Adrenal Glucocorticoids Modulate $[^3$H]$]Cyclic AMP Binding to Protein Kinase A (PKA), Cyclic AMP-Dependent PKA Activity, and Protein Levels of Selective Regulatory and Catalytic Subunit Isoforms of PKA in Rat Brain

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ABSTRACT
Alterations in hypothalamic-pituitary-adrenal (HPA) function are associated with changes in mood and behavior. Protein kinase A (PKA), on activation, phosphorylates many important intracellular proteins and thereby plays a major role in mediating various physiological functions in brain. We systematically examined the relationship of altered HPA function with PKA modifications in rat brain after administering corticosterone to normal rats and by first adrenalectomizing rats and then simultaneously treating them with different doses of corticosterone. Rats were decapitated on day 1, 4, or 14. Subcutaneously implanted 50- or 100-mg corticosterone pellets in normal rats for 4 or 14 days significantly decreased PKA activity, $B_{max}$ of $[^3$H]$]cyclic AMP binding, and protein levels of selective PKA regulatory (RI$_a$, RII$_b$) and catalytic (Cat$_b$) subunit isoforms in cortex and hippocampus in a dose-dependent manner without any significant changes at day 1; these changes were more pronounced at day 14. However, adrenalectomy caused the opposite changes in these measures at day 4 or 14 in both cortex and hippocampus, and the magnitude of the changes was more pronounced at day 14. Simultaneous treatment with implanted corticosterone at 50- or 100-mg doses in adrenalectomized rats reversed the adrenalectomy-induced increases in PKA measures in a dose-dependent manner. These results suggest that endogenous glucocorticoid modifies the expression of RI$_a$, RII$_a$, and Cat$_b$ subunit isoforms of PKA, as well as the catalytic and regulatory activities of PKA, and that these alterations in PKA may in part explain HPA axis-mediated changes in mood and behavior.

Adrenal steroids play an important role in modulating many aspects of central nervous system function, including regulation of mood, behavior, emotions, and learning (McEwen, 1987). This role of adrenal steroids extends to psychopathology as suggested from observations of an overactive hypothalamic-pituitary-adrenal (HPA) axis in patients with depression and other affective disorders as evidenced by a high cortisol level in plasma, increased levels of corticotropin-releasing hormone and adrenocorticotropic hormone in cerebrospinal fluid, and failure to suppress plasma cortisol levels after administration of dexamethasone (Halbreich et al., 1985; Van de Kar, 1989; Murphy, 1991; Holsboer et al., 1995). Also, protracted treatment with glucocorticoids may induce depression (Ling et al., 1981), whereas compounds that lower serum cortisol levels have been used as effective antidepressants (reviewed by Wolkowitz and Reus, 1999). Recently, Fernandes et al. (1997) demonstrated that protracted administration of corticosterone depresses motor activity and exploratory behavior in rats.

The precise mechanisms by which corticosteroids exert behavioral changes are not fully understood; however, one possibility could be the effects of glucocorticoids on the expression of ionotropic and metabotropic neurotransmitter receptors. For example, in rats, adrenalectomy, which eliminates the endogenous glucocorticoids, enhances the expression of serotonin (5-HT)$_{1A}$ receptor mRNA in brain, and this action is reversed by corticosterone treatment (reviewed by Chaouloff, 1995; Zhong and Ciarnello, 1995). Also, the expression of 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors as well as that of $\beta$-adrenergic receptors is modified in rat brain after treatment with glucocorticoids (reviewed by Chaouloff, 1995). Alterations in expression of these neurotransmitter receptors due to an abnormal HPA axis may lead to changes in receptor responsiveness to extracellular messages. For example, while studying the phosphoinositide signaling cascade, we observed that repeated administration of dexamethasone increased the activity as well as the expression of...
specific isozymes of protein kinase C (PKC) and of phospholipase C in rat brain (Dwivedi and Pandey, 1999a,b). However, in the adenyl cyclase-cyclic AMP signaling cascade, which is operative in signal transduction at β-adrenergic and 5-HT$_{1A}$ receptors, earlier studies have indicated that β-adrenergic receptor- or forskolin-stimulated cyclic AMP accumulation is increased by glucocorticoids in rat brain cortical slices (Duman et al., 1989). Moley et al. (1983) reported that adrenalectomy, hypophysectomy, or treatment with metopirone, an inhibitor of steroid synthesis, is associated with an increase in norepinephrine-stimulated cyclic AMP formation in rat brain and that this effect was reversed by administration of corticosterone. Furthermore, Rodan and Rodan (1986) and Chang and Bourne (1987) reported that isoproterenol-stimulated adenyl cyclase activity can be enhanced in osteosarcoma ROS 17/2.8 cells and GH3 cells, respectively, by dexamethasone.

