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Lithium-Induced Gene Expression of Inducible Cyclic Adenosine Monophosphate Early Repressor in the Rat Adrenal Gland

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Lithium has acute and chronic effects on the hypothalamic-pituitary-adrenal gland (HPA) axis that are important for both therapeutic (e.g., treatment of mood disorders) and experimental (e.g., as the toxin in conditioned taste aversion studies) applications. We visualized lithium-induced activation of the HPA axis in rats by the adrenal expression of inducible cAMP early repressor (ICER), which is activated by elevated intracellular cAMP. We have shown that 1) intraperitoneal lithium chloride (LiCl) induces transient expression of ICER and c-fos mRNAs in the rat adrenal cortex and increases plasma level of corticosterone; 2) the cortical expression of ICER mRNA by LiCl occurs in a dose-dependent manner; 3) adrenal induction of ICER expression is delayed compared with c-fos expression; 4) dexamethasone pretreatment (4 mg/kg) blocks corticosterone release and adrenocortical ICER induction either by systemic LiCl (76 mg/kg) or by restraint stress; and 5) intracerebroventricular LiCl (127 μg/5 μl) is sufficient for adrenocortical, but not medullary, ICER induction. These results suggest that adrenocortical ICER expression could serve as a reliable marker for lithium-induced activation of the HPA axis. Understanding the activation of immediate-early genes such as c-fos or ICER in response to a single LiCl injection is an important first step in understanding the long-term changes in gene expression elicited by lithium that are involved in its therapeutic and toxic effect. The pattern and mechanism by which lithium stimulates ICER transcription in the adrenal gland would serve as a useful model system in future studies of lithium.

Key words: hypothalamic-pituitary-adrenal gland axis; dexamethasone; c-fos; In situ hybridization

Although lithium has been used clinically for over 30 years and many studies related to its therapeutic effects have been done (Pilcher, 2003), the cellular and molecular bases of the therapeutic and toxic effects of lithium remain poorly understood. Lithium is one of the most effective therapeutic agents prescribed for the treatment of bipolar disorder and depression. The antidepressant effects of lithium require chronic, low-dose administration and result in many long-term changes, including alterations in multiple neurotransmitters, signal transduction systems, and gene expression (Lenox and Manji, 1998). Dysfunction of the hypothalamic-pituitary-adrenal gland (HPA) system is one of the major pathophysiological alterations observed in patients suffering from mood disorders, and the abnormalities of the HPA activity return to normal following successful pharmacotherapy with lithium and other antidepressants (Holsboer and Barden, 1996). It is suggested that interactions of lithium with the HPA system may, at least partially, contribute to its therapeutic effects.

Lithium has also been widely used as a conventional stimulus to produce conditioned taste aversion (CTA) for its toxic effect. Blockade of HPA activation with adrenalectomy impairs the acquisition of lithium-induced CTA in mice (Peeters and Broekkamp, 1994). Pharmacological manipulation of the HPA system alters the strength of lithium-induced CTA in rats (Smotherman et al., 1976; Hennessy et al., 1980; Revusky and Martin, 1988). Indeed, intraperitoneal administration of lithium chloride activates the HPA axis in rats, i.e., increases plasma levels of adrenocorticotrophic hormone (ACTH) and corticosterone (Hennessy et al., 1976; Smotherman, 1985; Sugawara et al., 1988). These studies suggest that activation of the HPA axis by lithium may be a part of its effects as an unconditioned stimulus in CTA learning. However, little is known about the mechanism by which lithium activates the HPA system.

Inducible cAMP early repressor (ICER) is an immediate-early gene exhibiting very low basal expression levels, and its expression is induced by elevated

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intracellular cAMP levels. Several investigators have shown that induction of ICER expression in the HPA axis is coupled to HPA axis activation, in the rat hypothalamic paraventricular nucleus following hypertonic saline injection (Luckman and Cox, 1995), in the pituitary gland after restraint stress (Mazzucchelli and Sassone-Corsi, 1999), and in the adrenal gland following surgical stress or ACTH injection (Della Fazia et al., 1998). These reports suggest that ICER expression in the adrenal cortex could serve as a marker for the activation of the HPA axis. ICER expression also provides, as a marker of cAMP-induced gene expression, insight into the intracellular signaling cascades of the adrenal gland. In this study, we examined the time course, dose-response curve, and central dependence of the adrenal ICER expression by acute LiCl in order to investigate the mechanism of lithium-induced HPA activation. The dexamethasone suppression test was also performed on the adrenal ICER expression by systemic lithium.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats were purchased (Charles River Laboratories, Wilmington, MA) and acclimated to the laboratory conditions under a 12-hr light/12-hr dark cycle (lights on 07:00) at 25°C. Rats (250–350 g) had free access to Purina rodent chow and water and were weighed daily for 5–7 days prior to treatment to minimize handling stress. Animal experiments were carried in accordance with the NIH guidelines for the care and use of laboratory animals (revised 1996). All experimental animal protocols were approved by the Florida State University Institutional Animal Care and Use Committee.

Time Course of LiCl-Induced ICER Expression

Rats (42 total) received an intraperitoneal injection of LiCl (0.15 M, 12 ml/kg; Sigma Chemical Co., St. Louis, MO) at 07:00 (just after lights on) and then of sodium pentobarbital (65 mg/kg; Nembutal; Butler, Columbus, OH) 0.3, 1, 3, 6, 9, or 12 hr later (n = 6 per each time point). Uninjected (i.e., noninjection of LiCl) rats were anesthetized with intraperitoneal injection of sodium pentobarbital (65 mg/kg) 1 hr after lights on for the 0-hr time point. When completely anesthetized, rats were perfused transcardially, first with 100 ml of isotonic saline/0.5% sodium nitrate/1,000 U heparin and then with 400 ml of 4% paraformaldehyde/0.1 M phosphate buffer. Adrenal glands were removed, postfixed for 2 hr, and then transferred into 30% sucrose at least 24 hr prior to sectioning. The adrenal sections were processed for ICER, and the alternate sections for c-fos, cDNA in situ hybridization.

