinin-induced $[Ca^{2+}]_i$ rise in PC12 cells. (A) Fura-2-loaded cells were challenged with 300 nM bradykinin (BK) in the presence (solid trace) or absence (hatched trace) of 30 μM chlorpromazine (CPZ). Typical Ca^{2+} traces obtained in more than five separate experiments are presented. The results were reproducible. (B) $[Ca^{2+}]_i$ levels at the time of the bradykinin treatment (2 s marked as a) and after 90 s (marked as b) in the data of A were quantitatively analysed. The levels of $[Ca^{2+}]_i$ are depicted as % of the bradykinin-induced $[Ca^{2+}]_i$ rise without chlorpromazine treatment. Each point was obtained and represents the mean$\pm$s.e.mean from triplicate experiments. *$P<0.01$. (C) The same experiments as in A were performed in the absence of extracellular free Ca^{2+}. After the challenge with bradykinin, 2.2 mM $CaCl_2$ (Ca^{2+}) was added. Typical Ca^{2+} traces obtained in more than five separate experiments are presented. The results were reproducible. (D) The $[Ca^{2+}]_i$ level after the bradykinin treatment (Release, marked as a) and after the $CaCl_2$ treatment (Influx, marked as b) in the data of C were quantitatively analysed. The levels of the $[Ca^{2+}]_i$ are depicted as % of the bradykinin-induced $[Ca^{2+}]_i$ rise without chlorpromazine treatment. Each point was obtained from triplicate experiments and represents the mean$\pm$s.e.mean. *$P<0.01$.

Ca^{2+} stores, because the bradykinin-induced depletion of the Ca^{2+} stores is transient and filling state could be fully recovered after 1 min and concomitantly with stopping SOCE, whereas bradykinin stimulation in Ca^{2+}-free medium could evoke drastic depletion of Ca^{2+} stores, which caused sustained SOCE when extracellular Ca^{2+} was reintroduced.

In order to confirm the lack of a chlorpromazine effect on PLC activity, we measured the production of $InsP_3$ when bradykinin was applied to the cells. As shown in Table 1, chlorpromazine had no effect on the bradykinin-induced $InsP_3$ production. The results, therefore, suggest that chlorpromazine did not affect the PLC activation process triggered by bradykinin stimulation.

In [^{3}H]-noradrenaline-preincubated PC12 cells, 300 nM bradykinin induced prominently the secretion of [^{3}H]-noradrenaline (Figure 2). Chlorpromazine inhibited the bradykinin-evoked [^{3}H]-noradrenaline secretion in a concentration-dependent manner with an IC_{50} of 24 ± 5 μM ($n = 3$). The results suggest that chlorpromazine, by inhibiting the bradykinin-induced Ca^{2+} influx, thereby causes a decrease in [^{3}H]-noradrenaline secretion. This is in good agreement with a previous report showing that the rise in $[Ca^{2+}]_i$ is generally correlated with neurotransmitter secretion in PC12 cells (Suh & Kim, 1994). Recently, Koizumi & Inoue (1998) reported that SOCE is critical to the bradykinin-mediated neurotransmitter secretion of PC12 cells. Therefore, it is likely that chlorpromazine may inhibit the triggering of SOCE subsequent to the bradykinin-mediated PLC activation.

We tested this possibility by looking for an inhibitory effect of chlorpromazine on the SOCE induced by thapsigargin, an inhibitor of microsomal Ca^{2+}-ATPase. Chlorpromazine strongly lowered the thapsigargin-induced Ca^{2+} level when added during the sustained phase of the Ca^{2+} elevation (Figure 3A). The inhibition occurred in a concentration-dependent manner with an IC_{50} of 24 ± 2 μM ($n = 3$), which is similar to that for the inhibitory effect on secretion (Figure 3B).

