ABSTRACT

The hypothalamic-pituitary-adrenal (HPA) axis has been shown to be involved in mood and behavior. The possibility that adrenal glucocorticoids regulate components of the phosphatidylinositol (PI) signal transduction pathway was investigated. Two different doses of corticosterone (CORT) pellets (50 or 100 mg) were implanted in normal and bilaterally adrenalectomized (ADX) rats, and CORT regulation of the expression of Gqα protein, phospholipase C (PLC) isozymes, inositol 1,4,5-trisphosphate receptor (IP3R) isoforms, and of PI-PLC activity, [3H]IP3 binding to IP3Rs, and IP3 levels were measured in various brain areas after 1 or 14 days. Fourteen days of CORT treatment of normal rats dose dependently decreased PI-PLC activity and selectively the mRNA and protein expression of PLCβ1 isozyme in cortex and hippocampus. Bilateral ADX caused the opposite changes in these measures, and simultaneous CORT pellet implantation into ADX rats reversed these effects. Furthermore, 14 days of CORT treatment of normal rats increased [3H]IP3 binding to IP3Rs and decreased IP3 levels in cortex, hippocampus, and cerebellum, without any changes in expression of IP3R-I, IP3R-II, or IP3R-III isoform. On the other hand, ADX decreased [3H]IP3 binding and increased levels of IP3, and simultaneous CORT treatment of ADX rats prevented these changes. ADX or CORT treatment had no significant effects on the expression of Gq/11α protein.

These results suggest that manipulation of the HPA axis alters various components of the PI signaling pathway in rat brain, which may have physiological relevance to the HPA axis-mediated changes in mood and behavior.

Several clinical and preclinical studies suggest that adrenal steroids are involved in various functional aspects of the central nervous system such as behavior, emotion, and learning (for review, see McEwen, 1987). This is evident from the studies suggesting that abnormal hypothalamic-pituitary-adrenal (HPA) functions are associated with affective disorders. For example, higher levels of cortisol in plasma and of corticotrophin-releasing hormone in cerebrospinal fluid, and failure to suppress plasma cortisol levels after administration of dexamethasone (also known as the dexamethasone suppression test) have been shown in a subgroup of depressed patients (for review, see Nelson and Davis, 1997). In addition, glucocorticoid treatment induces depression (Ling et al., 1981), and antidepressants (for review, see Wolkowitz and Reus, 1999). Also, Fernandes et al. (1997) recently showed that protracted treatment with corticosterone (CORT) induces behavioral depression in rats.

The precise mechanisms by which behavioral changes occur because of abnormal HPA functions are not clear; however, adrenal steroids have been shown to alter the expression of many neurotransmitter receptors, including 5-hydroxytryptamine (5HT)1A, 5HT2A, 5HT2C, γ-aminobutyric acid, and β-adrenergic receptors (Meyer, 1985; Kuroda et al., 1993; Chaouloff, 1995). Besides their genomic actions, emerging evidence suggests that adrenal steroids may use receptor-mediated signaling pathways to regulate a wide range of functions over a broader time scale (for review, see Moore and Evans, 1999). In this regard, we examined the possible involvement of the phosphatidylinositol (PI) signaling pathway, which has been shown to be linked with many receptors, including 5HT2A, 5HT2C, and α1-adrenergic receptors, in glucocorticoid-mediated action.
In the PI signaling pathway, agonist-induced interaction of surface receptors with guanine nucleotide binding proteins (G proteins) activates the enzyme PI-specific phospholipase C (PI-PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG) (Berridge and Irvine, 1989). Both IP$_3$ and DAG act as second messengers. Whereas IP$_3$ mobilizes Ca$^{2+}$ from intracellular sources after binding with IP$_3$ receptors (Berridge et al., 1998), DAG activates the phosphorylating enzyme protein kinase C (Dekker et al., 1995). These events mediate cellular activation and subsequent biological responses such as neurotransmitter release, cell growth, differentiation, neuronal development, and gene expression (Nishizuka, 1988; Berridge and Irvine, 1989). Our earlier studies suggested that chronic but not acute administration of dexamethasone, a synthetic glucocorticoid, increases the catalytic activity of PI-PLC and the mRNA and protein expression of a specific isozyme, PLC$_{b_1}$, in rat brain (Dwivedi and Pandey, 1999). Also, it was previously reported that in vitro addition of dexamethasone to C6 glioma cells mobilizes 5HT$_{2A}$ receptor-mediated Ca$^{2+}$ (Muraoka et al., 1993) and that chronic administration of dexamethasone to rats causes decreased norepinephrine-stimulated PI metabolism in the brain (Takahashi et al., 1996). Because administration of dexamethasone inhibits the release of CORT via a feedback mechanism and is a poor substitute for depleted endogenous glucocorticoids in the brain (Birmingham et al., 1993), from these studies it is not clear whether the effects produced by dexamethasone are in response to endogenous glucocorticoids and whether the administration of exogenous glucocorticoids has similar or opposite effects to those elicited by dexamethasone.

To clarify these issues, in the present investigation, we studied the effects of both endogenous and exogenous glucocorticoids on PI-PLC activity and on the expression of PLC isozymes in various brain areas by adrenalectomizing (ADX) rats and simultaneously implanting CORT pellets into ADX rats, as well as by implanting CORT pellets of various doses into normal rats. Because G$_a$ proteins are coupled to and activate the enzyme PI-PLC (Strathmann and Simon, 1990), we studied their expression in brain of these rats. Furthermore, given the significance of IP$_3$ in mobilizing Ca$^{2+}$ from intracellular sources and mediating the signaling further downstream, we examined the effects of exogenous and endogenous glucocorticoids on levels of IP$_3$, binding of [H]IP$_3$ to IP$_3$ receptors (IP$_3$R$_s$), and on the expression of various isoforms of IP$_3$R$_s$ in brain of these rats. This investigation not only clarifies whether changes in PI-PLC are associated with endogenous glucocorticoids but also helps elucidate the role of IP$_3$ in glucocorticoid-mediated actions and, ultimately, the involvement of these components of the PI signaling system in HPA axis-mediated changes in mood and behavior.

