Differential Effects of Haloperidol and Clozapine on \([3H]cAMP\) Binding, Protein Kinase A (PKA) Activity, and mRNA and Protein Expression of Selective Regulatory and Catalytic Subunit Isoforms of PKA in Rat Brain

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ABSTRACT

The present study was undertaken to examine whether the mechanism of action of typical and atypical antipsychotics is related in their ability to regulate key phosphorylating enzyme of adenylyl cyclase-cAMP pathway, i.e., protein kinase A (PKA). For this purpose, regulatory (R) and catalytic (Cat) activities of PKA and expression of various isoforms of regulatory and catalytic subunits were examined in rat brain after single or chronic (21-day) treatment with haloperidol (HAL, 1 mg/kg) or clozapine (CLOZ, 20 mg/kg). It was observed that chronic but not acute treatment of CLOZ significantly decreased \([3H]cAMP\) binding to the regulatory subunit of PKA as well as catalytic activity of PKA in particulate and cytosol fractions of the rat cortex, hippocampus, and striatum. In these fractions, CLOZ significantly decreased protein levels of selective RIIα, RIIβ, and Catβ-subunit isoforms of PKA. These decreases were accompanied by decreases in their respective mRNA expression. In contrast, chronic but not acute treatment of HAL significantly increased \([3H]cAMP\) binding and the catalytic activity of PKA in particulate and cytosol fractions of only the striatum brain area. In addition, chronic treatment of HAL significantly increased mRNA and protein levels of RIIα and RIIβ-subunit isoforms in the striatum. None of the antipsychotics caused any change in the expression of the Catα, Catβ, or RIβ-subunit isoform. These results, thus, suggest that HAL and CLOZ differentially regulate PKA catalytic and regulatory activities and the expression of selective catalytic and regulatory subunit isoforms of PKA, which may be associated with their mechanisms of action.

Haloperidol (HAL) and clozapine (CLOZ), the two most commonly used antipsychotic agents, share the common property of blocking dopamine D2 receptors (Deutsch et al., 1991; Dixon et al., 1995). Despite this common feature, the clinical and behavioral profiles of these drugs differ. For example, although effectively blocking psychoses, HAL causes extrapyramidal side effects (EPS), including a Parkinson’s-like syndrome, and Tardive Dyskinesia (TD). On the other hand, CLOZ is associated with low incidence of EPS and TD. Some studies even suggest that CLOZ reduces symptoms of TD (Safferman et al., 1991), which is effective at ameliorating motor dysfunction in patients with idiopathic Parkinson’s disease (Pakkenberg and Pakkenberg, 1986; Arevalo and Gershaniak, 1993) and in the treatment-resistant negative symptoms of schizophrenia (Kane et al., 1988).

The mechanisms of action of antipsychotics in alleviating the symptoms associated with psychoses and the mechanisms responsible for their differential effects on EPS are not clear. Whereas one hypothesis is that different affinities of these two drugs toward dopamine D2 receptors may be responsible for their different clinical efficacy and incidence of EPS, several other biological factors may also be associated with their actions. This is based upon reports that suggest that CLOZ binds to numerous neurotransmitter receptors besides dopamine D2, including 5HT2A, 5HT2C, 5HT1A, 5HT6, α1- and α2-adrenergic, and muscarinic receptors (Bolden et al., 1991; Baldessarini et al., 1992; Kuoppamaki et al., 1994; Millan, 2000; Zhukovskaya and Neumaier, 2000).

In search for the mechanisms of action of antipsychotic drugs, several studies have been performed at the postreceptor sites, particularly of their effects on receptor-mediated phosphoinositide hydrolysis and various components of this signaling transduction system such as PKC and phospholipase C (Hokin-Neaverson, 1980; Li et al., 1991; Kuoppa-
maki et al., 1994; Dwivedi and Pandey, 1999). On the other hand, in adenylyl cyclase-cAMP signaling pathway, most of the studies of the effects of antipsychotic drugs are confined either to receptor-stimulated adenylyl cyclase activity or to the levels of G proteins, such as G, and G, linked to this signaling system via stimulatory and inhibitory fashion, respectively. For example, HAL, which antagonizes dopamine D, receptor, leads to an increase in cAMP (Kaneko et al., 1992). More recent studies suggest that CLOZ decreases 5HT, -mediated (Assie et al., 1997) and muscarinic M,-mediated (Zeng et al., 1997) cAMP formation. On the other hand, Kaplan et al. (1999) showed that whereas HAL increases GTPyS-stimulated adenylyl cyclase activity in rat cortex, olanzapine decreases it. But in the striatum, olanzapine produces effects opposite those of the cortex, and HAL has no effects on GTPyS-stimulated adenylyl cyclase activity. Other significant observations are that whereas HAL decreases G, and G, in rat striatum, CLOZ increases them (Gupta and Mishra, 1992; Shin et al., 1995). In contrast, Kaplan et al. (1999) reported no change in the striatum but a decrease in G, levels in the cortex. No change in these two subunits after HAL (See et al., 1993; Meller and Bohmaker, 1996) or olanzapine (Kaplan et al., 1999) treatment has also been reported. In light of these observations, it is quite possible that the mechanism of action of typical and atypical antipsychotic drugs may lie in their ability to differentially regulate adenylyl cyclase-cAMP pathway at the level of functional response.

In the adenylyl cyclase-cAMP pathway, this functional response is mediated by phosphorylating enzyme protein kinase A (PKA), which is activated by cAMP generated by the conversion of ATP in response to the activation of adenylyl cyclase by receptor-activated G, or G, proteins. PKA then phosphorylates various substrate proteins in cells, thereby mediating a variety of hormonal and physiological responses (Nestler and Greengard, 1994). In a native state, PKA exists as a tetramer holoenzyme that consists of two regulatory and two catalytic subunits. In the holoenzyme state, PKA exists in an inactive form. After an increase in intracellular cAMP, the regulatory subunits bind to cAMP, which results in the dissociation of the holoenzyme into a regulatory dimer and two monomers of catalytic subunits. The free catalytic subunits can then phosphorylate various substrates. Thus, both catalytic and regulatory subunits are important in facilitating PKA-mediated functions (Skålhegg and Taskén, 1997).