Dose Effect of LiCl on ICER Induction

Rats received an intraperitoneal injection of 0.15 M LiCl at doses of 0, 10, 19, 38, and 76 mg/kg (n = 6 per dose). Injections were made isovolumetric (12 ml/kg) and isotonic (0.30 Osm) with 0.15 M NaCl and given during the first half of the light cycle. The 0-mg/kg group received 12 ml/kg of 0.15 M NaCl. One hour after drug injection, rats were overdosed with sodium pentobarbital and decapitated once anesthetized. Adrenal glands were rapidly removed and fixed in phosphate-buffered 4% paraformaldehyde for 24 hr, then transferred into 30% sucrose for at least 24 hr prior to sectioning. The adrenal tissue sections were then processed for in situ hybridization with ICER oligonucleotide probe.

Dexamethasone Suppression

Rats received subcutaneous injection of dexamethasone (4 mg/kg; n = 12; Sigma Chemical Co.) or NaCl vehicle (0.15 M; n = 12). Two hours later, six dexamethasone rats and six vehicle rats received intraperitoneal LiCl (0.15 M, 12 ml/kg), and the remaining six dexamethasone rats and six vehicle rats were mildly restrained inside snug-fitting, ventilated plastic bags. One hour later, all rats were rapidly anesthetized by carbon dioxide gas and then decapitated. Trunk blood was collected for radioimmunoassay of plasma corticosterone level. Adrenal glands were removed immediately after collecting the trunk blood and then fixed in phosphate-buffered 4% paraformaldehyde for 24 hr. After cryoprotection in 30% sucrose for at least 24 hr, the adrenal glands were processed for in situ hybridization with ICER oligonucleotide probe. An additional six rats were included as untreated controls. All rats were sacrificed between 4–6 hr after lights on (i.e., between 11:00 and 13:00) to minimize diurnal variation in the plasma corticosterone level and circadian induction of ICER expression.

Central Administration of LiCl

Under chloral hydrate (153 mg/kg) and pentobarbital (35 mg/kg) anesthesia, rats were stereotaxically implanted with a 22-gauge, stainless-steel guide cannula (Plastics One, Roanoke, VA) aimed toward the lateral ventricle (1.2 mm caudal to bregma, 1.5 mm lateral to the midline, and 4 mm below the skull surface). Guide cannulae were held in place with dental acrylic bonded to stainless-steel screws anchored to the skull. An obdurator was inserted into each guide cannula and remained in place except during injections, when it was removed and replaced with an injector that extended 1.0 mm beyond the tip of the guide cannula. After 1 week of postoperational recovery, patency and placement of the cannula were verified by injection of 100 ng human angiotensin II (Sigma Chemical Co.) dissolved in 5 μl of 0.15 M NaCl; rats with cannulas projecting into the lateral ventricle responded to the angiotensin injection by vigorously licking the water bottle within 2 min, whereas rats that failed to drink were dropped from the study. Cannula placements were also verified postmortem by sectioning through the brain.

Rats (n = 5 per group) were injected with either LiCl (127 μg, 0.6M) or isoosmotic NaCl (180 μg, 0.6M). The volume of all injections was 5 μl, delivered over 30 sec with a hand-held 50 μl syringe (Hamilton Co., Reno, NV). The injector was left in place for 30 sec after solution delivery. One hour after the injections, rats were overdosed with sodium pentobarbital and transcardially perfused with phosphate-buffered 4% paraformaldehyde. Adrenal glands were removed, postfixed for 2 hr, and then transferred into 30%

sucrose for at least 24 hr prior to sectioning. The adrenal sections were processed for in situ hybridization with ICER oligonucleotide probe.

Plasma Corticosterone Assay

Rats were rapidly anesthetized with carbon dioxide gas 0.3 or 1 hr after an intraperitoneal injection of LiCl (0.15 M, 12 ml/kg, n = 5) or isovolumetric saline (n = 4) and decapitated once anesthetized. Uninjected rats (n = 4) were included as 0-hr controls. Trunk blood was collected into 1.5-ml microcentrifuge tubes containing 5 μl heparin and centrifuged at 3,000*g* for 10 min at 4°C. Plasma was removed into new tubes, frozen in liquid nitrogen, and stored at –80°C until use. Plasma levels of corticosterone were determined by radioimmunoassay with Coat-A-Count kit (Diagnostic Products Corporation, Los Angeles, CA).

In Situ Hybridization

For cDNA probes, c-fos cDNA (a full-length 2.1-kb restriction fragment; Curran et al., 1987) and ICER cDNA (166-bp restriction fragment comprising the ICER-specific portion of CREM cDNA; Stehle et al., 1993) were used. A 48-mer oligonucleotide (5′-CAG TTT CAT CTC CAG TTA CAG CCA TGT TGG GCT TTT GCA TAC AGA GTG-3′) complementary to a portion of the ICER-specific exon of the CREM gene was used as ICER oligonucleotide probe. A similar but shorter oligonucleotide was used by Luckman and Cox (1995). cDNA probes were labeled with ^{35}S-α-dATP (NEN Perkin Elmer, Boston, MA) by using a random priming kit (Roche Molecular Biochemicals, Indianapolis, IN), and oligonucleotide probes were 3′ end-labeled with ^{35}S-α-dATP (NEN) with terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals).

