Modulation of Protein Kinase C Isozymes and Substrates by Lithium: The Role of Myo-inositol

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Lithium is the most effective treatment for reducing both the frequency and severity of recurrent affective episodes, but despite extensive research, the molecular mechanisms underlying its therapeutic actions have not been fully elucidated. Signal transduction pathways are in a pivotal postion in the central nervous system, able to affect the functional balance between neurotransmitter systems and have clearly been demonstrated to be targets of lithium's actions. We investigate the hypothesis that the action of chronic lithium on PKC isozymes and substrates may be secondary to its potent effect in inhibiting the recycling of inositol. Rats received lithium for 3 weeks and also myo-inositol or saline twice daily via intracerebroventricular (ICV) injections. There was a significant interaction between chronic lithium and myo-inositol administration, with the chronic ICV administration of myo-inositol attenuating lithium's effects on PKC α, PKC ϵ, and on pertussis toxin–catalyzed [^{32}P]ADP-ribosylation. These results suggest that the effects of chronic lithium on signal transduction pathways may stem initially from its inhibition of inositol-1-phosphatase. Given the critical role of PKC isozymes and G proteins in modulating intracellular cross-talk between neurotransmitter systems and thereby the integrative functions of the CNS, future studies using other inhibitors of inositol monophosphatases are warranted, and offer the hope for the development of more potent and more rapidly acting mood-stabilizing drugs. [Neuropsychopharmacology 15:370–381, 1996]

Lithium, a simple monovalent cation, has demonstrated efficacy in the treatment of manic-depressive illness and is the most effective treatment for reducing both the fre-

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quency and severity of recurrent affective episodes (reviewed in Goodwin and Jamison 1990). Despite lithium's remarkable efficacy and decades of use, the molecular mechanisms underlying its therapeutic actions have not fully been elucidated. In recent years, it has become increasingly clear that rather than any single neurotransmitter system being responsible for depression or mania, multiple interacting and overlapping systems are involved in regulating mood, and that most effective drugs such as lithium do not work on any particular neurotransmitter system in isolation but, rather, affect the functional balance between interacting systems (Janowsky et al. 1972; Avissar et al. 1988; Manji et al. 1995). Signal transduction pathways are in a pivotal position in the central nervous system (CNS), can affect the functional balance between neurotransmitter systems, and have therefore been postulated to represent the targets underlying lithium's efficacy in treating both poles of

manic-depressive illness (Manji 1992; Jope and Williams 1994).

There is now considerable evidence that the chronic administration of "therapeutic" concentrations of lithium affects the function of second messenger–generating systems in the brain (Ebstein et al. 1976; reviewed in Mork et al. 1992; Jope and Williams 1994; Manji et al. 1995). At therapeutically relevant concentrations, lithium, is a potent inhibitor of inositol monophosphatase (K_i, 0.8 mM) and results in an accumulation of inositol-1-monophosphate (Ins(1)P) as well as a reduction in free inositol (Allison and Stewart 1971; Hallcher and Sherman 1980). Because the ability of a cell to maintain sufficient supplies of myo-inositol is crucial to the resynthesis of the phosphoinositides and the maintenance and efficiency of signaling, and because the mode of enzyme inhibition is *uncompetitive*, lithium's effects have been postulated to be most pronounced in systems undergoing the highest rate of PIP_2 hydrolysis (Berridge et al. 1982, 1989). Because the brain has limited access to inositol other than that derived from recycling of inositol phosphates, it has been suggested that a physiological consequence of lithium's action is derived through a depletion of free inositol (Berridge et al., 1982, 1989). Chronic lithium administration has been demonstrated to reduce agonist-stimulated phosphoinositide hydrolysis in rat brain in some studies (Casebolt and Jope 1989; Kendall and Nahorski 1987; Godfrey et al. 1989) and more recently to have multiple effects on the PI second messenger–generating system, including at the level of protein kinase C (PKC; reviewed in Jope and Williams 1994 and Manji and Lenox 1994). In recent studies, chronic lithium administration has been demonstrated to significantly reduce the levels of membrane-associated PKC α and PKC ϵ (Manji et al. 1993) (Masana MI, Chen G, Shorts L, Potter WZ, Manji HK (1995): Attenuation of PKC induced cyclic AMP increases in rat brain by chronic lithium: An in vivo microdialysis study. Under revision.) and of a major PKC phosphoprotein substrate, MARCKS (myristoylated alanine-rich C kinase substrate) in the rat hippocampus (Lenox et al. 1992).

The mechanism(s) by which lithium produces the isozyme-selective decreases in the immunolabeling of PKC α and ϵ and in the levels of MARCKS is presently unclear. It is noteworthy, however, that as a result of inositol depletion in the presence of lithium, metabolites within the phospholipid portion of the hydrolytic pathway that include diacylglycerol (DAG), phosphatidic acid (PA), and cytidine monophosphate-phosphatidate (CMP-PA) have been shown to be significantly elevated. This effect can be prevented by the presence of high concentrations of inositol (Drummond and Raeburn 1984; Downes and Stone 1986; Brami et al. 1991a, b). This might be expected as the end metabolite in this pathway, (i.e., CMP-PA) requires free myo-inositol to resynthesize inositol phosphates for the regenera-

tion of PIP_2 (Rana and Hokin 1990; Fisher et al. 1992). Because PKC activation is often followed by its rapid proteolytic degradation (Young et al. 1987), a prolonged increase in DAG levels by chronic lithium may lead to an increased membrane translocation and subsequent degradation and downregulation of PKC isozymes. Moreover, recent studies in fibroblasts have demonstrated that activating PKC results in a significant reduction in the levels of both MARCKS protein and mRNA (Erusalimsky et al. 1991; Linder et al. 1992). Thus, the reduction in hippocampal MARCKS observed following chronic lithium administration may similarly be mediated via a lithium-induced increase in DAG levels and subsequent activation of PKC isozymes.

