Effects of Treatment with Haloperidol, Chlorpromazine, and Clozapine on Protein Kinase C (PKC) and Phosphoinositide-Specific Phospholipase C (PI-PLC) Activity and on mRNA and Protein Expression of PKC and PLC Isozymes in Rat Brain

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ABSTRACT
The effects of acute (single) and chronic (21-day) administration of haloperidol (HAL), chlorpromazine (CPZ), or clozapine (CLOZ) on components of the phosphoinositide (PI)-signaling pathway were studied in rat brain. Chronic administration of HAL decreased protein kinase C (PKC) activity and mRNA and protein levels of PKC\(\alpha\) and \(\epsilon\) isozymes in both membrane and cytosol fractions of cortex, hippocampus, and striatum. Chronic administration of CPZ, however, decreased PKC activity only in the membrane fraction of cortex, hippocampus, and striatum, and had no effect on the levels of any PKC isozymes. On the other hand, chronic administration of CLOZ decreased PKC activity and mRNA and protein levels of PKC\(\alpha\), \(\gamma\), and \(\epsilon\) isozymes in membrane and cytosol fractions of cortex, hippocampus, and cerebellum. Studies of the effects on phospholipase C (PLC) revealed that only chronic administration of CPZ significantly decreased PI-PLC activity and mRNA and protein levels of the specific PLC\(\beta_1\) isozyme in membrane and cytosol fractions of cortex, hippocampus, cerebellum, and striatum. Acute-treatment data suggest that CPZ or CLOZ had no significant effects on PI-PLC or PKC; however, HAL translocated PKC, as evidenced from increased PKC activity and protein levels of PKC\(\alpha\) and \(\epsilon\) isozymes in the membrane fraction and the decrease in these parameters in the cytosol fraction of cortex, hippocampus, and striatum. Our results thus suggest that the interaction of antipsychotic drugs with PKC and PLC may be associated with their mechanisms of action.

The therapeutic efficacy of antipsychotic drugs is generally believed to be due to their ability to block central dopamine D\(2\) receptors (Seeman, 1976; Farde et al., 1989; Deutche et al., 1991; Dixon et al., 1995). Despite this supposed commonality, typical and atypical antipsychotic drugs have been shown to have different clinical and behavioral profiles. Most typical antipsychotic drugs [e.g., haloperidol (HAL) and chlorpromazine (CPZ)] induce extrapyramidal side effects, including a parkinsonism-like syndrome, and tardive dyskinesia (McEvoy, 1983); but atypical antipsychotic drugs [e.g., clozapine (CLOZ)] are associated with a low incidence of extrapyramidal side effects and tardive dyskinesia. Also, CLOZ has been found to be effective for the treatment-resistant negative symptoms of schizophrenia (Deutsch et al., 1991). The molecular mechanisms for these different actions of typical and atypical antipsychotic drugs are not clear.

Many overlapping hypotheses have been proposed. For example, it has been believed that the blockade of dopamine D\(2\) receptors in either nigrostriatal (typical antipsychotic drugs) or mesolimbic (atypical antipsychotic drugs) dopaminergic systems may be responsible for the actions of antipsychotic drugs (Wilk et al., 1975). On the other hand, their higher affinity for serotonin 2A (5-HT\(2A\); Meltzer et al., 1989; Leysen et al., 1993), 5-HT\(2C\), \(\alpha_1\), and \(\alpha_2\)-adrenergic, and muscarinic receptors (Baldessarini et al., 1992; Hietala et al., 1992; Kuoppamaki et al., 1993; Schotte et al., 1996) has been postulated to be the mechanism responsible for the action of atypical antipsychotic drugs.

Because 5-HT\(2A\), 5-HT\(2C\), \(\alpha_1\)-adrenergic, muscarinic, and dopamine D\(2\) receptors have been shown to be altered after chronic administration of antipsychotic drugs, and because these receptors are coupled with the phosphatidylinositol (PI)-signaling system, several studies have been performed to evaluate whether typical and atypical antipsychotic drugs have a common mechanism of action—that of interacting with the PI-signaling pathway. These studies showed that chronic administration of HAL to rats causes a decrease in

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ABBREVIATIONS: HAL, haloperidol; IP\(_3\), inositol 1,4,5-trisphosphate; PDBu, phorbol 12,13-dibutyrate; PI, phosphatidylinositol; PI\(_{1,4,5}\), phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PKC, protein kinase C; CLOZ, clozapine; CPZ, chlorpromazine; G protein, guanine nucleotide binding protein; bp, base pair.
norepinephrine- and dopamine-induced PI hydrolysis (Li et al., 1991, 1993). In contrast, chronic administration of CPZ has been shown to cause an increase in PI metabolism in rat and guinea pig brain slices (Hokin-Neaverson, 1980) and in C6 glioma cells in vitro (Leli et al., 1989). On the other hand, CLOZ inhibits 5-HT\textsubscript{2A}/\textsubscript{C} receptor-mediated PI hydrolysis in rat brain (Canton et al., 1994; Kuoppamaki et al., 1994). From these studies, it appears that all these three different antipsychotic drugs interact with the PI-signaling pathway.

In the PI-signaling pathway, agonist-induced interaction of cell surface receptors with G proteins (G\textsubscript{q/11} α, G\textsubscript{i} α, and G β\textsubscript{δ}) causes activation of the phospholipase C (PLC) enzyme, which in turn causes hydrolysis of phosphatidylinositol 4,5-bisphosphate (P\textsubscript{i}P\textsubscript{2}) into inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and diacylglycerol. IP\textsubscript{3} mobilizes Ca\textsuperscript{2+} from intracellular sources, whereas diacylglycerol activates protein kinase C (PKC) (reviewed by Majewski and Iannazzo, 1998). Thus, all three components of the PI-signaling pathway, i.e., G proteins, PLC, and PKC, play important roles in mediating receptor-induced functional responsiveness.