Modulation of protein phosphorylation and dephosphorylation is important in several pathways of cellular signaling. In the adenyl cyclase-cyclic AMP signaling system, protein phosphorylation is mediated by the enzyme protein kinase A (PKA), which becomes activated by cyclic AMP that is generated in response to adenyl cyclase activation by $G_\alpha$ or $G_\beta$ protein. On activation, PKA phosphorylates various intracellular proteins and thereby modifies hormonal and neurotransmitter responses, including receptor down-regulation or desensitization, altered neurotransmitter release, and activation or repression of gene expression (Builder et al., 1980; Nestler and Greengard, 1984; Benovic et al., 1988; Borrelli et al., 1992; Spaulding, 1993). Thus, given the significance of PKA in cellular signaling, it is important to examine how PKA is affected by altered HPA function. Although, as mentioned above, the effects of glucocorticoids on 5-HT$_{1A}$ and β-adrenergic receptors and on receptor-mediated adenyl cyclase activity have been studied, their effects on the signal transduction cascade events located further downstream in the adenyl cyclase-cyclic AMP signaling pathway at the level of PKA are not known.

This investigation is driven by the hypothesis that glucocorticoids may modify the phosphorylation of specific substrates by altering the number of binding sites for cyclic AMP to regulatory subunits of PKA as well as its catalytic activity and that this modification may be caused by altered expression of specific catalytic and/or regulatory subunit isoform(s) of PKA. To test this hypothesis, we studied the effects of exogenous and endogenous glucocorticoids on affinity and number of [3H]cyclic AMP-binding sites, on PKA activity, and on protein levels of individual isoforms of catalytic and regulatory subunits of PKA in rat brain. The effects of exogenous glucocorticoid were examined by s.c. implantation of pellets containing different doses of corticosterone either acutely for 1 day or chronically for 4 or 14 days. The effects of endogenous corticosterone were examined by adrenalectomizing rats and simultaneously implanting different doses of corticosterone pellets in these rats for 1, 4, or 14 days.

**Experimental Procedures**

**Materials.** [3H]Cyclic AMP was obtained from New England Nuclear (Boston, MA). 3-Isobutyl-1-methylxanthine, 4-(2-aminoethyl)benzenesulfonfyl fluoride (AEBSF), cyclic AMP, ATP, leupeptin, 2-mercaptoethanol, and Nonidet P-40 were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-32P]ATP was purchased from Amersham (Arlington Heights, IL). Kemptide was obtained from Calbiochem (La Jolla, CA). Antibodies for PKA regulatory subunit isoforms (Rα, RIIα, 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 was purchased from Sigma Chemical Co. Corticosterone pellets were purchased from Innovative Research of America (Sarasota, FL). All other chemicals were of analytical grade.

**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 acclimatized for 1 week before the experiment started.

**Adrenalectomy and Treatment with Corticosterone.** The following two treatment protocols were used. 1) Rats under light halothane anesthesia were implanted s.c. with corticosterone pellets containing 50 or 100 mg of corticosterone in a cholesterol base. These corticosterone pellets can maintain physiological serum concentrations of corticosterone for 21 days. The release of corticosterone per day after implantation of 50- or 100-mg corticosterone pellets is 2.38 and 4.76 mg, respectively. Control rats underwent an identical surgery procedure with implantation of a cholesterol pellet or underwent no treatment; these two types of treatment did not differ in their results in the final determination of PKA activity, [3H]cyclic AMP binding to PKA, or protein levels of regulatory and catalytic subunit isoforms. Rats were decapitated 1, 4, or 14 days after pellet implantation. 2) Rats were anesthetized with halothane anesthesia. Bilateral adrenalectomy 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. These rats were decapitated on day 1, 4, or 14 after adrenalectomy. Some adrenalectomized rats were implanted s.c. with placebo or corticosterone pellets (containing 50 or 100 mg of corticosterone) immediately after adrenalectomy. These rats were decapitated 4 or 14 days after corticosterone pellet implantation. The trunk blood was also collected on ice at decapitation and was centrifuged, and then the serum was stored at ~80°C until the assays were performed. Serum corticosterone levels were measured by a commercially available radioimmunoassay kit (ICN Biomedical, Inc., Cleveland, OH). Brains were removed quickly after the blood was taken. Cortices and hippocampi were dissected out and immediately stored at ~80°C until analyzed. For both experimental protocols, rats were decapitated between 9:00 and 11:00 AM, corresponding to 3 to 5 h after lights on.