In addition to its effects on PKC outlined, there is also considerable evidence that chronic lithium administration affects G protein function in both rodents and human subjects (Mork et al. 1992; Jope and Williams 1994; Manji et al. 1995). In a recent series of studies, the attenuation of the function of the inhibitory G protein (G_i) was accompanied by a significant increase in pertussis toxin–catalyzed [^{32}P]ADP-ribosylation in rat brain (Masana et al. 1995). As with most other studies (reviewed in Manji et al. 1995), these investigators did not observe alterations in the amounts of α_s,α_{i1-3}, or α_o. Because pertussis toxin selectively ADP-ribosylates the undissociated, inactive $\alpha\beta\gamma$ heterotrimeric form of G_i and G_o (Ui 1990), it has been postulated that lithium inactivates G_i by stabilizing the inactive conformation (Hsiao et al. 1992; Masana et al. 1995, under revision). At present, the molecular mechanism(s) underlying lithium's effects on G proteins remains to be fully established. There is some evidence for acute in vitro effects of lithium on G proteins (Avissar et al. 1988; Greenwood and Jope 1994), but many of lithium's chronic ex vivo effects persist after washing of the membranes and are therefore likely attributable to an indirect posttranslational modification of the G proteins (Jope and Williams 1994; Manji et al. 1995). Because G protein function is known to be regulated by phosphorylation (Houslay 1991), and because considerable evidence has shown that lithium affects PKC, one potential mechanistic explanation for lithium's effects on G protein subunit dissociation is phosphorylation by PKC.

In the present study, we sought to investigate the hypothesis that the action of chronic lithium on PKC isozymes and substrates may be secondary to its potent effect in inhibiting the recycling of inositol. Previous studies have shown that the administration of pharmacological concentrations of extracellular inositol attenuates some of lithium's biochemical, behavioral, and toxic effects (Busa and Gimlich 1989; Godfrey et al. 1989; Pontzer and Crews 1990; Kofman and Belmaker 1993; Tricklebank et al. 1991; Kofman et al. 1991; 1993). We have therefore investigated the role of myo-inositol supplementation on lithium-induced alterations in PKC

isozymes and PKC substrates and on pertussis toxin–catalyzed [^{32}P]ADP-ribosylation. We have found that the daily intracerebroventricular administration of myo-inositol attenuates several of chronic lithium's effects on signal transduction pathways.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (weight 250 to 350 g) were housed four per cage and exposed to a 12-hour light/dark cycle. The control group ($n = 16$) was fed ordinary rat chow, and the treatment group ($n = 16$) was fed Li_2CO_3-supplemented chow (0.2% by weight) for 3 weeks. Serum lithium concentration were determined by flame photometry in carotid blood obtained at sacrifice by decapitation.

For the purposes of myo-inositol administration, all the rats ($n = 32$) were anesthetized with Nembutal (50 mg/Kg) and implanted with guide cannulae into the dorsal part of the lateral ventricle using standard stereotaxic procedures. Coordinates for the cannula were 0.8 mm posterior to the bregma, 1.4 mm lateral to midline, and 5 mm below skull surface. At least 7 days of recovery was allowed following surgery. Rats were allowed to recover and were observed to behave normally with the chronic indwelling intracerebroventricular (ICV) cannula prior to initiation of lithium and/or myo-inositol treatment. Myo-inositol was dissolved in warm artificial cerebrospinal fluid (CSF) (147 mM NaCl, 2.5 mM KCl, 1.6 mM $MgCl_2$, 1.7 mM $CaCl_2$, and 2.2 mM glucose). Rats were injected twice daily for 3 weeks with either 10 mg myo-inositol [250 μg/μl in a volume of 40 μl; ($n = 16$; 8 lithium treated, 8 control)] or artificial CSF ($n = 16$; 8 lithium treated, 8 control) over a period of 3 minutes using a Koehlin 100-μl microsyringe, via an injection cannula that protruded 0.5 mm below the guide cannula. This treatment paradigm resulted in the generation of four distinct groups: lithium-artificial cerebrospinal fluid (CSF; $n = 8$), lithium-inositol ($n = 8$), control-CSF ($n = 8$), and control-inositol ($n = 8$). The time course of twice daily ICV inositol administration and dose of 10 mg was based on studies of Bersudsky et al. (1993), where Li-pilocarpine seizures required 10 mg for full reversal of effects and the effect of inositol was present after 12 hours, but gone by 24 hours.

Immunoblotting

For the immunoblotting studies, the rat brains were removed immediately upon decapitation, dissected on ice, and hippocampi and frontal cortices were rapidly frozen in dry ice and stored at −80°C. For the subse-

quent studies, each animal was treated as an individual sample, and there was no pooling of tissues. For Western blot anlaysis, the tissue was homogenized in 20 mM Tris buffer (pH 7.4) containing 2 mM EDTA, 0.5 mM EGTA, 10 mg/ml leupeptin, 10 mg/ml apoprotinin, 0.2 mM phenylmethylsulfonylfluoride, using a glass-glass tissue grinder (15 strokes). The homogenate was centrifuged at 1,000 g for 5 minutes in a Beckman table centrifuge. The resultant supernatant was centrifuged at 45,000 g for 1 hour at 4°C in a Beckman TL-100 centrifuge. The pellet (particulate fraction) was resuspended in the original volume of homogenization buffer. The supernatant (soluble fraction) was centrifuged again at 45,000 g for 2 hours to remove residual particulate fraction. An aliquot was taken for protein determination, and the fractions were then frozen at −80°C in plastic tubes. Protein concentration was determined by Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA) using bovine serum albumin as standard. PKC isozyme and PKC substrate (MARCKS and GAP-43) immunoblotting was performed on both cytosolic and particulate fractions. The linearity of the particulate and soluble fraction protein concentration for Western blotting was previously ascertained by resolution of selected concentrations of protein (between 10 and 200 μg). Subsequent PKC isozyme, MARCKS, and GAP-43 immunoblotting was performed using particulate or soluble protein concentrations demonstrated to be within the linear range for Western blotting using previously described methods (Manji et al. 1993; Masana et al. 1995). In brief, 50 μg of particulate and soluble fraction samples were subjected to SDS-PAGE on 10% polyacrylamide gels (5% stacking gel) according to the method of Laemmli (1970). Proteins resolved on SDS-PAGE were then electrophoretically transferred to nitrocellulose membranes. Nonspecific binding on the nitrocellulose was blocked with low-detergent "blotto" containing 50 mM TRIS (pH 8.0), 2 mM $CaCl_2$, 80 mM NaCl, 5% nonfat dry milk, 0.2% NP-40, and 0.02% NaN_3. Blots were then incubated at room temperature overnight with monoclonal anti-PKC isozyme antibodies [anti-PKC α, anti-PKC β(I/II), anti-PKC γ, Seikagaku America, St. Petersburg, FL; anti-PKC δ, anti-PKC ε, anti-PKC ζ, GIBCO, BRL Bethesda, MD], anti-MARCKS antibody (kindly donated by Dr. Jitendra Patel), and anti-GAP 43 antibody (Boeringer, Mannheim). The antibodies for all PKC isoforms, MARCKS, and GAP-43 were all diluted 1:1,000 in low-detergent blotto prior to use. Blots were washed three times, and in the case of the anti-PKC α, anti-PKC β(I/II), anti-PKC γ antibodies, incubated with a second antibody, antimouse IgG from rabbit (1 mg/ml; 1:200 dilution; Cappel, Malvern, PA) for 2 hours. After three washes, the nitrocellulose sheets were incubated with 1 × 10^6 cpm [^{125}I] protein A (Amersham) for 1 hour. The labeled blots were then washed, dried, and exposed to

