Phosducin-like protein levels in leukocytes of patients with major depression and in rat cortex: The effect of chronic treatment with antidepressants

Angela Matuzany-Ruban a, Gabriel Schreiber b, Peter Farkash c, Sofia Avissar a, *

a Department of Pharmacology, P.O. Box 653, Ben Gurion University of the Negev, Beer Sheva 84105, Israel
b Division of Psychiatry, Ben Gurion University of the Negev, Israel
c Division of Neurology, Ben Gurion University of the Negev, Israel

Received 18 June 2005; received in revised form 9 August 2005; accepted 26 September 2005

Abstract

The importance of signal transduction processes beyond receptors involving receptor–G protein coupling, in both the pathophysiology and the treatment of mood disorders, is well documented. Thus, regulatory elements of G protein function may play a role in the molecular mechanisms underlying these alterations. Phosducin-like proteins, a family of regulators of G protein function expressed throughout brain and body, modulate G protein function by high affinity sequestration of G protein-βγ subunits, thus impeding G protein-mediated signal transmission by both Gα and Gβγ subunits. An important consequence of Gβγ neutralization is the prevention of G protein-coupled receptor kinase phosphorylation resulting in a temporary protection to agonist-bound receptor desensitization. Phosducin-like protein levels were measured in brain cortices of rats chronically treated with one of five classes of antidepressants: imipramine, venlafaxine, maprotiline, citalopram, and moclobemide. None of the antidepressant treatments had any significant effect on phosducin-like protein levels. Phosducin-like protein levels were evaluated in mononuclear leukocytes from a group of 15 patients diagnosed with major depressive episode, both before the initiation of antidepressant treatment and after 4 weeks of antidepressant medication. No protein changes were found in leukocytes of either untreated patients with major depressive disorder or after 4 weeks of the treatment in comparison with healthy volunteers.

© 2005 Elsevier Ireland Ltd. All rights reserved.

Keywords: Signal transduction; G protein regulation; Unipolar depression; Monoamine reuptake inhibitor; Gβγ subunits sequestration

1. Introduction

Biochemical research in mood disorders has focused, along the cascade of events involved in signal transduction, from studies at the level of...
the primary messenger: the monoamine neurotransmitter (Schildkraut, 1965; Schatzberg and Schildkraut, 1995; Lambert et al., 2000) to the level of the neurotransmitter receptors, and lately to information transduction mechanisms beyond receptors, involving the coupling of receptors with signal transducers. Growing evidence suggests that receptor–G protein coupling, and its regulation, may be involved in both the pathogenesis and treatment of mood disorders (for review, see Avissar and Schreiber, 2002; Schreiber and Avissar, 2003). The available evidence relies primarily on (a) the effects of lithium (Avissar et al., 1988; Wang and Friedman, 1999; Minadeo et al., 2001), other antipolar treatments and antidepressant treatments (Avissar and Schreiber, 1992a,b; Donati et al., 2001; Shen et al., 2002; Pejchal et al., 2002), ECT (Avissar et al., 1998), and light therapy (Avissar et al., 1999) on receptor–G protein coupling; (b) differential alterations in the concentration and/or function of G proteins in peripheral blood elements of patients with bipolar disorder, major depression, and seasonal affective disorder (for review, see Avissar and Schreiber, 2002; Schreiber and Avissar, 2003); (c) alterations in the levels of G proteins in postmortem tissues of patients with bipolar and other mood disorders (Young et al., 1991, 1993; Friedman and Wang, 1996); (d) findings concerning G protein-encoding genes: G(o1f) (Berrettini et al., 1994; Rice, 1997; Berrettini et al., 1998), and Gz (Saito et al., 1999) as susceptibility loci for bipolar disorder and G-beta3 as susceptibility locus for major depressive disorder and seasonal affective disorder and as an indicator for antidepressant treatment response (Zill et al., 2000; Serretti et al., 2003; Willeit et al., 2003; Lee et al., 2004); (e) G protein-coupled receptor kinases (GRKs) (Niculescu et al., 2000; Garcia-Sevilla et al., 1999; Miralles et al., 2002; Grange-Midroit et al., 2003) and beta-arrestins (Avissar et al., 2004) have been suggested to be involved in the pathophysiology and treatment of mood disorders.

The discovery of phosducin (Phd) in photoreceptor cells of the retina (Lee et al., 1987), and of the ubiquitous distribution of phosducin-like proteins (PhdLP) (Miles et al., 1993), uncovered the existence of a family of proteins characterized as cytosolic regulators of G protein function (Schulz, 2001). The most important characteristic of these proteins appears to be their high affinity sequestration of βγ subunits of G proteins (Muller et al., 1996; Thibault et al., 1997), leading to neutralization of Gβγ and thus impeding G protein-mediated signal transmission, since Ga cannot reassemble with Gβγ to provide a functional G protein trimer (Gaβγ) (Müller et al., 1996; Bauer and Lohse, 1998). A further consequence expected from the rapid binding of PhdLP to Gβγ dimers is the inhibition of Gβγ-mediated effects, like activation of GRK-2 (Schulz et al., 1996), which will carry the prevention of GRK2/3-phosphorylation of GPCR (Pitcher et al., 1992; Schulz et al., 1996; Garson et al., 2002). Thus, it has been proposed that following agonist-induced activation of GPCRs, the binding of PhdLP to Gβγ dimers offers a temporary protection to agonist-bound receptors not coupled to G-proteins against being acted upon by GRKs. This presumably prevents the uncoupling and internalization of a fraction of these silent, but potentially active, GPCR (Garson et al., 2002).