**Determination of $B_{max}$ and $K_B$ of [3H]Cyclic AMP Binding to Cytosol and Particulate PKA in Rat Brain.** Specific [3H]cyclic AMP binding was performed as described by Nishino et al. (1993) with slight modifications. 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 AEBSF, and 10 μg/ml leupeptin. The homogenate was centrifuged at 100,000 g for 60 min. The supernatant ($S_1$) was saved. The pellet was resuspended in the homogenizing buffer and centrifuged again at 100,000 g for 60 min. This supernatant ($S_2$) was combined with $S_1$ 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 BSA as a standard. [3H]Cyclic AMP binding was performed in triplicate in an incubation buffer containing PEM buffer (20 mM phosphate (pH 7.4 at 25°C), 2 mM EDTA, and 15 mM 2-mercaptoethanol); [3H]Cyclic AMP (0.25–10 nM); particulate or...
cytosol fraction (−25 μg of protein); 0.25 mg of BSA; and 1.5 mM 3-isobutyl-1-methylxanthine, in the presence or absence of 5 μM cyclic AMP 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 cyclic AMP, Bmax, and KE 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 cortex and hippocampus. 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 100,000 g for 60 min at 4°C. The resulting supernatant (S1) was saved. The pellet obtained was homogenized in the homogenizing buffer and recentrifuged at 100,000 g for 60 min at 4°C. The resultant supernatant (S2) was combined with S1 and used as the cytosol fraction. The resulting pellet was homogenized in the homogenizing buffer and used as the particulate fraction. The protein content of these two fractions was then determined by the procedure of Lowry et al. (1951). Aliquots of the fractions were then used for quantitation of PKA activity by standard assay (Witt and Roskoski, 1975) with some modifications. The procedure is based on the phosphorylation of a specific substrate (kemptide: Leu-Arg-Arg-Ala-Ser-Leu-Gly) using the transfer of γ-phosphate 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 μCi [γ-32P]ATP (~3000 Ci/mmol prepared in 75 mM MgCl2 and 500 μM ATP). PKA activity was determined in the presence and the absence of cyclic AMP (10 μM). Reactions were carried out at 30°C for 10 min. Aliquots (20 μl) were spotted in duplicate onto phosphocellulose filters (2 × 2 cm; Whatman P81), washed twice in 75 mM H3PO4 for 5 min and twice in water 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 [γ-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 and hippocampus was determined by Western blot as described earlier (Brandon et al., 1996; Divedi and Pandey, 1999a, b). Brain samples were Deunce 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 12,000 g 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 μM Tris-HCl, pH 6.8; 4% β-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. Protein samples were loaded onto 10% (w/v) polyacrylamide gels 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, Arlington Heights, IL) using the Mini TransBlot transfer unit (Bio-Rad) at 0.15-A 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 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 overnight at 4°C with primary antibody (anti-PKA RIIα, RIIβ, RIIα, RIIβ, Cat α, or Cat β) 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 concentrations 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 antibody as the primary antibody (1:5000 for 2 h) and antirabbit 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 also 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 corresponding β-actin band. The values are presented as a percentage of the control.

Statistics. Data were analyzed using the SPSS (Chicago, IL) version 8.0 statistical software package. All values are given as the means ± S.D. One-way ANOVA was used to compare the effects of corticosterone or adrenalectomy on various parameters in cortex and hippocampus. Bonferroni’s multiple comparison tests were used to evaluate pair-wise differences. To examine the effects of corticosterone or adrenalectomy on various parameters between cortex and hippocampus and between days 4 and 14, three-way ANOVA considering two brain areas (cortex and hippocampus), two different time intervals (4 and 14 days), and three (sham and two corticosterone treatment groups) or four (sham, adrenalectomy, and two adrenalec- tomy + corticosterone treatment groups) different treatment groups as variables was performed. An α-value lower than 0.05 was considered significant.

Results

Adrenalecctomy or Treatment with Corticosterone Fails to Change Body Weight of Rats. There were no significant differences in body weight gain 2 weeks after adrenalecctomy or after corticosterone treatment of adrenalectomized rats. Mean body weight gain among the different groups was as follows: control, 239 ± 12 g; adrenalecctomized, 232 ± 16 g; adrenalecctomized + corticosterone pellet (50 mg), 229 ± 15 g; and adrenalecctomized + corticosterone pellet (100 mg), 239 ± 12 g. We also did not find any significant differences in body weight gain after 2 weeks of corticosterone treatment in normal rats (control, 242 ± 19 g; corticosterone (50 mg), 235 ± 13 g; corticosterone (100 mg), 242 ± 14 g).

Serum Corticosterone Levels. Serum corticosterone levels at 4 and 14 days after adrenalecctomy and corticoste- rone treatment are given in Table 1. When the rats were implanted with a 50- or 100-mg corticosterone pellet, the level of corticosterone at day 14 was greater than at day 4 and was dose-dependent; with the 50-mg dose of corticosterone, the serum level of corticosterone was lower than with the 100-mg dose. We could not detect any endogenous corticosterone in adrenalecctomized rats at either 4 or 14 days after adrenalecctomy. However, after simultaneous implanta-
Corticosterone Decreases $[^3H]$Cyclic AMP Binding to PKA in Cytosol and Particulate Fractions of Rat Brain. We first characterized $[^3H]$cyclic AMP binding to the regulatory subunit of PKA in both particulate and cytosol fractions of cortex and hippocampus. The time course for $[^3H]$cyclic AMP binding was determined from 30 s up to 150 min. Binding of $[^3H]$cyclic AMP was rapid and reached the maximum at 60 min in both the particulate and the cytosol fractions. After 60 min, the specific binding remained constant until 150 min (data not shown). The effects of different concentrations of protein (5–100 μg) on $[^3H]$cyclic AMP binding were also determined. It was observed that specific binding was linear at protein concentrations between 5 and 100 mg/ml and exhibited a single class of binding site. Nonspecific binding was nonsaturable and was linear with concentrations and cytosol (B) PKA in rat cortex. Each point is the mean of triplicate determinations. Inset, Scatchard plot of the specific binding of $[^3H]$cyclic AMP. B = $[^3H]$cyclic AMP specifically bound (femtomoles per milligram of protein); B/F = bound/free $[^3H]$cyclic AMP (femtomoles per milligram of protein × nanomolar). For this particular experiment, the binding indices in the particulate fraction (A) were $K_D$ = 0.56 nM, $B_{\text{max}}$ = 134 fmol/mg of protein, and $r = 0.99$. For the cytosol fraction (B), they were $K_D$ = 0.66 nM, $B_{\text{max}}$ = 330 fmol/mg of protein, and $r = 0.99$.