Although chronic administration of antipsychotics causes changes in PI hydrolysis in rat brain, there have been no studies regarding their effects on components of the PI-signaling pathway except that in vitro addition of CPZ to rat brain slices inhibits PKC activity (Mori et al., 1980; Schatzman et al., 1981) and PKC-mediated phosphorylation of endogenous PKC substrates (Kumar et al., 1997).

The aim of this study was to elucidate the molecular mechanisms of the actions of typical and atypical antipsychotic drugs by studying the interactions of these drugs with various components of the PI-signaling pathway. Therefore, we systematically examined the effects of acute (single-dose) and chronic (daily for 21 days) administration of HAL, CPZ, or CLOZ to rats on various components of the PI-signaling system, i.e., PKC, PLC, and α subunits of G proteins linked to dopamine D1/D2 receptors in rat brain, and 1 mg of HAL is approximately clinically equivalent to 15 to 20 mg of CLOZ (Wilmot and Szczepanik, 1988). The selection of the doses of CLOZ and CPZ was based on previous studies that indicated sufficient effects of these antipsychotic drugs on 5-HT\textsubscript{2A}/\textsubscript{C} receptors and up-regulation of dopamine D1 and D2 receptors, thus showing appropriate central nervous system activity (Kuoppamaki et al., 1993). The animals were decapitated 24 h after the last injection, and brains were removed quickly. Cortices, hippocampi, cerebellums, and striata were dissected out and immediately stored at −80°C until analysis.

Biochemical determinations in striatum, the striata from two rats were pooled.

[H\textsubscript{3}]PDBu Binding to Membrane and Cytosol PKC in Rat Brain. [H\textsubscript{3}]PDBu binding to membrane and cytosol PKC was determined by a radioligand-binding technique described earlier (Dwivedi and Pandey, 1999a). The procedure is as follows.

Preparation of Membrane and Cytosol Fractions. Tissues were homogenized with a Polytron at a setting of 8 for 15 s in 10 volumes of homogenizing buffer (50 mM Tris-HCl, pH 7.4, 2 mM EGTA, 1.0 mM MgCl\textsubscript{2}, and 1.0 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 100,000 \( \times g \) for 60 min at 4°C. The supernatant (S\textsubscript{1}) was saved. The pellet was rehomogenized and recentrifuged as described above. The supernatant (S\textsubscript{2}) was mixed with S\textsubscript{1}. The resultant pellet and the combined supernatant (S\textsubscript{1} + S\textsubscript{2}) fractions were used to measure B\textsubscript{max} and K\textsubscript{d} of [H\textsubscript{3}]PDBu binding to membrane and cytosol PKC, respectively.

PDBu Binding to Membrane PKC. The final pellet obtained from the procedure was resuspended in the required amount of incubation buffer (50 mM Tris-HCl, pH 7.4, 1 mM CaCl\textsubscript{2}, 75 mM magnesium acetate, 0.1% BSA, and 50 μg/ml phosphatidylserine). The binding assay was carried out in duplicate tubes containing the incubation buffer, [H\textsubscript{3}]PDBu ranging in concentration from 0.8 to 30 nM (six different concentrations), and 150 μl of membrane suspension with or without 10 μM PMA in a total volume of 500 μl. The tubes were incubated for 30 min at 37°C. Bound [H\textsubscript{3}]PDBu was separated from free [H\textsubscript{3}]PDBu by addition of 0.5 ml of washing buffer (50 mM Tris-HCl, pH 7.4, 0.1% BSA), and rapid filtration through a Whatman GF/B filter. Air-dried filters were used for counting radioactivity by a liquid scintillation counter.

[H\textsubscript{3}]PDBu Binding to Cytosol PKC. The binding assays for the cytosol fraction were carried out in duplicate tubes containing incubation buffer (50 mM Tris-HCl, pH 7.4, 1 mM CaCl\textsubscript{2}, 75 mM magnesium acetate, and 0.1% BSA), 150 μl of cytosol fraction, [H\textsubscript{3}]PDBu (0.8–30.0 nM, six different concentrations), bovine γ-globulin (100 μg/ml), and phosphatidylserine (50 μg/ml) in a total volume of 500 μl. The tubes were incubated for 30 min at 37°C. The tubes were then chilled, and proteins were precipitated by addition of 200 μl of chilled 12% (w/v) polyethylene glycol (in 50 mM Tris-HCl, pH 7.4). To allow complete precipitation, the samples were kept for 15 min at 4°C. Bound [H\textsubscript{3}]PDBu was separated from free [H\textsubscript{3}]PDBu by the method described above.

The specific binding in membrane and cytosol fractions was de-
fined as the difference between the binding observed in the presence and absence of 10 μM PMA. B_{max} and K_D were calculated by Scatchard analysis (McPherson, 1985), and protein content was determined by the method of Lowry et al. (1951) with BSA as the standard.

**Determination of PKC Activity in Membrane and Cytosol Fractions of Rat Brain.** PKC activity in subcellular tissue fractions was measured by the procedure described earlier (Dwivedi and Pandey, 1999a). The Amersham enzyme assay system was used to determine PKC activity, and a PKC-specific target peptide and all reagents were prepared according to the manufacturer’s recommendations. PKC activity was measured in membrane and cytosol fractions by procedures described (Dwivedi and Pandey, 1999b) with 5 μg of protein per tube in an incubation buffer (20 mM Tris-HCl, 1 mM CaCl₂, and 100 mM KCl, pH 7.4) containing 10 mM lithium chloride, Pip₂ substrate (50 μM unlabelled Pip₂, 2.0 μCi/ml [³²P]Pip₂, and 0.5 mg/ml cerebromed) in a total volume of 100 μl at 37°C for 10 min. The reaction was terminated by addition of 500 μl of 1 M HCl and 500 μl of a mixture of chloroform/methanol (1:1 v/v). The tubes were vigorously mixed and centrifuged at 1,000g for 10 min. The aqueous (upper) phase was transferred to a scintillation vial containing scintillation liquid, and the radioactivity was counted in a liquid scintillation counter. Each experiment had its blank, in which the protein suspension was added after stopping the reaction with chloroform/methanol. PKC activity is expressed as the amount of [³²P]IP₃ formed (dpm) per milligram of protein.