X-ray film with intensifying screens at $-80°C$. Quantitation of the immunoblots was performed by densitometric scanning of the autoradiograms using an Image Analysis system (NIH 1.47). An aliquot of pooled "standard" rat brain soluble or particulate fraction was run on one lane of each gel, and the immunolabeling was calculated relative to this standard. Data were normalized against the standard rat brain fractions, which were run on all blots to minimize between-blot variability.

Pertussis Toxin–Catalyzed [^{32}P]ADP-Ribosylation

Pertussis toxin (PT)–catalyzed labeling of rat cortical or hippocampal membrane G_i/G_o was performed using previously described methods (Masana et al. 1995); in brief, rat brain membranes were prepared as described and were incubated at $37°C$ for 60 minutes in a final volume of 0.2 ml, containing 100 mM potassium phosphate buffer (pH 7.5), 100 mM thymidine, 10 mM arginine, 0.5 mM ATP, 50 μM GTP, and 10 μM [α-^{32}P]-NAD (25 Ci/mMol, New England Nuclear, Boston) with activated toxin. Before incubation, pertussis toxin (5 μg/tube, obtained from List Biological Laboratories, Campbell, CA) was activated with 10 mM dithiothreitol for 30 minutes at $37°C$. The linearity of the plasma membrane protein concentration for quantitation of PT-catalyzed [^{32}P]labeling was previously ascertained to be between 20 and 400 μg. Thus, the present studies were performed using protein concentrations of 50 to 100 μg, with PT-catalyzed [^{32}P]ADP-ribosylation of all samples performed in duplicate. Labeled membranes were washed in ice-cold buffer, and proteins were solubilized and subjected to SDS-PAGE on 10% polyacrylamide gels using the method of Laemmli (1970). The gels were dried and exposed to an X-ray film (Kodak X-omat, Rochester, NY) with an intensifying screen at $-80°C$. Molecular weights of labeled protein bands were estimated by comparison with molecular weight standards. An aliquot of pooled "standard" rat brain membranes was run on one lane of every gel, and the quantitation of [^{32}P]incorporation was calculated relative to this standard.

Materials

PDBu were obtained from Sigma Chemical Company (St. Louis, MO). PKC α, β, and γ antibodies were from Seikagaku Kogyo Co., Ltd (Rockville, MD). PKC δ, ζ, and ε antibodies were from GIBCO, BRL (Bethesda, MD). Rabbit anti-mouse IgG was from Organon Teknika Co. (Durham, NC). ^{125}I-Protein A was from Amersham (Arlington Heights, IL). The protein assay kit was from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of analytical grade.

RESULTS

Chronic ICV administration of myo-inositol was without effect on plasma lithium levels (ICV CSF group, 0.712 ± 0.08; ICV inositol group, 0.713 ± 0.07 mM; p NS). Visual examination of the rat brains did not reveal any gross damage of the frontal cortices or hippocampi or enlargement of ventricles from chronic cannula implantation or myo-inositol administration.

Lithium Regulation of PKC Isozymes in the Hippocampus

These experiments were designed to assess the effects of chronic lithium and/or myo-inositol administration on the levels of both membrane-associated (Table 1) and cytosolic PKC isozymes in the hippocampus. A two-way analysis of variance (ANOVA) was conducted for each of the PKC isozymes (α, β(I/II), γ, δ, ε, and ζ). A significant interaction between lithium and inositol was observed for membrane-associated PKC α ($F_{1,28} = 6.28$; $p < .02$). Post hoc analysis revealed a significant difference between the levels of membrane-associated PKC α in the myo-inositol–treated group compared to either the control animals ($t = 2.27$; df = 14; $p < .05$) or the animals coadministered lithium and myo-inositol ($t = 2.36$; df = 14; $p < .05$). The lithium-treated group exhibited modestly lower levels of membrane-associated

Table 1. Hippocampal Membrane PKC Isozymes

Measure	Control	Lithium	Lithium/Inositol	Inositol
PKC α	94.7 (29.15)	79.7 (23.6)	95.37 (23.69)	63.91 (29.70)
PKC β	89.25 (17.97)	83.99 (19.4)	87.64 (14.28)	78.63 (15.12)
PKC γ	107.0 (32.34)	76.48 (22.05)	110.88 (44.77)	77.17 (29.31)
PKC δ	119.41 (18.82)	125.97 (17.93)	128.95 (11.98)	119.34 (16.78)
PKC ε	106.2 (28.5)	76.5 (22.1)	118.1 (26.9)	76.49 (19.26)
PKC ζ	116.65 (21.87)	97.50 (24.57)	112.13 (22.3)	101.71 (32.74)

Rats were treated with lithium and/or myo-inositol for 3 weeks, and hippocampal cytosolic and membrane fractions were subjected to Western blotting with selective anti-PKC α, anti-PKC β(I,II), anti-PKC γ, anti-PKC δ, anti-PKC ε, and anti-PKC ζ isozyme antibodies. Quantitation of the immunoblots was performed by densitometric scanning of the autoradiograms and was normalized to a pooled standard.