The sustained Ca^{2+} increase produced by thapsigargin was not reversed after several minutes of washing in drug-free solution, which is in contrast to chlorpromazine where it produced an immediate fall (data not shown). Furthermore, chlorpromazine decreased the thapsigargin-induced Ca^{2+} increase immediately. This suggests that chlorpromazine does

Table 1 The chlorpromazine effect on $InsP_3$ production in PC12 cells

Preincubation	*Stimulation*	*$InsP_3$ production* (pmol/mg protein)
Vehicle	None	1.13 ± 0.04
	Bradykinin	6.90 ± 0.95
Chlorpromazine	None	1.41 ± 0.41
	Bradykinin	7.23 ± 0.73

PC12 cells were preincubated with or without 100 μM chlorpromazine for 3 min and then treated with 300 nM bradykinin for 15 s. Each result is the mean $\pm$ s.e.mean of triplicate assays. The experiments were performed three times independently, and the results were reproducible.

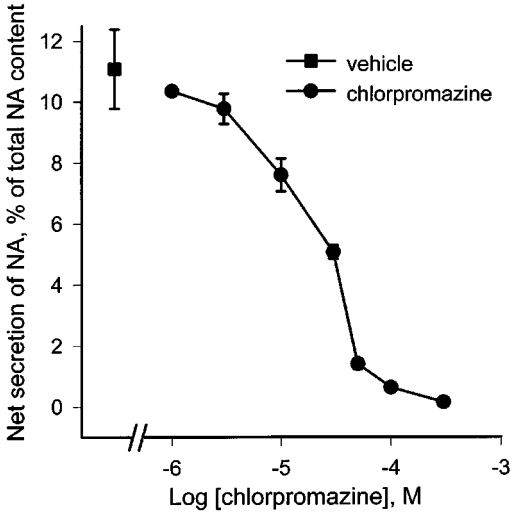

Figure 2 Inhibitory effect of chlorpromazine on the bradykinin-induced noradrenaline secretion of PC12 cells. [^{3}H]-noradrenaline-loaded PC12 cells were treated with 300 nM bradykinin for 10 min in the presence of the indicated concentrations of chlorpromazine. The secretion of [^{3}H]-noradrenaline induced by bradykinin in the absence of chlorpromazine is also presented. Three separate experiments were done, and each point represents a mean $\pm$ s.e.mean value. The results were reproducible.