**Determination of mRNA levels of PKC α, γ, and ε Isozymes and of PLC β₁ Isozyme by Competitive Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) in Rat Brain**

**RNA Isolation.** The procedures of RNA isolation and competitive RT-PCR analysis have been described previously (Dwivedi and Pandey, 1999b). Brain tissues were homogenized in 4 M guanidine isothiocyanate, 50 mM Tris-HCl (pH 7.4), and 25 mM EDTA, and the total RNA was isolated by CsCl ultracentrifugation. The yield of total RNA was determined by measuring the absorbency of an aliquot of the precipitated stock at a wavelength of 260/280 nm. To check for possible DNA contamination, after each extraction, tissue samples were run by RT-PCR without adding the RT enzyme.

**Oligonucleotides.** The primer pairs were designed to allow amplification for PKC α (659–982 base pairs (bp): forward, 5’-TGACCTCTCGTGAAGAATCTGCTCCT; reverse, 5’-ATGGCTGTCTTCTGCTTCTGAAAGATCTTCCCCGACC). The single-strand internal primers were designed by site-directed mutagenesis to introduce a 24-bp overlap of the primary PCR products. The primer pairs were designed to allow amplification for PKC α (659–982 base pairs (bp): forward, 5’-TGACCTCTCGTGAAGAATCTGCTCCT; reverse, 5’-ATGGCTGTCTTCTGCTTCTGAAAGATCTTCCCCGACC). The single-strand internal primers were designed by site-directed mutagenesis to introduce a 24-bp overlap of the primary PCR products.
products. Each of the internal standards was synthesized in two PCR steps, starting with a cDNA template reverse transcribed from rat brain RNA. The internal standard templates were linearized with Sp6I. The cRNA corresponding to sense strand was synthesized with linearized template and Sp6 RNA polymerase by means of an in vitro transcription kit.

Quantitative Analyses of PKC \( \alpha \), \( \gamma \), and \( \epsilon \) Isozymes and PLC \( \beta_1 \), isoyme mRNAs by Competitive RT-PCR. Decreasing concentrations of PKC \( \alpha \), \( \gamma \), \( \epsilon \), and of PLC \( \beta_1 \) internal standards (cRNA) were added to 1 \( \mu \)g of total RNA isolated from different areas of rat brain. The RNA-cRNA mixtures were denatured at 80°C for 6 min and then reverse transcribed with cloned Moloney murine leukemia virus reverse transcriptase (200 U) in RT buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl\(_2\), and 1 mM deoxynucleotide triphosphate with random hexamers (5 mM) and ribonuclease inhibitor (28 U) in a volume of 20 \( \mu \)l. The RT mixture was incubated at 37°C for 60 min to promote cDNA synthesis. The reaction was terminated by heating the tissue samples at 98°C for 5 min. In all assays, as a control, one RT reaction was performed in the absence of RNA.

Competitive PCR Amplification. After termination of the RT reaction, cDNA aliquots containing reverse-transcribed material were amplified with Hot Tub DNA polymerase in the Thermal Cycler (9600, Perkin-Elmer, Norwalk, CT). The amplification mixture contained cDNA, 0.5-\( \mu \)M specific primer pairs, 200 \( \mu \)M deoxynucleotide triphosphate, 1.5 mM MgCl\(_2\), 50 mM Tris-HCl (pH 9.0), 20 mM ammonium sulfate, 15 mM KCl, and 1.5 U of Hot Tub DNA polymerase in a 100-\( \mu \)l volume. Trace amounts of [\( ^3 \)H]dCTP (0.5–1.0 \( \mu \)Ci/sample) were included during the PCR step for subsequent quantification. The PCR mixture was amplified for 30 cycles with denaturation (94°C, 15 s), annealing (60°C, 30 s), and elongation (72°C, 30 s) amplification steps. The reaction was terminated with a 5-min final elongation step. After amplification, aliquots were digested with BglII in triplicate and run by 1.5% agarose gel electrophoresis.

To quantitate the amount of product corresponding to the reverse-transcribed and amplified mRNA, the ethidium bromide-stained bands were excised and counted. The results were calculated as the counts incorporated into the amplified cRNA standard divided by the counts incorporated into the corresponding isoyme mRNA amplification product versus a known amount of internal standard cRNA added to the test sample. The results are expressed as attomol PKC or PLC isoyme mRNA per microgram of total RNA.

