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Concurrent measures of protein kinase *C* and phosphoinositides in lithium-treated bipolar patients and healthy individuals: a preliminary study

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Abstract

This study examined the hypothesis that lithium inhibits the PI signaling pathway in humans during in vivo administration by concurrently measuring PKC isozymes and platelet membrane phosphoinositides in lithium-treated patients and healthy individuals. The platelet membrane and cytosolic levels of PKC alpha, beta I, beta II, delta, and epsilon were measured using Western blotting. The relative platelet membrane contents of phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4,5-bisphosphate (PIP_2) were measured with two-dimensional thin-layer chromatography. Nine euthymic lithium-treated bipolar subjects and 11 healthy control subjects were studied. Compared to control subjects, lithium-treated bipolar patients had significantly lower levels of cytosolic PKC alpha isozyme (t-test $= -3.24$, d.f. $= 17$, $P = 0.01$) and PIP_2 platelet membrane levels (t-test $= -2.51$, d.f. $= 18$, $P = 0.02$), and a trend toward reduced levels of cytosolic PKC beta II isozyme ($t = -2.17$, d.f. $= 17$, $P = 0.05$). There was no significant correlation between PIP_2 and any of the PKC isozymes. These preliminary findings suggest that chronic lithium treatment may decrease the levels of both cytosolic PKC alpha isozyme and membrane PIP_2 in platelets of bipolar disorder patients.

Keywords: Lithium; Phosphoinositides; Phosphatidylinositol-4,5-bisphosphate; Protein kinase C; Bipolar disorder; Psychopharmacology; Signal transduction

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1. Introduction

Abnormalities in intracellular signal transduction processes may be present in bipolar disorder subjects (Lachman and Papolos, 1989; Wachtel, 1989, 1990; Schreiber and Avissar, 1991; Hudson et al., 1993; Manji et al., 1995; Hahn and Friedman, 1999; Perez et al., 2000; Soares and Mallinger, 2000). In particular, the phosphatidylinositol (PI) pathway has been suggested as a possible site of dysfunction (Baraban et al., 1989; Snyder, 1992; Brown et al., 1993; Jope et al., 1996; Soares and Mallinger, 1996, 1997; Soares et al., 1999). Lithium and other medications effective in this disorder have been hypothesized to exert their effects by modulating these intracellular processes (Baraban et al., 1989; Manji et al., 1993, 1995, 1996a; Lenox and Watson, 1994; Bitran et al., 1995; Soares et al., 2000).

A hyperactivity of the PI pathway could be reflected by increased levels of phosphatidylinositol-4,5-bisphosphate (PIP_2), which is the major substrate for production of inositol trisphosphate (IP_3) and diacylglycerol (DAG). This could possibly result in increased activity of specific PKC isozymes (Soares and Mallinger, 1997). One preliminary report showed increased platelet membrane PIP_2 values in bipolar patients in the manic state (Brown et al., 1993), and subsequent reports suggested that lithium treatment at therapeutic concentrations may significantly decrease platelet membrane PIP_2 levels (Soares et al., 1997, 1999, 2000). Similarly, increased platelet PKC activity was found in drug-free bipolar disorder subjects in the manic state compared to healthy control subjects, and lithium treatment at therapeutic doses reduced its activity (Friedman et al., 1993). In postmortem brains of bipolar subjects, an altered subcellular distribution of PKC isozymes, compatible with increased activation of PKC, was also reported (Wang and Friedman, 1996). These findings are in general agreement with in vitro data showing that lithium treatment inhibits the PI pathway and interferes with PKC-mediated processes (Baraban et al., 1989; Wang and Friedman, 1989; Manji et al., 1993, 1995, 1996a; Lenox et al., 1992; Lenox and Watson, 1994; Manji and Lenox, 1994; Belmaker et al., 1995; Bitran et al., 1995; Lenox et al., 1998).