Forty-micrometer-thick sections of the adrenal glands were cut on a freezing, sliding microtome and collected into 20-ml glass scintillation vials containing ice-cold 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate). The SSC was pipetted off, and sections were suspended in 1 ml of prehybridization buffer (50% formamide, 10% dextran sulfate, 2× SSC, 1× Denhardt's solution, 50 mM dithiothreitol, and 0.5 mg/ml denatured herring sperm DNA) and incubated for 2 hr at 48°C for cDNA probes or at 37°C for oligonucleotide probes. cDNA or oligonucleotide probes labeled with ^{35}S-α-dATP were then added to the vials (1×10^7 cpm/vial) and hybridized overnight at 48°C for cDNA probes or at 37°C for oligonucleotide probes. After hybridization, the sections hybridized with oligonucleotide probe were washed sequentially in 2× SSC, 1× SSC, 0.5× SSC, and 0.5× SSC at 37°C for 10 min each. The sections hybridized with cDNA probes were washed in 2× SSC, 2× SSC, 1× SSC, 0.5× SSC, 0.25× SSC, 0.125× SSC, and 0.125× SSC at 48°C for 20 min each. The tissue sections were then mounted on gelatin-subbed slides, air dried, and apposed to Kodak BioMax film (Eastman Kodak Co., Rochester, NY) at 4°C. After film exposure, a few sets of slides were dipped in undiluted Kodak NTB-2 photoemulsion exposed at 4°C for 1 week. After development, slides were counterstained with cresyl violet and coverslipped.

In situ hybridization was carried out on the representative members of each experimental group at the same time under identical conditions, allowing direct comparison of mRNA expression. All adrenal sections from each experiment were exposed to the same piece of film at the same time, allowing simple comparison within each experiment. Several exposures were taken to ensure that films were not under- or overexposed. Exposure times varied from 12 to 48 hr to obtain autoradiographic images within a linear range of optical density after development in Kodak D-19 developer.

Image Analysis and Statistics

Pixel density was quantitated from the films by using a custom software program (MindsEye 1.26b, T. Houpt). Light levels were adjusted to standardize gray levels of film background, and images were captured in a 10-mm × 7.5-mm frame. Densitometry was restricted to hand-drawn outlines of the adrenal cortex or medulla. For each rat, average pixel densities were obtained from three to five adrenal sections. Individual mean values (mean pixel densities) for each region were then averaged across rats within experimental groups.

All data were analyzed by analysis of variance (ANOVA) and Tukey HSD post hoc comparisons in the SPSS statistical software package (SPSS, Chicago, IL) and presented by mean ± SEM. For all comparisons, the level of significance was set at $P \leq 0.05$.

RESULTS

Time Course of LiCl-Induced ICER and c-fos mRNA Expression

Rats were sacrificed 0, 0.3, 1, 3, 6, 9, or 12 hr after a single intraperitoneal LiCl at lights on (07:00), and the adrenal tissue sections were processed for ICER or c-fos mRNA in situ hybridization. Increases in mRNA level of ICER or c-fos in the adrenal glands were first observed 0.3 hr after the injection (Fig. 1a). Both ICER and c-fos mRNA in situ signals were detected in all three subregions, i.e., zona glomerulosa, zona fasciculata, and zona reticularis, of the adrenal glands (Fig. 1b). ICER and c-fos mRNA levels were quantified by measuring the in situ signals on X-ray films as mean pixel density (relative optical density). ICER mRNA level in the adrenal cortex was transiently increased after LiCl with the peak value at 1 hr ($P < 0.0005$ vs. 0-hr time point), and no significant increase was found at all other time points examined (Fig. 2a). ICER mRNA level in the adrenal medulla was not significantly changed under this experimental condition. The adrenocortical level of c-fos mRNA was significantly increased at 0.3 ($P = 0.037$) and 1 hr ($P = 0.025$) compared with the 0-hr time point, returned to its basal level by 3 hr after LiCl injection (Fig. 2b). Cortical c-fos expression appeared to reach its peak value earlier than cortical ICER expression, i.e., 0.3 hr for c-fos vs. 1 hr for ICER (Fig. 2a,b). The medullary expression level of c-fos mRNA was not significantly changed at all time points examined (Fig. 2b).