Quantitation of \( G_{\alpha_1} \) \( \alpha \) and \( G_{\alpha_0} \) \( \alpha \) Proteins in Rat Brain by Western Blot. Gel electrophoresis and immunolabeling of \( G_{\alpha_1} \) \( \alpha \) and \( G_{\alpha_0} \) \( \alpha \) proteins were performed by the Western blot technique with a method described earlier (Dwivedi and Pandey, 1997). The tissues were homogenized in a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM MgCl\(_2\), 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, and 0.2 U/ml aprotinin and centrifuged at 3000 rpm for 10 min. The supernatant was recentrifuged at 32,000 rpm for 15 min. The resultant pellet was resuspended in the same buffer. Protein content in the tissue samples was determined by the method of Lowry et al. (1951) with BSA as a standard. Aliquots (15 \( \mu \)l) of membrane suspension were added to 15 \( \mu \)l of 50 mM Tris-HCl (pH 6.8), 1 mM diithiothreitol, and 2% SDS and incubated for 5 min at 75°C. After incubation, 20 \( \mu \)l of 100 mM N-ethylmaleimide was added, and tissue samples were incubated for another 15 min at 21°C. Finally, 50 \( \mu \)l of gel-loading solution was added, and tissue samples were boiled for 3 min and kept on ice for 10 min. The tissue samples (20 \( \mu \)g of protein/lane) were loaded onto 10% (w/v) polyacrylamide gel. Gel electrophoresis and subsequent steps were performed similarly as described previously for the immunolabeling of PKC and PLC isoymes. The blots were incubated overnight at 4°C with primary monoclonal antibody (anti-\( G_{\alpha_1} \) \( \alpha \) or \( G_{\alpha_0} \) \( \alpha \)) at a dilution of 1:3000 with horseradish peroxidase-linked secondary antibody (anti-rabbit IgG, 1:3000) for 3 to 5 h at room temperature. The filters were extensively washed with TBST and exposed to ECL film. \( \beta \)-Actin antibody was probed in the same membrane. The bands on the autoradiograms were quantitated as described previously for the immunolabeling of PKC and PLC isoymes.

Statistics

Data were analyzed with the SPSS 8.0 (Chicago, IL) statistical software package. All values are given as the means ± S.D. Inter-group comparisons were made by ANOVA. Bonferroni multiple comparisons were used to evaluate pairwise differences. An \( \alpha \) value lower than .05 was considered significant.

Results

Effects of Chronic HAL, CPZ, or CLOZ Treatment on Body Weight. There were no significant differences in body weight gain among the three treatment groups compared with the saline-treated normal control rats. The body weights of rats before/after 3 weeks of treatment were 242 ± 16/290 ± 26, 248 ± 21/304 ± 29, 251 ± 19/293 ± 21, and 239 ± 23/280 ± 18 g in saline- , HAL-, CLOZ-, and CPZ-treated groups, respectively (\( n = 12 \) group, mean ± S.D.).

Effects of Acute and Chronic Administration of HAL, CPZ, or CLOZ on \(^3\)H]PDBu Binding to PKC in Membrane and Cytosol Fractions of Rat Brain. We observed that neither single nor chronic administration of HAL, CPZ, or CLOZ caused any significant effects on \( B_{\max } \) or \( K_D^D \) of \(^3\)H]PDBu binding to PKC in cytosol and membrane fractions obtained from cortex, hippocampus, cerebellum, and striatum (data not shown). \( B_{\max } \) and \( K_D^D \) for \(^3\)H]PDBu binding to PKC in membrane and cytosol fractions of various brain areas from control rat brain were as follows: cortex—\( B_{\max } \) membrane, 21.5 ± 3.1 fmol/mg of protein; cytosol, 32.8 ± 1.8 fmol/mg of protein; \( K_D^D \) membrane, 7.1 ± 0.7 nM; cytosol, 3.5 ± 1.2 nM; hippocampus—\( B_{\max } \) membrane, 26.2 ± 1.8 fmol/mg of protein; cytosol, 36.7 ± 4.13 fmol/mg of protein; \( K_D^D \) membrane, 6.5 ± 2.1 nM; cytosol, 3.1 ± 1.2 nM; cerebellum—\( B_{\max } \) membrane, 15.5 ± 2.1 fmol/mg of protein; cytosol, 24.8 ± 2.6 fmol/mg of protein; \( K_D^D \) membrane, 7.1 ± 1.2 nM; cytosol, 3.5 ± 0.9 nM.

Effects of Acute and Chronic Administration of HAL, CPZ, or CLOZ on PKC Activity in Membrane and Cytosol Fractions of Rat Brain. The results of the experiments examining the effects of acute administration of HAL, CPZ, or CLOZ on PKC activity in different brain areas are shown in Fig. 1. We observed that a single administration of HAL significantly decreased PKC activity in the cytosol fractions and significantly increased PKC activity in the membrane fractions obtained from the cortex, hippocampus, and striatum without any significant effects in the cerebellum. On the other hand, acute administration of CPZ or CLOZ had no significant effects on PKC activity in either the membrane or the cytosol fractions in any of the brain areas studied.

The effects of repeated administration of HAL, CLOZ, or CPZ on PKC activity in cortex, hippocampus, cerebellum, and striatum are depicted in Fig. 2. It was observed that chronic administration of HAL significantly decreased PKC activity in both membrane and cytosol fractions of cortex, hippocampus, and striatum but produced no significant changes in the cerebellum.

Chronic administration of CPZ, on the other hand, de-
Fig. 1. Effects of acute treatment with HAL, CPZ, or CLOZ on PKC activity in different areas of rat brain. Rats were given single i.p. injections with HAL (1 mg/kg), CPZ (10 mg/kg), or CLOZ (20 mg/kg) and decapitated 24 h after the injection. Values are means ± S.D. from six rats in each group. HAL-, CPZ-, and CLOZ-treated groups were compared with the control group. *p < .01–.001.
Fig. 2. Effects of chronic administration of HAL, CPZ, or CLOZ on PKC activity in different areas of rat brain. Rats were given i.p. injections with HAL (1 mg/kg), CPZ (10 mg/kg), or CLOZ (20 mg/kg) once daily for 21 days. Rats were decapitated 24 h after the last injection. Values are the means ± S.D. from six rats in each group. HAL-, CPZ-, and CLOZ-treated groups were compared with the control group. *p < .01–.001.
increased PKC activity in the membrane fractions of cortex, hippocampus, and striatum without having any effects in the cytosol fractions. Chronic administration of CPZ had no significant effect on PKC activity in either the membrane or the cytosol fraction of the cerebellum (Fig. 2).

CLOZ given for 3 weeks caused a significant decrease in PKC activity in both membrane and cytosol fractions of cerebral cortex, hippocampus, and cerebellum but had no significant effect in the striatum (Fig. 2).