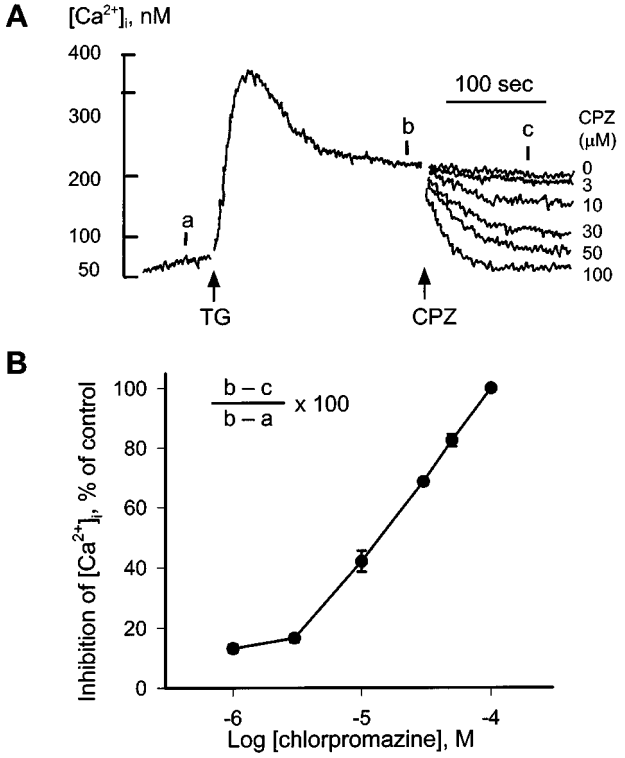

Figure 3 Effect of chlorpromazine on thapsigargin-induced store-operated Ca^{2+} entry (SOCE) in PC12 cells. (A) Fura-2-loaded cells were treated with the indicated concentrations of chlorpromazine (CPZ) after an incubation with 1 μM thapsigargin (TG). (B) Concentration-dependency of the chlorpromazine effect on thapsigargin-induced SOCE. Cells were treated with various concentrations of chlorpromazine after an incubation with 1 μM thapsigargin. Inhibition of $[Ca^{2+}]_i$ rise was calculated with reference to the $[Ca^{2+}]_i$ level at the point a, b, and c in A using the equation $(b-c)/(b-a) \times 100$. Data are depicted as % of the thapsigargin-induced Ca^{2+} level rise without chlorpromazine treatment. Each point was obtained from triplicate experiments and represents the mean $\pm$ s.e.mean. The results were reproducible.

not interfere with thapsigargin's action on Ca^{2+}-ATPase (SERCA) in the membranes of the intracellular Ca^{2+} stores, but that it rather inhibits SOCE. The effects of chlorpromazine on the bradykinin- and thapsigargin-induced Ca^{2+} influx were confirmed by its inhibitory effects on the influx of Mn^{2+}. The decrease in fluorescence of fura-2 is correlated with the amount of cytosolic Mn^{2+}, which also indicates how much of Mn^{2+} influx through Ca^{2+} channels has occurred. The fluorescence quenching caused by either bradykinin (Figure 4A,B) or thapsigargin (Figure 4C,D) treatment in the presence of 500 μM extracellular Mn^{2+} was dramatically inhibited by chlorpromazine.

SK&F96365, an antagonist of SOCE (Merritt *et al*., 1990), was used to confirm the inhibitory effect of chlorpromazine on the thapsigargin-induced SOCE. In order to test whether chlorpromazine and SK&F96365 had the same effect on SOCE, we sequentially treated the cells with chlorpromazine and SK&F96365 to measure their effects on the thapsigargin-induced Ca^{2+} rise. SK&F96365 (20 μM) decreased the level of the thapsigargin-induced Ca^{2+} elevation during the sustained phase just as chlorpromazine had done. Furthermore, subsequent addition of chlorpromazine did not add to the inhibition (Figure 5A,B) and *vice versa* (Figure 5C,D). Chlorpromazine mimics SK&F96365 in inhibiting the thapsigargin-induced Ca^{2+} elevation. The results, therefore, suggest that SK&F96365 and chlorpromazine share a target site linked to the inhibition of the cytosolic Ca^{2+} elevation, which implies that chlorpromazine inhibits SOCE. In addition, both SK&F96365 and chlorpromazine markedly inhibited the bradykinin-induced noradrenaline secretion (Table 2). When the cells were simultaneously treated with SK&F96365 and chlorpromazine, there was no additive inhibition. Since the data correlate well with the data of the chlorpromazine effect on the thapsigargin-induced Ca^{2+} rise, they confirm that chlorpromazine inhibits SOCE.

Discussion

Studies elucidating the nature and role of SOCE have been mainly done in non-excitable cells such as T cells and neutrophils; thus, the involvement of SOCE in neurotransmitter secretion in excitable cells remained relatively less well understood. However, recent investigations have uncovered a role of SOCE in neuronal cells. The experiments in PC12 cells (Koizumi & Inoue, 1998) and bovine adrenal chromaffin cells (Fomina & Nowycky, 1999) revealed that intracellular Ca^{2+} depletion induces store-operated currents, a secondary increase in the intracellular Ca^{2+} level, and the secretion of neurotransmitters.