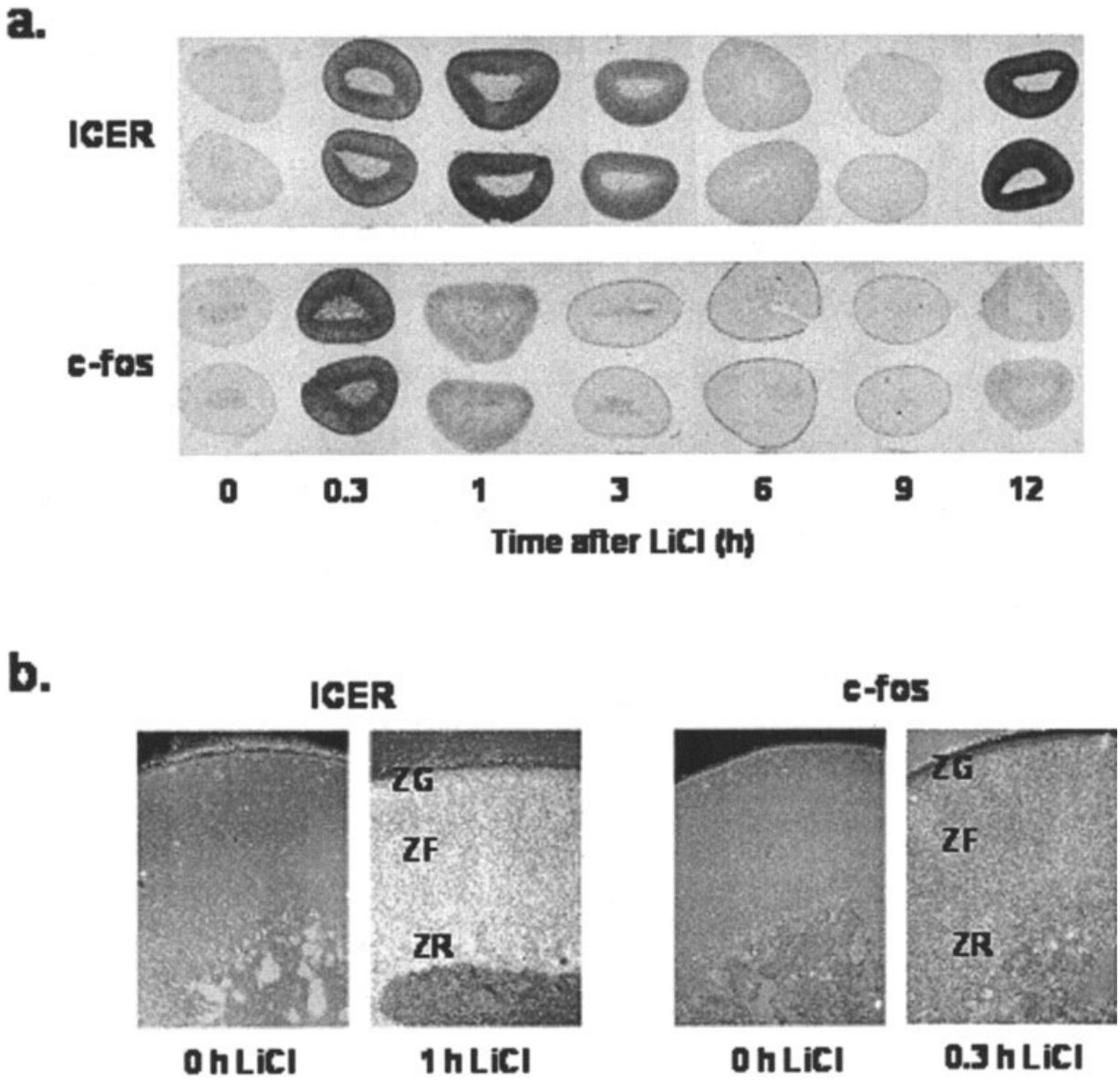

Fig. 1. ICER and c-fos mRNA in situ hybridization in the adrenal gland. **a:** Autoradiography of ICER and c-fos in situ hybridization signals in the adrenal gland. Rats were sacrificed at 0.3, 1, 3, 6, 9, and 12 hr following an intraperitoneal LiCl (0.15 M, 12 ml/kg) at 07:00 (lights on). For the 0-hr time point, the adrenal glands were collected from uninjected rats 1 hr after lights on. Two adrenal sections of each representative rat at each time point. **b:** Representative darkfield photomicrographs of ICER and c-fos in situ signals in all three subregions (ZG, zona glomerulus; ZF, zona fasciculate; ZR, zona reticularis) of the adrenal cortex. Marked increase in the cortical ICER expression was observed 1 hr, and c-fos 0.3 hr, after LiCl.

Correlation analyses were performed comparing c-fos and ICER expression in the cortex and the medulla, respectively, from the same animal at the same time point (Fig. 3). A significant correlation between c-fos expression and ICER expression ($P < 0.0005$) was found both in the cortex and in the medulla. The Pearson correlation R (0.764 in cortex; 0.810 in medulla) indicated that 58.4% of the total variation in cortical ICER expression is accounted for by cortical c-fos expression and that 65.6% of the total variation in medullary ICER expression is accounted for by medullary c-fos expression.

Plasma levels of corticosterone were measured 0.3 and 1 hr (20 min and 60 min) after an intraperitoneal injection of LiCl or NaCl (0.15 M, 12 ml/kg). Uninjected rats were included as 0 hr control. Plasma level of corticosterone was significantly elevated by LiCl, but not by NaCl, at both time points ($P < 0.01$ for 0.3 hr, $P < 0.001$ for 1 hr) compared with 0-hr control (Table I). Statistical significance for the elevated corticosterone

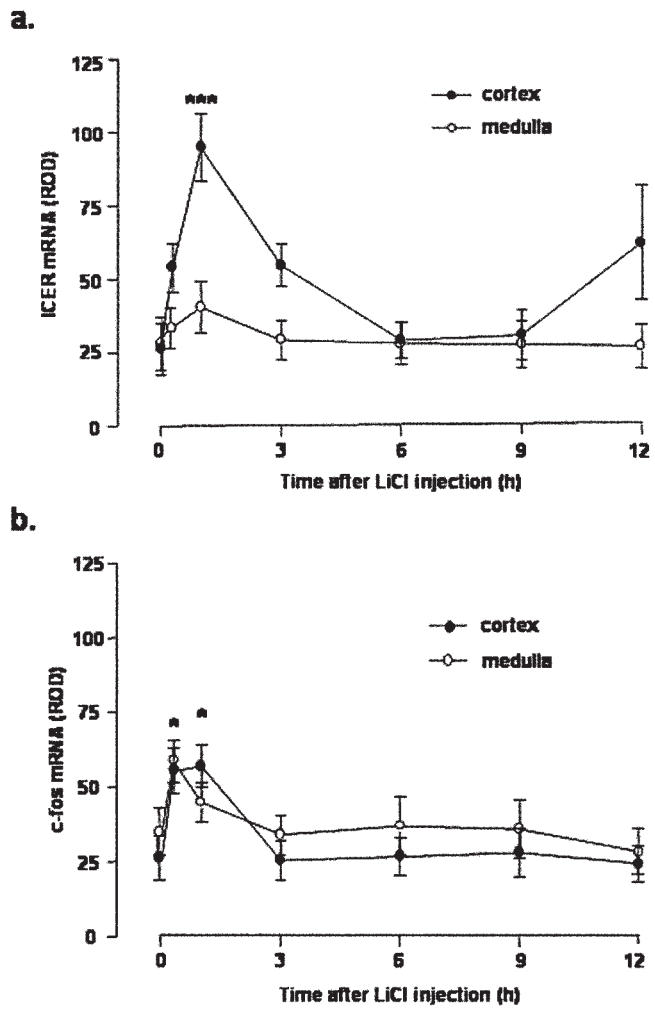

Fig. 2. Quantitative analyses of ICER and c-fos mRNA in situ signals on X-ray films. **a:** A significant increase in ICER mRNA expression ($P < 0.0005$ vs. 0 hr) was found at 1 hr after LiCl (0.15 M, 12 ml/kg) in the adrenal cortex (solid circles) and no significant increase in the medulla (open circle). **b:** c-fos mRNA expression significantly increased at 0.3 hr ($P = 0.037$ vs. 0 h) and 1 hr ($P = 0.025$ vs. 0 hr) after LiCl in the adrenal cortex (solid circles). c-fos Expression in the medulla (open circles) was not significantly induced by LiCl. The cortical c-fos expression by LiCl reached to its peak level earlier than the ICER expression (0.3 hr vs. 1 hr). Data represent means ± SEM for n = 6 per each time point. ROD, relative optical density.

