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Decreased Protein Kinase C (PKC) in Platelets of Pediatric Bipolar Patients: Effect of Treatment with Mood Stabilizing Drugs

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Abstract

Pediatric bipolar disorder (PBD) is a major public health concern, however, its neurobiology is poorly understood. We therefore studied the role of protein kinase C (PKC) in the pathophysiology of bipolar illness.

We determined PKC activity and immunolabeling of various PKC isozymes (i.e., PKC α , PKC β I, PKC β II, and PKC δ) in the cytosol and membrane fractions of platelets obtained from PBD patients and normal control subjects. PKC activity and PKC isozymes were also determined after 8 weeks of pharmacotherapy of PBD patients (n = 16) with mood stabilizers.

PKC activity and the protein expression of PKC β I and β II, but not PKC α or PKC δ , were significantly decreased in both membrane as well as cytosol fractions of platelets obtained from medication-free PBD patients compared with normal control subjects. Eight weeks of pharmacotherapy resulted in significantly increased PKC activity but no significant changes in any of the PKC isozymes in PBD patients.

These results indicate that decreases of specific PKC isozymes and decreased PKC activity may be associated with the pathophysiology of PBD and that pharmacotherapy with mood stabilizing drugs results in an increase and normalization of PKC activity along with improvement in clinical symptoms.

Keywords

Pediatric bipolar disorder; platelets; PKC isozymes; PKC activity; lithium; mood stabilizing drugs

1. Introduction

Pediatric bipolar disorder (PBD) is a major public health concern, with poor recovery between episodes and a high relapse rate (Geller et al., 2004). Effective treatments are limited: there is less than 50% response to monotherapy with mood stabilizers (Kafantaris et al., 2001a, b; Kowatch et al., 2000; Pavuluri et al., 2005). Understanding of the molecular neurobiology of PBD will augment our knowledge of its pathophysiology. This will lead to new knowledge about the therapeutic mechanisms of existing medications, such as lithium, and pave the way for new drug discoveries that can potentially affect or alter the intracellular aberrations and

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influence the signaling cascades, including enzymes, second messenger systems, and gene proteins (Manji et al., 1999).

Studies of patients with adult bipolar disorder (ABD) and of postmortem brain samples obtained from adult bipolar subjects indicate abnormalities of the phosphoinositide (PI) signaling system (Bezchlibnyk & Young, 2002; Jope et al., 1996; Pacheco & Jope, 1996;). In the PI signaling system, the interaction of an agonist with receptors, such as serotonin (5HT)_{2A} or 5HT_{2C}, causes the hydrolysis of the substrate phosphatidyl inositol 4,5-bisphosphate (PIP₂) by the enzyme phospholipase C (PLC), resulting in the formation of two second messengers known as inositol 1,4,5-tris phosphate (IP₃) and diacylglycerol (DAG) (Berridge & Irvine, 1989; Tanaka & Nishizuka, 1994). IP₃ stimulates the release of intracellular calcium from intracellular stores (Nishizuka, 1992), and DAG stimulates the enzyme protein kinase C (PKC), which is one of the major intracellular mediators of signals generated by the stimulation of cell surface receptors (Tanaka & Nishizuka, 1994). That abnormalities of the PI signaling system may be involved in bipolar illness is derived from both direct and indirect evidence, such as that lithium, an effective mood stabilizing drug, interferes with the PI signaling system by inhibiting the enzyme inositol monophosphatase, which converts IP₃ to IP₂, thus increasing the availability of the second messenger IP₃ in bipolar subjects (Allison & Stewart, 1971; Hallcher & Sherman, 1980).

As mentioned earlier, PKC is activated by the second messenger DAG and is an important component of the PI signaling system. PKC is involved in the phosphorylation of specific proteins, such as myristoylated alanine rich C kinase substrate (MARCKS), that perform important cellular functions (Aderem, 1992; Allen & Aderem, 1995; Nestler & Greengard, 1994; Nishizuka, et al., 1991). It is a key regulatory enzyme present in various tissues, including brain and platelets (Nishizuka, 1988; Shearman et al., 1989). It has been shown that PKC is a family of at least 12 structurally related isozymes (Casabona, 1997; Tanaka & Nishizuka, 1994). On the basis of molecular structure enzyme characterization, the PKC family has been subgrouped into three classes, known as conventional isozymes (α , β I, β II, γ), novel PKC isozymes (δ , ϵ , η , θ , μ), and atypical isozymes (ζ , λ , ι). PKC is involved in the modulation of many neuronal and cellular functions, such as neurotransmitter synthesis and release, regulation of receptors and ion channels, neuronal excitability, gene expression, and secretion and cell proliferation (Berridge & Irvine, 1989; Nestler & Greengard, 1994; Nishizuka, 1988). Both direct and indirect evidence suggests that PKC may play a crucial role in the pathophysiology of bipolar disorders.