In our study, we demonstrated that chlorpromazine inhibited SOCE which occurs subsequent to PLC activation

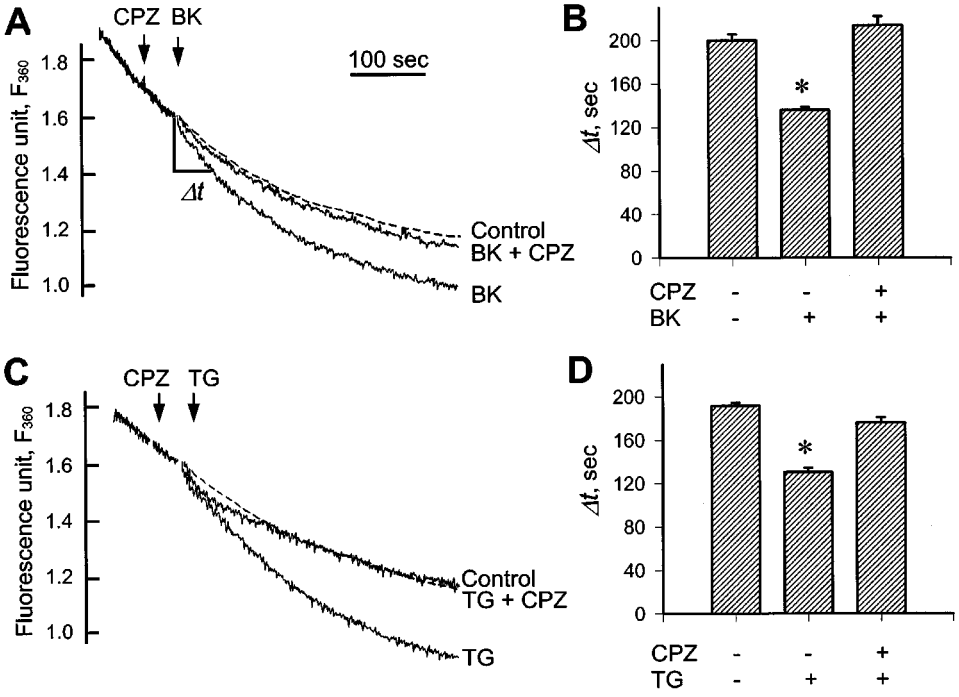

Figure 4 Effect of chlorpromazine on bradykinin- and thapsigargin-induced Mn^{2+} influx into PC12 cells. Mn^{2+}-induced fura-2 fluorescence quenching was recorded for fura-2-loaded cells preincubated with 500 μM Mn^{2+} and drug addition at the indicated times (arrow). The influx of Mn^{2+} was measured as described in the Methods. The data represent fluorescence intensities at 360 nm (F_{360}). (A) Bradykinin-induced Mn^{2+} influx was monitored with the stimulation with 300 nM bradykinin (BK) in the presence or absence of 50 μM chlorpromazine (CPZ). The trace without stimuli was depicted as dotted trace (Control). (B) The times (Δt) during the changes in fluorescence (from 1.6 to 1.4, arbitrary units) were quantitatively analysed using the results in A. Each point was obtained from triplicate experiments and represents the mean$\pm$s.e.mean. *$P<0.01$. (C) Thapsigargin-induced Mn^{2+} influx was monitored with or without the stimulation with 1 μM thapsigargin (TG) in the presence or absence of 50 μM chlorpromazine (CPZ). The trace without stimuli was depicted as dotted trace (Control). They are representative of four independent experiments. The results were reproducible. (D) The times (Δt) during the changes in fluorescence (from 1.6 to 1.4, arbitrary units) were quantitatively analysed using the results in C. Each point was obtained from triplicate experiments and represents the mean$\pm$s.e.mean. *$P<0.01$.