The maximum number of binding sites ($B_{\text{max}}$) and the apparent dissociation constant ($K_D$) in both particulate and cytosol fractions were determined by using different concentrations of $[^3H]$cyclic AMP (0.25–10 nM). Nonspecific binding was determined in the presence of 5 μM cyclic AMP. In particulate and cytosol (Fig. 1B) fractions obtained from the cortex of control rats. It was observed that specific binding 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 $[^3H]$cyclic AMP. The specific binding was in the range of 95 to 72%, depending on the concentration of $[^3H]$cyclic AMP used (0.25–10 nM). It was observed that $B_{\text{max}}$ of $[^3H]$cyclic AMP binding to PKA was greater in the cytosol than in the particulate fractions, which is in agreement with reports in the literature (Rahman et al., 1997). Also, we found that $B_{\text{max}}$ was greater in the hippocampus than in the cortex; however, $K_D$ values were similar in particulate and cytosol fractions of both brain areas.

When we determined $[^3H]$cyclic AMP binding after corticosterone treatment (50- or 100-mg pellets), it was observed that the effect of corticosterone was both time- and dose-dependent. For example, after 1 day of treatment with corticosterone (50- or 100-mg dose) neither $B_{\text{max}}$ nor $K_D$ of $[^3H]$cyclic AMP binding was altered in particulate or cytosol fractions of cortex or hippocampus (data not shown). However, 4 days of treatment with corticosterone dose dependently decreased $B_{\text{max}}$ of $[^3H]$cyclic AMP binding in both particulate and cytosol fractions of cortex and hippocampus (Fig. 2A). Although the decreases in $B_{\text{max}}$ of $[^3H]$cyclic AMP binding were slightly greater in hippocampus than in cortex, these differences were not significant. When $[^3H]$cyclic AMP binding was determined 14 days after the implantation of the corticosterone pellet, it was observed that the magnitude of the decreases in $B_{\text{max}}$ was significantly greater in both particulate and cytosol fractions of cortex and hippocampus (Fig. 2B) than after 4 days of treatment. Again, as was observed after 4 days of treatment, these changes were more profound in hippocampus than in cortex at both doses of corticosterone, but these differences were not significant.

We did not observe any significant effects of 4 or 14 days of corticosterone treatment on $K_D$ values either in cortex or in hippocampus. $K_D$ values in cortex and hippocampus of sham-operated rats were as follows: cortex: particulate, 0.67 ± 0.10 nM; cytosol, 0.73 ± 0.11 nM; hippocampus: particulate, 0.66 ± 0.15 nM; cytosol, 0.80 ± 0.09 nM.

After Adrenalectomy, $[^3H]$Cyclic AMP Binding to PKA Increases in Cytosol and Particulate Fractions of Rat Brain, and Simultaneous Treatment with Corticosterone Prevents this Action. To examine if the changes

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**Table 1**: Serum corticosterone levels in different treatment groups. Values are mean ± S.D.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>4 Days</th>
<th>14 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham-operated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosterone (50 mg)</td>
<td>42.1 ± 9.5</td>
<td>12.2 ± 2.3</td>
</tr>
<tr>
<td>Corticosterone (100 mg)</td>
<td>103.6 ± 29.4</td>
<td>25.6 ± 9.8</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham-operated</td>
<td>46.8 ± 11.4</td>
<td>ND</td>
</tr>
<tr>
<td>Adrenalectomy</td>
<td>0.5 ± 0.4</td>
<td>ND</td>
</tr>
<tr>
<td>Adrenalectomy + corticosterone (50 mg)</td>
<td>17.8 ± 5.5</td>
<td>25.6 ± 9.8</td>
</tr>
<tr>
<td>Adrenalectomy + corticosterone (100 mg)</td>
<td>36.4 ± 12.2</td>
<td>49.7 ± 17.7</td>
</tr>
</tbody>
</table>