The existent knowledge concerning the involvement of alterations in receptor–G protein coupling in the pathogenesis of mood disorders and in the mechanism of action of antidepressant and mood stabilizing drugs, together with the recent findings concerning the regulation of receptor–G protein coupling by phosducin-like proteins, raised the following research questions: (a) Is PhdLP involved in the mechanism of action of antidepressant medications? (b) Is PhdLP involved in the pathophysiology of major depressive disorder? To suggest possible answers to these questions in the present study, we undertook (i) measurements of the effects of chronic treatments of various types of antidepressants on PhdLP in rat cortex; (ii) measurements of PhdLP in mononuclear leukocytes of a group of patients diagnosed with major depressive episode before and after 4 weeks of antidepressant treatment in comparison with a group of healthy volunteer subjects.

2. Materials and methods

2.1. Patients

All subjects (patients with major depression and healthy controls) were evaluated using the
Structured Clinical Interview for Axis I DSM-IV Disorders (SCID DSM-IV). Inclusion criteria for patients and healthy subjects were (1) 18 years of age or older; (2) in good general health with no clinically significant systemic abnormalities and no major findings in physical examination; (3) normal laboratory test results for renal, hepatic, hematologic, and thyroid function, and normal electrocardiogram; (4) being untreated with antidepressants for at least 20 weeks; (5) willing and able to give informed consent. For the patient group only: (6) diagnosis of a current existence of a major depressive episode according to DSM-IV criteria; (7) have not yet been started on antidepressant medication; and (8) have a score of at least 18 on the 17-item Hamilton Rating Scale for Depression. Exclusion criteria for all subjects were (1) history or evidence of clinically significant physical disorders; (2) current existence or history of a clinically significant diagnosable major psychiatric disorder (for the patient group only: other than a major depressive episode); (3) current or recent treatment (within the past 20 weeks) with psychopharmacological or other medications; and (4) alcohol or drug dependency or abuse within the previous 12 months. After a complete description of the study to the subjects, written informed consent was obtained for a 20–60-ml blood donation. In all cases, blood was drawn between 8:00 and 10:00 a.m. The Hamilton Depression and Beck Inventories were administered before blood donation. The study was carried out in accordance with the latest version of the Helsinki Declaration and approved by the Institutional Review Board.

The group of 15 untreated patients with major depression consisted of 8 female and 7 male patients (average age 36.6, S.D.=15.3, range 18–58) with Hamilton scores >20. Patients were examined before the initiation of treatment and 4 weeks after treatment began. Patients were blindly assigned in advance to receive treatment with either a selective serotonin reuptake inhibitor (SSRI) (citalopram, 20–40 mg/day) or a selective norepinephrine reuptake inhibitor (SNRI) (venlafaxine, 150–225 mg/day). The healthy volunteer group consisted of 15 subjects (8 female and 7 male), average age 37.1 (S.D.=12.6, range 19–57) years, from the students and staff of Ben Gurion University.

2.2. Isolation of mononuclear leukocytes and preparation of subcellular fractions

Mononuclear leukocytes (MNL) were isolated from 60 ml heparinized fresh blood of patients or adult donors, using Ficoll-Paque gradient (Boyum, 1967). The samples were diluted in at least 3 volumes of PBS and centrifuged at 100×g for 10 min. The supernatant was removed and the lymphocytes were suspended in 1 ml ice-cold homogenizing buffer (25 mM Tris HCl, pH 7.4, 1 mM EGTA, 1 mM DTT and antiprotease cocktail 1:100 (Sigma)) followed by Polytron homogenization at 2500 rpm (PT 1200, Kinematica AG, Switzerland). The homogenate was centrifuged at 18,000×g for 20 min, 4 °C, to separate between the soluble and insoluble fractions. All fractions were kept at −70 °C until assayed by the quantitative measures.

2.3. Rat chronic treatment with antidepressant drugs

Male rats (Sprague–Dawley, 250 g) were chronically intragastrically treated for 21 days once daily with imipramine, moclobemide, venlafaxine, citalopram and maprotiline, 10 mg/kg/day (6 rats per drug treatment). Control rats were intragastrically treated with distilled water, used as vehicle. On day 22 of treatment, rats were decapitated and the brain cortices were immediately dissected and prepared for immunoblot analysis. All animal experiments were carried out according to NIH guidelines and had Institutional Review Board approval. All efforts were made to minimize animal suffering.