In the present study, we tested whether different clinical and behavioral profiles of HAL and CLOZ are associated with their ability to differentially regulate adenylyl cyclase pathway at the level of PKA. For this purpose, we examined [H]cAMP binding, catalytic activity, and expression of various regulatory and catalytic subunits of PKA after acute and chronic treatment of HAL and CLOZ to rats.

**Experimental Procedures**

**Materials**

[3H]cAMP was obtained from PerkinElmer Life Sciences (Boston, MA). 3-Isobutyl-1-methyllumithine, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), cAMP, ATP, leupeptin, 2-mercaptoethanol, and NP-40 were purchased from Sigma-Aldrich (St. Louis, MO). Hot Tub-DNA polymerase, RNase inhibitor, BglII, (α-32P)dCTP, [γ-32P]ATP, horseradish peroxidase-linked secondary anti-mouse and anti-rabbit antibodies were purchased from Amersham BioSciences (Arlington Heights, IL). EcoRI, HindIII, and in vitro transcription kit were purchased from Promega (Madison, WI). Kemp tide was obtained from Calbiochem (La Jolla, CA). Antibodies for PKA regulatory subunit isoforms (RIα, RIβ, RIIα, and RIIβ) were purchased from Chemicon International Inc. (Temecula, CA), whereas antibodies for catalytic subunit isoforms (Catα and Catβ) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PKA and PKC inhibitor peptides were obtained from Upstate Biotechnology (Lake Placid, NY), whereas compound R24571 and monoclonal β-actin antibody were purchased from Sigma. HAL was obtained from Roxane Laboratory (Columbus, OH), and CLOZ was obtained from Research Biochemical International (Natick, MA). All other chemicals were of analytical grade obtained from Sigma-Aldrich.

**Animals**

Virus-free Sprague-Dawley male rats, initially weighing 220 to 250 g, were used. Rats were housed in groups of three under standard laboratory conditions (temperature 21 ± 1°C humidity 55 ± 5%, 12-h light/dark cycle). Animals were provided free access to food and water. Rats were acclimatized for 1 week before starting the experiment.

**Drugs and Treatments**

The Internal Review Board of the University of Illinois at Chicago approved this study. HAL was diluted with saline to 0.5 mg/ml. CLOZ was dissolved in a minimum of 0.1 M hydrochloric acid and diluted with distilled water, and pH was adjusted to 5.5 to 6.0 with 1 M sodium hydroxide. The stock solution was further diluted in saline to 10 mg/ml. Rats were given i.p. injections (2 ml/kg/day) of CLOZ (20 mg/kg) or HAL (2 mg/kg) either as a single dose or once daily for 21 days. Control rats were given i.p. injections of an equal volume of normal saline (0.9% w/v). Each of the three groups contained 12 rats each. The same six rats from each group were used for biochemical determination in the cortex and hippocampus. For biochemical determination in the striatum, the striata from two rats were pooled. The dose of HAL used in this study was selected because the level of HAL in rat plasma at this dose is similar to human therapeutic plasma levels (Kaneda et al., 1992). Also, this dose level of HAL has been shown to affect dopamine D/D receptors in rat brain, and 1 mg of HAL is clinically equivalent to approximately 15 to 20 mg of CLOZ (Wilmot and Szczepanik, 1989). The selection of the dose for CLOZ was based on previous studies that indicated sufficient effects of these antipsychotic drugs on 5HT, , 5HT, receptor and up-regulation of dopamine D/D, receptors, thus, showing appropriate central nervous system activity (Kuoppamaki et al., 1994). In our previous study, we showed that these doses of HAL and CLOZ caused significant effects on the levels of PKC and phospholipase C in rat brain (Dwivedi and Pandey, 1999). The animals were decapitated 24 h after the last injection, and the brains were removed quickly. Cortices, hippocampi, and striata were dissected out and immediately stored at −80°C until analysis.

**Determination of B and K of [H]cAMP Binding to Cytosol and Particulate PKA in Rat Brain**

Specific [H]cAMP binding was performed as described previously (Dwivedi and Pandey, 2000). Brain samples were homogenized in 10 volumes of ice-cold buffer containing 20 mM Tris-HCl (pH 7.4 at 25°C), 2 mM EDTA, 25 mM 2-mercaptoethanol, 0.5 mM AEBSF, and 10 μg/ml leupeptin. The homogenate was centrifuged at 100,000 g for 60 min. The supernatant (S,) was saved. The pellet was resuspended in the homogenizing buffer and centrifuged again at 100,000 g for 60 min. This supernatant (S,) was combined with S, and used as the cytosol fraction; the pellet was homogenized in the homogenizing buffer and used as the particulate fraction. The protein content was determined in these two fractions according to the procedure of Lowry et al. (1951) using bovine serum albumin as a standard.
(3)H)cAMP binding was performed in triplicate in an incubation buffer (containing 20 mM phosphate buffer, pH 7.4 at 25°C, 2 mM EDTA, and 15 mM 2-mercaptoethanol [PEM buffer]), (3)H)cAMP (0.25–10 nM), particulate or cytosol fraction (25 μg of protein), 0.25 mg of bovine serum albumin, and 1.5 mM 3-isobutyl-1-methylxanthine, in the presence or absence of 5 μM cAMP, in a total volume of 500 μl. The incubation was carried out at 25°C for 60 min and terminated by rapid filtration under vacuum using a Brandel Cell Harvester (Biomedical Research and Development Laboratories, Inc., Gaithersburg, MD) followed by three washes with 2 ml of ice-cold PEM buffer. The radioactivity retained on the filter was counted using a liquid scintillation counter. Nonspecific binding was defined as the radioactivity bound in the presence of 5 μM cAMP. Bmax and Kd were calculated by Scatchard plots using the EBDA program (McPherson, 1985).