ND, none detected.
Fig. 2. Effects of 4 (A) or 14 (B) days of corticosterone treatment (implanted 50- or 100-mg pellet) on $B_{\text{max}}$ of $[^3\text{H}]$cyclic AMP binding in particulate and cytosol fractions of cortex and hippocampus. Values are the means $\pm$ S.D. from six rats in each group. Corticosterone-treated groups (50 mg, $\square$; 100 mg, $\blacksquare$) were compared with the sham-operated group (■). *$P < .001$. 
in \(B_{\text{max}}\) of \([\text{H}]\text{cyclic AMP}\) binding are also regulated by the endogenous glucocorticoid in vivo, we studied the effects of adrenalectomy with and without simultaneous corticosterone treatment. It was observed that there were no significant differences in \(B_{\text{max}}\) or \(K_{D}\) values 1 day after the adrenalectomy in either particulate or cytosol fractions of cortex or hippocampus (data not shown). However, after a time lapse of 4 days after adrenalectomy, there was a significant increase (29–45%) in \(B_{\text{max}}\) of \([\text{H}]\text{cyclic AMP}\) binding to both particulate and cytosol PKA in both cortex and hippocampus (Fig. 3A). Two weeks after adrenalectomy, \(B_{\text{max}}\) of \([\text{H}]\text{cyclic AMP}\) binding was further increased (49–61%) in both particulate and cytosol fractions of cortex and hippocampus (Fig. 3B). When we compared the differences in \(B_{\text{max}}\) between days 4 and 14 after adrenalectomy, the degree of the increases was significantly higher at day 14 in both cortex and hippocampus. Comparison of \(B_{\text{max}}\) after 4 or 14 days of adrenalectomy between cortex and hippocampus showed that although the magnitude of the increases in \(B_{\text{max}}\) was greater in hippocampus than in cortex at both the time intervals, the differences were not statistically significant. The increase in \(B_{\text{max}}\) either after 4 days (Fig. 3A) or 14 days (Fig. 3B) was reversed in a dose-dependent manner by administration of corticosterone. With the 50-mg dose of corticosterone, the adrenalectomy-induced increase in \(B_{\text{max}}\) was partially prevented, but with the 100-mg dose, \(B_{\text{max}}\) was nearly the same as the control value. There were no significant differences in \(K_{D}\) values between controls and adrenalectomized or adrenalectomized + corticosterone-treated rats (data not shown).

**Corticosterone Treatment Decreases PKA Activity in Cytosol and Particulate Fractions of Rat Brain.** PKA activity was determined in both particulate and cytosol fractions of cortex and hippocampus using kemptide, a heptapeptide, which is a highly potent and efficacious PKA substrate (Kemp et al., 1977). Initially, we characterized PKA activity using various concentrations of cyclic AMP and noted that the maximum stimulation was observed at 10 \(\mu\)M cyclic AMP. For example, in particulate and cytosol fractions of cortex, basal and cyclic AMP-stimulated (10 \(\mu\)M) PKA activity was as follows: particulate: basal, 140 ± 11 pmol/min/mg of protein; cyclic AMP-stimulated, 560 ± 25 pmol/min/mg of protein; cytosol: basal, 280 ± 17 pmol/min/mg of protein; cyclic AMP-stimulated, 950 ± 41 pmol/min/mg of protein. We next examined the ability of a selective PKA inhibitor (a 17-residue synthetic peptide: TYADFIASGRTGRRNAI-NH\(_2\)) to block the activity in both particulate and cytosol fractions (Glass et al., 1989). We observed that PKA activity was completely inhibited in the presence of the PKA inhibitor in both particulate and cytosol fractions (data not shown). To ensure that PKA activity is specific and that the activities of other kinases do not account for our results, in each assay we added two protein kinase inhibitors, namely, a PKC inhibitor peptide [a 13-amino-acid synthetic peptide: RFARKGALRQKNV (Smith et al., 1990)] and compound R2457 [a calmodulin kinase II inhibitor: 1-[bis-(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-[2,4-dichlorobenzyloxy]ethyl]-1H-imidazolium chloride (Fischer et al., 1987)]. In addition, we also determined the effects of time and protein concentration on PKA activity. We observed that PKA activity was linear between 2 and 50 \(\mu\)g of protein and between 5 and 30 min (data not shown) of incubation. After this initial characterization, we determined PKA activity in normal rat brain and observed that PKA activity was greater in the cytosol than in the particulate fractions and that it was greater in hippocampus than in cortex.

When the effects of 1 day of treatment with corticosterone on PKA activity were determined, it was observed that PKA activity was not significantly different in particulate or cytosol fractions of cortex and hippocampus at either dose of corticosterone (50- or 100-mg pellet) from that of sham-operated rats (data not shown). However, 4 days of treatment with corticosterone (50 or 100 mg) caused a significant decrease in PKA activity in particulate and cytosol fractions of both cortex and hippocampus in a dose-dependent manner, i.e., at the 50-mg dose, the decrease was 17 to 25%, whereas at the 100-mg dose, the decrease was 35 to 45% (Fig. 4A). When rats were treated with 50- or 100-mg doses of corticosterone for 14 days, there was a further decrease (28–69%) in PKA activity in both particulate and cytosol fractions of cortex and hippocampus, and this decrease was also dose-dependent (Fig. 4B). Furthermore, we observed that the magnitude of changes in PKA activity in both cortex and hippocampus was significantly greater after 14 days of corticosterone treatment compared with 4 days of treatment. When we compared PKA activity between cortex and hippocampus after 4 or 14 days of corticosterone treatment, we observed that although the magnitude of the decrease in PKA activity was greater in hippocampus than in cortex at both time intervals, the difference was not statistically significant.

**Adrenalectomy Increases PKA Activity in Cytosol and Particulate Fractions of Rat Brain, and Simultaneous Treatment with Corticosterone Prevents This Action.** There were no significant differences in PKA activity in particulate or cytosol fractions of cortex or hippocampus 24 h after adrenalectomy (data not shown).

