Abnormalities of Cyclic Adenosine Monophosphate Signaling in Platelets From Untreated Patients With Bipolar Disorder

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Background: Abnormalities in the cyclic adenosine monophosphate (cAMP)–dependent phosphorylation system have been recently reported in patients with bipolar disorder. We evaluated the immunoreactivity of the regulatory and catalytic subunits of cAMP-dependent protein kinase (protein kinase A) and 1 of its substrates, Rap1, in platelets from untreated euthymic, manic, and depressed patients with bipolar disorder and healthy subjects.

Methods: Platelets were collected from 112 drug-free patients with bipolar disorder (52 euthymic, 29 depressed, and 31 manic) and 62 healthy subjects. The levels of cAMP-dependent protein kinase and Rap1 were assessed by Western blot analysis, immunostaining, and computer-assisted imaging.

Results: The immunolabeling of the catalytic subunit of cAMP-dependent protein kinase was significantly different among groups (P<.001), with higher values in untreated depressed and manic patients with bipolar disorder compared with untreated euthymic patients with bipolar disorder and healthy subjects. No significant differences were found in the immunolabeling of the regulatory subunits (type I and type II) of cAMP-dependent protein kinase. The immunolabeling of Rap1 was significantly higher (P<.001) in untreated euthymic, depressed, and manic patients than in healthy persons.

Conclusions: Levels of Rap1 and the catalytic subunit of cAMP-dependent protein kinase are altered in the platelets of bipolar patients. These findings may provide clues toward understanding the involvement of cAMP signaling in the pathogenesis of bipolar disorder.

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SUBJECTS AND METHODS

SUBJECTS

After providing written informed consent, 112 patients (60 men and 52 women; age, 37.2 ± 9.92 [mean ± SD] years) and 62 age- and sex-comparable healthy volunteers (36 men and 26 women; age, 40.3 ± 12.52 years) were included in this study. None of the subjects were part of previous studies. All patients, recruited at the Mood Disorders Clinical and Research Unit, San Raffaele Hospital, Milan, Italy, met the full criteria for bipolar I disorder according to the DSM-IV. Those with a history of important acute or chronic medical illness or alcohol or psychotropic drug abuse were excluded. The diagnosis was assessed by clinical interview. Clinical information was collected directly from each patient and from at least 1 close relative as a coinformant. Any definitive documentation—eg, discharge summaries of prior hospital admissions for episodes of illness—that could be obtained were also considered in assessing the diagnosis. Medical health was documented by the medical history, physical examination, electrocardiography, blood and serum chemical analyses (including hepatic and renal profiles), and thyroid function tests.

The group with bipolar disorder comprised 52 outpatients in euthymic condition for at least 3 months before entering the study (a score of ≤8 on the 21-item Hamilton Depression Rating Scale, and a score of ≤9 on the Young Mania Rating Scale), 29 inpatients with bipolar disorder with a depressive episode (score, > 21 on the Hamilton Depression Rating Scale), and 31 inpatients with bipolar disorder with a manic episode (score, > 21 on the Young Mania Rating Scale). The mean ± SD age of the 52 euthymic patients was 39.2 ± 12.5 years; of the 29 depressed patients, 41.9 ± 11.3 years; and of the 31 manic patients, 40.6 ± 13.8 years. The ages at onset were 29.3 ± 8.2 years, 29.1 ± 8.2 years, and 28.6 ± 8.9 years, respectively. The mean ± SD number of previous episodes was 3.5 ± 2.0 in euthymic patients, 4.2 ± 2.3 in depressed patients, and 3.3 ± 2.1 in manic patients. The sex distribution (male:female) in the 3 groups was 35:17, 16:13, and 9:22, respectively.

All patients had been medication free for at least 1 month before blood specimens were drawn. In particular, most patients (58%) had never taken lithium salts: 31 (59.6%) of 52 euthymic patients, 17 (58.6%) of 29 depressed patients, and 17 (54.8%) of 31 manic patients. The remaining patients had been treated with lithium only during previous manic episodes that occurred at least 1 year before entering the study and never as long-term therapy. Other mood-stabilizing agents used to treat previous episodes were carbamazepine in 20 (17.9%) of 112 patients and valproate sodium in 7 (6.2%) of 112 patients.