The present study was conducted by concurrently measuring PKC isozymes and PIP_2 in platelets of lithium-treated bipolar patients and healthy individuals, in order to further examine the hypothesis of inhibition of these signal transduction processes by lithium. We hypothesized that the levels of PKC alpha and PIP_2 would be significantly lower in lithium-treated patients as compared to healthy control subjects. In an exploratory fashion, we also examined cross-sectionally the possible existence of a correlation between platelet PKC isozymes and PIP_2 levels in lithium-treated subjects and healthy individuals.

2. Method

2.1. Subjects

Nine bipolar disorder type I patients (4 males, 5 females; mean $\pm$ S.D. age = 42.0 ± 8.2 years) were studied. They did not have any current medical problems, no psychiatric comorbidity, and no significant history of substance use. All patients were euthymic, and had been on lithium treatment for at least 1 month. Patients were on lithium doses ranging from 900 to 1650 mg/day (mean dose = 1200 mg/day), resulting in 12-h serum levels from 0.32 to 0.94 mEq/l (mean $\pm$ S.D. = 0.60 ± 0.19 mEq/l). All patients were being treated with lithium as monotherapy, and had not been on any other medications for at least 2 weeks, with the exception of one patient who received a single dose of over-the-counter ibuprofen (or NSAID) 1 day before the study.

Eleven healthy control subjects were studied (4 males, 7 females; mean age $\pm$ S.D. = 33.6 ± 8.5 years); they did not have any history of psychiatric disorders, and had no history of significant substance use or current medical problems. Psychiatric disorders were excluded by having the control subjects undergo a SCID-IV interview, nonpatient version. They did not have any history of psychiatric disorders in first-degree relatives.

2.2. Clinical procedures

Patients were followed through the Depression

and Manic Depression Prevention Program (DMDPP), an outpatient research program at the Western Psychiatric Institute and Clinic, University of Pittsburgh Medical Center. All patients met DSM-IV diagnostic criteria for bipolar disorder, type I, as determined by a SCID interview (Spitzer et al., 1994), and confirmed in a clinical evaluation conducted by an attending psychiatrist. The diagnosis was subsequently reviewed in a consensus meeting with the clinician who completed the SCID, the clinic research coordinator, and a senior investigator. After complete description of the study to the subjects, written consent was obtained. A blood sample was obtained from a peripheral vein for conduct of platelet PKC isozyme and phosphoinositide determinations. Blood samples were obtained in the time interval between 10.00 and 13.00 h. Patients were asked to sit on a chair for 15 min, and thereafter the blood samples were collected. Blood samples were collected with a 21-gauge butterfly needle into plastic syringes.

2.3. PKC assays

Blood samples (2×25 ml) were collected into 60-ml plastic syringes containing 3 ml of acid citrate dextrose anticoagulant (Sigma Chemical Company, USA). The platelet membrane preparations were obtained immediately after blood collection, and stored at -80°C. Homogenates were centrifuged at $14\,000 \times g$ for 10 min to remove undissolved debris. The linearity of the protein concentration for immunoblotting of PKC isozymes was ascertained by resolution of selected concentrations of protein. Subsequent PKC isozyme immunoblotting was performed using protein concentrations demonstrated to be within the linear range for Western blotting using previously described methods (Molchan et al., 1993; Chen et al., 1998). In brief, samples were subjected to SDS-PAGE on 10% polyacrylamide gels. Proteins thus resolved were then electrophoretically transferred to nitrocellulose membranes. Non-specific binding in the nitrocellulose membranes was blocked with low detergent 'blotto' which contained 50 mM Tris (pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 5% non-fat dry milk, 0.2% NP-40, and 0.02% NaN_3). The blots were incubated overnight at room temperature with anti-PKC isozyme antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:100); five PKC isozymes (alpha, beta I, beta II, delta, and epsilon) were measured in the cytosol and membrane fractions. The immunocomplexes were detected with an Amersham ECL or ECL plus kit, and quantitation was done with densitometric scanning using an Image Analysis system (NIH 1.55). An aliquot of pooled 'standard' platelet membrane or cytosol fraction was run on one lane of each gel, to allow normalization of the data against the pooled standards, in order to minimize variability between blots. The final PKC isozyme values are expressed as normalized optical density units.