Friedman et al. (1993) studied PKC activity in platelets of adult bipolar patients, before and after lithium therapy. They found an increase in the ratio of membrane-bound to cytosolic PKC activities, which was attenuated by lithium treatment. More recently, we have reported that PKC activity, as well as the expression of specific PKC isozymes, is significantly decreased in the platelets of ABD patients (Pandey et al., 2002). The other evidence that PKC may be involved in the pathophysiology of bipolar disorder is derived from the observation that lithium treatment causes a decrease in PKC activity in the rat brain (Wang et al., 2001). Taken together, these studies do suggest the involvement of PKC not only in the pathophysiology of bipolar disorder but also in the therapeutic mechanism of action of lithium and other mood stabilizing drugs.

While the neurobiological abnormalities associated with ABD are beginning to unravel, the neurobiological abnormalities of PBD are poorly understood. Given that early intervention is critical and developmental neurobiological changes in PBD can be potentially different from the ABD, it is imperative to study PBD. Therefore, we studied PKC activity and the protein expression of specific PKC isozymes in the platelets of PBD patients pre- and post-treatment with mood stabilizing drugs to examine if abnormalities of any specific PKC isozymes are

associated with the pathophysiology of bipolar illness and if treatment with mood stabilizing drugs would alter or normalize these abnormalities in PBD patients.

2. Methods

2.1. Subjects

This protocol was approved by the Institutional Review Board (IRB) of the University of Illinois at Chicago. The matched normal control subjects (n=23) were recruited through the research program through community advertisement.

Matched normal controls recruited for this study were free of any history of psychiatric or major medical disorders, such as neurological, cardiovascular, pulmonary, endocrine, and renal disorders. They abstained from medication for at least two weeks prior to their assessment and gave informed consent for the study. Subjects were matched with regard to age, gender, and race. All parents and children gave consent and assent, respectively, unless the subjects were over 16, when they also gave consent. PBD patients were unmedicated for a period of at least one week prior to the blood draw and were either in manic or mixed phase at the time of recruitment. Clinical screening and structured interviews were performed during this initial medication-free period. Blood was drawn again from the patients after 8 weeks of treatment with mood stabilizers or second generation antipsychotics (SGAs).

2.2. Clinical Assessments

Patients were diagnosed according to the DSM-IV diagnostic criteria using the Washington University at St. Louis Schedule for Affective Disorders and Schizophrenia (WASH-U-KSADS; Geller et al., 1998), the latest and expanded version for the use in pediatric population. The final diagnosis was derived based on additional information from clinical charts, clinical interviews and information from the WASH-U-KSADS. Clinical ratings were obtained at baseline and after 8 weeks of treatment. Young Mania Rating Scale (YMRS; Young et al., 1978) was used to rate manic symptoms and the Children's Depression Rating Scale (CDRS-R; Poznanski et al., 1984) was used to rate depressive symptoms at baseline and after the completion of treatment.

2.3. Isolation of Platelets

Human venous blood was collected into a tube containing 3.8 % (w/v) sodium citrate (1 vol: 9 vol blood). The blood was centrifuged immediately at 210 g for 10 min at 4°C to obtain platelet-rich plasma, which was centrifuged at 4000 g for 10 min at 4°C to obtain the platelet pellet. This pellet was resuspended in Tris-HCl buffer (pH 7.4) and centrifuged at 4000 g to obtain the final platelet pellet.

2.4. Preparation of Membrane and Cytosol Fractions

The platelet pellet was homogenized in homogenizing buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 5 mM ethylenediamine tetraacetic acid, 1.5 mM pepstatin, 2 mM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 units/ml aprotinin, and 2 mM dithiothreitol using the Polytron®. The supernatant was centrifuged at 100,000 g for 60 min at 4°C. The resulting supernatant was the cytosol fraction, and the pellet was resuspended in the homogenizing buffer containing 0.2% (w/v) Triton x-100. The homogenate was kept at 4°C for 60 min with occasional stirring and then centrifuged at 100,000 g for 60 min at 4°C. The resulting supernatant was used as the membrane fraction. The concentration of protein in the cytosol and the membrane fractions was determined using the procedure of Lowry et al. (1951).