**Effects of Acute and Chronic Administration of HAL, CPZ, or CLOZ on Immunolabeling of PKC Isozymes in Membrane and Cytosol Fractions of Rat Brain.** The steady-state concentrations of protein levels of α, βI, βII, γ, δ, ε, and ζ isoforms of PKC in both membrane and cytosol fractions of cortex, hippocampus, cerebellum, and striatum were determined after single and chronic administration of HAL, CPZ, or CLOZ. Representative Western blots showing immunolabeling of PKC isozymes in the cortical brain area are given in Fig. 3. Similar to our earlier report (Dwivedi and Pandey, 1999a), in this study, we also observed that the molecular mass for PKC α and δ were 80 kDa, whereas the molecular mass for PKC γ and ε were 78 and 90 kDa, respectively. The molecular mass for PKC βI, βII, and ζ were 80 kDa. β-Actin was used as a housekeeping protein and migrated to 46 kDa.

The effects of acute administration of HAL, CPZ, or CLOZ on the expression of PKC α, γ, and ε isoforms (where we observed changes) are given in Fig. 4. It was observed that the acute administration of HAL increased the immunolabeling of PKC α and ε isoforms in the membrane fractions along with a decrease in the immunolabeling of these isoforms in the cytosolic fractions of cortex, hippocampus, and striatum. Acute administration of HAL had no significant effects on the immunolabeling of PKC βI, βII, δ, γ, or ζ isoforms (data not shown). Moreover, acute administration of HAL had no significant effect on the immunolabeling of any of the PKC isozymes in the cerebellum. Acute administration of CLOZ or CPZ produced no significant effect on the immunolabeling of PKC α, γ, δ, ε (Fig. 4), βI, βII, δ, or ζ (data not shown) isoforms in the membrane or cytosolic fractions of cortex, hippocampus, cerebellum, or striatum.

The effects of chronic administration of HAL, CPZ, or CLOZ on steady-state levels of PKC α, γ, and ε isoforms in different brain areas are depicted in Fig. 5, and representative Western blots showing the effects of chronic administration of HAL, CPZ, or CLOZ on the immunolabeling of PKC isozymes in the cortex is given in Fig. 3. It was observed that chronic administration of HAL significantly decreased the expressed protein levels of PKC α and ε isoforms in both membrane and cytosol fractions of cortex, hippocampus, and striatum (Fig. 5) without producing any significant changes in the protein levels of PKC βI, βII, δ, γ, or ζ isoforms (data not shown), whereas HAL had no significant effect on the protein levels of any of the PKC isozymes in the cerebellum. Chronic administration of CPZ to rats did not cause any significant changes in steady-state levels of any of the PKC isoforms either in membrane or in cytosol fractions obtained from cortex, hippocampus, striatum, and cerebellum (Fig. 5).

As shown in Fig. 5, we observed that CLOZ significantly decreased the protein levels of PKC α, γ, and ε isoforms in both membrane and cytosol fractions of cortex, hippocampus, and cerebellum without producing any effects on the protein levels of PKC βI, βII, δ, or ζ isoforms. Administration of CLOZ had no significant effect on the immunolabeling of any of the PKC isozymes in the striatum.

**Effects of Chronic Administration of HAL, CPZ, or CLOZ on mRNA Levels of PKC α, γ, and ε Isozymes.** To determine whether the decrease in protein levels of PKC α and ε isoforms caused by chronic treatment with HAL and the decrease in levels of PKC α, γ, and ε isoforms by CLOZ were due to a decrease in gene expression of these isoforms, we determined mRNA levels of PKC α, γ, and ε isoforms with the quantitative RT-PCR technique in all the brain areas in which we determined protein levels of PKC α, γ, and ε isoforms. Representative gel electrophoreses showing competitive RT-PCR for PKC α, γ, and ε isoforms in the cortex are given in Figs. 6A, 7A, and 8A, respectively. In addition, representative graphs showing the quantitation of mRNA for PKC α, γ, and ε isoforms are given in Figs. 6B, 7B, and 8B, respectively. As expected, we observed the amplification products for PKC α arising from the mRNA template at 327 bp and the corresponding digestion products arising from cRNA at 174 + 153 bp (Fig. 6A); for PKC γ template at 307 bp and cRNA at 157 + 147 bp (Fig. 7A); for PKC ε template at 321 bp and cRNA at 164 + 157 bp (Fig. 8A). Using this approach, we determined the absolute amounts of PKC α, γ, and ε isoform mRNAs after chronic administration of HAL, CLZ, or CLOZ. We observed that chronic administration of HAL significantly decreased mRNA levels of PKC α (Fig. 6C) and ε (Fig. 8C) in cortex, hippocampus, and striatum, but it had no effect in the cerebellum. On the other hand, chronic treatment with CLOZ significantly decreased the levels of PKC α (Fig. 6C), γ (Fig. 7C), and ε (Fig. 8C) isoforms in cortex, hippocampus, and cerebellum but not in striatum. We did not observe any significant effects of CPZ on the mRNA levels.