When rats were adrenalectomized and simultaneously implanted with corticosterone pellets, however, we observed significant effects of adrenalectomy and corticosterone replacement on PKA activity at both days 4 and 14. Four days after adrenalectomy there was significantly increased PKA activity in both particulate and cytosol fractions of cortex and hippocampus (Fig. 5A). This increase was slightly but non-significantly greater in hippocampus than in cortex. Simultaneous replacement by implanting corticosterone pellets in adrenalectomized rats prevented the adrenalectomy-induced increase in PKA activity in both particulate and cytosol fractions of cortex and hippocampus in a dose-dependent manner. With the 50-mg corticosterone pellet, the reversal was partial; however, with the 100-mg dose, PKA activity was almost completely reversed to normal values (Fig. 5A).

At fourteen days after adrenalectomy there was a much greater increase (45–55%) in PKA activity in both particulate and cytosol fractions of cortex and hippocampus (Fig. 5B) than at day 4. The extent of the increase in PKA activity was slightly greater in hippocampus than in cortex; however, this difference was not statistically significant. Treatment of adrenalectomized rats with two different doses of corticosterone for 14 days caused a significant dose-dependent reversal of the adrenalectomy-induced increase in PKA activity: ~40 to 50% with the 50-mg dose of corticosterone and ~90 to 100% with the 100-mg dose of corticosterone (Fig. 5B).

**Corticosterone Treatment Decreases the Protein Levels of Selective Regulatory (RI\(_{\alpha}\) and RII\(_{\beta}\)) and Cat-
Fig. 3. Effects of 4 (A) or 14 (B) days of adrenalectomy and adrenalectomy + corticosterone treatment on $B_{\text{max}}$ of $[^{3}H]$cyclic AMP binding in particulate and cytosol fractions of cortex and hippocampus. Corticosterone pellet (50 or 100 mg) was implanted immediately after adrenalectomy. Values are the means ± S.D. from six rats in each group. The adrenalectomy group (■) was compared with the sham-operated group (□), whereas the adrenalectomy + corticosterone-treated groups (50 mg, □; 100 mg, ■) were compared with the adrenalectomy group (■). *, compared with sham, $P < .001$; **, compared with adrenalectomy, $P < .001$. 

**Bmax of $[^{3}H]$cyclic AMP Binding (fmol/mg protein)**
Fig. 4. Effects of 4 (A) or 14 (B) days of corticosterone treatment (implanted 50- or 100-mg pellet) on PKA activity in particulate and cytosol fractions of cortex and hippocampus. Values are the means ± S.D. from six rats in each group. Corticosterone-treated groups (50 mg, □; 100 mg, □) were compared with the sham-operated group (○). *P < .001.
Fig. 5. Effects of 4 (A) or 14 (B) days of adrenalectomy and adrenalectomy + corticosterone treatment on PKA activity in particulate and cytosol fractions of cortex and hippocampus. A corticosterone pellet (50 or 100 mg) was implanted immediately after adrenalectomy. Values are the means ± S.D. from six rats in each group. The adrenalectomy group (II) was compared with the sham-operated group (□), whereas the adrenalectomy + corticosterone-treated groups (50 mg, □; 100 mg, ■) were compared with the adrenalectomy group (II). *, compared with sham, P < .001; **, compared with adrenalectomy, P < .001.
Adrenalectomy Increases the Protein Levels of PKA Regulatory and Catalytic Subunit Isoforms in Rat Brain, and Simultaneous Treatment with Corticosterone Reverses This Action. One day after adrenalectomy, we did not observe any significant effects on the protein levels of regulatory or catalytic subunit isoforms of PKA in cortex and hippocampus (data not shown). However, 4 days after adrenalectomy, we observed significantly increased protein levels of RIα, RIIβ, and Catβ subunit isoforms in cortex and hippocampus (Fig. 8A). Representative Western blots showing the effects 14 days after adrenalectomy on the protein levels of regulatory and catalytic subunit isoforms in rat cortex are depicted in Fig. 6B, and its effects in cortex and hippocampus are summarized in Fig. 8B. It was observed that 14 days of adrenalectomy caused a significant increase in the protein levels of the same catalytic and regulatory subunit isoforms in both the cortex and hippocampus as observed at day 4; however, these changes were much more robust 14 days after adrenalectomy (Fig. 8B). When we compared the magnitude of the changes between cortex and hippocampus, although there was a greater degree of change in hippocampus, the difference was not significant. We did not observe any significant effects at either 4 or 14 days after adrenalectomy on the protein levels of RIIα, RIIβ, and Catα subunit isoforms in cortex or hippocampus.