**Determination of PKA Activity in Cytosol and Particulate Fractions of Rat Brain**

PKA activity was determined in both particulate and cytosol fractions obtained from the cortex, hippocampus, and striatum as described previously (Dwivedi and Pandey, 2000). The brain tissues were homogenized in a homogenizing buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1 mM dithiothreitol, 110 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 8.7 μg/ml phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 100,000g for 60 min at 4°C. The resulting supernatant (S1) was saved. The pellet obtained was homogenized in the homogenizing buffer and recentrifuged at 100,000g for 60 min at 4°C. The resultant supernatant (S2) was combined with S1 and used as the cytosol fraction. The pellet was homogenized in the homogenizing buffer and used as the particulate fraction. The protein content of these two fractions was determined by the procedure of Lowry et al. (1951). The procedure is based on the phosphorylation of a specific substrate (kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly) using the transfer of [γ-32P]ATP by PKA. PKA activity was determined in duplicate in a final volume of 50 μl containing 50 mM Tris (pH 7.4), 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 0.05% nonidet P-40, 10 mM dithiothreitol, 1 mM sodium orthovanadate, 500 μM kemptide (PKA substrate), 2 μM PKC inhibitor peptide, 20 μM compound R24571 (calmodulin kinase II inhibitor), and 100 μCl of [γ-32P]ATP (3000 Ci/mmol prepared in 75 mM MgCl2 and 500 μM ATP). PKA activity was determined in the presence (cAMP-stimulated) and the absence (basal) of cAMP (10 μM). Reactions were carried out at 30°C for 10 min. Aliquots (20 μl) were spotted in duplicate onto phosphocellulose filters (2 x 2 cm, Whatman P81), washed in 75 mM H3PO4 twice for 5 min and again in water twice for 5 min, and air-dried. The [32P] contained in the filter papers was then quantitated by liquid scintillation spectrometry. Background counts, calculated for each sample from a parallel reaction that did not contain kemptide, were subtracted. Data are expressed as picomoles of [32P]phosphate transferred to kemptide substrate per minute per milligram of protein.

**Quantitation of Catalytic and Regulatory Subunit Isoforms of PKA in Rat Brain by Western Blot**

Immunolabeling of catalytic and regulatory subunit isoforms of PKA in cortex, hippocampus, and striatum was determined by Western blot as described previously (Dwivedi and Pandey, 2000). Brain samples were Dounce homogenized in 10 volumes of ice-cold buffer containing 20 mM Tris-HCl (pH 7.4 at 25°C), 2 mM EDTA, 25 mM 2-mercaptoethanol, 0.5 mM AEBSF, plus 0.5% Triton X-100, 2 μg/ml leupeptin, 3 μg/ml aprotinin, and 0.2 mg/ml soybean trypsin inhibitor and were sonicated. The homogenate was centrifuged at 12,000g for 10 min at 4°C. The supernatant fraction was used for immunolabeling. Equal volumes of supernatant (20 μl containing 30 μg of protein) and gel loading solution (50 mM Tris-HCl, pH 6.8, 4% β-mercaptoethanol, 1% sodium dodecyl sulfate [SDS], 40% glycerol, and a trace amount of bromphenol blue) were mixed, and the samples were boiled for 3 min and kept on ice for 10 min. Protein samples were loaded onto 10% (w/v) SDS-polyacrylamide gel using the Mini Protein II gel apparatus (Bio-Rad, Hercules, CA). The gels were run using 25 mM Tris-base, 192 mM glycine, and 0.1% (w/v) SDS at 150 V. The proteins were subsequently transferred electrophoretically to an enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham) using the Mini TransBlot transfer unit (Bio-Rad) at 0.15-amp constant current. Membranes were washed with TBST buffer (10 mM Tris-base, 0.15 M NaCl, and 0.05% Tween 20) for 10 min. The blots were blocked by incubation with 5% (w/v) powdered nonfat milk in TBST, 0.2% (v/v) nonidet P-40, and 0.02% (w/v) SDS, pH 8.0. The blots were incubated overnight at 4°C with primary antibody (anti-PKA RIIα, RIIβ, RIIβ, Catα, or Catβ) at a dilution of 1:3000 to 1:5000 depending on the antibody used. The membranes were then washed with TBST and incubated with horseradish peroxidase-linked secondary antibody (anti-rabbit IgG; 1:3000) for 3 h at room temperature. The membranes were extensively washed with TBST and exposed to ECL film. Before starting the immunolabeling, the procedure was standardized using 10 to 100 μg of protein. We found that the optical density of the bands varied linearly with a concentration of up to 100 μg of protein. To normalize our data, we used β-actin as a housekeeping protein. The protein levels of β-actin were determined after stripping the membrane and probing with β-actin monoclonal as primary antibody (anti-PKA RIIα, RIIβ, or Catβ) at a dilution of 1:5000 for 2 h and anti-mouse IgG (1:5000 for 2 h) as the secondary antibody. The dilution of the antibodies and the duration of exposure of the nitrocellulose membranes on autoradiographic film were standardized. The optical densities of the bands on the autoradiograms were quantified using the Loats Image Analysis System (Westminster, MD), and the optical density of each band was corrected by the optical density of the activated protein kinase A.