2.5. Determination of PKC Activity in Membrane and Cytosol Fractions

PKC activity in the membrane and cytosol fractions was measured by the procedure described earlier by us (Dwivedi & Pandey, 1999). An Amersham enzyme assay kit was used to determine PKC activity. A PKC-specific target peptide and all the necessary cofactors were provided in the kit. Assay tubes (with a final incubation volume of 75 μ l) contained 25 μ l of a component mixture [3 mM Ca ($C_3H_3O_2$)₂, 75 μ g/ml L- α -phosphatidyl-L-serine, 6 μ g/ml phorbol 12-myristate 13-acetate, 225 μ M substrate peptide, and 7.5 mM dithiothreitol in 50 mM Tris-HCl containing 0.05% sodium azide, pH 7.5] and 25 μ l of the membrane or cytosol fraction. The reaction was initiated by addition of 25 μ l of Mg-ATP buffer (10 μ Ci/ml [γ ³²P]ATP, 1.2 mM ATP, 72 mM MgCl₂, and 30 mM HEPES, pH 7.4) to each tube. The tubes were incubated for 15 min at 37°C, and the reaction was terminated by addition of 100 μ l of the “stop” reagent (300 mM orthophosphoric acid containing corrosive acid) to each tube. An aliquot of the solution from each tube (35 μ l) was blotted onto individual peptide-binding papers. Papers were washed with 75 mM phosphoric acid twice for 5 min. Papers were dried, and the retained radioactivity was counted by a liquid scintillation counter. The result was expressed as nanomoles per minute per milligram of protein. Before starting our experiments, the specificity of the PKC assay in membrane and cytosol fractions was determined using staurosporine (100 nM) as the PKC inhibitor. It was observed that in the presence of staurosporine, PKC activity was inhibited by 99.94%.

2.6. Quantitation of PKC Isozymes in Membrane and Cytosol Fractions by Western Blot

Immunolabeling of PKC α , β I, β II, and δ isozymes was determined as described previously (Pandey et al., 2002). Equal volumes of membrane or cytosol fraction samples containing 25 μ g protein and gel loading solution (50 mM Tris-HCl [pH 6.8], 4% β -mercaptoethanol, 1% sodium dodecylsulfate (SDS), 40% glycerol, and bromphenol blue) were mixed and the tissue samples were boiled for 3 min and then kept on ice for 10 min. Samples were loaded onto 7.5% (w/v) acrylamide gel using the Mini Protein II gel apparatus (BioRad, Hercules, CA, USA). The gels were run using 25 mM Tris-base, 192 mM glycine, and 0.1 % (w/v) SDS at 150 volts. The proteins were subsequently transferred electrophoretically to an ECL nitrocellulose membrane (Amersham, IL, USA) using the Mini Trans Blot transfer unit (BioRad, Hercules, CA, USA) at 0.150 amp constant current. Membranes were washed with TBST buffer (10 mM Tris-base, 0.15 M NaCl, and 0.05% [v/v] Tween 20) for 10 min. The blots were blocked by incubating with 5% (w/v) powdered nonfat milk in TBST, 2 ml nonidet P-40, and 0.02% (w/v) SDS (pH 8.0). Then the blots were incubated with anti-PKC α , β I, β II, or δ antibodies overnight at 4°C. The dilution of antibodies ranged from 1:3000 to 1:5000 depending on the antibody used. Membranes were washed with TBST and incubated with horseradish peroxidase-linked secondary antibody (anti-rabbit or anti-mouse IgG) for 1 to 5 hr at room temperature. Membranes were extensively washed with TBST and exposed to enhanced chemiluminescent (ECL) film. Membranes were stripped using stripping solution (Chemicon International, Temecula, CA, USA), and monoclonal β -actin antibody (1:5000 for 2 hr) was probed, followed by secondary anti-mouse IgG antibody (1:5000 for 2 hr). The bands on the autoradiogram were quantified using the Loats Image Analysis System (Westminster, MD, USA), and the optical density of each band of the PKC was corrected by the optical density of the corresponding β -actin band. The values are represented as a percentage of the control. β -actin was used as an internal control to reduce interblot variability.