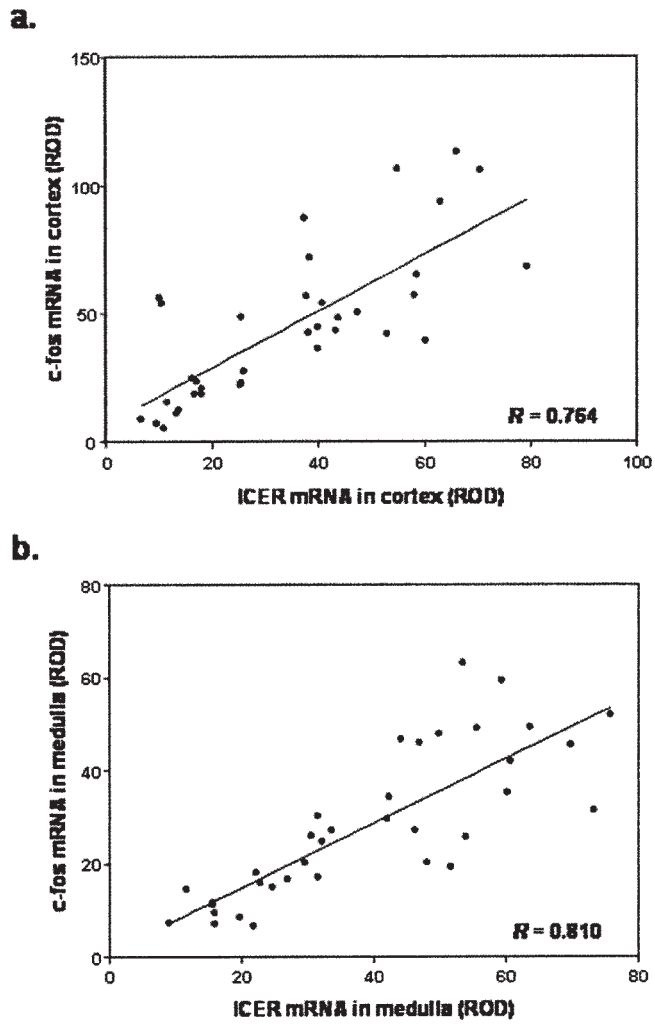

Fig. 3. Correlation analyses between the adrenal c-fos mRNA expression and the adrenal ICER mRNA expression. Data from the time course experiment were plotted comparing c-fos and ICER expression in the cortex (**a**) and the medulla (**b**), respectively, from the same animal at the same time point. Data from the 12-hr time point were not included because of the circadian effect on the cortical ICER expression. a: The Pearson correlation R between c-fos expression and ICER expression in the adrenal cortex was 0.764, which is highly significant (N = 36; $P < 0.0005$). R^2 is 0.584, indicating that 58.4% of the total variation in cortical ICER expression is accounted for by cortical c-fos expression. b: The Pearson correlation R between c-fos expression and ICER expression in the adrenal medulla was 0.810, which is highly significant (N = 36; $P < 0.0005$). R^2 is 0.656, indicating that 65.6% of the total variation in medullary ICER expression is accounted for by medullary c-fos expression.

level was found only in the 1-hr LiCl group ($P < 0.001$) when it was compared with NaCl groups at each time point.

Dose-Dependent Induction of ICER mRNA Expression by LiCl

Rats were given with an intraperitoneal injection of 0.15 M LiCl at doses of 0, 10, 19, 38, or 76 mg/kg and sacrificed 1 hr later for ICER mRNA in situ hybridization. The injections were made isovolumetric (12 ml/kg) and isotonic (0.30 Osm) with 0.15 M NaCl, so the 0-mg/kg LiCl group received 12 ml/kg of 0.15 M NaCl per se. LiCl appeared to increase ICER mRNA expression in the adrenal cortex in a dose-dependent manner (Fig. 4a). Significant inductions in the cortical ICER expression occurred with 38 mg/kg (0.15 M, 6 ml/kg) and 76 mg/kg (0.15 M, 12 ml/kg) of

TABLE I. Plasma Corticosterone Levels (ng/ml)[†]

Minutes	Uninjected (n = 5)	NaCl (n = 4)	LiCl (n = 5)
0	28.481 ± 0.000		
20		116.343 ± 11.584	314.338 ± 52.716[★]
60		51.598 ± 21.967	477.473 ± 104.899[★★,★★★]

[†]Rats received intraperitoneal injection of LiCl (0.15 M, 12 ml/kg) or the same volume of physiologic saline at lights on (07:00 hr), and trunk bloods were collected 0.3 hr (20 min) or 1 hr after the injection. Plasma corticosterone levels were determined by radioimmunoassay. Uninjected rats were sacrificed at 07:00 hr as 0-hr controls. Data represent means ± SEM.
★$P < 0.01$ vs. 0-hr control.
★★$P < 0.001$ vs. 0-hr control.
★★★$P < 0.001$ vs. 1-hr NaCl.