![Fig. 3. Representative Western blots showing the effects of chronic administration (21 days) of HAL (1 mg/kg), CPZ (10 mg/kg), or CLOZ (20 mg/kg) on immunolabeling of PKC isozymes in rat cortex. Protein samples (25 μg) were subjected to 7.5% polyacrylamide gel electrophoresis and transferred to ECL-intracellulose membranes, which were then incubated with primary antibodies specific for PKC α, βI, βII, γ, δ, ε, or β-actin, and secondary anti-rabbit (for PKC isozymes) or anti-mouse (for β-actin) antibody. The bands were quantified as described in Experimental Procedures. The ratios of optical densities of the PKC isozymes to that of β-actin were calculated.](image-url)
Fig. 4. Effects of acute treatment (single i.p. injection) with HAL (1 mg/kg), CPZ (10 mg/kg), or CLOZ (20 mg/kg) on immunolabeling of PKC isozymes in membrane and cytosol fractions obtained from different areas of rat brain. Values are the means ± S.D. from six rats in each group. HAL-, CPZ-, and CLOZ-treated groups were compared with the control group. *p < .001. Note that we did not observe any significant effects of acute treatment with HAL, CPZ, or CLOZ on immunolabeling of PKC βI, βII, δ, and ε isozymes in cortex, hippocampus, cerebellum, or striatum.
Fig. 5. Effects of chronic treatment (i.p. injections once daily for 21 days, 24 h after the last injection) of HAL (1 mg/kg), CPZ (10 mg/kg), or CLOZ (20 mg/kg) on immunolabeling of PKC isozymes in different areas of rat brain. Values are the means ± S.D. from six rats in each group. HAL-, CPZ-, and CLOZ-treated groups were compared with the control group. *p < 0.01–0.001. Note that we did not observe any significant effects of acute treatment with HAL, CPZ, or CLOZ on immunolabeling of PKC βI, βII, δ, and ε isozymes in cortex, hippocampus, cerebellum, or striatum.
levels of PKC α, γ, or ε isozymes in any of the brain areas studied.

Effects of Acute and Chronic Administration of HAL, CPZ, or CLOZ on PI-PLC Activity in Rat Brain. PI-PLC activity was determined in membrane and cytosol fractions of cortex, hippocampus, cerebellum, and striatum brain regions after acute and chronic administration of HAL, CPZ, or CLOZ. PI-PLC activities in various areas of control rat brain were as follows: cortex—membrane, 21,580 ± 6186 dpm · min⁻¹ · mg⁻¹ of protein, cytosol, 22,618 ± 1385 dpm · min⁻¹ · mg⁻¹ of protein; hippocampus—membrane, 30,115 ± 1886 dpm · min⁻¹ · mg⁻¹ of protein, cytosol, 32,316 ± 1867 dpm · min⁻¹ · mg⁻¹ of protein; cerebellum—membrane, 17,365 ± 1678 dpm · min⁻¹ · mg⁻¹ of protein, cytosol, 23,159 ± 2148 dpm · min⁻¹ · mg⁻¹ of protein; and striatum—membrane, 13,581 ± 967 dpm · min⁻¹ · mg⁻¹ of protein, cytosol, 21,266 ± 1358 dpm · min⁻¹ · mg⁻¹ of protein. We did not observe any significant effects of acute administration of HAL, CPZ, or CLOZ on PI-PLC activity in membrane and cytosol fractions of cortex, hippocampus, cerebellum, or striatum. The results of chronic administration of HAL, CPZ, or CLOZ on PI-PLC activity in cortex, hippocampus, cerebellum, and striatum are provided in Fig. 8. We observed that CPZ significantly decreased PI-PLC activity in both membrane and cytosol fractions of cortex, hippocampus, striatum, and cerebellum. Chronic administration of CLOZ and HAL, however, failed to produce any significant effects on PI-PLC activity in membrane or cytosol fractions in any of the brain regions studied (Fig. 9).

Effects of Acute and Chronic Administration of HAL, CPZ, or CLOZ on the Immunolabeling of PLC Isozymes. Consistent with what we reported earlier (Dwivedi and Pandey, 1999b), Western blot analysis showed that PLC β₁ migrated to 150 kDa, whereas PLC γ₁ and δ₁ migrated to 145 and 85 kDa, respectively (Fig. 10). Acute administration of HAL, CPZ, or CLOZ did not cause any significant effects on steady-state levels of PLC β₁, δ₁, and γ₁ isozymes in either membrane or cytosol fractions obtained from cortex, hippocampus, cerebellum, or striatum (data not provided).
The results of repeated administration of HAL, CPZ, or CLOZ on the immunolabeling of PLC isozymes in the various brain areas are given in Fig. 11, and representative Western blots showing the immunolabeling of the various PLC isozymes in the cortex are given in Fig. 10. It was observed that chronic administration of CPZ significantly decreased the expression of the PLC \( \beta_1 \) isozyme in both membrane and cytosol fractions obtained from cortex, hippocampus, cerebellum, and striatum without any significant effects on the levels of PLC \( \delta_1 \) and \( \gamma_1 \) isozymes. In contrast to the results with CPZ, chronic administration of HAL or CLOZ had no significant effects on the immunolabeling of PLC \( \beta_1 \), \( \delta_1 \), and \( \gamma_1 \) isozymes in any of the brain areas studied.

**Effects of Chronic Administration of HAL, CPZ, or CLOZ on mRNA Levels of the PLC \( \beta_1 \) Isozyme.** To examine whether the decrease in protein levels of PLC \( \beta_1 \) after chronic administration of CPZ was due to reduced expression of the PLC \( \beta_1 \) gene, we determined the mRNA levels of the PLC \( \beta_1 \) isozyme in the various brain areas. A representative gel electrophoresis of the PLC \( \beta_1 \) isozyme in rat hippocampus is given in Fig. 12A. As expected, we observed the amplification product arising from the mRNA template at 316 bp and the digestion product at 214 bp. A representative graph showing the quantitation of PLC \( \beta_1 \) mRNA is given in Fig. 12B. The effects of chronic administration of HAL, CPZ, or CLOZ on the mRNA expression of PLC \( \beta_1 \) in various brain areas are given in Fig. 12C. We observed that chronic administration of CPZ significantly decreased the mRNA expression of PLC \( \beta_1 \) in cortex, hippocampus, cerebellum, and striatum. However, the chronic administration of HAL or CLOZ had no significant effect on the mRNA levels of the PLC \( \beta_1 \) isozyme in any of the brain areas studied (Fig. 12C).