2.7. Statistical Analysis

The statistical analysis of the data was performed using SPSS software for Windows version 12 (SPSS). Results are expressed as the mean $\pm$ standard deviation. The comparison of the data between normal control and bipolar subjects was performed using an independent sample t-test for equal or unequal values as appropriate. One way ANOVA was performed to compare

normal controls and PBD patients before and after treatment and $p \leq 0.05$ was considered significant. Pearson correlation matrix was used to determine the effect of age, gender and behavioral rating scores with PKC variables

3. Results

3.1. Effect of Age and Gender on PKC Activity and PKC Isozymes

We determined PKC activity and PKC isozymes in 23 patients with PBD and 23 matched normal control subjects, referred to as normal controls. The demographic and clinical characteristics of the study subjects are presented in Table 1. The symptom scores on CDRS and YMRS are also provided in Table 1. As can be seen from the table, the mean YMRS for the PBD patients was comparable to those reported in the literature (Pavuluri et al., 2005). After 8 weeks of treatment there was a significant reduction in the YMRS in PBD patients. There were no significant differences in the mean age between patients and normal control subjects. The male/female ratio was very similar in both the control as well as the PBD group. To examine if age or gender had any significant effect on PKC activity or PKC isozymes, we determined the correlations between age or gender and PKC activity and PKC isozymes. However, we did not observe any significant effect of age or gender on either PKC activity or any of the PKC isozymes.

3.2. PKC Activity in Pediatric Bipolar Patients

We determined PKC activity in membrane and cytosol fractions of platelets from PBD and normal control subjects, and the results are shown in Figure 1. There was a significant decrease in the PKC activity in membrane and cytosol fractions of platelets obtained from PBD patients compared with normal control subjects. In order to determine if there were any alterations in the ratio of PKC activity in platelets of PBD patients compared with normal control subjects, we calculated the ratio of PKC activity in membrane and cytosol fractions. Although there was a decrease in PKC activity in both membrane and cytosol fractions of platelets from PBD patients, there were no significant differences in the membrane to cytosol ratio of PKC activity between PBD and control subjects (Fig. 1). These results indicate that the decrease in PKC activity in membrane and cytosol fractions observed in platelets of PBD patients is not related to an abnormal translocation of PKC.

Because we observed a decrease in PKC activity in the membrane, as well as cytosol, fractions of platelets from PBD compared with normal control subjects, we examined if this decrease in PKC activity was related to an altered protein expression of any of the PKC isozymes. We therefore determined the immunolabeling of PKC isozymes known to be present in platelets (i.e., PKC α , PKC β I, PKC β II, and PKC δ) in the membrane and cytosol fractions obtained from PBD and normal control subjects (Fig. 2, 4–6). Immunoblots of PKC isozymes from two control subjects and two bipolar subjects are shown in Figure 3. As reported in the literature, PKC α , β I, and β II isozymes migrated to 80 kD whereas PKC δ migrated to 78 kD. As can be seen from the figure, there was an apparent decrease in the levels of the PKC isozymes in the membrane and/or cytosol fractions of PBD. The mean levels of PKC isozymes in PBD and normal control subjects are shown graphically in Figures 2, 4–6. When we compared the levels of PKC isozymes as corrected by β -actin, we found that the protein expression of PKC β I (Fig. 4) and β II (Fig. 5) was significantly decreased in both membrane and cytosol fraction of PBD compared with normal control subjects. On the other hand, there was no significant decrease in the protein expression of either PKC α (Fig. 2) or PKC δ (Fig. 6) in either the membrane or the cytosol fraction obtained from PBD compared with normal control subjects.

3.3. Effect of Treatment with Mood Stabilizing Drugs on PKC Activity and Protein Levels of PKC Isozymes

Since it has been observed that treatment with mood stabilizing drugs causes a decrease in PKC activity, we determined the effect of mood stabilizing drugs on the PKC activity and the protein levels of PKC isozymes in the platelets of PBD.

PKC activity and immunolabeling of PKC isozymes was determined in 16 out of 23 patients during the medication-free period and after 8 weeks of treatment with lithium ($n = 2$), anticonvulsant mood stabilizers, such as valproic acid, lamotrigine or oxcarbazepine ($n = 11$), or SGAs, such as risperidone or aripiprazole ($n = 3$). The results are shown in Figures 2, 4–7. As can be seen in the figure, there was no significant difference in the levels of any of the PKC isozymes, i.e., PKC α , PKC β I, or PKC β II or PKC γ in the membrane or in the cytosol fractions of platelets obtained from these patients before and after 8 weeks of treatment and clinical improvement.