**Experimental Procedures**

**Materials**

- D-IP$_3$, phosphatidylinositol 4,5-bisphosphate (PIP$_2$), hexadecyltrimethylammonium bromide (cetrimide), and monoclonal antibody for β-actin were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibodies for PLC $b_1$, δ$_1$, and γ$_1$ were obtained from Upstate Biotechnology (Lake Placid, NY). Antibodies for IP$_3$R-I, IP$_3$R-II, and IP$_3$R-III were purchased from Santa Cruz Biotechnol-ogy (Santa Cruz, CA).
- [H]PIP$_2$, Hot Tm DNA polymerase, RNase inhibitor, BgIII, [α-$^{32}$P]dCTP, horseradish peroxidase-linked secondary antibodies (anti-mouse/rabbit), enhanced chemiluminescence (ECL)-nitrocellulose membrane, chemiluminesence detection agent, and the IP$_3$ assay kit were obtained from Amersham (Arlington Heights, IL). EcoRI, HindIII, and the in vitro transcription kit were purchased from Promega (Madison, WI). CORT pellets were from Innovative Research of America (Sarasota, FL).

**Animals**

Virus-free Sprague-Dawley male rats initially weighing 220 to 220 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. Rats were acclimated for 1 week before the experiment started.

**ADX and Treatment with CORT**

Two treatment protocols were used. In the first protocol, rats under light halothane anesthesia were s.c. implanted with CORT pellets containing 50 or 100 mg of CORT in a cholesterol base. These CORT pellets can maintain a physiological serum concentration of CORT for 21 days. The release of CORT after implantation of a 50- or 100-mg CORT pellet is 2.38 or 4.76 mg/day, respectively. Control rats underwent an identical surgery procedure with implantation of a cholesterol base pellet or underwent no treatment; these two types of treatment did not differ in their results in the final determinations of PI-PLC activity, of [H]IP$_3$ receptor binding, or of protein levels of PLC isozymes or IP$_3$R isoforms. Rats were decapitated 1 or 14 days after pellet implantation. In the second protocol, rats were anesthetized with halothane anesthesia. Bilateral ADX was performed by making a small incision (0.5 in.) in the skin and the muscle wall just below the ribcage. The adrenal glands were visualized and removed. The muscle wall was sutured, and the skin incision was closed with wound clips. Control rats were sham-operated; the adrenal glands were visualized but not removed. These rats were given drinking water containing 0.9% (w/v) saline ad libitum. They were decapitated on day 1 or 14 after ADX. Some ADX rats were implanted s.c. with placebo or a CORT pellet (containing 50 or 100 mg of CORT) immediately after ADX. The rats were decapitated 1 or 14 days after CORT pellet implantation.

The trunk blood was collected on ice at decapitation and was centrifuged, and then the serum was stored at −80°C until the assays were performed. Serum CORT levels were measured by a commercially available radioimmunoassay kit (ICN Biomedical, Inc., Cleveland, OH). Brains were removed quickly after the blood was taken. Cortex, hippocampus, and cerebellum were dissected out and immediately stored at −80°C until analyzed. For both experimental protocols, rats were decapitated between 9:00 AM and 11:00 AM, corresponding to 3 to 5 h after lights on.

**Preparation of Membrane and Cytosol Fractions**

Cortices and hippocampi were homogenized in a homogenizing buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EGTA, 5 mM EDTA, 1.5 mM pepstatin, 2 mM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 U/ml aprotinin, and 2 mM dithiothreitol, using the Polytron. The supernatant was centrifuged at 100,000g for 60 min at 4°C. The resulting supernatant was the cytosol fraction, and the pellet was resuspended in the homogenizing buffer containing 0.2% (w/v) Triton X-100. The homogenate was kept at 4°C for 60 min with occasional stirring and then centrifuged at 100,000g for 60 min at 4°C. The resulting supernatant was used as the membrane fraction. Both the membrane and the cytosol fractions were used to determine the PI-PLC activity and the immunolabeling of PLC isozymes. The concentration of protein in these fractions was determined using the procedure of Lowry et al. (1951).
Determination of PI-PLC Activity

PI-PLC activity was measured in membrane and cytosol fractions of cortex and hippocampus by the enzymatic assay procedures described by Dwivedi and Pandey (1999a) using 5 μg of protein/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 unlabeled PIP₂, 2 μCi/ml [³²P]PIP₂, and 0.5 mg/ml cetrimide) in a total volume of 100 μl at 37°C for 10 min. The reaction was terminated by the 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 1000g 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. PI-PLC activity is expressed as the amount of [³²P]IP₃ formed (dpm) per milligram of protein.