**Effects of Acute and Chronic Administration of HAL, CPZ, or CLOZ on the Immunolabeling of \( \text{G}_{q/11} \alpha \) and \( \text{G}_0 \).**
Proteins. Because \(G_{q/11}\) and \(G_\alpha\) proteins have been shown to be coupled to PLC, we determined whether the antipsychotic drugs had any effect on the expression of these G proteins in rat brain. As reported earlier (Dwivedi and Pandey, 1997), we again observed that \(G_{q/11}\) and \(G_\alpha\) proteins migrated to 42 and 40 kDa, respectively (Fig. 13).

In all brain areas studied, the administration of HAL, CPZ, or CLOZ either at acute or chronic doses had no significant effect on the steady-state levels of \(G_{q/11}\) or \(G_\alpha\) proteins (data not shown).

Discussion

To examine whether the mode of action of antipsychotic drugs is related to their interaction with the PI-signaling system, we studied the effects of three different antipsychotic drugs (i.e., a butyrophenone, HAL; a phenothiazine, CPZ; and an atypical, CLOZ) on the expressed levels of G proteins coupled to PLC; on the catalytic and the regulatory domains of PKC, by measuring PKC activity and \([3H]PDBu\) binding to PKC, respectively; on protein and mRNA expression of PKC and PLC isozymes; and on the catalytic activity of PI-PLC in the rat brain.

As summarized in Table 1, the following observations emerged from this study: 1) none of the antipsychotic drugs administered acutely or chronically had any significant effects on \([3H]PDBu\) binding to PKC. 2) Chronic treatment with CLOZ or HAL inhibited PKC activity in membrane and cytosol fractions of the cortex and the hippocampus. In addition, HAL decreased PKC activity in the striatum and CLOZ decreased PKC activity in the cerebellum. On the other hand, chronic CPZ decreased PKC activity only in the membrane fraction of cortex, hippocampus, and striatum. 3) For both HAL and CLOZ, the decrease in PKC activity was associated with decreased mRNA and protein levels of PKC \(\alpha\) and \(\epsilon\) isozymes, as well as of the PKC \(\gamma\) isozyme in the case of CLOZ; CPZ had no significant effects on the levels of any of the PKC isozymes. 4) Acute treatment with HAL translocated PKC \(\alpha\) and \(\epsilon\) isozymes from cytosol to membrane in
Fig. 9. Effects of chronic administration (i.p. injections once daily for 21 days) of HAL (1 mg/kg), CPZ (10 mg/kg), or CLOZ (20 mg/kg) on PI-PLC activity in rat brain. Values are the means ± S.D. from six rats in each group. HAL-, CPZ-, and CLOZ-treated groups were compared with the control group. *p < .001.
PKCs are insensitive to activation by phorbol esters. PLC has been divided into \( \beta, \delta, \) and \( \gamma \) isoforms (Cockcroft and Thomas, 1992). PLC \( \beta \) has been shown to be activated by receptors that activate the \( G \) family of \( G \) proteins, whereas PLC \( \gamma \) is regulated by receptor and nonreceptor kinases of the Src family (Cockcroft and Thomas, 1992). The regulation of PLC \( \delta \) is not known. Here, we observed that HAL and CLOZ decreased specific PKC isoforms and that CPZ decreased the PLC \( \beta_1 \) isozyme. The mechanisms by which these antipsychotic drugs down-regulated specific PKC and PLC isoforms are not clear; however, this does not seem to be related to the translocation of PKC or PLC isoforms from cytosol to membrane, because we observed decreased PKC and PLC isoforms in both membrane and cytosol fractions of rat brain. Earlier studies reported that CPZ, a pheno-thiazine cationic amphiphilic drug, interacts with membrane phospholipids, thereby reducing PKC activity (Mori et al., 1980; Kumar et al., 1997); however, we observed that, although CPZ decreased PKC activity in the membrane fractions, it reduced levels of the PLC \( \beta_1 \) isoform in both membrane and cytosol fractions. In addition, acute treatment with CPZ had no significant effect on PKC or PLC. These observations raise the possibility that the changes in PKC and/or PLC may be due to factors other than the interaction of CPZ with membrane phospholipids. One possibility could be that the PKC and/or the PLC isoforms were selectively degraded by phorbol esters or proteolytic enzymes. This is supported by recent studies showing a selective down-regulation of PKC \( \alpha \) by phorbol esters in human neuroblastoma cell lines (Leli et al., 1993) and a subcellular distribution of specific PKC \( \alpha \) and \( \varepsilon \) isoforms by phorbol esters in NIH 3T3 fibroblasts (Goodnight et al., 1995). Whether such a mechanism is responsible for the down-regulation of selective PKC or PLC isoforms by antipsychotic drugs is currently conjectural.

In this context, note that the effects of these antipsychotic drugs on PKC are specific to certain brain areas. For example, HAL decreased PKC activity in cortex, hippocampus, and striatum, whereas CLOZ produced changes in PKC activity in cortex, hippocampus, and cerebellum. Also note that 5-HT\(_2\) receptors are abundant in cortex, hippocampus, and cerebellum, whereas dopamine receptors are abundant in the striatum. On the other hand, PKC and PLC are present in all of these brain areas. Thus, the changes in PKC and PLC may be indirectly associated with changes in the density of the above-mentioned receptors. Although we have used crude membrane and cytosol fractions to determine PKC and PLC, it is unlikely that the changes in PKC are due to residual effects of antipsychotic drugs, because we did not observe any significant effects of these drugs on \( K_m \) of \(^{3}H\)PDHbu binding to PKC.