However, we observed that the post-treatment platelet PKC activity was significantly increased in both cytosol as well as membrane fractions in 16 PBD patients compared with the baseline pretreatment levels as shown in Figure 7, and the post-treatment PKC activity levels were almost similar to those observed in the normal control subjects.

In order to examine the effect of treatment with mood stabilizing drugs and lithium on PKC activity and PKC isozymes, we separated patients into groups treated with anticonvulsant mood stabilizers ($n = 11$), lithium ($n = 2$) and SGAs ($n = 3$), and found that there were no significant differences in the protein expression of any of the PKC isozymes, either in the membrane or cytosol fractions before and after treatment (Fig. 7).

These results, in summary, thus indicate that 8 weeks of treatment with mood stabilizing drugs or SGAs significantly increases PKC activity, both in the membrane and cytosol fraction without causing any changes in the expression levels of PKC α , PKC β I, PKC β II or PKC δ .

The other issue is if the increase in PKC activity observed in PBD patients after treatment was related either to the severity of illness, as determined by YMRS, or to treatment response, as determined by change in the YMRS scores after 8 weeks of treatment. We found no significant correlation between the PKC activity and the baseline YMRS in the PBD patients suggesting that although the PKC activity is decreased in these patients, there is no one-to-one correlation with the severity of the illness. Next, we compared the change in YMRS scores with the change in PKC activity in these patients. We did not find any significant correlation between the changes in YMRS scores in these patients with the change in PKC activity. This suggested that although decreased YMRS scores were associated with an increase and normalization of PKC activity there was no one-to-one correlation between YMRS scores and the PKC activity.

In order to examine if the PKC activity or protein expression of PKC isozymes was related to depressive symptoms or change in depressive symptoms we also examined the correlation between baseline CDRS scores and change in baseline CDRS scores after treatment with PKC activity and protein expression of PKC isozymes (PKC α , PKC β I, PKC β II and PKC δ). However, we did not observe any significant correlation between PKC activity and PKC isozymes before and after treatment with either baseline CDRS or change in CDRS scores after treatment. These data thus indicates that PKC measures are not related either to the severity of depression or to improvement of depressive symptoms in these bipolar patients.

4. Discussion

In order to examine the role of PKC in the pathophysiology of PBD, in this study we determined the protein expression of PKC isozymes in the membrane, as well as the cytosol, fractions of platelets obtained from PBD (during a medication-free period) and matched normal control subjects. We found a selective decrease in the protein expression of PKC β I and PKC β II in the membrane, as well as the cytosol, fractions of platelets from PBD subjects compared with normal control subjects; whereas, there was no significant decrease in the protein expression of PKC α or PKC δ , either in the cytosol or the membrane fractions. We also found a decrease in PKC activity in both membrane and cytosol fractions of platelets of PBD subjects compared with normal control subjects. These studies, thus, for the first time demonstrated alterations of PKC isozymes and PKC activity in the platelets of PBD subjects, which suggest that abnormalities of PKC may be associated with the pathophysiology of PBD.

As stated earlier, this is the first study of PKC in PBD, since, to our knowledge, there are no comparable studies of PBD in the literature. However, the role of PKC has been studied both in the platelets, as well as the postmortem brain, obtained from adult bipolar subjects (Friedman et al., 1993; Wang & Friedman, 1996). Although the findings are inconsistent due to various methodological and other confounding variables, these studies in general do show an alteration of PKC in bipolar illness. For example, Friedman et al. (1993) and Wang & Friedman (1996) observed that PKC activity is increased in the platelets of adult bipolar subjects during the manic phase, whereas Wang and Friedman (1996) did not find any differences in PKC α either in membrane or cytosol fractions of platelets between bipolar adult patients and normal control subjects. Soares et al. (2000) found a significant decrease in PKC α but not PKC β I, PKC β II, or PKC δ in platelets of lithium-treated adult bipolar patients compared with control subjects.

The most comparable study to the present study of PKC in the platelets of PBD patients is the one in ABD patients recently reported by our group (Pandey et al. 2002). In that study, we determined the protein levels of PKC α , PKC β I, PKC β II, and PKC δ , as well as PKC activity, in the cytosol and membrane fractions of platelets obtained from adult bipolar patients and normal control subjects. We found a selective decrease in the protein expression of PKC α , PKC β I, and PKC β II but not PKC δ in the platelets of adult bipolar patients compared with normal control subjects. We also found a decrease in PKC activity in the platelets of adult bipolar patients in that study. The results of our present study are thus comparable to some extent to those reported by our group in ABD, with minor differences. For example, the decreases in PKC activity, as well as in the expression of PKC β I and PKC β II, observed by us in PBD are similar to those reported by us in adult bipolar patients (Pandey et al., 2002). However, whereas there appears to be a decrease in PKC α in ABD, our current study does not show any difference in PKC α between PBD and normal control subjects. The observation that there were no changes in PKC δ in PBD patients is similar to that reported by us in adult bipolar patients.