**TABLE 1**

<table>
<thead>
<tr>
<th>Primers</th>
<th>GenBank Accession No.</th>
<th>Nucleotide Position</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>External primer sequences PKA RII α, RIIβ, and Catβ-subunit isoforms for amplification</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIα</td>
<td>F: 5'-AGAGTGAATCGGACACGCGAGCG ACG</td>
<td>J02934</td>
</tr>
<tr>
<td>R: 5'-GCCACGCCTTCCCATCTGAACC</td>
<td></td>
<td>490-510</td>
</tr>
<tr>
<td>RIβ</td>
<td>F: 5'-TAAACCGGTCCACAGAGCGGTTG</td>
<td>M12492</td>
</tr>
<tr>
<td>R: 5'-GGTACACCCCGCTTCCACAC</td>
<td></td>
<td>618-638</td>
</tr>
<tr>
<td>Catβ</td>
<td>F: 5'-GGGAGAAGCGGCAGGCTGCCA</td>
<td>D10770</td>
</tr>
<tr>
<td>R: 5'-CCAGAAGCAGTTTCCATAACC</td>
<td></td>
<td>360-331</td>
</tr>
<tr>
<td><strong>Internal primer sequences</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIα</td>
<td>5'-CATTCTGTTCTGTCCGAACCATGCTGCATC</td>
<td>438-461</td>
</tr>
<tr>
<td>RIIβ</td>
<td>5'-GATCCAGGTTCTCTGAAGGCAGAGTGG</td>
<td>438-461</td>
</tr>
<tr>
<td>Catβ</td>
<td>5'-TGGAGATCCAGATGCTGATCTA</td>
<td>544-568</td>
</tr>
</tbody>
</table>

F, forward; R, reverse; bold and italicized letters indicate the mutated bases; underlined bases indicate the BgII or XhoI cleavage site.
corresponding β-actin band. The values are presented as a percentage of the control.

Because Catα- and Catβ-subunits are quite homologous, we determined the specificity of the antiserum by using 100-fold excess blocking peptide (relative to the molarity of the antiserum) corresponding to the epitope used to generate the Catα- or Catβ-subunit. We found that corresponding peptides for Catα and Catβ blocked the bands observed after incubation with antibodies for Catα or Catβ (Fig. 4A), suggesting that the antibodies for Catα and Catβ, in fact, recognize these subunits, and they do not cross-react with each other.

**Determination of mRNA Levels of PKA RIIα, PKA RIIβ, and PKA Catβ 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; 1999; Dwivedi et al., 2000) 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 CsCl2 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 reverse-transcriptase enzyme.

**Oligonucleotides.** Amplification primers for PKA RIIα, PKA RIIβ, and PKA Catβ were synthesized on the model 381A DNA synthesizer (Applied Biosystems, Foster City, CA) by using phosphoramidite chemistry, leaving the terminal dimethoxytrityl group intact. All primers were purified by reverse-phase chromatography using oligonucleotide purification columns (Applied Biosystems) according to the manufacturer’s manual. The primer pairs were designed to allow amplification for PKA RIIα (128–510 bp; Scott et al.,

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**Fig. 1.** Saturation isotherm of [3H]cAMP binding to particulate (A) and cytosol (B) PKA in rat cortex. Each point is the mean of triplicate determinations. Inset, Scatchard plot of the specific binding of [3H]cAMP. B. [3H]cAMP specifically bound (femtomoles per milligram of protein); and B/F, bound/free [3H]cAMP (femtomoles per milligram of protein × nanomolar). For this particular experiment, the binding indices in the particulate fraction (A) were: \( K_D = 0.65 \) nM, \( B_{max} = 129 \) fmol/mg protein, and correlation coefficient \( r = 0.99 \). For the cytosol fraction (B): \( K_D = 0.67 \) nM, \( B_{max} = 348 \) fmol/mg protein, and \( r = 0.99 \).
1987), PKA RII (313–638 bp; Jahnsen et al., 1986), and PKA Catβ (3–378 bp; Shuntoh et al., 1992). Each primer contained a comparable G/C content to minimize variability in hybridization efficiency at the annealing temperature. The sequences and the positions of external primers for each primer are given in Table 1. The specificity of PKA RIIα, PKA RIIβ, and PKA Catβ-products was checked by sequencing the amplified area with the Sequenase version 2.0 DNA Sequencing Kit using HindIII and EcoRI, which produced fragments of the expected sizes.

**Synthesis and Cloning of Internal Standards.** Internal standard templates were generated by site-directed mutagenesis using PCR overlap extension. Each standard was designed to introduce a BglII or XhoI restriction site midway between the amplification primers so that the digestion of the amplicon would generate two fragments of approximately equal molecular size. The designs for each internal primer are provided in Table 1. The single-strand internal primers were designed and synthesized so that the restriction site was introduced with only a minimal number of base substitutions, and also such that there was a 24- to 26-bp overlap of the two fragments from the first PCR step. Each of the internal primers were designed and synthesized so that the restriction site would be preserved. The designs for each internal primer are provided in Table 1. The single-strand internal primers were designed and synthesized so that the restriction site was introduced with only a minimal number of base substitutions, and also such that there was a 24- to 26-bp overlap of the two fragments from the first PCR step. Each of the internal primers were designed and synthesized so that the restriction site would be preserved.

**The First PCR Step.** Different concentrations of heat-denatured linear starting templates (from 1 to 100 ng) were amplified with 1 µmol of either 5'-external and 3'-internal primers or 3'-external and 5'-internal primers. PCR was performed with 1.5 U of Hot Tub DNA polymerase in a 100-µl reaction volume containing 200 µM dNTPs, 1.5 mM MgCl₂, 50 mM Tris/HCl (pH 9.0), 20 mM ammonium sulfate, and 15 mM KCl. The amplicons from the first PCR step were extracted and purified from low-melting-point agarose.