Simultaneous treatment with corticosterone pellets (50 or 100 mg) of adrenalectomized rats either for 4 (Fig. 8A) or 14 (Fig. 8B) days prevented the adrenalectomy-induced increase in protein expression of PKA RIα, RIIβ, and Catβ subunit isoforms in both cortex and hippocampus. The reversal was partial with the lower dose (50 mg) of corticosterone; however, the higher dose (100 mg) was fully effective in reversing these changes to normal levels.
Discussion

This investigation was driven by the hypothesis that glucocorticoids regulate the catalytic and regulatory properties of PKA in brain under physiological conditions. This regulation appears to be dependent on the duration of corticosterone exposure. For example, we found that corticosterone treatment for 1 day had no significant effects, but 4 days of corticosterone treatment significantly decreased PKA activity and Bmax of [3H]cyclic AMP binding in both cytosol and particulate fractions of rat cortex and hippocampus. These changes were even more pronounced after 14 days of corticosterone treatment. Furthermore, these changes were dependent on the corticosterone dose: although even a low dose of corticosterone (50-mg) pellet caused significant decreases in PKA activity and in Bmax of [3H]cyclic AMP binding, these changes were much more profound at a larger dose (100 mg).

To examine whether PKA is regulated by the endogenous glucocorticoid, we studied PKA activity and [3H]cyclic AMP binding in rat brain at different time intervals after bilateral adrenalectomy, i.e., days 1, 4, and 14. On day 1, although the level of corticosterone in the serum was negligible, there were no significant changes in Bmax of [3H]cyclic AMP binding or in PKA activity in particulate or cytosol fractions of cortex or hippocampus. However, 4 days after adrenalectomy, there was a significant increase in PKA activity and in Bmax of [3H]cyclic AMP binding in both cortex and hippocampus, and at day 14, the magnitude of these changes was much more robust. When adrenalectomized rats were simultaneously implanted with a corticosterone pellet, a dose-dependent reversal of the adrenalectomy-induced increases in PKA activity and in Bmax of [3H]cyclic AMP binding in both particulate and cytosol fractions of cortex and hippocampus was observed. The 100-mg dose of corticosterone was able to completely reverse these changes.

We next examined whether the modifications in number of [3H]cyclic AMP-binding sites and in catalytic activity of PKA by adrenal glucocorticoids were due to altered expression of specific regulatory and/or catalytic subunit isoform(s) of PKA. Structurally, PKA is a holoenzyme, composed of two homodimeric regulatory (R) and two catalytic (Cat) subunits (Francis and Corbin, 1994). In the holoenzyme state, PKA is inactive. After an increase in intracellular cyclic AMP, the regulatory PKA subunits bind cyclic AMP, which results in the dissociation of the holoenzyme into a dimeric regulatory unit and two monomers of catalytic subunits (Flockhart and Corbin, 1982; Gettys and Corbin, 1989). The free catalytic

Fig. 7. Effects of 4 (A) or 14 (B) days of corticosterone treatment (implanted 50- or 100-mg pellet) on protein levels of catalytic and regulatory PKA subunit isoforms in cortex and hippocampus. Values are the means ± S.D. from six rats in each group. Corticosterone-treated groups (50 mg, ■; 100 mg, □) were compared with the sham-operated group (□). *P < .01 to < .001. Note that we did not observe any significant effects of 4 or 14 days of corticosterone treatment on immunolabeling of PKA R1β, R1α, or Cat subunit isoforms in cortex or hippocampus.
subunits then phosphorylate substrates or translocate into the nucleus and phosphorylate nuclear proteins (Wen et al., 1994). Thus, both the catalytic and the regulatory subunits are important in facilitating PKA-mediated functions. 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 (Showers and Maurer, 1986). Type I PKA is primarily cytoplasmic, whereas type II PKA is mainly particulate (Leiser et al., 1986). Multiple isoforms of regulatory (RIα, RIβ, RIIα, RIIβ) and catalytic (Catα, Catβ, Catγ) subunits exist and are encoded by separate genes (Scott et al., 1987; Clegg and McKnight, 1988). The tissue distribution of regulatory subunits is such that RIα and RIIα are present ubiquitously, whereas RIβ is present in brain and in developing sperms. However, RIIβ is the predominant isofrom and principal mediator of cAMP-mediated activity in the central nervous system (Sarkar et al., 1984). The catalytic subunit isoforms Catα and Catβ are ubiquitously expressed, although Catβ is the predominant isofrom in brain (Uhler et al., 1986), and Catγ is a testis-specific isofrom. When we determined the protein levels of Catα, Catβ, RIα, RIβ, RIIα, and RIIβ subunit isoforms in brain of rats after 4 and 14 days of corticosterone treatment, we observed that the expression of the specific RIα, RIIβ, and Catβ isoforms was significantly decreased in cortex and hippocampus at both time intervals, but these changes were much more pronounced at day 14. However, 1 day of corticosterone treatment had no significant effects on the protein levels of either regulatory or catalytic subunit isoforms. Furthermore, removal of the adrenal gland increased the expression of these same PKA isoforms, and this was dependent on the duration of removal of the adrenal gland. Simultaneous treatment with corticosterone prevented these increases in both cortex and hippocampus in a dose-dependent manner. Our results thus suggest that under physiological conditions, endogenous glucocorticoids play an important role in regulating and maintaining the expression of these particular regulatory and catalytic subunit isoforms.