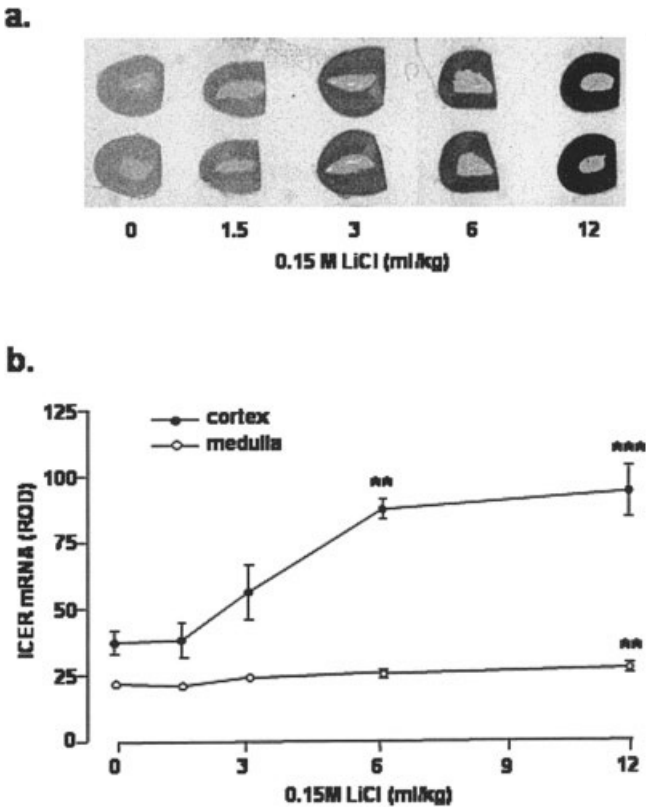

Fig. 4. ICER mRNA in situ hybridization in the adrenal gland. Rats were sacrificed 1 hr after an intraperitoneal LiCl at doses of 0, 10, 19, 38, or 76 mg/kg (0.15 M LiCl; 0, 1.5, 3, 6, or 12 ml/kg). **a:** Autoradiography of ICER mRNA in situ signals on X-ray films. Two adrenal sections of each representative rat at each dose. **b:** ICER mRNA expression in the adrenal cortex (solid circles) significantly increased by 38 mg/kg (0.15 M, 6 ml/kg) and 76 mg/kg (0.15 M, 12 ml/kg) of LiCl ($P = 0.001$ and $P < 0.0005$, respectively), and in the medulla (open circles) only by 76 mg/kg ($P = 0.003$), compared with 0 mg/kg of LiCl. Data represent means ± SEM for n = 6 per each dose. ROD, relative optical density.

LiCl ($P = 0.001$ and $P < 0.0005$, respectively) compared with 0 mg/kg of LiCl (Fig. 4b). ICER mRNA expression in the adrenal medulla was significantly increased only by 76 mg/kg LiCl, the highest dose ($P = 0.003$ vs. 0 mg/kg LiCl).

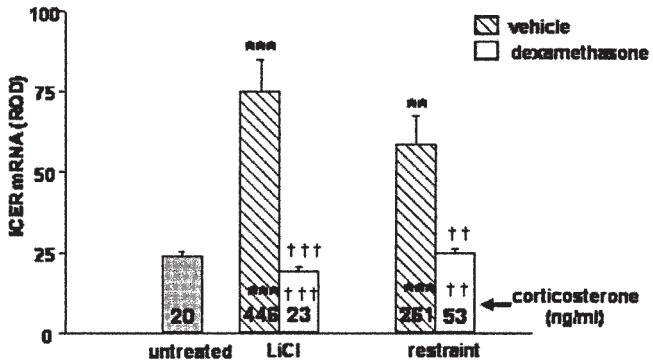

Fig. 5. Induction of ICER mRNA expression 1 hr following intraperitoneal LiCl injection or after 1 hr of restraint stress in the adrenal cortex of rats pretreated with dexamethasone (4 mg/kg, 2 hr prior to LiCl or restraint). Dexamethasone successfully blocked the cortical ICER inductions either by LiCl or by restraint stress. Plasma corticosterone levels were significantly increased by LiCl or restraint, and this increase was blocked by dexamethasone pretreatment. Mean plasma corticosterone levels for each treatment group are shown within the bar representing the ICER data for that group. ICER data represent means ± SEM for n = 6 per each group. ★★★$P < 0.0005$, ★★$P = 0.002$ vs. untreated group, †††$P < 0.0005$, ††$P = 0.003$ for ICER, $P = 0.001$ for corticosterone vs. vehicle-injected group.

Dexamethasone Suppression of LiCl-Induced ICER mRNA Expression

To determine whether the adrenocortical ICER induction by LiCl is mediated by activation of the HPA axis, rats were pretreated with dexamethasone to suppress release of ACTH. Rats received a subcutaneous injection of dexamethasone (4 mg/kg) or vehicle and then either LiCl (0.15 M, 12 ml/kg) or restraint stress 2 hr later. Rats were sacrificed 1 hr after LiCl or immediately after I hr of restraint. Untreated controls received a vehicle injection instead of dexamethasone. Plasma corticosterone level was markedly increased either by LiCl or by restraint stress ($P < 0.0005$ vs. untreated control); however, these increases were blunted by dexamethasone (Fig. 5; data within bars). This result suggests that dexamethasone successfully blocked both LiCl- and restraint-induced activation of the HPA axis. The adrenocortical expression of ICER mRNA was significantly increased either by LiCl or by restraint stress ($P < 0.0005$, $P = 0.002$ vs. untreated control), and these