Immunolabeling of PLC β₁, γ₁, and δ₁ Isozymes

Equal volumes of protein samples (membrane or cytosol fractions; 20 μl containing 30 μg of protein) and gel loading solution (50 mM Tris-HCl (pH = 6.8), 4% 2-mercaptoethanol, 1% 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. The samples (30 μg of protein in each lane) were loaded onto 7.5% (w/v) 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 ECL-nitrocellulose membrane (Amersham) using the Mini Trans Blot transfer unit (Bio-Rad) at 1.050-amp constant current. Membranes were washed with Tris-buffered saline/Tween 20 (TBST) buffer (10 mM Tris base, 0.15 M NaCl, and 0.05% Tween 20) for 10 min. The blots were blocked by incubating 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). Then the blots were incubated for 1 h with primary monoclonal antibody (anti-PLC β₁, γ₁, or δ₁) at a dilution of 1:1000 (Dwivedi and Pandey, 1999a). The membranes were then washed with TBST and incubated with horseradish peroxidase-linked secondary antibody (anti-mouse IgG; 1:3000) for 1 h at room temperature. The membranes were then extensively washed with TBST and exposed to ECL film. Before starting the immunolabeling experiments with the samples, the procedure was standardized with TBST and then exposed to film. For further details, see Dwivedi and Pandey (1999a). The membranes were then incubated with horseradish peroxidase-linked secondary antibody (anti-mouse IgG; 1:3000) for 1 h at room temperature. The membranes were then extensively washed with TBST and exposed to ECL film. Before starting the immunolabeling experiments with the samples, the procedure was standardized.

Synthesis and Cloning of Internal Standards. The detailed procedures for the preparation of internal standards (cRNA) have been described by Dwivedi and Pandey (1999a). Each internal standard targeted by the same primers used to amplify the canonic sequence was generated by site-directed mutagenesis to introduce a BglII restriction site between the amplification primers so that the digestion of the amplicon would generate two fragments of approximately equal molecular size. The internal primer sequence was as follows: (645–668 bp) 5'-CTACTTTCCGAGACCCGAGG-3', and reverse, 5'-TOCTTGTTGGCTGATCTTTCT-3' (Suh et al., 1988; GenBank accession no. M208636). Each primer contained a comparable GC content to minimize variability in hybridization efficiency at the annealing temperature. The specificity of the PLC β₁ mRNA product 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 size.

Quantitative Analyses of PLC β₁ Isozyme mRNA by Competitive RT-PCR. Decreasing concentrations of PLC β₁ internal standard cRNA were added to 1 μg of total RNA isolated from cortices or hippocampi. The RNA/cRNA mixtures were denatured at 80°C for 5 min and then reverse-transcribed with cloned Moloney murine leukemia virus and reverse transcriptase (200 U; Life Technologies, Grand Island, NY) in RT buffer containing 50 mM Tris-HCl (pH = 8.3), 75 mM KCl, 3 mM MgCl₂, and 1 mM dNTPs using random hexamers (2.5 mM) and human placenta ribonuclease inhibitor (HPRI) (8.3 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 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 amplified mixture contained cDNA, 0.5 μM specific primer pairs, 1.5 mM MgCl₂, 50 mM Tris-HCl (pH = 9.0), 20 mM ammonium sulfate, 15 mM KCl, and 0.5 U of Hot Tub DNA polymerase in a 100-μl volume. Trace amounts of [³²P]dCTP (0.5–1 Ci/sample) were included during the PCR step for subsequent quantification. The PCR mixture was amplified for 32 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 mRNA amplification product versus the known amount of internal standard (cRNA) added to the test sample. The results are expressed as attomoles per microgram of total RNA.

Preparation of Membranes for [3H]IP$_3$ Binding Assay and Immunolabeling of IP$_3$R Isoforms

Brain samples were homogenized by Polytron at setting 7 for 30 s in a homogenizing buffer containing 50 mM Tris-HCl (pH = 7.7), 1 mM EDTA, and 2 mM 2-mercaptoethanol. The homogenate was centrifuged at 40,000g for 15 min at 4°C. The supernatant was discarded and the pellet was homogenized once again in the homogenizing buffer and centrifuged as described above. This process was repeated one more time and the resulting pellet was resuspended in a buffer containing 50 mM Tris-HCl (pH = 8.4), 1 mM EDTA, and 1 mM 2-mercaptoethanol. This fraction was used for the [3H]IP$_3$ binding assay, as well as for the immunolabeling of IP$_3$Rs.

[3H]IP$_3$ Binding Assay

Binding of [3H]IP$_3$ was carried out in duplicate by the procedures described in Dwivedi et al. (1998). The incubation medium contained incubation buffer (50 mM Tris-HCl, pH = 7.7, 1 mM 2-mercapto-ethanol, 1 mM EDTA), [3H]IP$_3$ (specific activity 21 Ci/mmol) ranging from 10 to 100 nM (six different concentrations), and 40 µl of membrane suspension in a total volume of 100 µl. Nonspecific binding was determined in the presence of 10 µM D-IP$_3$. The incubation was performed at 4°C for 10 min and rapidly terminated by the addition of 5 ml of cold washing buffer [50 mM Tris-HCl, pH = 7.7, 1 mM EDTA, and 0.1% (w/v) BSA] and filtration under vacuum through Whatman GF/B filters. The filters were dried and the filter-bound radioactivity was analyzed by a liquid scintillation counter. Specific binding was defined as the difference between total binding and binding observed in the presence of D-IP$_3$. The maximum number of binding sites ($B_{max}$) and the apparent dissociation constant ($K_d$) were computed by Scatchard Analysis using the EBDA program (McPherson, 1985). The protein concentration in membrane fraction was determined by the method of Lowry et al. (1951).

Immunolabeling of IP$_3$R Isoforms

The immunodetectable levels of IP$_3$R-I, IP$_3$R-II, and IP$_3$R-III isoforms were determined by Western blot as described above for PLC isoforms and also in our previous publication (Dwivedi et al., 1998). Equal amounts of protein samples (25 µg) were subjected to a 7.5% polyacrylamide gel and subsequently transferred to an ECL nitrocellulose membrane. The blots were incubated with primary antibodies (anti-Gq/11 α) and the subsequent steps were performed similarly as described previously for the immunolabeling of PLC isoforms. The bands were developed using a chemiluminescence detection system (Amersham). The bands on the autoradiograms were quantified as described previously for the immunolabeling of PLC isoforms.