The comparison group included 62 consenting volunteers recruited from the staff of our hospital, who had no personal or familial history of mental disorders, alcoholism, or drug abuse and no active medical problems, as determined by clinical interview. To exclude the presence of a personal or familial history of mental disorders, the proband and, when possible, other members of the proband’s family or social group were interviewed. Information regarding first- and second-degree relatives was obtained for each subject.

PLATELET ISOLATION AND IMMUNOBLOT ANALYSIS

A morning blood specimen (50 mL) was obtained from all subjects by venipuncture and placed in tubes containing anticoagulant.

Continued on next page

RESULTS

Figure 2 illustrates representative immunoblots of PKA subunits, Rap1, and actin immunoreactivities in total platelet proteins from untreated patients with bipolar disorder and healthy subjects. The monoclonal antibodies against the regulatory (type I or type II) or the catalytic subunits of PKA revealed the presence of major platelet protein bands migrating with apparent molecular masses of 49, 52, and 40 kd, respectively. The anti-Rap1 antibody recognized a single band with an apparent molecular mass of 21 kd and the antiactin antibody a single band with an apparent molecular mass of 42 kd. Quantitation of immunoblots was obtained through densitometric scanning of autoradiographs. Data were then normalized and analyzed as described in the “Statistical Analysis” subsection of the “Subjects and Methods” section. The Table summarizes the results obtained in each group of untreated patients with bipolar disorder and healthy subjects.

The analysis of variance revealed no significant difference in the levels of regulatory subunit type I ($F_{3,142} = 0.77; P = .51$) or type II ($F_{3,162} = 0.24; P = .86$) of PKA in platelets from the 4 groups (Table). Significant differences were found in the levels of the catalytic subunit of the enzyme ($F_{2,106} = 15.57; P < .001$). Post hoc comparisons showed that during illness episodes, patients with bipolar disorder had about 11% and 16% greater immunoreactivity of catalytic subunit than euthymic patients with bipolar disorder and controls, respectively: manic patients vs euthymic patients, $P = .003$; manic patients vs control subjects, $P < .001$; depressed patients vs euthymic patients, $P < .001$; and depressed patients vs controls, $P < .001$. No differences were found when compar-
sodium citrate (3.8%) as an anticoagulant. Platelets were isolated from the fresh blood specimen as previously described.\textsuperscript{30,31} Pellets were then washed 3 times with ice-cold Tris hydrochloride isotonic buffer and then frozen at 
\textsuperscript{\textminus}80°C until subsequent use. On the day of the assay, platelets were homogenized by sonication with two 15-second pulses in ice-cold Tris hydrochloride buffer (10 mmol/L) containing egtazic acid (1 mmol/L), ethylenediaminetetraacetic acid (1 mmol/L), dithiothreitol (1 mmol/L), peptatin (2 mg/mL), aprotinin (50 U/mL), and phenylmethylsulfonyl fluoride (0.1 mmol/L), pH 7.4. The platelets were prepared and then analyzed by personnel unaware of the diagnosis.

Protein concentrations were determined according to the method of Bradford.\textsuperscript{38}

The linearity of the platelet protein concentration for the Western blotting experiments was ascertained by resolving selected quantities—between 2 and 25 µg—of proteins. The relationship between the total amount of proteins loaded into the gels and the integration of area \texttimes\ optical density was linear within 2 to 15 µg of protein range.