The possible mechanisms of altered expression of RIα, RIIβ, and Catβ isoforms by glucocorticoids are not known at the present time. However, the observed changes do not appear to be due to the direct effects of glucocorticoids be-

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**Fig. 8.** Effects of 4 (A) or 14 (B) days of adrenalectomy and adrenalectomy + corticosterone treatment on protein levels of catalytic and regulatory PKA subunit isoforms in cortex and hippocampus. Values are the means ± S.D. from six rats in each group. The adrenalectomy group (□) was compared with the sham-operated group (□) whereas the adrenalectomy + corticosterone-treated groups (50 mg, □; 100 mg, ■) were compared with the adrenalectomy group (□). * compared with sham, *P < .001; ** compared with adrenalectomy, *P < .001. Note that we did not observe any significant effects of adrenalectomy on immunolabeling of PKA RIβ, RIIα, or Catα subunit isoforms in cortex or hippocampus.

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cause we did not observe any significant effects of acute administration of corticosterone or adrenalectomy. It is quite possible that sustained stimulation of receptors, G proteins, or effectors by glucocorticoids as observed by earlier investigators (Johnson and Jaworski, 1983; Duman et al., 1989; Saito et al., 1989; Chaoullof, 1995) may cause adaptive changes in the expression of these isoforms. This is not surprising because there are reports that suggest that sustained stimulation of receptors or G proteins can cause desensitization of receptors or effectors (Milligan, 1993; Galas and Harden, 1995).

Earlier studies have suggested that glucocorticoids can up- or down-regulate adenylyl cyclase activity depending on the type of neurotransmitter receptors affected. For example, Moley et al. (1983) and Harrelson and McEwen (1987) reported that glucocorticoids increase the responsibility of norepinephrine-stimulated adenylyl cyclase activity in the rat cerebral cortex 2 weeks after bilateral adrenalectomy and that this response is reversed after 3 days of treatment with corticosterone. Similarly, Gannon and McEwen (1990) reported an increase in forskolin-stimulated adenylyl cyclase activity in rat brain after adrenalectomy. Contrary to these reports, Duman et al. (1989) and Rodan and Rodan (1986) found that prolonged treatment with glucocorticoids increased isoproterenol- or forskolin-stimulated adenylyl cyclase activity in the rat cerebral cortex and in ROS 17/2.8 cells, respectively, and Johnson and Jaworski (1983) reported that glucocorticoid treatment of cultured fibroblasts increased isoproterenol-stimulated intracellular cyclic AMP accumulation. Furthermore, Saito et al. (1989) reported that levels of Gα are increased and levels of Gβγ are decreased in rat cortex after 7 days of corticosterone treatment, suggesting an up-regulated adenylyl cyclase-cyclic AMP system. In this investigation, we observed that the number of [3H]cyclic AMP-binding sites, PKA activity, and expression of specific PKA regulatory and catalytic subunit isoforms are decreased after corticosterone administration. Thus, it appears that whether glucocorticoids up- or down-regulate adenylyl cyclase activity, the overall response at the level of PKA is decreased.

The functional significance of decreased PKA after corticosterone treatment remains to be elucidated; however, as mentioned earlier, dysregulation of HPA function might have an important role in the vulnerability to depressive behavior (Halbreich et al., 1985; Maes et al., 1991; Holsber et al., 1995). In this context, given the role of PKA in phosphorylating crucial proteins and thereby modulating cellular functions, our observations of altered protein expression of the selective RIIα, RIIβ, and Catβ subunit isoforms as well as changes in PKA activity and in the number of [3H]cyclic AMP-binding sites after manipulation of the HPA axis appear to have physiological significance, especially because RIIβ and Catβ are the predominant isoforms and principal mediators of cAMP-mediated activity in the central nervous system. Interestingly, there have been few studies that suggest a role for PKA in depressive behavior. For example, Manier et al. (1995) and Shelton et al. (1996) reported decreased cyclic AMP-dependent PKA activity in fibroblasts of depressed patients, whereas Rahman et al. (1997) showed that [3H]cyclic AMP binding is decreased in postmortem brain of bipolar depressed subjects. Also, we have recently observed that Bmax of [3H]cyclic AMP binding and PKA activity are reduced in postmortem brain of depressed suicide subjects (Dwivedi et al., 1999). Because depressed patients often show increased cortisol levels, it is quite possible that the changes in PKA in these subjects may be related to abnormal HPA function; however, to fully understand the implications of altered PKA in human mood and behavior to elucidate the interrelationship of altered HPA function and PKA, further clinical investigations are needed.

In summary, our results show that removal of corticosterone by adrenalectomy increased PKA activity and number of [3H]cyclic AMP-binding sites in the rat cortex and hippocampus along with an increase in protein expression of the specific RIIα, RIIβ, and Catβ isoforms of PKA. These increases were preventable by simultaneous treatment with corticosterone. In addition, treatment with corticosterone caused the opposite changes in PKA from those observed after adrenalectomy. These results suggest that the expression of selective isoforms of PKA regulatory and catalytic subunits is under the regulation of glucocorticoids, which may in turn be associated with the alterations in cyclic AMP binding as well as in the catalytic properties of PKA. These alterations in PKA may be relevant in glucocorticoid-mediated changes in mood and behavior.

References