Statistics

Data were analyzed using the SPSS 9.0 (Chicago, IL) statistical software package. All values are given as the mean ± S.D. Data were subjected to one-way ANOVA followed by Dunnett's test for multiple comparisons (Dunnett, 1980). An α-value lower than .05 was considered significant.

Results

Serum CORT Levels. The serum CORT levels 1 day after ADX (acute) were as follows: sham, 52.5 ± 14.1; ADX, 0.3 ± 0.2 ng/ml, whereas at day 14 (chronic), we could not detect CORT in ADX rats. The levels of CORT in serum 14 days after CORT pellet implantation into ADX rats were as follows: ADX + CORT (50-mg pellet), 25.2 ± 6.7; ADX + CORT (100-mg pellet), 47.8 ± 15.1 ng/ml. CORT pellet implantation into normal rats yielded the following serum CORT levels at day 14: CORT (50-mg pellet), 60.1 ± 17.5; CORT (100-mg pellet), 110 ± 19.5 ng/ml. This is in agreement with our previous observations (Dwivedi and Pandey, 2000).

Effects of ADX and of Simultaneous CORT Pellet Implantation into ADX Rats on PI-PLC Activity. It was observed that 1 day after ADX there were no significant effects on PI-PLC activity either in cortex (membrane: sham, 20,659 ± 1,967; ADX, 21,137 ± 2,034 dpm/min/mg of protein; cytosol: sham, 22,128 ± 2,153; ADX, 21,865 ± 1,553 dpm/min/mg of protein) or in hippocampus (membrane: sham, 29,511 ± 2,175; ADX, 31,165 ± 2,017 dpm/min/mg of protein; cytosol: sham, 19,960 ± 1,967; ADX, 20,343 ± 2,021 dpm/min/mg of protein).
The molecular mass of the PLC isoform was 46 kDa (Fig. 3). The immunolabeling of the various PLC isozymes (PLC-1, PLC-2, and PLC-3) in both membrane and cytosol fractions of cortex or hippocampus (data not shown). However, as shown in Fig. 2, at 14 days both doses of CORT had significantly decreased the catalytic activity of PI-PLC, the higher dose (100 mg) being more effective in both membrane and cytosol fractions of cortex (Fig. 2A) and hippocampus (Fig. 2B) than the lower dose (50 mg).

**Effects of ADX and of Simultaneous CORT Pellet Implantation into ADX Rats on Immunolabeling of PLC Isozymes (PLC-1, PLC-2, and PLC-3) as Well as on mRNA Levels of the PLC β1 Isozyme.** We determined the immunolabeling of the various PLC isoforms (PLC-1, PLC-2, and PLC-3) in both membrane and cytosol fractions of cortex and hippocampus. As reported in Dwivedi and Pandey (1999a), it was observed that PLC-1 migrated to 150 kDa, whereas PLC-2 and PLC-3 migrated to 85 and 145 kDa, respectively (Fig. 3). The molecular mass of β-actin, used as a housekeeping protein, was 46 kDa (Fig. 3). The immunolabeling of β-actin in cortex and hippocampus did not change either after ADX or after CORT pellet implantation into normal rats. This is in agreement with our previous reports suggesting that levels of β-actin remain unaltered in rat brain after treatment with dexamethasone or CORT (Dwivedi and Pandey, 1999a,b, 2000).

We did not observe any significant effects 1 day after ADX on the immunodetectable levels of PLC-1, PLC-2, or PLC-3 isozyme in membrane or cytosol fractions of cortex or hippocampus (data not shown). Representative Western blots showing the effects of ADX and of CORT pellet implantation into ADX rats are given in Fig. 3 and diagrammatically depicted in Fig. 4. The results indicate that 14 days after ADX there was a significant increase in levels of the PLC-1 isozyme in both membrane and cytosol fractions of cortex and hippocampus (Fig. 4), without any significant changes in the levels of PLC-2 or PLC-3 isozyme. Simultaneous implantation of CORT pellets, either 50 or 100 mg, was able to prevent the ADX-induced increase in immunolabeling of the PLC-1 isozyme in
both membrane and cytosol fractions of cortex and hippocampus (Fig. 4). Implantation of the 100-mg CORT pellet completely reversed the levels of PLCβ1 to normal levels, whereas the 50-mg CORT pellet produced significant reversal but only partial reversal (Fig. 4).

Because we observed that ADX specifically increased the protein levels of only the PLCβ1 isozyme, we sought to determine whether this increase was associated with an increase in its mRNA levels. We had earlier characterized the determination of mRNA levels of the PLCβ1 isozyme by quantitative RT-PCR in rat cortex and hippocampus. As reported by Dwivedi and Pandey (1999a), we observed an amplification product of PLCβ1 arising from the mRNA template at 316 bp and the corresponding digestion product arising from the cRNA at 214 + 122 bp (Fig. 5A). It was observed that mRNA levels of the PLCβ1 isozyme followed a similar pattern of changes after ADX as was observed with its protein levels, i.e., 1 day after ADX there were no significant effects on mRNA levels of the PLCβ1 isozyme either in cortex or in hippocampus (data not shown). However, by 14 days after ADX, there was a significant increase in the expression of the PLCβ1 isozyme in both cortex and hippocampus (Fig. 6). Furthermore, the ADX-induced increase in mRNA levels of the PLCβ1 isozyme was reduced by both doses of CORT pellets. The higher dose (100 mg) was more effective and almost completely abolished the ADX-induced increase in PLCβ1 mRNA levels, whereas the reversal was only partial by the lower dose (50 mg) in both cortex and hippocampus (Fig. 6).