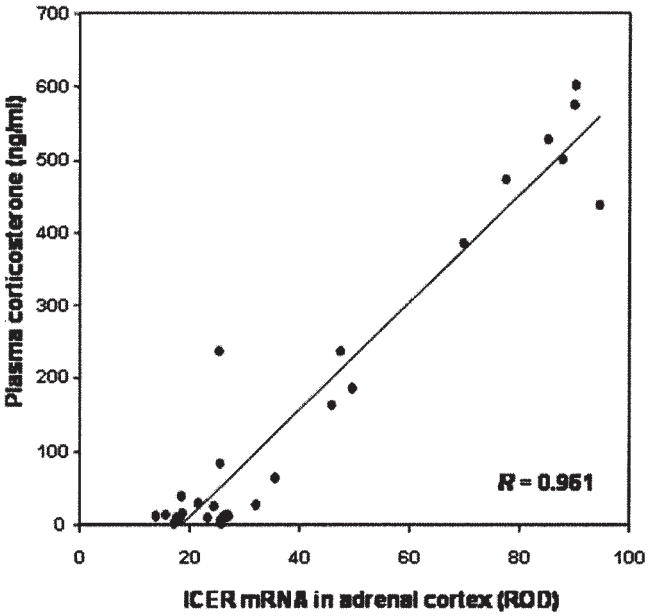

Fig. 6. Plot showing the correlation between plasma concentrations of corticosterone and ICER mRNA expression in the adrenal cortex. The Pearson correlation R between plasma corticosterone level and ICER expression in the adrenal cortex was 0.961, which is highly significant (N = 30; $P < 0.0005$). R^2 is 0.923, indicating that 92.3% of the total variation in cortical ICER expression is accounted for by plasma corticosterone concentration.

increases were blocked in the dexamethasone-pretreated rats (Fig. 5). These results suggest that lithium, as an interoceptive stressor, may induce ICER mRNA expression in the adrenal cortex via activation of the HPA axis. A correlation analysis was performed to examine the nature of the relationship between plasma corticosterone level and the cortical ICER expression (Fig. 6). The Pearson correlation R between plasma corticosterone level and ICER expression in the adrenal cortex was 0.961, which is highly significant (N = 30, $P < 0.0005$). R^2 was 0.923, indicating that 92.3% of the total variation in cortical ICER expression is accounted for by plasma corticosterone concentration.

Induction of ICER mRNA Expression by Centrally Administered LiCl

To determine whether the central action of lithium is sufficient to induce adrenocortical ICER expression, we examined cortical ICER mRNA levels 1 hr after intracerebroventricular LiCl (127 μg/5 μl, 0.6 M). The central administration of LiCl significantly ($P = 0.016$) increased ICER mRNA level in the adrenal cortex compared with central isoosmotic NaCl (180 μg/5 μl, 0.6 M; Fig. 7). ICER mRNA level in the adrenal medulla of LiCl-treated rats did not differ from that of the NaCl-treated rats. This result indicates that the central action of lithium is sufficient to induce the cortical, but not the medullary, ICER mRNA expression.

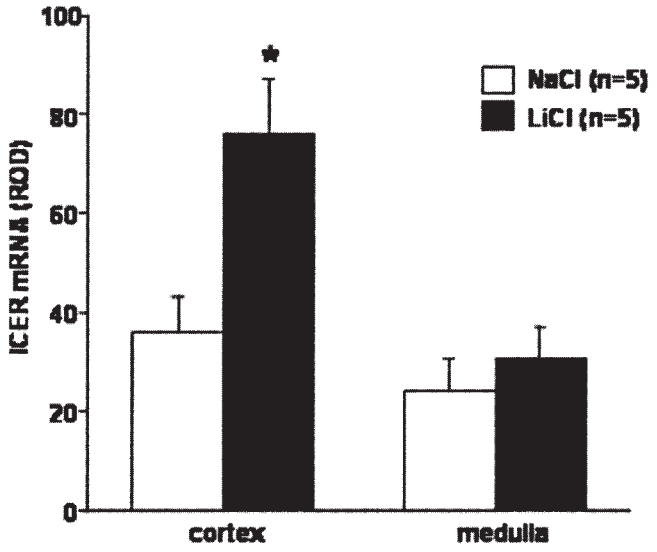

Fig. 7. ICER mRNA expression in the adrenal gland 1 hr after an intracerebroventricular LiCl (127 μg/5 μl, 0.6 M) or isoosmotic NaCl (180 μg/5 μl, 0.6 M). Central LiCl significantly induced ICER mRNA expression in the adrenal cortex, but not in the medulla. Data represent means ± SEM. *$P = 0.016$ vs. NaCl group.

DISCUSSION

We demonstrated that the expression of ICER and c-fos is transiently induced by intraperitoneal lithium in the adrenal cortex. ICER is the only inducible member of cAMP response element modulator (CREM), a family of transcription factors binding to cAMP response elements (CREs) in the promoter regions of genes and potently inhibiting CRE-mediated gene transcription (Sassone-Corsi, 1998). CREM proteins have been shown to bind to CRE elements in the c-fos promoter and inhibit cAMP-stimulated c-fos transcription, leading to the proposal that ICER may be responsible for terminating c-fos transcription (Foulkes et al., 1991; Mao et al., 1998; Spencer and Houpt, 2001). Thus, it was hypothesized that ICER expression may correlate with c-fos expression during lithium-induced activation of adrenal gland. Indeed, the correlation analyses of c-fos and ICER expression by lithium showed that the adrenocortical c-fos expression is highly correlated with the adrenocortical ICER expression. Our hypothesis is further supported by the finding that peak induction of the cortical ICER expression by lithium was slightly delayed with respect to peak c-fos induction; i.e., the peak induction for c-fos was 0.3 hr after LiCl and for ICER 1 hr after LiCl. We previously showed a similar phase relationship between c-fos and ICER inductions after LiCl in the hypothalamic paraventricular nucleus, the center of the HPA axis, although ICER induction peaked even later (between 1 and 3 hr) in the forebrain region (Spencer and Houpt, 2001). Given the limited number of cell types in the adrenal cortex, it is probable that c-fos and ICER expression are colocalized in the adrenocortical cells, likely as a part of lithium-induced

HPA activation. Thus, it is possible that ICER induction may serve as a negative-feedback signal after cortical stimulation by LiCl to induce a refractory period of transcriptional response. Several studies have shown that the amplitude of corticosterone released in response to a stressful event is diminished when preceded by an earlier stress event (Keim and Sigg, 1976; Ma and Lightman, 1998; Cole et al., 2000; Bhatnagar et al., 2002), suggestive of a refractory period for the HPA activation in general. However, further studies are still required to prove our hypothesis.