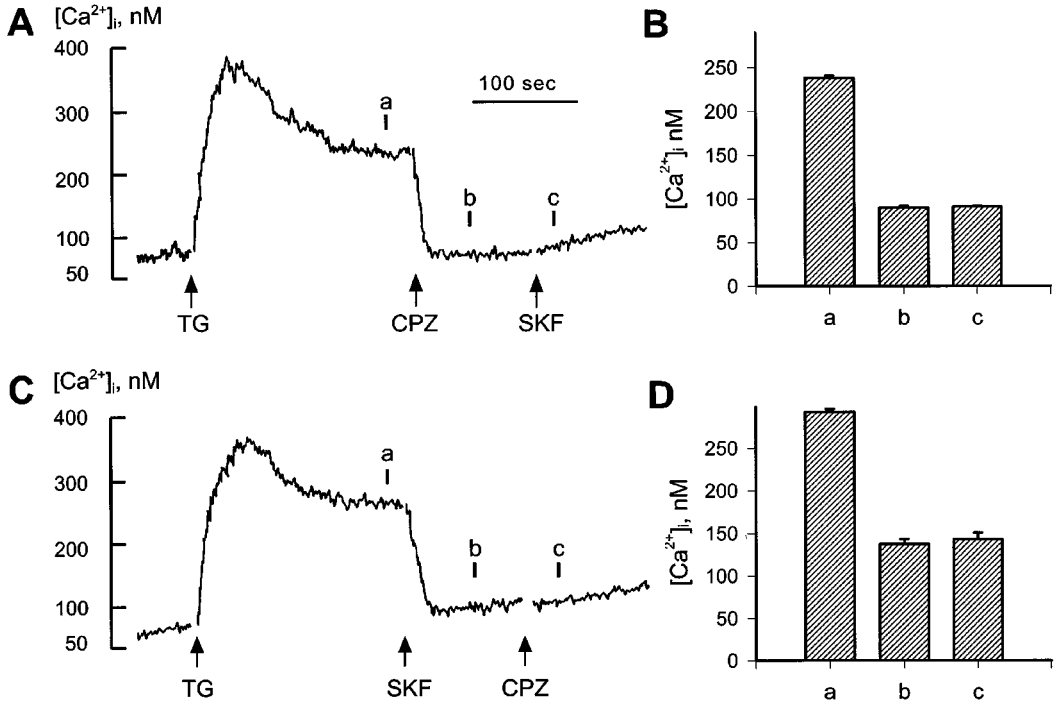

Figure 5 Effect of SK&F96365 on the inhibition of the thapsigargin-induced SOCE by chlorpromazine. (A) Fura-2-loaded PC12 cells were treated with 1 μM thapsigargin (TG), then challenged with 50 μM chlorpromazine (CPZ) in the presence of 20 μM SK&F96365 (SKF). (B) The $[Ca^{2+}]_i$ level at point a, b, and c were quantitatively analysed using the results in A. Each point was obtained from triplicate experiments and represents the mean $\pm$ s.e.mean. No statistical significance was evident between the data of b and c. (C) Cells were treated with 1 μM thapsigargin (TG), then challenged with 20 μM SK&F96365 (SKF) in the presence of 50 μM chlorpromazine (CPZ). The data are representative of more than four independent experiments. The results were reproducible. (D) The $[Ca^{2+}]_i$ level at point a, b, and c were quantitatively analysed using the results in C. Each point was obtained from triplicate experiments and represents the mean $\pm$ s.e.mean. No statistical significance could be seen between the data of b and c.

Table 2 The inhibitory effect of chlorpromazine and SK&F96365 on bradykinin-induced noradrenaline secretion by PC12 cells

Preincubation	*Stimulation*	*Noradrenaline secretion* (% of control)
Vehicle	None	0.62 ± 0.14
Vehicle	Bradykinin	11.08 ± 1.30
Chlorpromazine	Bradykinin	0.60 ± 0.23
SK&96365	Bradykinin	0.47 ± 0.20
Chlorpromazine + SK&F96365	Bradykinin	0.35 ± 0.03

[^{3}H]-Noradrenaline-loaded PC12 cells were preincubated with 20 μM SK&F96365 or 100 μM chlorpromazine, then treated with 300 nM bradykinin. The data are representative of three independent experiments, and each point is the mean $\pm$ s.e.mean of triplicate results. The results were reproducible.