PKC α than control animals, which did not reach statistical significance in the present study (Con, 94.7 $\pm$ 29.15; Lith, 79.7 $\pm$ 23.6 OD units; p = .2). The largest interaction between lithium and inositol was observed for membrane-associated PKC ϵ ($F_{1,27}$ = 9.53; p = .005). The lithium-treated animals demonstrated lower levels of membrane-associated PKC ϵ (Lith, 76.5 $\pm$ 22.1 OD units) than either the control animals (Con, 106.2 $\pm$ 28.5; p = .07) or the animals coadministered lithium and myo-inositol (Lith + Inos, 118.1 $\pm$ 26.93; p = .01). The levels of membrane-associated PKC ϵ in the myo-inositol–treated group was also significantly different from that in the animals coadministered lithium and myo-inositol (t = 2.84; df = 14; p = .02). There were no significant interactions between lithium and myo-inositol observed for hippocampal membrane-associated PKC β(I/II), δ, or ζ. Similarly, there were no significant interactions between lithium and myo-inositol observed for the levels of any of the PKC isozymes (α, β (I/II), γ, δ, ϵ, and ζ) in the cytosolic fraction (data not shown).

Lithium Regulation of PKC Isozymes in the Frontal Cortex

These experiments were designed to assess the effects of chronic lithium and/or myo-inositol administration on the levels of both membrane-associated (Table 2) and cytosolic PKC isozymes in the frontal cortex. A two-way ANOVA was conducted for each of the PKC isozymes (α, β (I/II), γ, δ, ϵ, and ζ). In contrast to the results seen in the hippocampus, a significant interaction between lithium and inositol was observed only for membrane-associated PKC (β(I/II) in the frontal cortex ($F_{1,28}$ = 4.61; p < .05); post hoc analyses revealed that this was due to the significantly higher levels of membrane-associated PKC β(I/II) in the animals coadministered lithium and myo-inositol treated compared to any of the other groups. In this brain region, although the lithium-treated group exhibited lower levels of membrane-associated PKC α (Con; 99.67 $\pm$ 40.3; Lith, 83.7 $\pm$ 27.7 OD units) and PKC ϵ (Con, 95.8 $\pm$ 31.3; Lith, 79.1 $\pm$ 20.3 OD units) than for control animals, these decreases did not

reach statistical significance. Similar to what was observed in the hippocampus, there were no significant interactions between lithium and myo-inositol observed for the levels of any of the cystosolic PKC isozymes (α, β, (I/II), γ, δ, ϵ, ζ) in the frontal cortex (data not shown).

Lithium Regulation of PKC Substrates in Hippocampus

These experiments sought to investigate the effects of chronic lithium and/or myo-inositol administration on the levels of the major PKC substrates MARCKS and GAP-43 in the hippocampus (Figure 1A shows a representative immunoblot). A two-way ANOVA revealed a significant interaction between lithium and inositol with respect to MARCKS immunolabeling in the particulate fraction ($F_{1,28}$ = 4.89; p < .04). Post hoc analyses revealed a significant difference between the levels of MARCKS in the myo-inositol–treated group compared to the animals coadministered lithium and myo-inositol (t = 2.087; df = 14; p < .05). The lithium-treated group exhibited lower levels of MARCKS than did control animals, which did not reach statistical significance (Con, 104.45 $\pm$ 15.54; Lith, 81.57 $\pm$ 15.55 OD units; p = .3). There were no significant interactions between lithium and inositol treatment on the levels of GAP-43 in the particulate fraction (Figure 1B). There were no significant interactions between lithium and inositol treatment on the levels of either MARCKS or GAP-43 in the hippocampal soluble fraction (not shown).

Lithium Regulation of PKC Substrates in Frontal Cortex

We investigated the effects of chronic lithium and/or myo-inositol administration on the levels of the major PKC substrates MARCKS and GAP-43 in the frontal cortex (see Figure 2A for a representative immunoblot). There were no significant interactions between lithium and inositol treatment on the levels of MARCKS in the particulate fraction ($F_{1,28}$ = 0.242; p = .6). A near-significant interaction between lithium and inositol was ob-

Table 2. Cortical Membrane PKC Isozymes

Measure	Control	Lithium	Lithium/Inositol	Inositol
PKC α	99.67(40.3)	83.7 (27.7)	95.37 (23.69)	83.91 (29.70)
PKC β	82.83(18.3)	83.23 (9.64)	112.69 (14.28)	74.56 (15.37)
PKC γ	113.16 (38.76)	118.88 (16.9)	116.58 (44.45)	101.08 (29.31)
PKC δ	90.1 (24.65)	92.03 (38.04)	103.15 (38.88)	88.67 (18.15)
PKC ϵ	95.8 (31.3)	79.01 (20.3)	118.1 (26.9)	76.49 (19.26)
PKC ζ	106.20 (39.64)	111.79 (27.72)	110.79 (22.3)	100.58 (21.69)

Rats were treated with lithium and/or myo-inositol for 3 weeks, and frontal cortical cytosolic and membrane fractions were subjected to Western blotting with selective anti-PKC α, anti-PKC β(I,II), anti-PKC γ, anti-PKC δ, anti-PKC ϵ, and anti-PKC ζ isozyme antibodies. Quantitation of the immunoblot was performed by densitometric scanning of the autoradiograms and was normalized to a pooled standard.