Whole-platelet homogenates from each subject were solubilized in denaturing buffer and boiled for 2 minutes. Aliquots with equal amounts of proteins (5 µg) were loaded on 12% polyacrylamide gels and subjected to 1-dimensional electrophoresis (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Patients' specimens were always run in duplicate or triplicate on the same gel with those of their respective controls. Proteins, thus resolved in bands, were electrophoretically transferred onto Hybond polyvinylidene difluoride membrane (Amersham International, Buckinghamshire, England) for 90 minutes at 100 V. Blots were then preincubated for 1 hour in Tris buffered saline-Tween 20 buffer, pH 7.6 (Tris hydrochloride, 25 mmol/L; sodium chloride, 137 mmol/L; and 0.1% Tween 20), containing 1% bovine serum albumin (blocking buffer). All incubations were performed at room temperature. After a thorough washing with TBS-T, the blots were incubated in blocking buffer for 90 minutes with monoclonal antibodies against the regulatory type I (Transduction Laboratories, Lexington, Ky; dilution 1:500), the regulatory type II (Biomol, Plymouth Meeting, Pa; dilution 1:500), and the catalytic (Transduction Laboratories; dilution 1:500) subunits of PKA, Rap1 (Transduction Laboratories; dilution 1:2500), and actin (clone DC40, Sigma-Aldrich Corp, St Louis, Mo; dilution 1:1000) in blocking buffer for 1 hour. The labeled blots were then washed with TBS-T, and immunoreactivity was detected with the Western blot detection system (Enhanced Chemiluminescent, Amersham International), followed by exposure to film. Quantitation of the immunoblots was performed by densitometric scanning of the autoradiograms using an image analysis system (National Institutes of Health Image, Version 1.47, Bethesda, Md). Not all patients and controls had assays for the various antibodies because a satisfactory amount of platelet proteins was not obtained for each subject.

An aliquot of pooled standard platelet proteins was run on 1 lane of every gel to minimize the interassay variation, as previously described.\textsuperscript{6,10} The optical density units obtained from each subject were normalized against those from a pooled platelet standard.

**STATISTICAL ANALYSIS**

Data were analyzed using a 1-way analysis of variance (2-tailed), followed by Newman-Keuls post hoc comparison tests. Results are expressed as mean ± SD. The level of significance was $P<.05$.

Furthermore, only the levels of catalytic subunits of PKA were significantly higher in depressed and manic patients than in euthymic patients and controls. Overall, these data suggest that altered levels of Rap1 observed in the whole sample of patients with bipolar disorder could be considered a state-independent biochemical abnormality, whereas those of PKA observed only in depressed and manic patients could be considered a state-related abnormality.

The main limitation of our findings is that they come from platelets that are exposed to a potentially different neurohormonal environment than that of brain tissue. Transposing platelet findings to those that might be found only in a subset of neurons in the brain in this disorder can be difficult. Furthermore, it is not possible to exclude the role of a previous exposure to psychotropic drugs in producing these changes, even though patients were apparently drug free for at least 1 month. Taking into account these limitations, our data complement previous reports\textsuperscript{12-14,30-32,39,40} demonstrating alterations in the cAMP signaling in patients with affective disorders.

The observed abnormalities are unlikely to be the result of a general alteration in platelet proteins because the immunorelabeling of both types of regulatory subunits and of actin, the protein used as an internal control, showed no change among groups.

### Components of the cAMP-dependent phosphorylation system are altered in platelets from patients with bipolar disorder

More specifically, the levels of Rap1 were significantly higher in euthymic, depressed, and manic patients with bipolar disorder than in healthy subjects. Furthermore, only the levels of catalytic subunits of PKA were significantly higher in depressed and manic patients than in euthymic patients and controls. Overall, these data suggest that altered levels of Rap1 observed in the whole sample of patients with bipolar disorder could be considered a state-independent biochemical abnormality, whereas those of PKA observed only in depressed and manic patients could be considered a state-related abnormality.

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Previous studies30-32 have reported higher cAMP-dependent phosphorylation of Rap1 in platelets of untreated euthymic patients with bipolar disorder. Our data showing higher Rap1 and unvaried PKA levels in euthymic patients with bipolar disorder suggest that the abnormal phosphorylation in the former can be attributed to the increase in its own levels. This is also supported by the fact that the 20% increment in Rap1 immunoreactivity is comparable to that previously reported30-32 in the cAMP-stimulated phosphorylation of this protein.