The functional significance of the decreases in PKC and PLC by these antipsychotics is not yet known, but it is well established that a wide variety of extracellular signals produce many biological responses by regulating the state of phosphorylation-dephosphorylation of specific proteins, which are then involved in the regulation of cellular functions. It has been shown that PKC and PLC are involved in various neuronal functions, such as synthesis and release of neurotransmitters, regulation of receptors and ion channels, neuronal excitability, long-term potentiation, and gene expression. Thus, modulation of the expression of specific PKC and PLC isoforms by these drugs may cause changes in

![Fig. 10. Representative Western blots showing the effects of chronic administration (21 days) of HAL (1 mg/kg), CPZ (10 mg/kg), or CLOZ (20 mg/kg) on immunolabeling of PLC isozymes in rat cortex. Protein samples (25 \( \mu \)g) were subjected to 7.5% polyacrylamide gel electrophoresis and transferred to ECL-intracellulose membranes, which were then incubated with primary antibodies specific for PLC \( \beta_1, \delta, \gamma_1, \) or \( \beta \)-actin and secondary anti-mouse antibody. The bands were quantified, and the ratios of optical densities of the PLC isozymes to that of \( \beta \)-actin were calculated.](image)
Fig. 11. Effects of chronic administration (i.p. injections once daily for 21 days) of HAL (1 mg/kg), CPZ (10 mg/kg), or CLOZ (20 mg/kg) on the immunolabeling of PLC isozymes in rat brain. Values are the means ± S.D. from six rats in each group. *p < .001.
various physiological functions, which in turn may be associated with their antipsychotic properties. Interestingly, PI hydrolysis has been reported to be elevated in platelets (Kaiya, 1992) and in postmortem brain (Jope et al., 1998) of schizophrenia patients. In addition, it has been shown that PI-PLC activity is increased in platelets of schizophrenia patients (Yao and van Kammen, 1996). It is possible that the effects of the antipsychotic drugs on PKC and PLC in rat brain may be of relevance to the effects of these drugs in decreasing schizophrenia symptoms, although, from our results, it is unclear whether these effects on PKC and PLC are specific to antipsychotic drugs or whether they are also shared by antidepressants and other classes of psychoactive drugs. In this context, note that lithium and valproic acid, which are effective antimanic agents, have been shown to affect PKC and their substrate MARCKS (Manji et al., 1999). Because antipsychotic drugs are effective antimanic agents, the observed effects of these antipsychotic drugs on PKC may be more relevant to their antimanic than to their antipsychotic effects.

In conclusion, this is the first in vivo study that systematically examines the effects of typical and atypical antipsychotic drugs on the components of the PI-signaling system. It

Fig. 12. A, a representative experiment showing a competitive PCR analysis for PLC β1 mRNA content in the hippocampus of a normal rat brain. Decreasing concentrations of PLC β1 standard cRNA (400 to 25 pg) were added to a constant amount (1 µg) of total RNA isolated from the hippocampus. The mixtures were reverse transcribed and PCR amplified in the presence of trace amounts of [32P]dCTP; aliquots were digested by BgIII and electrophoresed on 1.5% agarose gel. The higher molecular size band (316 bp) corresponds to the amplification products arising from the mRNA, whereas the lower bands (214 ± 122 bp) arise from cRNA generated from the internal standard digested by BgIII. B, data derived from the agarose gel are plotted as the counts incorporated into the amplified cRNA standard divided by the counts incorporated into the corresponding isozyme mRNA amplification product versus the known amount of internal standard cRNA added to the test sample. The point of equivalence represents the amount of PLC β1 mRNA. C, effects of chronic administration (i.p. injections once daily for 21 days) of HAL (1 mg/kg), CPZ (10 mg/kg), or CLOZ (20 mg/kg) on mRNA levels of the PLC β1 isozyme in the rat brain. Data are the means ± S.D. from six rats in each group. HAL-, CPZ-, and CLOZ-treated groups were compared with the control group. *p < .001.

Fig. 13. Representative Western blots showing the effects of chronic administration (21 days) of HAL (1 mg/kg), CPZ (10 mg/kg), or CLOZ (20 mg/kg) on immunolabeling of Gq/11 α and Go α proteins in rat cortex. Protein samples (20 µg) were subjected to 10% polyacrylamide gel electrophoresis and transferred to ECL-intracellulose membranes, which were then incubated with primary antibodies specific for Gq/11 α, Go α, or β-actin and secondary anti-rabbit (for Gq/11 α and Go α) or anti-mouse (for β-actin) antibody. The bands were quantified, and the ratios of optical densities of Gq/11 α and Go α to that of β-actin were calculated.
TABLE 1
Summary of effects of psychoactive drugs after chronic treatment (21 days) on measures of PKC, PLC, and G proteins in rat brain

<table>
<thead>
<tr>
<th>Antipsychotic Drugs</th>
<th>(^{3} \text{H} \text{PI-PLC} \text{ binding} ) ( (B_{max}/K_{D}) )</th>
<th>PKC activity</th>
<th>Immunolabeling of PKC isoforms</th>
<th>mRNA levels of PKC isoforms</th>
<th>PI-PLC activity</th>
<th>Immunolabeling of PLC ( \beta_{1} ) isozyme</th>
<th>mRNA levels of PLC ( \beta_{1} ) isozyme</th>
<th>Immunolabeling of G Proteins</th>
<th>( G_{a_1}, \alpha )</th>
<th>( G_{a_3} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAL</td>
<td>0/0</td>
<td>0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>CPZ</td>
<td>0/0</td>
<td>0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>CLOZ</td>
<td>0/0</td>
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<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
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</tr>
</tbody>
</table>

0 = No change; ↓ = decrease. Changes in \(^{3} \text{H} \text{PI-PLC} \text{ binding}, \text{ PKC activity, immunolabeling of PKC and PLC isozymes, and PI-PLC activity are in both membrane and cytosol fractions except where marked (\( \ast \)).

\( \ast \) Only in membrane fractions.

\( \ast \ast \) CLOZ also decreased immunolabeling and mRNA levels of the PKC \( \gamma \) isoform.

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