Figure 1. Effects of chronic lithium and/or myo-inositol on immunolabeling of PKC substrates in the hippocampus. Rats were treated with lithium ± myo-inositol for 3 weeks, and cytosolic and membrane fractions from hippocampi were prepared and subjected to SDS-PAGE on 10% polyacrylamide gels. Proteins thus resolved were electrophoretically transferred to nitrocellulose membranes; blots were incubated overnight with anti-MARCKS and anti-GAP-43 antibodies, followed by [125I]protein A. Dried blots were exposed to X-ray film with intensifying screens at −80°c. Quantitation of the immunoblots was performed by densitometric scanning of the autoradiograms. An aliquot of pooled "standard" rat brain cytosolic or particulate fraction was run on one lane of each gel, and the immunolabeling was normalized to this standard. (A) Representative immunoblot of MARCKS and GAP-43. (B) Effects of chronic lithium ± myo-inositol administration on immunolabeling of MARCKS and GAP-43 ($n = 8$ in each group). *, Significant interaction between lithium and inositol with respect to MARCKS immunolabeling in the particulate fraction (two-way ANOVA: $F_{1,28} = 4.89$; $p < .04$), li, lithium treated; Con, control; inos, myo-inositol treated; MARCKS, myristoylated alanine rich C kinase substrate; GAP-43, growth-associated protein.
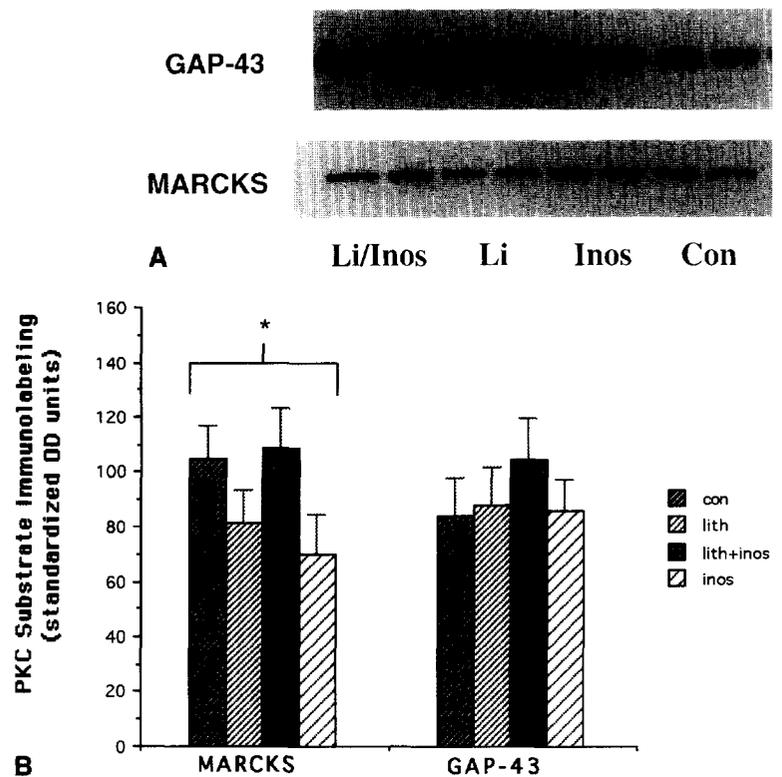
served for GAP-43 in the particulate fraction ($F_{1,28} = 3.86$; $p = .06$). Post hoc analyses revealed a significant difference between the levels of GAP-43 in the group of animals coadministered lithium and myo-inositol compared to the myo-inositol–treated group ($t = 2.188$; df = 14; $p < .05$) and a trend toward statistical significance compared to the lithium-treated group ($t = 1.702$; df = 14; $p = .09$; Figure 2B). There were no significant interactions between lithium and inositol treatment on the levels of either MARCKS or GAP-43 in the soluble fraction (not shown).

Effects of Chronic Lithium on Pertussis-Catalyzed [32P]ADP-Ribosylation

These experiments looked into the effects of chronic lithium and/or myo-inositol administration on pertussis-catalyzed [32P]ADP-ribosylation (an indirect measure of the conformational state of G_i/G_o).

Hippocampus. A representative autoradiogram of PT-catalyzed [32P]ADP-ribosylation (ADPR) in hippocampal membranes is shown in Figure 3A. A two-way ANOVA revealed a highly significant interaction between chronic lithium and inositol treatment with respect to PT-catalyzed [32P]ADPR in the hippocampus ($F_{1,28} = 9.40$; $p < .004$). The lithium-treated group exhibited a significantly higher PT-catalyzed [32P]ADPR than

for either the control group (Con; 2,140 ± 1015; Lith, 3,694 ± 1,236 OD units; $t = 2.76$, df = 14, $p = .01$), or the myo-inositol treated group (INOS: 2,083 ± 1,037 OD units; $t = 2.86$, df = 14, $p < .01$). PT-catalyzed [32P]ADPR in the group coadministered myo-inositol and lithium was not significantly different from the control group, suggesting that myo-inositol attenuates lithium's effects. Myo-inositol administration alone was almost completely without effect on PT-catalyzed [32P]ADPR in the hippocampus (Con; 2,140 ± 1,015; Inos, 2,083 ± 1,037 OD units; $t = 0.1$, df = 14, $p = .92$; Figure 3B).