**The Second PCR Step.** Equivalent and increasing amounts of the two amplicons from the first PCR step were pooled, and a second PCR reaction was performed with the two external primers containing the cloning site EcoRI and HindIII. The final product was purified from low-melting-point agarose and digested with BglII or XhoI to verify the presence of the restriction site. After digestion with EcoRI-HindIII, the material was extracted from low-melting-point agarose and cloned into the corresponding sites of pGem-4Z using standard cloning methodology.

**In Vitro cRNA Synthesis.** The internal standard templates were linearized with SpI, which cuts 601 bp downstream of the EcoRI cloning site. The cRNA corresponding to the sense strand was synthesized using 4 to 8 µg of linearized template with T7 RNA polymerase using an in vitro transcription kit. Aliquots of the stock cRNA were used to obtain reproducible concentration measurements based on its optical density at the 260/280 nm wavelength.

**Quantitative Analyses of PKA RIIα, PKA RIIβ, and PKA Catβ-mRNA by Competitive RT-PCR.** Decreasing concentrations of PKA RIIα, PKA RIIβ, and PKA Catβ internal standard cRNA were added to 1 µg 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 and reverse-transcriptase (200 U) in RT buffer containing 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, and 1 mM dNTPs using random hexamers (5 mM) and ribonuclease inhibitor (28 U) in a volume of 20 µl. 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, PerkinElmer Life Sciences, Boston, MA). The amplification mixture contained cDNA, 0.5 μM specific primer pairs, 200 μM dNTPs, 15 mM MgCl₂, 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-μl volume. Trace amounts of [³²P]dCTP (0.5–1 μCi/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 Bgl II (RII) or XhoI (RII and Catβ) 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 isozyme mRNA amplification product versus a known amount of internal standard cRNA added to the test sample. The results are expressed as attomoles of mRNA per microgram of total RNA.

Statistics
Data were analyzed with SPSS 9.0 (Chicago, IL) statistical package. All values are the mean ± S.D. Intergroup comparisons were made by analysis of variance. Bonferroni’s multiple comparisons were used to evaluate pair-wise differences. An α-value lower than 0.05 was considered significant.

Results
Effects of Acute and Chronic Administration of HAL and CLOZ [³H]cAMP Binding to Regulatory Subunits

Fig. 3. Effects of chronic treatment with HAL and CLOZ on PKA activity in particulate and cytosol fractions of cortex, hippocampus, and striatum. Rats were given i.p. injections of HAL (1 mg/kg) or CLOZ (20 mg/kg) for 21 days and decapitated 24 h after the last injection. Values are means ± S.D. from six rats in each group. HAL- and CLOZ-treated groups were compared with the control group. *, p < 0.001.

of PKA in Particulate and Cytosol Fractions of Rat Brain. The characterization of [³H]cAMP binding to regulatory subunits of PKA in particulate and cytosol fractions of rat brain has been reported in our previous publication (Dwivedi and Pandey, 2000). The maximum number of binding sites (Bmax) and the apparent dissociation constant (Kd) in both particulate and cytosol fractions were determined by using different concentrations of [³H]cAMP (0.25–10 nM). Nonspecific binding was determined in the presence of 5 μM cAMP. Figure 1 represents a typical saturation isotherm and a Scatchard plot (inset) of [³H]cAMP binding to particulate (Fig. 1A) and cytosol (Fig. 1B) fractions obtained from the cortex of a control rat. It was observed that specific binding site was saturable and exhibited a single class of binding site. Nonspecific binding was nonsaturable and was linear with concentrations of 0.25 to 10 nM [³H]cAMP. The specific binding was in the range of 92 to 78% depending upon the concentration of [³H]cAMP used (0.25–10 nM). We found that Bmax of [³H]cAMP binding to PKA was greater in the cytosol than in the membrane. In addition, Bmax was greater in the hippocampus than in the cortex and striatum; however, Kd values were similar in all brain areas.

When we determined [³H]cAMP binding after acute or chronic treatment of HAL or CLOZ, we observed that acute treatment of both of these antipsychotics had no significant effects on either Bmax or Kd in particulate and cytosol fractions obtained from the cortex, hippocampus, or striatum (data not shown). Chronic administration of HAL, however, increased Bmax of [³H]cAMP binding in both particulate and
and absence (basal) of 10 M cAMP. This concentration of cAMP produces maximal stimulation of PKA (Dwivedi and Pandey, 2000). The specificity of PKA activity was determined by examining the ability of selective PKA inhibitor (a 17-residue synthetic peptide: TYADFIASGRTGRNAI-NH2) to block the activity in both membrane and cytosol fractions. We observed that PKA activity was completely inhibited in the presence of PKA inhibitor in both particulate and cytosol fraction (data not shown). To ensure that PKA activity is specific and that the activities of other kinases do not account for our results, we added two protein kinase inhibitors in each assay, a PKC inhibitor peptide (a 13-amino acid synthetic peptide: RFARKGLARQKNV) and compound R2457 (a calmodulin kinase II inhibitor: 1-[bis-(4-chlorophenyl) methyl]-3-[2-(2,4-dichlorophenyl)]-2-[2,4-dichloro benzoyloxyethyl]-1H-imidazolium chloride.

Acute administration of HAL or CLOZ had no significant effects on basal or cAMP-stimulated PKA activity in particulate or cytosol fraction of the cortex, hippocampus, and striatum (data not shown). Chronic treatment of HAL or CLOZ also did not cause significant change in basal PKA activity in the cortex, hippocampus, or striatum. Basal PKA activity (picomoles per minute per milligram of protein) in these brain areas after HAL and CLOZ treatment were as follows. Cortex: control, particulate = 128 ± 18 and cytosol = 270 ± 12; HAL-treated: particulate = 134 ± 21 and cytosol = 277 ± 15; CLOZ-treated: particulate = 131 ± 24 and cytosol = 250 ± 21. Hippocampus: control, particulate = 133 ± 15 and cytosol = 266 ± 22; HAL-treated: particulate = 141 ± 19 and cytosol = 285 ± 27; CLOZ-treated: particulate = 119 ± 16 and cytosol = 244 ± 17. Striatum: control, particulate = 116 ± 15 and cytosol = 255 ± 17; HAL-treated: particulate = 125 ± 24 and cytosol = 281 ± 16; CLOZ-treated: particulate = 119 ± 18 and cytosol = 262 ± 28.