2.4. Phosphoinositide assays

Platelet membrane phosphoinositides were measured as previously reported (Mallinger et al., 1993). Blood samples (2×34 ml) were collected with plastic syringes containing 6 ml of acid citrate dextrose anticoagulant each (Sigma Chemical Company, USA). The platelet membrane preparations were obtained immediately after blood collection, and stored at -80°C. Subsequently, the platelet membrane suspensions were thawed at room temperature, and a biphasic lipid extraction was performed. The membrane phospholipids were separated using two-dimensional thin-layer chromatography on high performance plates, and quantitated by scanning laser densitometry. The phospholipid spots were outlined and quantitated with the GelScan XL 2400 software (version 2.1, Pharmacia LKB Biotechnology), by a research assistant who was blind to sample identity. The values were expressed as absorbance units times area ($AU \times mm^2$), and the relative absorbances for each individual phosphoinositide were calculated as a percentage of the total phospholipid absorbance.

2.5. Statistical analysis

The statistical analysis was performed with the software SPSS for Windows, version 7.5 (SPSS,

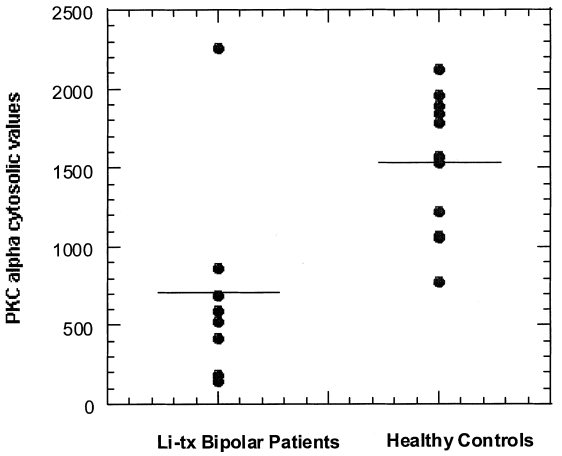

Fig. 1. The figure presents platelet values of cytosolic protein kinase C (PKC) alpha isozyme in lithium-treated euthymic bipolar patients and healthy individuals. The values were significantly reduced in patients (t-test = −3.24, d.f. = 17; P = 0.01).

Inc.). Individual t-tests were performed for each dependent variable. Pearson correlation coefficients were calculated to examine the existence of correlation among the PKC isozymes, phosphoinositides, and clinical variables.

3. Results

Lithium-treated bipolar patients had significantly lower PKC alpha values in cytosol compared to healthy control subjects (mean ± S.D. = 708 ± 670 and 1529 ± 438, median = 556 and 1568, respectively; t-test = −3.24, d.f. = 17, P = 0.01) (see Fig. 1). There was also a trend for lower cytosolic PKC beta II isozyme (mean ± S.D. = 1578 ± 1468 and 2711 ± 798, median = 1397 and 3023, respectively; t-test = −2.17, d.f. = 17, P = 0.05). There were no statistically significant differences between patients and control subjects for the other PKC isozymes measured (Table 1). Fig. 2 contains a representative PKC immunoblot from a lithium-treated patient and a healthy control subject, which illustrates our method.

Bipolar patients had significantly reduced levels of PIP_2 compared to healthy control subjects (0.3 ± 0.1% and 0.4 ± 0.1%, respectively; t-test = −2.51, d.f. = 18, P = 0.02), but there were no statistically significant differences between patients and control subjects for PI (4.9 ± 0.8% and 5.2 ± 1.7%, respectively; t = −0.38, d.f. = 18, P = 0.71) or PIP (0.6 ± 0.2% and 0.7 ± 0.2%, respectively; t = −0.86, d.f. = 18, P = 0.40) levels. The phosphoinositide data which are included in

Table 1
Protein kinase C isozymes in lithium-treated bipolar patients and healthy individuals[a]