Frontal Cortex. A representative of autoradiogram of PT-catalyzed [32P]ADP-ribosylation in frontal cortical membranes is shown in Figure 4A. A two-way ANOVA also revealed a significant interaction between chronic lithium and inositol treatment with respect to PT-catalyzed [32P]ADPR in the frontal cortex ($F_{1,28} = 6.74$; $p < .05$). The lithium-treated group exhibited a significantly higher PT-catalyzed [32P]ADPR labeling than the myo-inositol–treated group (Lith, 2,865 ± 913; Inos, 1,815 ± 717 OD units; $t = 2.09$, df = 14, $p < .05$) and near-significant increases compared to the control group (Con, 1,982 ± 899 OD units; $t = 1.75$, df = 14, $p = .09$). Myo-inositol administration alone was almost completely without effect on PT-catalyzed [32P]ADPR (Con, 1,982 ± 899; Inos, 1,815 ± 717 OD units; $t = 0.33$, df = 14, $p = .74$; Figure 4B).
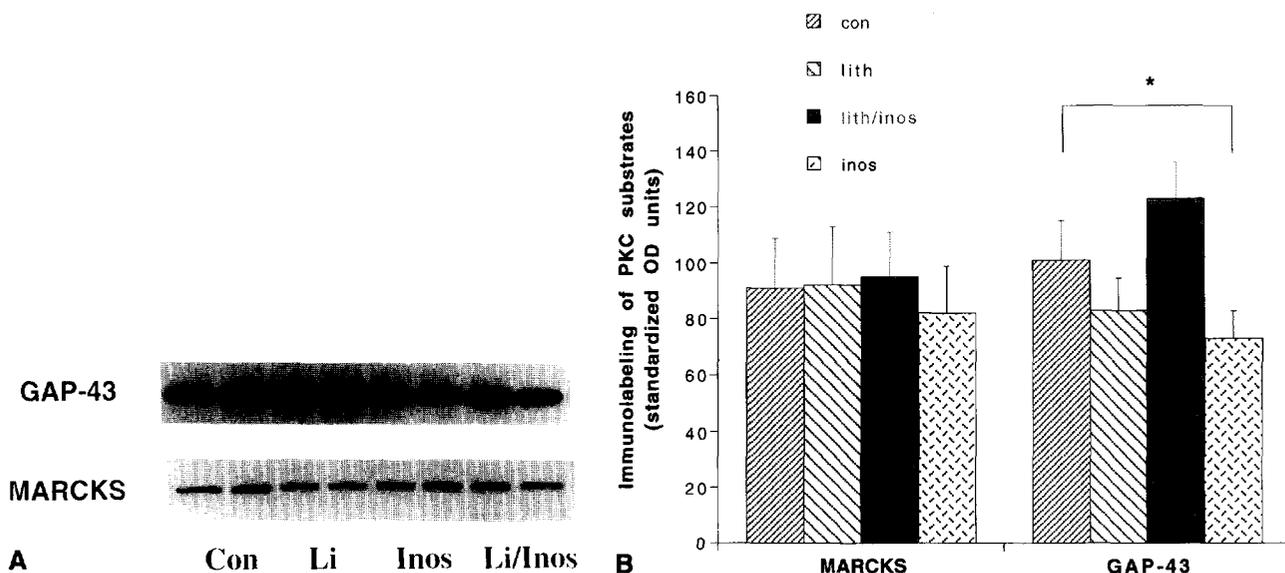
Figure 2. Effects of chronic lithium and/or myo-inositol on immunolabeling of PKC substrates in the frontal cortex. Rats were treated with lithium ± myo-inositol for 3 weeks, and cytosolic and membrane fractions from frontal cortices were prepared and subjected to SDS-PAGE on 10% polyacrylamide gels. Proteins thus resolved were electrophoretically transferred to nitrocellulose membranes; blots were incubated overnight with anti-MARCKS or anti GAP-43 antibodies. Quantitation of the immunoblots was performed by densitometric scanning of the autoradiograms, and the immunolabeling was normalized to a pooled standard. **(A)** Representative immunoblot of MARCKS and GAP-43. **(B)** Effects of chronic lithium ± myo-inositol administration on immunolabeling of MARCKS and GAP-43 (n = 8 in each group). *, Near-significant interaction between lithium and inositol on the levels of GAP-43 in the particulate fraction (two-way ANOVA; $F_{1,28}$ = 3.86; p ± .06). Li, lithium treated; Con, control; Inos, myo-inositol treated; **MARCKS**, myristoylated alanine-rich C kinase substrate; **GAP-43**, growth-associated protein.

DISCUSSION

In the present study, we have obtained partial support for the proposal that the action of chronic lithium on

PKC isozymes and substrates may stem initially from its potent effect in inhibiting the recycling of inositol; however, the effects observed with the chronic ICV administration of myo-inositol *alone* make an unequivocal
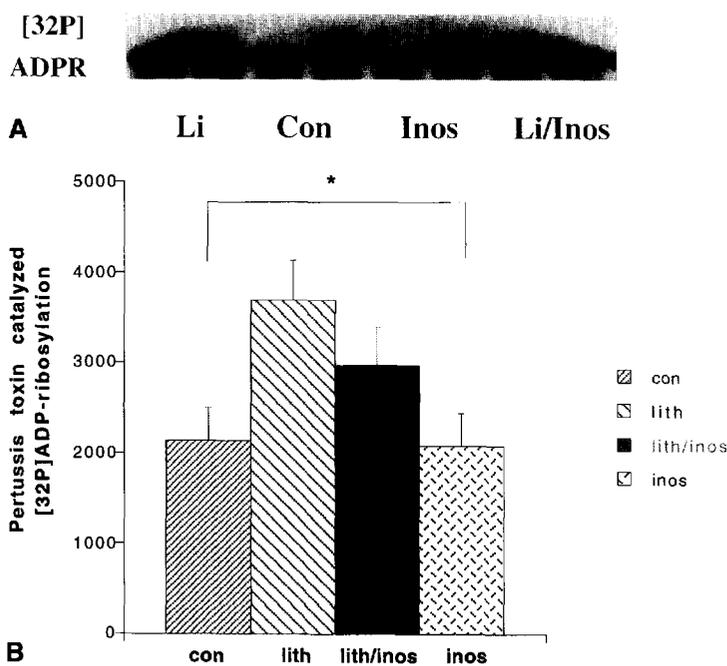
Figure 3. Effects of chronic lithium on PT-catalyzed [32P]ADP-ribosylation in the hippocampus. Rats were treated with lithium ± myo-inositol for 3 weeks, membranes were prepared from hippocampi and were subjected to PT-catalyzed labeling with 10 µM [α-32P]-NAD (25 Ci/mmol) and activated toxin. Pertussis toxin catalyzed [32P]ADP-ribosylation of all samples was performed in duplicate. Labeled membranes were washed in ice-cold buffer, proteins were solubilized and subjected to SDS-PAGE on 10% polyacrylamide gels; the gels were dried and exposed to an X-ray film with an intensifying screen at −80°C. Quantitation of [32P]incorporation was performed by densitometric scanning of the autoradiograms. **(A)**, Representative autoradiogram of PT-catalyzed [32P]ADP-ribosylation. **(B)**, Effects of chronic lithium ± myo-inositol administration on PT-catalyzed [32P]ADP-ribosylation (n = 8 in each group). *Highly significant interaction between chronic lithium and inositol treatment on PT-catalyzed [32P]ADPR in the hippocampus (two-way ANOVA; $F_{1,28}$ = 9.40; P < .004). *[32P]ADPR*, PT-catalyzed [32P]ADP-ribosylation; Li, lithium treated; Con, control; Inos, myo-inositol treated.