On the other hand, chronic administration of HAL significantly increased cAMP-stimulated PKA activity in both particulate and cytosol fraction of striatum without any change in cortex or hippocampus (Fig. 3), whereas chronic administration of CLOZ significantly decreased cAMP-stimulated PKA activity in both particulate and cytosol fractions of the cortex, hippocampus, and striatum (Fig. 3).

Effect of Acute and Chronic Treatment of HAL and CLOZ on Protein Levels of Regulatory and Catalytic Subunit Isoforms of PKA in Rat Brain. Representative Western blots of regulatory and catalytic subunit isoforms of PKA in rat striatum are shown in Fig. 4. The apparent molecular masses for PKA RIα-, RIβ-, and Cα-isoforms were 45, 51, 54, and 42 kDa, respectively, whereas PKA RIβ- and Catβ-isoforms migrated to 55 kDa. To normalize our data, we probed the same membrane with β-actin antibody. The apparent molecular mass for β-actin protein was 46 kDa. We did not find any significant effects of antipsychotic drug treatment on protein levels of β-actin in any brain areas studied. The optical density of each regulatory and catalytic subunit isoform protein was corrected with the optical density of corresponding β-actin band on the same immunoblot. This procedure has been used previously in our laboratory (Dwivedi and Pandey, 1999, 2000). To validate our data, we initially determined the immunolabeling of each regulatory and catalytic subunit isoform of PKA in rat brain using five different concentrations of protein from control and drug-treated groups. It was observed that the optical density increased linearly with increasing concentrations of protein (10–100 μM) and that the curve shifted toward the right or the left, respectively, for those isoforms in which changes were observed, depending on whether their protein levels decreased or increased. Acute treatment of HAL or CLOZ did not cause any significant effects on either catalytic or regulatory subunit isoforms in the cortex, hippocampus, or striatum (data not shown). Western blot showing effects of chronic administration of HAL and CLOZ on immunolabeling of PKA Catα- and Catβ-subunits in presence (2) and absence (1) of peptides corresponding to Catα- and Catβ-antibodies. Protein sample (30 μg) from one control rat cortex was subjected to gel electrophoresis. The membranes were incubated with anti-PKA Catα- or Catβ-subunit antibody (1). The same membranes were stripped and incubated with 100-fold excess peptides (relative to the molar concentration) corresponding to Catα- or Catβ-antiserum along with respective antibodies. Note that incubation of peptides blocked the bands that were initially recognized by Catα- and Catβ-antibodies. B, representative Western blots showing the effects of chronic treatment with HAL or CLOZ on immunolabeling of PKA regulatory (RIα, RIβ, and RIIβ) and catalytic (Catα and Catβ) subunit isoforms in rat striatum. Lane 1, control; lane 2, HAL treated; and lane 3, CLOZ treated. Protein samples (30 μg) were subjected to 10% polyacrylamide gel electrophoresis and transferred to ECL-nitrocellulose membranes, which were then incubated with primary antibodies specific for each regulatory and catalytic subunit isoform of PKA and secondary anti-rabbit antibody. The membranes were stripped and probed with β-actin primary and anti-mouse secondary antibody. The bands were quantified as described under Experimental Procedures. Ratios of the optical densities of PKA subunit isoforms to that of β-actin were calculated.
ing of PKA regulatory and catalytic subunit isoforms in rat striatum is depicted in Fig. 4B, and their effects in the cortex, hippocampus, and striatum are diagrammatically represented in Fig. 5. It was observed that the administration of HAL significantly increased protein levels of the RIIβ- and RIIα-subunits in striatum, without any change in cortex and hippocampus brain area. On the other hand, chronic administration of CLOZ decreased expression of RIIα, RIIβ, and Catβ-subunit expression in the cortex, hippocampus, and striatum. HAL did not affect immunolabeling of RIIα, Catα, or Catβ, whereas CLOZ was ineffective in causing changes in immunolabeling of RIIα and Catβ-subunits.

Effects of HAL or CLOZ Administration on mRNA levels of RIIα, RIIβ, and Catβ-Subunit Isoforms in Rat Brain. To examine whether altered immunolabeling of RIIα and RIIβ by HAL and RIIα, RIIβ, and Catβ by CLOZ was because of altered gene expression, we determined mRNA levels of these regulatory and catalytic subunit isoforms by quantitative RT-PCR after chronic HAL and CLOZ administration. Representative gel electrophoreses showing competitive RT-PCR for PKA RIIα, RIIβ, and Catβ in the cortex are given in Figs. 6A, 7A, and 8A, respectively. In addition, representative graphs showing the quantitation of mRNA for PKA RIIα, RIIβ, and Catβ are given in Figs. 6B, 7B, and 8B, respectively. As expected, we observed that the amplification products for PKA RIIα arise from the mRNA template at 383 bp and the corresponding digestion products arise from cRNA at 191 + 192 bp (Fig. 6A); for RIIβ, template at 326 bp and cRNA at 166 + 160 bp (Fig. 7A); and for Catβ, template at 375 bp and cRNA at 178 + 197 bp (Fig. 8A). We observed that mRNA expression of Catβ, RIIα, and RIIβ were quite similar in all the brain areas studied. However, mRNA expression of RIIβ was higher than RIIα- and Catβ-subunits.