Effects of CORT Treatment of Normal Rats on Immunolabeling of PLC Isozymes (PLCβ1, δ1, and γ1) and on mRNA Levels of the PLCβ1 Isozyme. Acute (1 day) implantation of a CORT pellet into normal rats, either 50 or 100 mg, failed to cause any changes in the immunolabeling of PLCβ1, δ1, or γ1 isozyme in cortex or hippocampus (data not shown).

Representative Western blots showing the effects of ADX and of CORT pellet implantation into ADX rats are given in Fig. 7 and diagrammatically depicted in Fig. 8. Chronic (14 days) implantation of a CORT pellet into normal rats, 50 or 100 mg, caused a significant decrease in the immunolabeling of the PLCβ1 isozyme in membrane and cytosol fractions of cortex and hippocampus (Fig. 8). This effect was dose dependent, i.e., the higher dose of CORT (100 mg) caused a greater decrease than the lower dose (50 mg). Implantation of the CORT pellets did not cause any significant effects on the immunolabeling of PLCδ1 or γ1 isozyme either in cortex or in hippocampus.

Fig. 4. Effects of 14 days of ADX and of ADX + CORT pellet implantation (50 or 100 mg) on protein levels of PLC isozymes in membrane and cytosol fractions of cortex (A) and hippocampus (B). □, sham-operated; □, ADX; □, ADX + CORT (50 mg); □, ADX + CORT (100 mg). Values are the mean ± S.D. from six rats in each group. The ADX group was compared with the sham-operated group, whereas the ADX + CORT-treated groups were compared with the ADX group. *, as compared with sham; **, as compared with ADX. *P < .01, **P < .001. Note that we did not observe any significant effects of ADX on protein levels of PLCδ1 or γ1 isozyme in cortex or hippocampus.

Fig. 5. A, representative experiment showing a competitive PCR analysis for PLCβ1 mRNA content in the cortex of a normal rat brain. Decreasing concentrations of PLCβ1 standard cRNA (400–25 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]dCPT; 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 band (214 + 122 bp) arises 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 PLCβ1 mRNA.
The effects of CORT treatment on mRNA levels of the PLC $\beta_1$ isozyme are shown in Fig. 9. Similarly as was observed regarding protein levels of the PLC $\beta_1$ isozyme, we found that mRNA levels of the PLC $\beta_1$ isozyme were significantly decreased with both doses of CORT in both cortex and hippocampus (Fig. 9). This effect was dose dependent, i.e., the magnitude of the decrease was greater with the 100-mg dose of CORT than the 50-mg dose.

Effects of ADX and of Simultaneous CORT Pellet Implantation into ADX Rats on $[3H]$IP$_3$ Binding and on Immunolabeling of IP$_3$ Rs.
In our initial experiments, we characterized $[3H]$IP$_3$ binding in various areas of rat brain and found that $B_{max}$ of $[3H]$IP$_3$ binding was highest in cerebellum, whereas in cortex and hippocampus, $B_{max}$ was much lower. However, the affinity of $[3H]$IP$_3$ binding was higher in cortex and hippocampus than in cerebellum, which is in agreement with reports in the literature (Snyder and Supattapone, 1989); therefore, besides cortex and hippocampus, we also determined $[3H]$IP$_3$ binding in the cerebellum.

It was observed that 1 day after ADX there were no significant effects either on $B_{max}$ or $K_D$ of $[3H]$IP$_3$ binding in cortex, hippocampus, or cerebellum (data not shown). Fourteen days of ADX, however, significantly decreased $B_{max}$ of $[3H]$IP$_3$ binding in cortex, hippocampus, and cerebellum (Table 1). The extent of the decrease was similar in all three brain areas. When ADX rats were simultaneously implanted with a CORT pellet (50 or 100 mg), it was observed that the ADX-induced decrease in $B_{max}$ was reversed significantly at both doses, with the higher dose of CORT being more effective and almost completely normalizing $B_{max}$ in cortex, hippocampus, and cerebellum. Neither ADX nor CORT implantation had any significant effects on $K_D$ of $[3H]$IP$_3$ binding in cortex, hippocampus, or cerebellum (Table 1).

Because $[3H]$IP$_3$ binds to all the isoforms of IP$_3$ Rs without discriminating between them, we next determined whether the decrease in number of IP$_3$ Rs binding sites by ADX was due to altered expression of a specific isoform(s) of IP$_3$ Rs. Ini-
Fig. 10. Representative Western blots showing the effects of 14 days of ADX and CORT pellet implantation (50 or 100 mg) into ADX rats on [3H]IP$_3$ binding in rat brain. Values are expressed as the mean ± S.D. n = 6 in each group. CORT-treated groups were compared with sham.

Table 1

Effects of 14 days of ADX and of CORT pellet implantation (50 or 100 mg) into ADX rats on [3H]IP$_3$ binding in rat brain.

<table>
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<th>$B_{\text{max}}$ (pmol/mg protein)</th>
<th>$K_D$ (nM)</th>
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<tr>
<td>Cortex</td>
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<tr>
<td>Sham-operated</td>
<td>0.52 ± 0.01</td>
<td>5.7 ± 0.81</td>
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<td>ADX</td>
<td>0.21 ± 0.03$^{*,**,†}$</td>
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<td>ADX + CORT (50 mg)</td>
<td>0.33 ± 0.04$^{*,**,†}$</td>
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<td>ADX + CORT (100 mg)</td>
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<td>Hippocampus</td>
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<tr>
<td>Sham-operated</td>
<td>0.69 ± 0.03</td>
<td>6.1 ± 0.92</td>
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<tr>
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<td>0.33 ± 0.08$^{*,**,†}$</td>
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<td>13.5 ± 0.98</td>
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</tr>
<tr>
<td>ADX + CORT (50 mg)</td>
<td>11.7 ± 1.8$^{*,**,†}$</td>
<td>14.6 ± 1.7</td>
</tr>
<tr>
<td>ADX + CORT (100 mg)</td>
<td>15.2 ± 0.9$^{*,**,†}$</td>
<td>13.8 ± 1.5</td>
</tr>
</tbody>
</table>

* As compared with sham; †† as compared with ADX; $^*$ $P < .01$, $^{**} P < .001$.