Figure 4. Effects of chronic lithium on PT-catalyzed [^{32}P]ADP-ribosylation in the frontal cortex. Rats were treated with lithium ± myo-inositol for 3 weeks, membranes were prepared from hippocampi and were subjected to PT-catalyzed labeling with 10 μM [α-^{32}P]-NAD (25 Ci/mmol) and activated toxin. Labeled membranes were washed in ice-cold buffer, proteins were solubilized and subjected to SDS-PAGE on 10% polyacrylamide gels; the gels were dried and exposed to X-ray film with an intensifying screen at −80°C. Quantitation of [^{32}P]incorporation was performed by densitometric scanning of the autoradiograms. **(A)**, Representative autoradiogram of PT-catalyzed [^{32}P]ADP-ribosylation. **(B)**, Effects of chronic lithium ± myo-inositol administration on PT-catalyzed [^{32}P]ADP-ribosylation ($n = 8$ in each group). * Significant interaction between chronic lithium and inositol treatment on PT-catalyzed [^{32}P]ADPR ($F_{1,28} = 6.74; p < .05$). *[^{32}P]ADPR*, PT-catalyzed [^{32}P]ADP-ribosylation; *Li*, lithium treated; *Con*, control; *Inos*, myo-inositol treated.
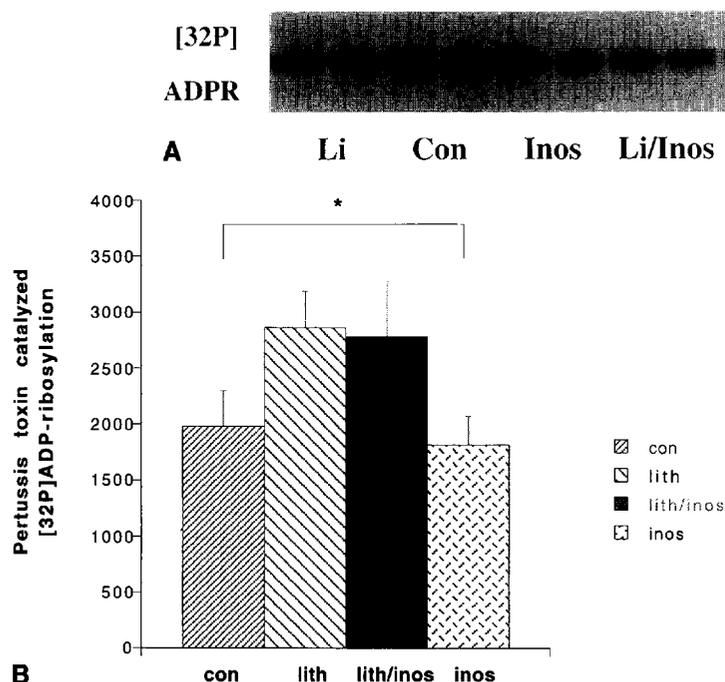
interpretation of the results difficult. Several laboratories have now determined that lithium exerts significant effects on PKC and its substrates (reviewed in Jope and Williams 1994; Manji and Lenox 1994); in the present study, we observed a significant interaction between chronic myo-inositol and lithium administration with respect to the immunolabeling of membrane associated PKC α and PKC ε in the hippocampus. It should be noted that, although our hypothesis was that myo-inositol would attenuate lithium's effects, the significant interactions that we have observed are also compatible with a lithium-induced attenuation of *myo-inositol's effects*. Similar to what we (Masana et al. 1995, under revision) and others (Jope RS, personal communication) have previously observed in ex vivo or in vitro studies, respectively, we found a significant decrease in the immunolabeling of PKC ε following chronic lithium administration. This lithium-induced decrease in the levels of membrane-associated PKC ε was attenuated by the coadministration of myo-inositol (Table 1). Although more modest, a similar pattern of effects was observed for the immunolabeling of membrane-associated PKC α. As with previous studies (Manji et al. 1993; Masana et al. 1995, under revision), lithium's effects were very selective for membrane-associated PKC α and ε, with minor alterations observed in the levels of the other isozymes (Table 1). We also observed a significant interaction between chronic lithium and myo-inositol administration on the levels of MARCKS and GAP-43, with chronic myo-inositol attenuating the lithium-induced decreases in particulate MARCKS and GAP-43 in the rat hippocampus and frontal cortex, respectively.

The mechanism(s) by which chronic lithium treatment produces the isozyme-specific effects on PKC and regulates the levels of its major substrates, MARCKS and GAP-43, remains to be fully elucidated. PKC subspecies exhibit subtle differences in their biochemical characteristics and cellular locations and also differ in their susceptibility to degradation by phorbol esters, which potently cause activation and membrane-insertion of the isozymes, thereby rendering them more susceptible to proteolytic degradation (Young et al. 1987; Kishimoto et al. 1989; Isakov et al. 1990; Borner et al. 1992). It is also noteworthy that exposure of neuroblastoma cells (Leli and Hauser 1992) or PC12 cells (Jope RS, personal communication) to 1 mM lithium in vitro produces isozyme-selective decreases in PKC α and (in the case of PC12 cells) PKC ε; these resutls are strikingly similar to those we have observed in ex vivo (that is after chronic treatment of the animals) studies (Manji et al. 1993; Masana et al. 1995, under revision). Lithium, possibly via its inhibition of inositol-1-phosphatase, increases the levels of diacylglycerol (DAG), an endogenous activator of PKC, and its metabolites (phosphatidic acid and cytidine monophosphate-phosphatidic acid) (Drummond and Raeburn 1984; Downes and Stone 1986; Godfrey et al. 1989; Brami et al. 1991; Varney et al. 1992). Because prolonged PKC activation is often followed by its proteolytic degradation (Young et al. 1987; Kishimoto et al. 1989), a prolonged increase in DAG levels by lithium may lead to an increased membrane translocation and subsequent degradation of PKC (Manji et al. 1993). Moreover, PKC activation also produces a reduction in the levels of MARCKS (Erusalimsky et al.

1991; Linder et al. 1992), suggesting that the reduction in hippocampal MARCKS observed following chronic lithium administration may also be mediated via a lithium-induced increase in DAG levels and subsequent activation of PKC isozymes; the results of the present study support such a contention.