We showed that dexamethasone pretreatment effectively suppresses the adrenocortical ICER expression by acute LiCl as well as by restraint stress. This result suggests that cortical ICER induction either by acute lithium or by restraint is dependent on activation of the HPA axis and concurs with a previous report by Della Fazia and coworkers (1998) that induction of ICER expression in the adrenal gland by surgical stress is coupled to the release of adrenocorticotrophic hormone (ACTH). Indeed, a correlation analysis of the dexamethasone suppression test showed that plasma corticosterone concentration is highly correlated with the adrenocortical ICER expression. We demonstrated that plasma corticosterone levels are increased shortly after intraperitoneal LiCl, as previously reported by others (Hennessy et al., 1976; Smotherman, 1985; Sugawara et al., 1988). It has been reported that lithium stimulates ACTH release (Hennessy et al., 1976; Smotherman, 1985; Sugawara et al., 1988) and that ACTH injection induces robust ICER expression in the adrenal cortex (Della Fazia et al., 1998). These reports together with our results support the idea that lithium-induced ICER mRNA expression in the adrenal cortex is a part of the HPA activation, and adrenocortical ICER expression could serve as a reliable marker of lithium-induced HPA activation. We showed that central action of lithium may be sufficient to induce HPA activation by demonstrating that central administration of LiCl significantly induces adrenocortical ICER expression. This is supported by previous reports that central administration of LiCl reliably induces CTA in rats (Barranco et al., 2001) and that lithium-induced CTA is accompanied by HPA activation (Hennessy et al., 1976, 1980; Revusky and Martin, 1988; Sugawara et al., 1988; Peeters and Broekkamp, 1994; Smotherman et al., 1976).

We confirmed ICER expression in the adrenal cortex to be a good marker of HPA activity. Adrenocortical ICER expression as a part of HPA activation may be related to the cortical steroidogenesis. ACTH induces adrenocortical ICER expression (Della Fazia et al., 1998), and acute stimulation of steroidogenesis in the adrenal cortex by ACTH occurs through a cAMP-dependent mechanism (Haynes et al., 1959). ICER, after induction by elevated cAMP, forms heterodimers with other cAMP response element-binding proteins (CREBs), binds to consensus CRE sites, and potently inhibits CRE-mediated gene transcription (Sassone-Corsi, 1998). The promoter of neuronal nitric oxide synthase (nNOS) carries CRE in its upstream region (Jeong et al., 2000), and nNOS expression is known to be regulated by calcium influx through a CREB family transcription factor-dependent mechanism (Sasaki et al., 2000). Restraint stress induces nNOS expression in the adrenal cortex (Kishimoto et al., 1996; Tsuchiya et al., 1996), and nitric oxide has been reported to be implicated in the adrenocortical steroidogenesis (Cymeryng et al., 1998). We and others have shown that plasma corticosterone level is markedly increased by acute lithium (Hennessy et al., 1976; Smotherman, 1985; Sugawara et al., 1988). Taken together, it appears that induction of a cAMP-dependent transcriptional repressor ICER by lithium in the adrenal cortex may serve to limit the duration of cAMP-stimulated gene transcription, such as nNOS expression, which is likely implicated in the adrenocortical steroidogenesis.

We demonstrated that intraperitoneal lithium induces a transient expression of c-fos and ICER in the adrenal medulla. It has been reported that lithium administration activates the adrenomedullary catecholaminergic system (Fontela et al., 1986; O'Conner et al., 1988; Terao et al., 1992). A single injection of LiCl alters norepinephrine levels in the brain (Otero Losada and Rubio, 1984, 1992) and increases the plasma epinephrine, norepinephrine, and glucose levels (Fontela et al., 1986, 1990; Chaouloff et al., 1992). We previously reported that intraperitoneal LiCl increases mRNA expression of tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine biosynthesis, in the midbrain locus coeruleus and the adrenal medulla, as well as ICER expression in the adrenal medulla (Kim et al., 2003). It has been reported that ICER expression regulates transsynaptic induction of TH gene in the adrenal medulla of reserpine-treated rats (Tinti et al., 1996; Trocme et al., 2001). It was proposed that the increase in TH expression with lithium likely occurs through the activator protein-1 transcription factor pathway (Chen et al., 1998) and that ICER expression may be responsible for terminating c-fos transcription (Foulkes et al., 1991; Mao et al., 1998; Spencer and Houpt, 2001). In this study, the adrenomedullary c-fos expression by lithium was found to be correlated with the adrenomedullary ICER expression. With the results taken together, we suggest that lithium, as an interoceptive stressor, may activate the sympathetic adrenomedullary system as well and that lithium-induced ICER expression in the adrenal medulla may result from sympathetic adrenomedullary activation. A possible local effect of systemic lithium on the adrenomedullary expression of ICER still cannot be ruled out, because the medullary ICER induction by central LiCl did not show statistical significance in this study.

Additionally, there was a circadian increase in the adrenocortical ICER expression at 12 hr after LiCl, i.e., at lights off, although statistical significance was not found (Fig. 2). This may be linked to the circadian rhythm in glucocorticoid secretion (Weitzman, 1976). ICER expression also shows a circadian variation in the

pineal gland (Stehle et al., 1993) and in the hypothalamic paraventricular and supraoptic nuclei (Spencer and Houpt, 2001).

In conclusion, understanding the activation of immediate-early genes such as c-fos and ICER in response to a single LiCl injection is an important first step in understanding the long-term changes in gene expression elicited by lithium that are involved in its therapeutic or toxic effect. It has been suggested that at least part of the therapeutic or toxic actions of lithium includes its interaction with the HPA system. Our results suggest that the adrenocortical expression of ICER mRNA could serve as a reliable marker for lithium-induced activation of the HPA axis. The mechanism by which lithium acutely stimulates or chronically modulates the HPA axis activity, and perhaps sympathetic outflow as well, remains to be fully elucidated. The pattern and mechanism by which lithium stimulates ICER transcription in the adrenal gland would serve as a useful model system in future studies of lithium.

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