What could be the reasons for the subtle differences in the abnormal pattern of PKC isozymes between the adolescent and the adult bipolar disorder? As stated earlier in the Introduction section, although the manifestations of major clinical symptoms between ABD and PBD are similar, some of the symptoms appear to be dissimilar, and it is possible that these differences in the manifestations of the symptoms may reflect developmental variations.

The role and functions of specific PKC isozymes are not fully understood; however, experiments with knockout mice with specific PKC isozyme deficits do indicate that each isozyme may be associated with some specific functions. For example, the knockout mice deficient in PKC α show long-term synaptic depression (Leitges et al., 2004). On the other

hand, in the mice specifically lacking PKC γ , spatial memory is mildly affected (Hayashi et al., 2005) and long-term synaptic potentiation in hippocampus is impaired (Abeliovich et al., 1993). The mice lacking PKC β suffer from a deficit in contextual fear conditioning (Weeber et al., 2000). Although these specific behavioral functions of PKC isozymes cannot be fully translated in terms of human behavior or the symptoms of the illness, this does indicate that the deficit observed by us in PKC β may be related to some specific behavioral impairment in both PBD as well as ABD. On the other hand, the specific behavior impairments or abnormalities related to PKC α may be present only in ABD but not in PBD.

The next question was, therefore, what might be the functional significance of the observed decrease in the protein expression of specific PKC isozymes in the membrane, as well as the cytosol, fractions? Since we found a decrease in both membrane and cytosol fractions, since the ratio of PKC activity in the membrane and cytosol fractions was not different from that observed in normal control subjects, this indicated that the decrease was not related to an abnormal translocation of PKC from the cytosol to the membrane. Even though the decrease was found in two specific PKC isozymes, that is, PKC β I and PKC β II, and in both the cytosol and the membrane fraction, it did cause a decrease in the PKC activity. PKC is involved in important physiological and behavioral function and this is related to its ability to phosphorylate important substrates and transcription factors. One of the important substrates for PKC is MARCKS, which has also been shown to be abnormal in bipolar disorder. PKC is also involved in the phosphorylation of transcription factors. So a decrease in the phosphorylation of MARCKS by PKC will thus alter some of the physiological functions associated with MARCKS, and the decrease in phosphorylation of transcription factors, such as CREB, may cause decreased transcription of some of the important target genes, such as BDNF, that are involved in important physiological and behavioral functions.

The mechanisms that cause an alteration of PKC activity and of protein expression of specific PKC isozymes are not clear, but they may be related to alterations in the effector, PLC, resulting in abnormal formation of DAG. They may also be related to some of the receptors linked to the PI signaling system, such as 5HT_{2A} or 5HT_{2C}. An increase in the number of 5HT_{2A} receptors has been reported by us in the platelets of adult bipolar patients (Pandey et al., 2003), and increased agonist activation of platelet 5HT_{2A} receptors may increase the formation of the second messenger DAG and result in persistently increased activation of PKC isozymes. However, it is not clear why this activation would cause a specific change in one of the PKC isozymes. Another possibility is that PKC isozyme abnormalities may be related to genetic changes.

Another mechanism that may cause changes or downregulation in the PKC activity and protein expression of some of its isozymes may be related to alterations in the levels of PIP₂. PIP₂ is a substrate for the formation of the second messenger (DAG) and IP₃ as a result of the activation of the effector for phospholipase C (PLC) (Berridge & Irvine, 1989; Tanaka & Nishizuka, 1994). A change in the level of PIP₂ may cause alterations in the formation and levels of DAG and hence altered activation of PKC. That these alterations in the levels of DAG and changes in the activation of PKC may be responsible for the observed decreased PKC activity and PKC isozymes therefore appears quite plausible because of the observation by some investigators that the levels of PIP₂ is changed in the platelets of bipolar patients (Soares et al., 1999). This change in PIP₂ may be related possibility to altered oxidative stress as reported in bipolar patients (Frey et al., 2006 a, b; Ranjekar et al., 2003).