	Bipolar patients (n = 9) (mean ± S.D.; median)	Healthy control subjects (n = 11) (mean ± S.D.; median)	Statistical analysis (t-tests)
Alpha cytosolic	708 ± 670; 556	1529 ± 438; 1568	t = −3.24, d.f. = 17, P = 0.01*
Alpha membrane	1127 ± 463; 1191	1145 ± 663; 943	t = −0.07, d.f. = 17, P = 0.95
Beta I cytosolic	1942 ± 1999; 1498	3043 ± 888; 3117	t = −1.63, d.f. = 17, P = 0.12
Beta I membrane	1683 ± 1025; 1639	1626 ± 1027; 1281	t = 0.12, d.f. = 17, P = 0.91
Beta II cytosolic	1578 ± 1468; 1397	2711 ± 798; 3023	t = −2.17, d.f. = 17, P = 0.05
Beta II membrane	2358 ± 1053; 2100	2389 ± 831; 2395	t = −0.07, d.f. = 17, P = 0.94
Delta cytosolic	1220 ± 2359; 380	1432 ± 1117; 938	t = −0.26, d.f. = 17, P = 0.80
Delta membrane	2598 ± 1682; 2664	2417 ± 1081; 2660	t = 0.29, d.f. = 17, P = 0.78
Epsilon cytosolic	1070 ± 862; 952	1575 ± 966; 1442	t = −1.18, d.f. = 17, P = 0.26
Epsilon membrane	1812 ± 1109; 2146	1566 ± 1622; 1039	t = 0.37, d.f. = 17, P = 0.72

[a] *Notes.* Cytosolic, cytosolic fraction; membrane, membrane fraction. The PKC isozyme values are expressed as normalized optical density units.

*Statistically significant as per defined significance level of $P < 0.05$. Statistical analysis done with t-tests. No Bonferroni correction was applied for the variable cytosolic PKC alpha, as a reduction in PKC alpha was part of the primary study hypothesis, based on findings from previous studies.