When we determined the absolute amounts of RIIα, RIIβ, and Catβ mRNA after chronic administration of HAL or CLOZ, we observed that HAL significantly increased mRNA expression of RIIα (Fig. 6C)- and RIIβ (Fig. 7C)-subunits in the striatum without any change in the cortex and hippocampus. On the other hand, CLOZ significantly decreased mRNA levels of RIIα (Fig. 6C)-, RIIβ (Fig. 7C)-, and Catβ (Fig. 8C)-subunits in the cortex, hippocampus, and striatum. HAL failed to change mRNA levels of Catβ in any brain region studied (Fig. 8C).

Discussion

The results of the present study suggest that chronic treatment of HAL and CLOZ differentially regulate adenylyl cyclic-cAMP signaling pathway at the level of PKA. For example, chronic treatment of HAL significantly increased [3H]cAMP binding to regulatory subunits of PKA and catalytic activity of PKA in particulate and cytosol fractions of striatum. In contrast, chronic administration of CLOZ de-
creased [3H]cAMP binding to regulatory subunits of PKA and catalytic activity of PKA in particulate and cytosol fractions of not only the striatum, but also the cortical and hippocampal brain areas.

Two major categories of PKA holoenzyme have been identified, i.e., type I and type II, which differ in structure depending on the regulatory subunit incorporated, whereas the catalytic subunits are either identical or very similar. Type I PKA is primarily cytoplasmic, whereas type II PKA is mainly particulate. Multiple isoforms of PKA regulatory and catalytic subunits exist and are encoded by separate genes. For example, four regulatory (RIα, RIβ, RIIα, and RIIβ) and three catalytic (Catα, Catβ, and Catγ) subunits of PKA exist. The tissue distribution of regulatory subunits is such that RIα and RIIα are present ubiquitously, whereas RIβ is present in the brain and in developing sperms. However, RIIβ is the predominant isoform and principal mediator of cAMP-mediated activity in the central nervous system. The catalytic subunit isoforms Catα and Catβ are ubiquitously expressed, although Catβ is the predominant isoform in brain, and Catγ is a testis-specific isoform (for a recent review, see Skålhegg and Tasken, 1997). To examine whether observed changes in [3H]cAMP binding to regulatory subunits and catalytic activity of PKA were related to the expression of specific regulatory and/or catalytic subunits of PKA, we determined mRNA and protein levels of various isoforms of regulatory and catalytic subunits of PKA after treatment with HAL or CLOZ. We found an interesting pattern of changes in the expression of catalytic and regulatory subunits. Chronic administration of HAL increased the levels of PKA RIIα- and RIIβ-subunits specifically without any change in Catα, Catβ, or other regulatory RIα- or RIβ-subunit isoforms. This effect was specific to striatum brain area where changes in [3H]cAMP binding and PKA activity were observed. Chronic administration of CLOZ, on the other hand, decreased not only protein levels of RIIα- and RIIβ-subunits, but also that of Catβ-subunit in all the brain areas, i.e., the cortex, hippocampus, and striatum. CLOZ, as with

Fig. 6. A, a representative experiment showing a competitive PCR analysis for PKA RIIα mRNA content in cortex of a normal rat. Decreasing concentrations of PKA RIIα standard cRNA (100–6.25 pg) were added to a constant amount (1 μg) of total RNA isolated from cortex. The mixtures were reverse transcribed and PCR amplified in the presence of trace amounts of [32P]dCTP; aliquots were digested by XhoI and electrophoresed on 1.5% agarose gel. The higher molecular size band (383 bp) corresponds to amplification product arising from the mRNA, whereas the lower band (191 + 192 bp) arises from cRNA generated from the internal standard digested by XhoI. Bl, blank. 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 RIIα mRNA amplification product versus the known amount of internal standard cRNA added to the test sample. The point of equivalence represents the amount of RIIα mRNA. C, effects of chronic administration (i.p. injections once daily for 21 days) of HAL (1 mg/kg) or CLOZ (20 mg/kg) on mRNA levels of PKA RIIα-subunit isoform in rat cortex, hippocampus, and striatum. Data are the mean ± S.D. from six rats in each group. HAL- and CLOZ-treated groups were compared with the control group. *p < 0.001.
HAL, had no significant effects on RIα-, RIβ-, or Catα-subunits. These changes were accompanied by alterations in mRNA expression of respective regulatory and catalytic subunits as determined by quantitative RT-PCR using specific primers. These results, thus, suggest that observed alterations in [3H]cAMP binding to regulatory subunits and catalytic activity of PKA are related to altered expression of specific regulatory and catalytic subunits, respectively.