Treatment with lithium and other mood stabilizers may restore the levels of PIP₂ as a result of their normalizing effect not only on bipolar illness but also on oxidative stress due to an increase in antioxidants and a decrease in oxidative stress, since it has been reported by some investigators that treatment with mood stabilizing drugs results in decreased oxidative stress

(Shao et al., 2005). The increased levels of PIP₂ may also account in the restoration of PKC activity. It is therefore quite possible that the changes in the PKC activity and some of the PKC isozymes observed by us, may not be directly related to the diseases process itself but may be intermediate in the chain of cascade of events which may have important functional consequences.

4.1. Effect of Treatment with Mood Stabilizing Drugs on PKC Activity and PKC Isozymes in Platelets of PBD

One of the major lines of evidence suggesting the involvement of PKC in the pathophysiology of bipolar disorders is derived from the observation that mood stabilizing drugs, such as lithium or valproic acid (VPA), cause changes in the activity of PKC and the expression of PKC isozymes, both under in-vitro and ex-vivo conditions (Jope, 1999; Manji et al., 1993; Manji & Lenox, 1994; Morishita & Watanabe, 1994), thus suggesting that alterations in PKC may be involved in the pathophysiology of bipolar disorders (Friedman et al., 1993; Ikonomov & Manji, 1999; Manji et al., 1995; Manji & Lenox, 1999; Wang et al., 1999; Pandey et al., 2002). We therefore examined if pharmacotherapy with mood stabilizing drugs and/or clinical improvement are associated with changes in PKC activity or in the expression of PKC isozymes in the platelets of PBD patients. We thus determined the protein expression of PKC isozymes and PKC activity during the drug-free baseline period and after 8 weeks of treatment with mood stabilizing drugs.

When we determined the PKC isozymes and PKC activity in the membrane and cytosol fractions of platelets obtained from PBD patients, we observed a significant increase and normalization of PKC activity in the cytosol as well as membrane fractions of platelets of PBD patients compared with pretreatment values.

Although we found a significant increase in the PKC activity both in the membrane as well as the cytosolic fractions after 8 weeks of treatment, we did not find any significant differences in the protein expression levels of any of the PKC isozymes either in the cytosol or membrane fraction before and after treatment with mood stabilizing drugs. It was even more surprising that there was no change even in the protein expression levels of PKC β I and PKC β II which were found to be significantly reduced in the PBD patients during the medication-free baseline period prior to the initiation of treatment. The reasons for this dissociation in the changes after treatment between PKC activity and PKC isozymes are not clear. Since PKC activity represents the functional aspects of the PKC isozymes, it does indicate that treatment and clinical response are associated with an increase in PKC function. However, this increase does not appear to be caused by increases in the protein levels of any of the PKC isozymes we determined. How the PKC activity changed without any changes in PKC isozymes remains to be determined.

The other question which these observations raised is if changes in PKC activity after treatment are the pharmacological effects of treatment or are related to clinical improvement. Effect of treatment with lithium and valproic acid has been studied by several groups of investigators both in the animals and *in vitro*; however, the results appear to be inconsistent. Both Jope et al. (1996) and Young et al. (1999) determined the effect of treatment with lithium on PKC activity and protein expression of PKC isozymes on rat brain. Young et al. (1999) did not find changes in the protein expression levels of PKC α after treatment with lithium and similarly Casebolt & Jope (1991) found no changes in the PKC activity after treatment with lithium although PKC-mediated phosphorylation of some proteins was increased. Friedman et al. (1993) determined the levels of PKC activity in bipolar patients after treatment with lithium and found increased PKC activity and translocation of PKC from cytosol to membrane in lithium treated patients. In our study, patients were treated with different types of mood stabilizing drugs which included predominantly anti-epileptic drugs such as valproic acid (n = 11), neuroleptic, such as aripiprazole or risperidone (n = 3), and 2 patients were treated with

lithium. When we compared each group with each other, we did not find significant differences between each group either before or after treatment (Fig. 7). Thus, the increase in PKC activity doesn't appear to be related to the treatment either with lithium, SGAs or anticonvulsant mood stabilizers. Consequently, it is possible that the increase in PKC activity after treatment may be related to clinical improvement or clinical response. We therefore determined the correlation between changes in PKC activity and changes in the YMRS scores; however, we did not find any significant correlation between them. Although we did not find significant correlation between changes in YMRS and changes in PKC activity it is still possible that the changes in PKC activity may be related to clinical response. Follow-up studies of changes in PKC activity after treatment with mood stabilizing drugs is needed to further examine the significance of this finding which may suggest that PKC may be used as a target for the therapeutic action of mood stabilizing drugs.