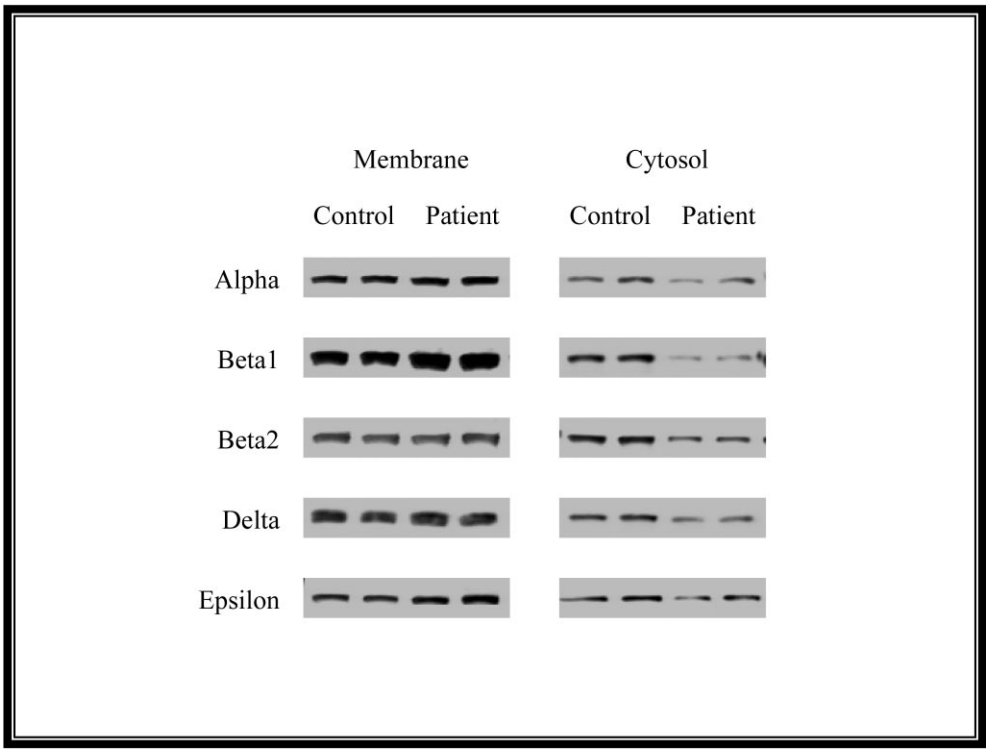

Fig. 2. This figure presents a representative immunoblot with the measured protein kinase C (PKC) isozymes in one lithium-treated patient and one healthy control subject. It illustrates our main finding of reduced cytosolic alpha PKC fraction in lithium-treated individuals.

this report originate from a sample of bipolar patients and healthy control subjects that largely overlaps with the sample previously reported in another report (Soares et al., 1999), but it is also included here for the purpose of examining possible correlations with the PKC data, which are the focus of the present report.

We have redone all *t*-test comparisons after log transformation of the data, to compare the PKC isozymes and phosphoinositide platelet values between lithium-treated patients and healthy controls. The results of the analyses were very similar, and the P value for cytosolic PKC alpha was 0.01, and for PIP_2 was 0.02. The P value for cytosolic PKC beta II was 0.05, and that for cytosolic PKC beta I was 0.05.

There was no significant correlation among any of the PKC isozymes and the phosphoinositide platelet measures in either bipolar patients or healthy individuals (Pearson correlation coefficients, $P > 0.05$). Of interest, cytosolic PKC alpha and PIP_2 were not significantly correlated either in lithium-treated patients ($n = 8$; Pearson correlation coefficient $= -0.610$; $P = 0.108$) or in healthy control subjects ($n = 11$; Pearson correlation coefficient $= -0.352$; $P = 0.289$) (see Fig. 3). Age was not significantly correlated with any of the phosphoinositide or PKC isozymes measured in either patients or healthy control subjects (Pearson correlation coefficients, $P > 0.05$).

The 12-h serum lithium levels were not significantly correlated with any of the PKC isozymes or phosphoinositide measures (Pearson correlation coefficients, $P > 0.05$). This analysis was repeated after excluding three subjects who had 12-h lithium levels in the sub-therapeutic range, which for the purpose of this analysis was considered to be < 0.5 mEq/l, and there was still no significant

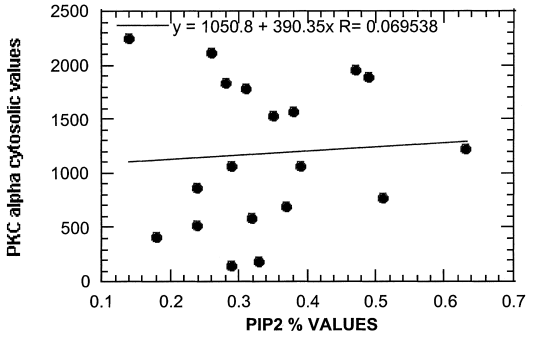

Fig. 3. The figure presents the plotted values of platelet cytosolic protein kinase C (PKC) alpha isozyme and phosphatidylinositol-4,5-bisphosphate (PIP_2) in a sample of lithium-treated euthymic bipolar patients and healthy individuals. No significant correlation was found in the total sample ($n = 19$; Pearson correlation coefficient = 0.070; $P = 0.777$), or in the patient ($n = 8$; Pearson correlation coefficient = -0.610; $P = 0.108$) or control ($n = 11$; Pearson correlation coefficient = -0.352; $P = 0.289$) groups.

correlation. Three of the nine bipolar patients had been on lithium for less than 3 months at the time their blood samples were obtained (all were on lithium for at least 1 month); all others had been on lithium for at least several months or years. The average $\pm$ S.D. length of time on lithium for the patient sample was 130 ± 92 weeks, median = 167 weeks. There was no significant correlation between length of time on lithium and PKC isozymes or phosphoinositide values (Pearson correlation coefficients, $P > 0.05$).