We determined $B_{\text{max}}$ and $K_D$ of [3H]IP$_3$ binding in cortex, hippocampus, and cerebellum after acute or chronic implantation of a CORT pellet into normal rats. Acute implantation of CORT (1 day) had no significant effects on $B_{\text{max}}$ or $K_D$ of [3H]IP$_3$ binding in any of the brain areas (data not shown). The results of $B_{\text{max}}$ of [3H]IP$_3$ binding after 14 days of CORT pellet implantation are given in Table 2. It was observed that chronic implantation of CORT at both the 50- and the 100-mg dose significantly increased $B_{\text{max}}$ of [3H]IP$_3$ binding in cortex, hippocampus, and cerebellum, and this effect was dose dependent, i.e., the magnitude of increase was greater with the 100-mg CORT pellet. Furthermore, CORT implantation, of either 50 or 100 mg, had no significant effects on $K_D$ values in any of the brain areas studied (Table 2).

Our immunolabeling studies of IP$_3$R-I, IP$_3$R-II, and IP$_3$R-III isoforms indicate that acute or chronic implantation of a CORT pellet into normal rats did not cause any significant effects on the levels of any of the IP$_3$R isoforms in cortex, hippocampus, or cerebellum, as is indicated in representative Western blots (Fig. 10B) from the cortex brain area showing no changes in the protein levels of IP$_3$R isoforms after 14 days of CORT pellet implantation (50 or 100 mg) into normal rats.

Effects of ADX and Simultaneous CORT Pellet Implantation into ADX Rats on IP$_3$ Levels. To examine whether the changes in IP$_3$ were responsible for the observed changes in [3H]IP$_3$ binding sites, we determined IP$_3$ levels in cortex, hippocampus, and cerebellum. One day after ADX there were no significant effects on the levels of IP$_3$ in cortex, hippocampus, or cerebellum. However, 14 days of ADX caused a substantial and significant increase in levels of IP$_3$.

Table 2

Effects of 14 days of CORT pellet implantation (50 or 100 mg) into normal rats on [3H]IP$_3$ binding in rat brain.

<table>
<thead>
<tr>
<th></th>
<th>$B_{\text{max}}$ (pmol/mg protein)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham-operated</td>
<td>0.57 ± 0.03</td>
<td>5.9 ± 0.99</td>
</tr>
<tr>
<td>CORT (50 mg)</td>
<td>0.75 ± 0.04$^*$</td>
<td>5.3 ± 0.76</td>
</tr>
<tr>
<td>CORT (100 mg)</td>
<td>0.88 ± 0.06$^*$</td>
<td>5.7 ± 0.68</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham-operated</td>
<td>0.73 ± 0.05</td>
<td>5.8 ± 1.1</td>
</tr>
<tr>
<td>CORT (50 mg)</td>
<td>0.91 ± 0.04$^*$</td>
<td>6.3 ± 0.97</td>
</tr>
<tr>
<td>CORT (100 mg)</td>
<td>1.08 ± 0.02$^*$</td>
<td>6.1 ± 0.86</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham-operated</td>
<td>17.5 ± 0.88</td>
<td>12.6 ± 1.3</td>
</tr>
<tr>
<td>CORT (50 mg)</td>
<td>23.7 ± 1.7$^*$</td>
<td>13.9 ± 1.0</td>
</tr>
<tr>
<td>CORT (100 mg)</td>
<td>27.4 ± 1.0$^*$</td>
<td>12.2 ± 0.95</td>
</tr>
</tbody>
</table>

* $P < .001$.  

The effects of 14 days of ADX and CORT pellet implantation into ADX rats (50 or 100 mg) on the expressed levels of IP$_3$R isoforms are given in Fig. 10A, which indicates that neither ADX nor CORT treatment of ADX rats had any significant effects on the levels of IP$_3$R isoforms in the cortex. Similarly, we did not find any significant effects of ADX or ADX + CORT (50 or 100 mg) on the immunolabeling of IP$_3$R isoforms in hippocampus or cerebellum (data not shown).
in cortex, hippocampus, and cerebellum (Table 3). Implantation of a 50- or 100-mg CORT pellet into ADX rats decreased the levels of IP₃ in all these brain areas. The 100-mg CORT pellet was able to completely prevent the ADX-induced increase in IP₃ levels in cortex, hippocampus, and cerebellum (Table 3).

**Effects of CORT Pellet Implantation into Normal Rats on IP₃ Levels.** IP₃ levels were measured in cortex, hippocampus, and cerebellum after acute and chronic implantation of a CORT pellet into normal rats. Acute (1-day) implantation of CORT had no significant effects on the levels of IP₃ in cortex, hippocampus, or cerebellum. When the levels of IP₃ were determined 14 days after CORT pellet implantation into normal rats, we observed that both the 50- and the 100-mg CORT pellet significantly decreased IP₃ levels in all the brain areas studied (Table 4). This effect was much more pronounced with the 100-mg dose than the 50-mg dose of CORT.

**Effects of ADX and of CORT Pellet Implantation into ADX Rats and into Normal Rats on Levels of Gq/11 α Proteins.** Because Gq/11 α protein has been shown to be coupled with PI-PLC (Stratham and Simon, 1990), we determined whether ADX or CORT pellet implantation affects the levels of Gq/11 α protein in rat brain. Acute or chronic ADX, or CORT pellet implantation into normal or ADX rats, did not cause any significant effects on the immunolabeling of Gq/11 α protein in cortex or hippocampus (data not shown).