and depletion of intracellular Ca^{2+} stores. The primary evidence was obtained from the data showing the chlorpromazine-induced inhibition was more obvious in the 'Ca^{2+}-decreasing state' rather than in the 'Ca^{2+}-increasing state. That is, the peak in the bradykinin-induced Ca^{2+} increase was not affected, whereas the sustained phase was inhibited by chlorpromazine. In addition, chlorpromazine inhibited the bradykinin-induced Ca^{2+} influx without affecting the initial Ca^{2+} release from internal stores in the absence of external Ca^{2+}. The results can be interpreted as chlorpromazine inhibited SOCE without affecting the pathways before the Ca^{2+} release. Secondly, chlorpromazine did not inhibit bradykinin-induced $InsP_3$ production, although chlorpromazine did inhibit the Ca^{2+} signalling mediated by PLC-linked receptors. Thirdly, chlorpromazine inhibited the sustained phase of Ca^{2+} elevation, when cells were treated with thapsigargin. Fourthly, the bradykinin- and thapsigargin-induced Mn^{2+} influx was also inhibited by chlorpromazine. Finally, in SK&F96365-treated cells, chlorpromazine did not add to the inhibition of the bradykinin- or thapsigargin-induced SOCE and noradrenaline secretion.

The mechanism of action of chlorpromazine still needs further studies. This is in part due to our limited understanding about Ca^{2+} release activated channels, the channels for SOCE. The Ca^{2+} release-activated channel, which is the target of chlorpromazine, is suggested to consist of *Trp* (Phillips *et al*., 1992; Scott *et al*., 1997). Until now, eight isoforms of *Trp* have been cloned and analysed. But it is still unclear which *Trp* actually acts as Ca^{2+} release activated channel. The opening mechanism of *Trp* remains also a subject of debate. Some studies suggest direct interaction between *Trp* and the $InsP_3$ receptor, while others suggest the involvement of a *Trp*-opening soluble factor which is generated upon Ca^{2+} store depletion. In addition, although several cytosolic factors, including tyrosine kinase (Lee *et al*., 1993), phosphatase (Meucci *et al*., 1995), protein kinase A (Song *et al*., 1998), and protein kinase C (Montero *et al*., 1994), have been suggested, the regulatory mechanisms of

SOCE remains unclear. Interestingly, chlorpromazine has been reported to affect calmodulin and to inhibit Ca^{2+}/calmodulin-sensitive protein kinase (Prozialeck *et al*., 1982; Klockner & Isenberg, 1987). However, this cannot explain the chlorpromazine-mediated inhibition of SOCE, because inhibition of the Ca^{2+}/calmodulin-sensitive protein kinase leads to the activation of SOCE. Thus, the study of SOCE events and their regulation must be further pursued in order to explain the target of chlorpromazine. A series of SOCE inhibitors have been reported. Their relative potency order can be listed as: arachidonic acid (IC_{50}: 8 μM; Alonso-Torre and Garcia-Sancho, 1997) = SK&F96365 (IC_{50}: 8.5 μM; Merritt *et al*., 1990) > capsaicin (IC_{50}: 25 μM; Choi and Kim, 1999) $\geqslant$ 2-aminophenyl butane (maximal effect at 75 μM; Rossum *et al*., 2000) > neomycin (maximal effect at 1 mM; Sipma *et al*., 1996). Apparently the mechanism of 2-aminophenyl butane involves disruption of the interaction between the $InsP_3$ receptor and *Trp* without affecting other channels of receptors. Most inhibitors are thought to act nonspecifically, because they also affect other channels, and their exact mechanisms of action are unclear with many possibilities such as direct interaction with the channel pore, changes in a property of the lipid bilayer, or inhibition of a cellular component affecting SOCE. Because of the high lipid-solubility of chlorpromazine, it is difficult to figure out whether it acts from the extracellular space or the intracellular space. Therefore, the precise action mechanism of chlorpromazine remains to be determined.

Many PLC-linked neurotransmitters or hormones induce SOCE as part of their cellular mechanism of action. Our results demonstrate that chlorpromazine inhibits noradrenaline secretion induced by the activation of PLC-linked receptors. Our results of the chlorpromazine-induced inhibition of SOCE suggest that chlorpromazine may act on other tissues and modulate their physiological responses mediated by PLC-linked receptors. Chlorpromazine's effect on SOCE could thus help us to better understand the pharmacological and toxicological effects of chlorpromazine.

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