Considering the lack of evidence with respect to protein phosphorylation in patients with bipolar disorder in different mood states, further studies are needed to clarify the relationship between the levels of Rap1 and catalytic subunits observed in depressed and manic patients.

Bearing in mind that lithium and antidepressant drugs have many dissimilar clinical effects and could affect components of cAMP signaling differently,17-29,31,41 it will be interesting to test whether these compounds could cause the levels of PKA and Rap1 in patients with bipolar disorder to return to normal.

Whether the results presented here are primary disease-related changes or reflect adaptive responses consequent to other dysfunctions in cell signaling is still unclear. It has been documented12 that patients with bipolar disorder have disturbances in G proteins coupled to cAMP signaling, which may lead to dysfunctions in the cAMP-dependent phosphorylation. Furthermore, keeping in mind the cross-talk between the signal transduction pathways,42,43 the observed modifications may be joined to disturbances in Ca++ signaling and the phosphoinositides cycle, including protein kinase C activity, which were found altered in patients with bipolar disorder.44-51

Rap1 is a small guanosine triphosphate–binding protein present in different tissues, including the brain.51 Although its role remains to be fully elucidated, evidence indicates that Rap1 could be involved in cellular events, such as calcium mobilization, cytoskeleton organization, and phosphoinositides metabolism, most of which

<table>
<thead>
<tr>
<th>Platelet Proteins</th>
<th>Controls (n = 62)</th>
<th>Euthymia (n = 52)</th>
<th>Depression (n = 29)</th>
<th>Mania (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulatory subunit, type I†</td>
<td>92.13 ± 11.60</td>
<td>87.72 ± 18.66</td>
<td>87.10 ± 16.66</td>
<td>91.44 ± 22.55</td>
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<tr>
<td>Regulatory subunit, type II‡</td>
<td>102.61 ± 20.45</td>
<td>104.42 ± 20.35</td>
<td>104.09 ± 15.76</td>
<td>100.81 ± 19.34</td>
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<td>Catalytic subunit§</td>
<td>100.96 ± 10.51</td>
<td>104.87 ± 15.63</td>
<td>119.59 ± 10.63</td>
<td>113.81 ± 15.01</td>
</tr>
<tr>
<td>Rap1</td>
<td>98.27 ± 10.03</td>
<td>118.11 ± 14.31#</td>
<td>116.98 ± 11.47#</td>
<td>121.17 ± 15.79#</td>
</tr>
<tr>
<td>Actin</td>
<td>97.22 ± 9.54</td>
<td>99.90 ± 13.09</td>
<td>98.07 ± 10.31</td>
<td>100.61 ± 13.28</td>
</tr>
</tbody>
</table>

*Data are given as mean densitometric standardized units ± SD.
†Controls, n = 43; euthymic patients, n = 47; depressed patients, n = 26; and manic patients, n = 30.
‡Controls, n = 55; euthymic patients, n = 51.
§Controls, n = 54; euthymic patients, n = 50.
#P < .001 vs euthymic patients and P < .001 vs control subjects.
*P = .003 vs euthymic patients and P < .001 vs control subjects.
have been found to be also altered in patients with bipolar disorder.\(^4,5,13-15\) More recently, Rap1 was found to be involved in the regulation of signal cascade coupled to neurotrophic factors.\(^5,9\) This is an intriguing finding considering recent data,\(^2,27,60\) suggesting an involvement of neurotrophic factors in mood disorders.

At present, it is difficult to envisage the molecular mechanism(s) underlying the alterations of Rap1 and PKA. A recent study\(^61\) demonstrated no structural or regulatory abnormalities in the gene encoding for the G protein stimulatory α subunit, even if its levels were altered in patients with bipolar disorder.\(^3,12\) These findings suggest that alterations in the transcriptional, posttranscriptional, translational, or posttranslational processes that are known to regulate the levels of proteins should be taken into account.

**CONCLUSIONS**

Levels of Rap1 and the catalytic subunit of PKA are altered in platelets of patients with bipolar disorder. These findings may provide clues toward understanding the involvement of cAMP signaling in the pathogenesis of bipolar disorder.

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