**Discussion**

This investigation has led to the following observations: 1) bilateral ADX increased PI-PLC activity and, selectively, the mRNA and protein expression of only the PLC β₁ isozyme, and simultaneous CORT treatment of ADX rats prevented these changes; 2) CORT treatment of normal rats decreased PI-PLC activity and mRNA and protein levels of the PLC β₁ isozyme without any changes in the expression of PLC δ₁ and γ₁ isozymes; 3) ADX decreased the number of [³H]IP₃ binding sites, and CORT treatment of ADX rats reversed this decrease; 4) CORT pellet implantation increased [³H]IP₃ binding sites; 5) ADX or CORT treatment of ADX or normal rats had no significant effects on the immunolabeling of IP₃R isoforms; 6) ADX increased the levels of IP₃, whereas CORT treatment of ADX rats prevented this increase; 7) CORT treatment of normal rats decreased the levels of IP₃; and 8) ADX or CORT treatment had no significant effects on the expression of Gq/11 α proteins. These results thus suggest that adrenal glucocorticoids affect various components of the PI signaling cascade in the central nervous system.

In a previous study, we demonstrated that chronic but not acute dexamethasone treatment of rats caused a significant increase in PI-PLC activity along with an increase in mRNA and protein levels of the PLC β₁ isozyme in cortex and hippocampus (Dwivedi and Pandey, 1999a). Interestingly, in the present study similar observations were noted in ADX rats, namely, ADX increased PI-PLC activity as well as mRNA and protein levels of the PLC β₁ isozyme in rat cortex and hippocampus. On the other hand, simultaneous CORT treatment of ADX rats dose dependently reversed these increases, and implantation of a CORT pellet into normal rats produced effects opposite to those elicited by ADX on PI-PLC activity and on mRNA and protein levels of the PLC β₁ isozyme. It thus appears that PI-PLC is under the regulation of endogenous glucocorticoids; however, why dexamethasone and CORT produce opposite changes is currently unclear. Some evidence suggests that dexamethasone penetrates poorly into the brain; however, uptake and retention of dexamethasone are very high in the pituitary (Birmingham et al., 1993). Recently, Meijer et al. (1998) reported that the multiple-drug-resistance gene's P-glycoprotein binds to dexamethasone and causes poor cell nuclear retention, and suggested that dexamethasone is a poor substitute for depleted endogenous glucocorticoids in brain. In view of these observations, it is possible that administration of dexamethasone might be inhibiting the release of CORT by a feedback mechanism; however, because dexamethasone penetrates poorly into the brain and does not substitute the endogenous glucocorticoids in brain, it may be producing effects similar to those of ADX.

Another important aspect is the selective action of CORT in regulating the expression of only the PLC β₁ isozyme. As is well known, PLC has been categorized into three major families: PLC β, PLC δ, and PLC γ (Cockcroft and Thomas, 1992). All PLC isozymes recognizePIP₂ as a substrate and carry out Ca²⁺-dependent hydrolysis of inositol lipids; however, these isozymes are differentially regulated and expressed (Cockcroft and Thomas, 1992). PLC β is activated by receptors that activate the Gq family of G proteins (Taylor et al., 1991), whereas PLC γ is regulated by receptor and non-receptor tyrosine kinases (Rhee and Bae, 1997). Little is known about the regulation of PLC δ. Our observation of altered expression of the PLC β₁ isozyme, without any changes in PLC γ₁ or δ₁, suggests that glucocorticoid action selectively affects the PI signal transduction system, in which PLC β is the principal mediator of PIP₂ hydrolysis. Furthermore, it appears that the decrease in PI-PLC activity could be due to a decrease in the expression of the PLC β₁ isozyme. This is supported by the notion that CORT treatment causes a generalized decrease in both membrane and

**TABLE 3**

IP₃ levels in rat brain 14 days after ADX and simultaneous implantation of CORT pellets (50 or 100 mg) into ADX rats

<table>
<thead>
<tr>
<th></th>
<th>Cortex</th>
<th>Hippocampus</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>64.8 ± 11.2</td>
<td>75.6 ± 12.4</td>
<td>90.4 ± 17.5</td>
</tr>
<tr>
<td>ADX</td>
<td>103 ± 11.7**</td>
<td>110.2 ± 9.1††</td>
<td>135.2 ± 10.2**</td>
</tr>
<tr>
<td>ADX + CORT (50 mg)</td>
<td>81.2 ± 9.7† †</td>
<td>91.6 ± 8.4††</td>
<td>110.6 ± 11.4† †</td>
</tr>
<tr>
<td>ADX + CORT (100 mg)</td>
<td>59.8 ± 8.5**</td>
<td>72.5 ± 7.6**</td>
<td>95.3 ± 9.8**</td>
</tr>
</tbody>
</table>

* As compared with sham; † as compared with ADX; ** P < .01, †† P < .001.

**TABLE 4**

IP₃ levels in rat brain 14 days after CORT pellet implantation (50 or 100 mg) into normal rats

<table>
<thead>
<tr>
<th></th>
<th>Cortex</th>
<th>Hippocampus</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>60.7 ± 6.2</td>
<td>71.8 ± 11.8</td>
<td>93.1 ± 14.9</td>
</tr>
<tr>
<td>CORT (50 mg)</td>
<td>41.1 ± 3.5*</td>
<td>53.2 ± 9.9*</td>
<td>62.7 ± 8.1*</td>
</tr>
<tr>
<td>CORT (100 mg)</td>
<td>25.4 ± 3.1*</td>
<td>29.5 ± 5.3*</td>
<td>35.1 ± 7.6*</td>
</tr>
</tbody>
</table>

* P < .001.
cytosol PI-PLC activity, which suggests that this reduction in activity may be due to reduced synthesis of the PLC $\beta_1$ isoyme. In this context it is important to mention that the expression of $G_\alpha$, $\alpha$ protein remains unaltered, which supports the concept of selective action of glucocorticoids in brain.