4. Discussion

Our preliminary findings are, to our knowledge, the first report of concurrent determinations of PKC isozymes and phosphoinositide levels in platelets of bipolar patients and healthy individuals. We have found reduced platelet levels of cytosolic PKC alpha and membrane PIP_2 in lithium-treated bipolar subjects compared to normal control subjects, suggesting that lithium treatment may significantly decrease the levels of both of these intermediates in vivo in bipolar patients, and more generally, may reduce the activity of the inositol phospholid signal transduction system.

The findings of reduced platelet PKC alpha in the cytosolic fraction in lithium-treated bipolar patients are consistent with a previous report that found a reduction in PKC platelet activity in bipolar patients after 2 weeks of lithium treatment (Friedman et al., 1993). These findings are also in agreement with preclinical rodent and cell culture studies, which have demonstrated that lithium exerts significant effects on PKC and its substrates (Zatz and Reisine, 1985; Reisine and Zatz, 1987; Wang and Friedman, 1989; Casebolt and Jope, 1991; Lenox et al., 1992; Manji et al., 1993; Watson and Lenox, 1996; Jensen and Mork, 1997; Jope, 1999; Manji and Lenox, 1999). In contrast to many of the previous rodent and cell culture studies, our present study found that the lithium-treated bipolar patients exhibited lower levels of PKC alpha in the platelet cytosolic fractions only. This may reflect potential baseline differences in cytosolic/membrane PKC isozyme subcellular localization in bipolar patients, the non-nucleated nature of platelets compared to other tissues investigated, or the combination thereof. The potential differences in lithium's effects in platelets vs. brain neurons should be further investigated in future studies.

In this preliminary study we also found a non-statistically significant trend toward reduced platelet levels of the cytosolic PKC beta I and beta II fractions in lithium-treated individuals. These are also not in agreement with prior brain findings, as reviewed above, and should be further examined in larger patient samples.

The PIP_2 values were significantly reduced in lithium-treated bipolar patients compared to healthy individuals, as previously reported (Soares et al., 1999). Six of the 10 bipolar patients and 10 of the 11 healthy individuals included in that previous report are among the subjects who have been included in this new study. The phosphoinositide data on those subjects are also included here in order to examine, in an exploratory fashion, possible PKC and PIP_2 correlations. Decreased PIP_2 platelet levels could be consistent with in vitro and in vivo animal literature showing

significant effects of lithium treatment in the PI pathway (Baraban et al., 1989; Snyder, 1992; Lenox and Watson, 1994; Manji et al., 1995, 1996b; Lenox et al., 1998); however, they are not in agreement with animal studies which failed to demonstrate specific effects of lithium on PIP_2 in rodent brains (Sherman et al., 1985, 1986; Ishima et al., 1993). The reduction in PIP_2 levels in platelets of euthymic lithium-treated bipolar patients compared to healthy control subjects reported here may reflect the effects of lithium treatment. The cross-sectional design utilized in this study does not allow us to establish that with certainty; however, this would be in agreement with a related follow-up study involving seven drug-free bipolar patients studied while unmedicated, and again after at least 3 subsequent weeks of lithium treatment, during which the post-lithium PIP_2 values were significantly reduced (Soares et al., 2000). These findings would also be in agreement with recent MRS findings suggesting that lithium treatment decreases the myo-inositol concentrations in the frontal lobe of bipolar patients (Moore et al., 1999). These results also follow the preliminary demonstration of the antimanic effects of a PKC inhibitor (tamoxifen) (Bebchuk et al., 2000), and add to the growing body of data implicating the PKC signaling pathway in the treatment of bipolar disorder (Manji and Lenox, 1999). Nonetheless, these data should be clearly regarded as preliminary at this point, and replication in larger patient samples is warranted.

The lack of a significant correlation between the platelet levels of PKC isozymes and phosphoinositides, if further confirmed, would be consistent with the fact that there is extensive cross-regulation between these related signal transduction pathways (as reviewed in Manji et al., 1995). Alternatively, it could eventually be consistent with the hypothesis of these being independent steps in lithium's mechanism of action. However, a definitive answer to this question should come from longitudinal studies involving larger patient samples that specifically examine lithium-induced changes in a pre- and post-lithium treatment design.