An intriguing aspect of the study worthy of further comment is the observation that chronic administration of myo-inositol *alone* also had significant effects on PKC isozymes and substrates; the myo-inositol effects were generally similar to those observed with lithium administration alone, but rather than being additive, coadministration of lithium and myo-inositol *attenuated* lithium's effects. Interestingly, a somewhat analogous interaction between lithium and inositol has been observed in behavioral studies (Kofman et al. 1991); thus these investigators found that, although inositol alone produced a nonsignificant decrease in rodent rearing behavior, it markedly attenuated the lithium-induced decreases in rearing. Recently, oral inositol has been reported to have antidepressant effects (Levine et al. 1995) and antipanic effects in humans. It may seem somewhat paradoxical that lithium effects are reversible with inositol, and inositol itself has psychoactive effects and biochemical effects in the same direction. However, inositol may have effects at several points in the PI cycle; thus, it can be a substrate for PI synthase and conceivably increase PI levels when PI synthase is not fully saturated. It may inhibit phospholipase C or regulate it in a complex fashion and thus modulate second-messenger function. In addition, the literature on the use of inositol to enhance surfactant synthesis in neonates with respiratory distress syndrome suggests that inositol can shift the ratio of phosphatidylcholine and phosphatidylinositol in membranes. Recent studies by Eichberg and co-workers (Zhu and Eichberg 1990; Eichberg et al., 1993) also have provided evidence that a specific pool of myo-inositol used for PI synthesis is depleted in sciatic nerves of diabetic rats; interestingly, in such model systems, there is a decrease in PKC activity, and most important for the present study, PKC activity is increased in response to exogenously applied myo-inositol, possibly because of an increase in PI-derived DAG levels (Kim et al. 1991; discussed in Eichberg et al. 1993).

Although the results of the present study as well as the studies outlined suggest that chronic myo-inositol has significant effects on PKC, the precise mechanisms underlying these effects remain to be elucidated. One potential mechanism is the "driving" of the PI cycle and the generation of DAG by high concentrations of exogenously applied myo-inositol. DAG is metabolized via phosphatidic acid to the liponucleotide CDP-diacylglycerol (CDP-DAG), which in the presence of inositol is converted to PI in a reaction catalyzed by PI synthase (Agranoff et al. 1958). The latter enzyme is reported to have a relatively low affinity for inositol (Benjamins

and Agranoff 1969; Ghalayani and Eichberg 1985), and thus under conditions where inositol concentrations are reduced, PI synthesis may be compromised. There is at present, however, no evidence to suggest that PI synthase is not saturated with myo-inositol under "basal" conditions (that is, conditions in which PI synthesis has not been compromised by the administration of a drug-like lithium), making it difficult to reconcile the proposal that myo-inositol can drive PI turnover under such conditions. It remains possible, however, that inositol concentrations are lower in biochemically and behaviorally relevant brain compartment (e.g., in neurons) or alternatively that there is a PI-synthase enzyme that has a higher K_m for inositol (Imai and Gershengorn 1987). Indeed, it has been proposed that much of the variation seen in different experimental preparations for the study of brain lithium effects may be explained on the basis of differences in inositol content (Sherman et al. 1985; Stubbs and Agranoff 1993).

An additional hypothesis is suggested by the fact that myo-inositol is an endogenous organic osmolyte and a very hyperosmotic solution of inositol was used chronically in this study. The possibility that at least some of the effects of myo-inositol administration alone may be mediated by its osmolytic effects is suggested by a recent study that compared the effects of epi-inositol (an isomer that cannot be used for phosphoinositide synthesis) on the lithium/pilocarpine seizure paradigm (Williams and Jope 1995); however, because two other isomers of inositol, namely, chiro (Kofman et al. 1993) or scyllo (Tricklebank et al. 1991) are ineffective in this model, the effects of epi-inositol do not appear simply to represent nonspecific osmotic effects. It is thus clear that further studies are necessary to elucidate the mechanim(s) by which myo-inositol produces its various biological effects.

In the present study, similar to what we have previously observed (Masana et al. 1995, under revision), we found significantly higher levels of PT-catalyzed [^{32}P]ADP-ribosylation in brain membranes prepared from rats chronically treated with lithium. Because pertussis toxin selectively ADP-ribosylates the undissociated, inactive $\alpha\beta\gamma$ heterotrimeric form of G_i, it has been postulated that lithium inactivates G_i via a stabilization of the inactive conformation (Hsiao et al. 1992; Masana et al. 1995, under revision). Interestingly, in the present study, there was a highly significant interaction between chronic lithium and inositol treatment with respect to PT-catalyzed [^{32}P]ADPR in the hippocampus, with myo-inositol markedly attenuating lithium's effects. At present, the molecular mechanism(s) underlying lithium's effects on G proteins remains to be fully established. There is some evidence to suggest that lithium has acute in vitro effects on G proteins (Avissar et al. 1988; Greenwood and Jope 1994), but many of chronic lithium's effects persist after washing of the membranes and are there-

fore likely attributable to an indirect posttranslational modification of the G proteins (Jope and Williams 1994; Manji et al. 1995). Because G protein function is known to be regulated by phosphorylation (Houslay 1991) and considerable evidence has shown that lithium affects PKC, one potential mechanistic explanation for lithium's effects on G protein subunit dissociation is phosphorylation by PKC. The results of the present study, in which coadministration of myo-inositol attenuated lithium-induced increases in PT-catalyzed [^{32}P]ADP labeling provides indirect support for a role of PKC. It should be noted, however, that in contrast to the alterations of PKC isozymes observed with chronic myo-inositol alone, no such alterations in PT-catalyzed [^{32}P]ADP labeling were observed with chronic myo-inositol administration, precluding a simple unequivocal interpretation of the results.

In conclusion, we have shown that chronic lithium treatment produces complex effects on PKC isozymes and on the conformational state of G_i/G_o, and that these effects are attenuated by the coadministration of myo-inositol. The precise mechanism(s) underlying these effects remains to be fully elucidated; given the critical role of PKC isozymes and G proteins in modulating intracellular cross-talk between neurotransmitter systems, and thereby the integrative functions of the CNS, these effects may play a major role in lithium's mood-stabilizing effects. Thus, future studies using the bisphosphonate inhibitors of inositol monophosphatases (Atack et al. 1993, 1994) are warranted, because they may delineate the role of inositol depletion on lithium's complex long-term effects and offer the hope for the development of more potent and more rapidly acting mood-stabilizing drugs.

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