IP$_3$ is one of the important second messengers produced from the hydrolysis of PIP$_2$ by the enzyme PI-PLC. The IP$_3$ signal is physiologically effective only on IP$_3$Rs, which transduce this intermediate signal to a Ca$^{2+}$ signal, by mobilizing Ca$^{2+}$ from intracellular stores, which then participate in various physiological functions (Berridge et al., 1998). To examine whether the decreases in activity and expression of the PLC $\beta_1$ isoyme had any further effects downstream, at the level of IP$_3$, we measured IP$_3$ levels, [H]IP$_3$ binding to IP$_3$Rs, and the expression of individual IP$_3$R isoforms. It was observed that the number of [H]IP$_3$ binding sites in cortex, hippocampus, and cerebellum was decreased by ADX, and that CORT treatment of ADX rats reversed these changes. Furthermore, CORT treatment of normal rats produced changes opposite to those elicited by ADX. Because IP$_3$ binds to all the IP$_3$R isoforms and because specificity and binding affinity of IP$_3$ do not differ in different classes of IP$_3$Rs (Yamada et al., 1994), we further examined whether the decrease in number of IP$_3$ binding sites was due to altered expression of IP$_3$R isoform(s). Three distinct types of the IP$_3$R family have been molecularly cloned: IP$_3$R-I, IP$_3$R-II, and IP$_3$R-III (Furuchi et al., 1994). IP$_3$R-I is the most characterized isoform (Ferris and Snyder, 1992; Mikoshiba, 1993) and is the predominant type in rodent brain. Our immunolabeling studies suggest that CORT treatment of normal or ADX rats has no significant effects on the protein expression of any of the IP$_3$R isoforms, which suggests that the decrease in IP$_3$ binding to IP$_3$R is not due to altered expression of IP$_3$R(s). However, it was observed that CORT treatment of normal rats decreased IP$_3$ levels, whereas ADX caused the opposite effect. Thus, it appears that the decrease in IP$_3$ levels by CORT could be the consequence of decreased PI-PLC activity, and that the number of binding sites for IP$_3$Rs could have been increased as a compensatory event in response to the decreased IP$_3$ levels. Another possibility could be that glucocorticoids may modify the activity of protein kinases, which may in turn cause altered phosphorylation of IP$_3$Rs, thereby alter the binding characteristics of IP$_3$Rs. In fact, it has been demonstrated that the binding characteristics of IP$_3$Rs are altered after phosphorylation mediated by protein kinase A (Snyder and Supattapone, 1989).

The mechanism(s) by which glucocorticoids cause changes in the expression of PLC $\beta_1$ and further downstream, at the level of IP$_3$, appears to be complex because both genomic and nongenomic actions of glucocorticoids have been proposed (Moore and Evans, 1999). Because we did not find significant changes in the expression of PLC $\beta_1$ after acute CORT treatment, whereas chronic treatment of CORT decreased its expression, it indicates that the action of CORT on PI-PLC is a delayed event and this effect may be indirect. There is a possibility that subsequent changes in the signaling cascade or in the brain, which could be genomic in nature, may be required to cause the alterations in the expression of PLC $\beta_1$. In this regard, it has been demonstrated that chronic administration of dexamethasone decreases noradrenaline-stimulated PI metabolism in rat brain (Takahashi et al., 1996). Furthermore, in the brain of aged rats, where glucocorticoids have been demonstrated to participate in aging (Landfield and Eldridge, 1991; Stein-Behrens and Sapolsky, 1992), it has been shown that $\alpha_1$-adrenergic- and muscarinic receptor-stimulated PI hydrolysis is decreased (for review, see Bothmer and Jolles, 1994). Recently, Nicole et al. (1999) have shown that glutamate receptor-mediated PI turnover is decreased in the brain of aged rats along with a decrease in the expression of PLC $\beta_1$. In addition, the expression of 5HT$_2A$ and 5HT$_2C$ receptors has been shown to be increased in rat brain after chronic glucocorticoid administration (Chauolf, 1995). From these studies it appears that glucocorticoids cause an increase in the expression of receptors and a decrease in the receptor-mediated PI turnover. In the present study, we did not examine receptor-mediated IP$_3$ formation; however, in accordance with the previous studies, it appears that the decrease in IP$_3$ levels could be a consequence of decreased PI turnover in response to the decreased activity of PI-PLC and decreased expression of the PLC $\beta_1$ isoyme, which may have been desensitized in response to sustained stimulation of receptors and receptor-mediated PI hydrolysis.

Further studies are needed to clarify these issues.

The functional implications of altered PI-PLC and IP$_3$ by glucocorticoids remain to be elucidated; however, as mentioned above, abnormal HPA function has been reported in affective disorders (Nelson and Davis, 1997). Furthermore, we have previously reported that the number of [H]IP$_3$ binding sites is increased in platelets of depressed patients (Dwivedi et al., 1998), and that the expression of the PLC $\beta_1$ isoyme and PI-PLC activity are decreased in the prefrontal cortex of suicide subjects (Pandey et al., 1999). It is possible that these changes in depressed and/or suicide subjects could be related to abnormal HPA function. However, to fully understand the implications of altered PI-PLC and IP$_3$ in human mood and behavior and to elucidate the inter-relationship of altered HPA function with PI-PLC and IP$_3$, further clinical investigations need to be conducted.

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