In conclusion, our studies of PKC in the platelets of PBD patients have several important findings and implications. They indicate (1) that PBD is associated with a specific decrease, or abnormalities, of PKC β I and PKC β II, both in the membrane, as well as in the cytosol; (2) that this may cause changes in the functions of PKC since PKC activity is also found to be decreased in these patients; and (3) that this is not related to an abnormality of the translocation of PKC from the cytosol to the membrane. Our studies also suggest that treatment with mood stabilizing drugs does cause adaptive changes in PKC activity but not PKC isozymes, and that this abnormality of PKC in PBD is normalized after treatment and clinical improvement.

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Fig. 1.

Mean PKC activity in the membrane and cytosol fractions of platelets obtained from medication-free PBD patients ($n = 23$) and normal control (NC) subjects ($n = 23$) and in medication-free PBD patients before the initiation of therapy ("Pre"; $n = 16$) and after 8 weeks of treatment ("Post"; $n = 16$). **Note:** The 16 patients are also included in the 23 medication-free patients. Values are the mean $\pm$ S.D.

^a $P = .002$; ^b $P = .001$.

Fig. 2.

Mean protein levels of PKC α isozyme in membrane and cytosol fractions of platelets obtained from medication-free PBD patients ($n = 23$) and normal control (NC) subjects ($n = 23$) and in the PBD patients between the initiation of therapy ("Pre"; $n = 16$) and after 8 weeks of treatment ("Post"; $n = 16$). **Note:** The 16 patients are also included in the 23 medication-free patients. Values are the mean $\pm$ S.D.

Fig. 3. Representative Western blots showing the immunolabeling of PKC β I, PKC β II isozymes and β -actin in membrane and cytosol fractions from two PBD patients and 2 normal control subjects.

Fig. 4.

Mean protein levels of PKC β I isozyme in membrane and cytosol fractions of platelets obtained from medication-free PBD patients ($n = 23$) and normal control (NC) subjects ($n = 23$) and in medication-free before the initiation of therapy ("Pre"; $n = 16$) and after 8 weeks of treatment ("Post"; $n = 16$). **Note:** The 16 patients are also included in the 23 medication-free patients. Values are the mean $\pm$ S.D.

^a $P = .002$

Fig. 5.

Mean protein levels of PKC β II isozyme in membrane and cytosol fractions of platelets obtained from medication-free PBD patients ($n = 23$) and normal control (NC) subjects ($n = 23$) and in medication-free PBD patients before the initiation of therapy ("Pre"; $n = 16$) and after 8 weeks of treatment ("Post"; $n = 16$). **Note:** The 16 patients are also included in the 23 medication-free patients. Values are the mean $\pm$ S.D.

^a $P = .002$

Fig. 6.

Mean protein levels of PKC δ isozyme in membrane and cytosol fractions of platelets obtained from PBD patients (n = 23) and normal control (NC) subjects (n = 23) and in medication-free PBD patients before the initiation of therapy (n = 23) and after 8 weeks of treatment (n = 16).

Note: The 16 patients are also included in the 23 medication-free patients. Values are the mean $\pm$ S.D.

Fig. 7.

Mean PKC activity in the membrane and cytosol fractions of platelets obtained from medication-free PBD patients before the initiation of therapy ("Pre"; $n = 16$) and after 8 weeks of treatment ("Post"; $n = 16$). The samples obtained from PBD patients after 8 weeks of treatment were subdivided into three groups: i) PBD patients treated with anti-epileptic medication ("AEp"; $n = 11$); ii) PBD patients treated with lithium ("Li"; $n = 2$) and PBD patients treated with SGAs ("NL"; $n = 3$). Values are the mean $\pm$ S.D.

^b $P = .001$

Table 1
Demographic Characteristics of Pediatric Bipolar Patients and Normal Control Subjects

Group	Age (Years)	Gender (M/F)	Race	CDRS Pre-treatment	CDRS Post-treatment	YMRS Pre-treatment	YMRS Post-treatment
Normal Controls	12.39 ± 3.03	13M/10F	11 White 2 Black 10 Other	--	--	--	--
PBD Patients	13.04 ± 2.93	12M/11F	12 White 9 Black 2 Other	45.35 ± 18.87	22.94 ± 4.58	24.87 ± 7.80	6.44 ± 6.90

Values are the mean ± SD.