Our results of the opposite effects of HAL and CLOZ on regulatory and catalytic activities of PKA and the specificity in the expression of PKA subunits appear to be quite interesting. Earlier studies suggested that antipsychotic drugs modulate adenyl cyclase-cAMP pathway upstream at the level of cAMP formation or the expression of G-protein subunits, involved in the inhibition (Giα) or stimulation (Gsα) of adenyl cyclase. For example, in vitro studies using HeLa cells expressing 5HT1A receptors (Assie et al., 1997) or Chinese hamster ovary cells expressing muscarinic M4 receptors (Zeng et al., 1997) showed decreased cAMP formation by CLOZ. On the other hand, Kaplan et al. (1999) reported that chronic administration of HAL to rats increased GTPγ-S-stimulated adenyl cyclase activity in cortex, whereas olanzapine decreased this activity. However, in the striatum brain area, HAL was ineffective in causing changes in adenyl cyclase activity, whereas olanzapine decreased adenyl cyclase activity in this brain area (Kaplan et al., 1999). In contrast, Kaneko et al. (1992) showed that intravenous injection of HAL to rats increased cAMP levels in striatum. Several other studies suggested that the expression of Giα or Gsα-protein is altered in rat brain after chronic treatment of HAL or CLOZ to rats. For example, expression of Giα and Gsα were decreased by HAL and increased by CLOZ in the striatum brain region (Gupta and Mishra, 1992; Shin et al., 1995). Kaplan et al. (1999) recently showed that the level of Gsα decreased in the cortex but not in the striatum. No change in the expression of these two G-protein subunits after HAL treatment has also been reported (See et al., 1993; Meller and Bohmaker, 1996). The reports of expression of G-protein subunits after HAL or CLOZ treatment, therefore, are quite inconsistent. On the other hand, from the results of
adenylyl cyclase activity or cAMP formation, one can infer that HAL increases, whereas CLOZ decreases, cAMP formation in rat brain. At functional level, increased cAMP by HAL should activate PKA and CLOZ should inactivate PKA; however, that does not appear to be the case. We have observed that HAL increases expression of regulatory subunits and catalytic activity of PKA specifically in the striatum but not in the cortex. If observed PKA activation was related to cAMP formation, we would have seen this effect also in cortex, where increased GTPγ-S-mediated cAMP formation by HAL was found (Kaplan et al., 1999). These effects, thus, appear to be unrelated to changes in G proteins or cAMP levels upstream in this pathway. Furthermore, these changes are not due to the direct effects of drugs, because acute treatment of HAL or CLOZ was ineffective in causing any alterations in PKA. One speculation could be that these effects might be indirect, secondary to the changes in the number and/or expression of receptors, such as dopamine D₁ and D₂, α₂-adrenergic, 5HT₁A, or 5HT₁D, linked to adenylyl cyclase-cAMP signaling transduction system in inhibitory or stimulatory fashion by HAL or CLOZ. This is further supported by the observation that dopamine D₂ receptors are rich in the striatum, whereas the cortex and hippocampus are rich in serotonin and adrenergic receptors. On the other hand, PKA subunits are abundantly expressed in all of these brain areas.

At the neuroanatomical level, the brain region specificity of altered expression of selective RIIα- and RIIβ-subunits by HAL in the striatum and RIIα-, RIIβ-, and Catβ-subunits by CLOZ in the cortex, hippocampus, and striatum is quite intriguing. This specificity could be related to the superior clinical efficacy of CLOZ over HAL, because numerous reports have implicated striatal (Pearce et al., 1990), cortical (Goldman-Rakic, 1991; Shenton et al., 1992; Selemion et al., 1995), and hippocampal (Luchins, 1990) structures in the pathophysiology of schizophrenia. It is quite possible that

Fig. 8. A, a representative experiment showing a competitive PCR analysis for PKA Catβ-mRNA content in cortex of a normal rat. Decreasing concentrations of PKA Catβ-standard cRNA (50–3.125 pg) were added to a constant amount (1 μg) of total RNA isolated from cortex. The mixtures were reverse transcribed and PCR amplified in the presence of trace amounts of [32P]dCTP; aliquots were digested by XhoI and electrophoresed on 1.5% agarose gel. The higher molecular size band (375 bp) corresponds to amplification product arising from the mRNA, whereas the lower band (178 + 197 bp) arises from cRNA generated from the internal standard digested by XhoI. Bl, blank. 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 RIIα-mRNA amplification product versus the known amount of internal standard cRNA added to the test sample. The point of equivalence represents the amount of Catβ-mRNA. C, effects of chronic administration (i.p. injections once daily for 21 days) of HAL (1 mg/kg) or CLOZ (20 mg/kg) on mRNA levels of PKA Catβ-subunit isoform in rat cortex, hippocampus, and striatum. Data are the mean ± S.D. from six rats in each group. HAL- and CLOZ-treated groups were compared with the control group. * p < 0.001.
chronic treatment of CLOZ may be ameliorating schizophrenia symptoms by reducing activation and expression of specific PKA Catβ-, RIα-, and RIβ-subunits in these brain regions. On the other hand, increased RIα- and RIβ-subunit expression in the striatum by HAL may be related to its extrapyramidal side effect, the brain area in which antagonism of D2 receptors is generally believed to be associated with this property. Thus, activation of PKA in striatum could partially be involved in mediating extrapyramidal side effect by HAL, whereas CLOZ, which has opposite effects on PKA in this and other brain areas, may not only be preventing extrapyramidal side effect but may be associated with its antipsychotic properties. Interestingly, a recent study suggests that the expression of RI and RIα subunits of PKA are decreased in the platelets of schizophrenia subjects (Tardito et al., 2000), raising the possibility that schizophrenia may be related to PKA activation and that CLOZ may alleviate the symptoms by deactivating PKA.

The mechanism by which PKA is involved in HAL- and CLOZ-mediated actions is a matter of further study; however, it is well established that many biological functions are regulated by the state of phosphorylation of specific proteins. PKA is an important regulatory enzyme that phosphorylates various substrates, including transcription factors, involved in synthesis and release of neurotransmitters, receptor down-regulation and desensitization, and expression of genes implicated in survival and maintenance of neurons. Differential modulation in the expression of regulatory and/or catalytic subunit(s) of PKA by HAL and CLOZ may, thus, result in significant alterations in various physiological functions, which may in turn be associated with mechanism of action of these two antipsychotic drugs.

In conclusion, we observed that HAL and CLOZ differentially regulated the gene expression of selective regulatory and catalytic subunit isoforms such that HAL increased and CLOZ decreased the expression of RIα and RIβ. In addition, CLOZ also decreased the expression of Catβ-subunit isoform. These changes were accompanied with increased (by HAL) or decreased (by CLOZ) cAMP-dependent PKA activity and [3H]AMP binding to regulatory subunits of PKA. These changes were confined to specific brain areas. For example, HAL was effective only in the striatum, whereas CLOZ caused changes in the cortex, hippocampus, and striatum. This differential effect and brain region selectivity, thus, may be relevant in the mechanisms of action of typical and atypical antipsychotics; however, further studies are needed to examine whether these effects are common to all typical and atypical antipsychotics or are restricted to HAL and CLOZ.

References


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