Increased platelet membrane PKC activity in the manic state has been previously reported (Friedman et al., 1993, 1995). If present, it could reflect a DAG action from hyperactivation of the PI pathway in bipolar disorder (Soares and Mallinger, 1997). Increased activity of the PI pathway in the manic state may also be reflected by findings of increased membrane content of PIP_2, the main precursors for IP_3 and DAG production, in drug-free patients (Brown et al., 1993). Unmedicated bipolar patients in various mood states should be studied in order to clarify the role of mood state in eventual PIP_2 or PKC abnormalities, and to determine whether any existing abnormalities are trait or state findings associated with this disorder. These longitudinal studies would also help clarify whether reduced levels of platelet PIP_2 or cytosolic PKC alpha are a result of lithium treatment, or whether they also reflect mood normalization. In a recent report involving unipolar depressed subjects, Pandey et al. (1998) found evidence of increased formation of PKC in platelets of drug-free patients compared to healthy individuals, using a method with [^{3}H]phorbol dibutyrate binding, suggesting PKC abnormalities in the depressed state. However, another recent report (Young et al., 1999) involving unmedicated bipolar subjects, mostly in the depressed state, and also a group of euthymic bipolar individuals who had been on lithium for over 6 months did not find significant differences in platelet PKC alpha levels compared to healthy control subjects. Future studies should further attempt to clarify these conflicting findings.

A potential limitation of our current study is that the patient group was older than the control subjects (42.0 ± 8.2 vs. 33.6 ± 8.5 years old, respectively). However, no significant correlation was found between age and PKC isozymes or phosphoinositides. Also, one of our bipolar subjects received a single dose of ibuprofen on the day before the study. This is a potential confounding factor, as non-steroidal anti-inflammatory agents have known effects on platelet function. Nonetheless, the data on this individual were in a range consistent with the other individuals in this group, and we elected to maintain this subject in the data set. We repeated the analysis without that subject, and the significant results

for reduction of PIP_2 and cytosolic PKC alpha in bipolar patients remained unchanged.

An important potential limitation is the fact that these studies used a platelet model, and it is not known to what extent platelets reflect the actual in vivo brain processes. Platelets have been used as a model in a number of human neuropharmacology studies, given the lack of direct means to study signal transduction processes in the human brain in vivo (Rotman, 1983; Stahl, 1985; Pletscher, 1986). A major consideration in such studies is, of course, the question of whether or not the peripheral circulating cells are an adequate model system for the CNS biochemical pathways under investigation. A recent study therefore investigated the correlation between lithium-induced changes in PKC alpha in rodent frontal cortex and platelets in the same animal. Consistent with findings in cultured cells and brain (vide supra), chronic lithium administration markedly reduced the levels of membrane-associated PKC alpha in frontal cortex and in platelets (Manji and Lenox, 1999). Furthermore, there was a strong correlation between lithium-induced changes in rat platelet membrane PKC alpha and rat frontal cortical membrane PKC alpha. These results suggest that lithium-induced changes in PKC alpha in the platelet may provide meaningful information about similar changes in the brain. However, future approaches that allow direct measures of signal transduction processes in the in vivo human brain should be developed, in order to further investigate these preliminary findings.

As our study involved a relatively small sample of subjects, the results reported here should be viewed as preliminary. Future studies in larger samples of bipolar disorder patients should attempt to replicate these findings, and further characterize the effects and time course of lithium's action in PIP_2 and PKC-mediated processes in bipolar disorder patients, as well as investigate a possible relationship with therapeutic response.

In conclusion, the study of signal transduction mechanisms in bipolar disorder patients may provide a better understanding of the pathophysiology of the disorder and the mechanisms of action of lithium. These approaches may potentially result in new pharmacological interventions for this severe disorder.

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