2.4. Rat brain cortex preparation

Rat brain cortex homogenization was carried out in a glass Teflon homogenizer with ice-cold buffer containing 25 mM Tris–Cl, pH 7.4, 2 mM EDTA, 1 mM DTT and antiprotease cocktail (1:100, Sigma). After initial centrifugation at 800×g for 5 min, the supernatant was collected and further centrifuged at 48,000×g for 30 min using a Beckman TI80 rotor. The resulting soluble and insoluble fractions were separated. The soluble fraction was collected and further centrifuged at 120,000×g for 45 min; the resulting supernatant was used for all measurements.
2.5. *Generation of antiserum against phosducin-like protein*

An antiserum against phosducin-like protein (PhdLP) was generated at Genemed Systems, Inc., by immunization of rabbits using the C-terminal sequence fragment PEREVHLEHTKIEE (265–279). A serum was collected after two boost injections. Similar results were obtained in a preliminary immunoblot analysis of human MNL and rat brain cortex carried out with this antibody and compared with an established PhdLP antibody (Humrich et al., 2003).

2.6. *Immunoblot analysis*

As PhdLP is essentially a cytosolic protein, only the soluble cellular fraction was used in all measurements. On the day of assay, 20 μg of total protein were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose filter. Blots were blocked by incubation with 5% milk for 1 h in Tris-buffered saline containing 0.1% Tween-20 (TTBS) and incubated overnight with antisera directed to PhdLP (dilution 1:500), followed by incubation with anti-rabbit IgG labeled with horseradish peroxidase in 2% milk. Immunoreactivity was detected with the ECL Western Blot Detection System (Amersham, Oakville, Ont., Canada) followed by exposure to X-ray film. Semi-quantitative analysis was carried on using a computerized image analysis system (B.I.S. 202D, Pharmacia).

2.7. *Statistical analyses*

Bonferroni’s *t*-test of multiple comparisons against a single control group was used for statistical comparisons of PhdLP levels in MNL of patients with major depressive disorder while untreated and after 4 weeks of treatment in comparison with healthy volunteers (two groups of 15 patients before and after treatment against a single control group of 15 subjects). A similar test was used for statistical comparisons of PhdLP levels in cerebral cortices between control rats and rats under chronic treatment with antidepressants (five groups of six animals each against a single control group of six animals). In tests where no statistically significant differences were observed, we evaluated the possibility of type II errors by determining the power of the test, calculating the non-centrality parameter and using power charts (Glantz, 1992).

3. *Results*

3.1. *Animal studies*

PhdLP levels were measured in brain cortices of rats chronically treated for 3 weeks with one of

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PhdLP (%)</th>
<th>S.D. (%)</th>
<th>T</th>
<th><em>P</em></th>
<th>Non-centrality parameter (ϕ)</th>
<th>Power of test</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100.0</td>
<td>8.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Imipramine</td>
<td>93.9</td>
<td>8.1</td>
<td>1.16</td>
<td>&gt;0.2</td>
<td>2.13</td>
<td>0.97</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>93.1</td>
<td>2.4</td>
<td>1.75</td>
<td>&gt;0.2</td>
<td>2.81</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Citalopram</td>
<td>99.8</td>
<td>10.4</td>
<td>0.06</td>
<td>&gt;0.5</td>
<td>1.86</td>
<td>0.91</td>
</tr>
<tr>
<td>Maprotiline</td>
<td>89.6</td>
<td>4.1</td>
<td>1.89</td>
<td>&gt;0.2</td>
<td>2.63</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Moclobemide</td>
<td>98.4</td>
<td>6.9</td>
<td>0.32</td>
<td>&gt;0.5</td>
<td>2.28</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Bonferroni’s *t*-test of multiple comparisons against a single control group was used for statistical comparisons of PhdLP levels in cerebral cortices between control rats and rats under chronic treatment with antidepressants. In tests where no statistically significant differences were observed, the non-centrality parameter (ϕ) was calculated and power charts were used to determine the power of the test and the possibility for type II errors.

* PhdLP levels are given as percent of level in control rats.
* Bonferroni’s *t*-test of multiple comparisons against a single control group (df=35 for each comparison).
* Power of test is calculated for detecting a 25% change at alpha=0.05 in the measured parameter among control rats and antidepressant-treated rats.
five types of antidepressants: the non-selective mono-
amine reuptake inhibitors imipramine and venlaf-
xine; the norepinephrine-specific reuptake inhibitor
maprotiline; the serotonin-specific reuptake inhibitor
citalopram; and the reversible MAO inhibitor moclo-
bemide. As shown in Table 1, none of the various
antidepressant treatments had any significant effect
on PhdLP level. As no statistically significant differ-
ences were observed, we evaluated the possibility of
type II errors by determining the power of the tests,
calculating the non-centrality parameter, and using
power charts (Glantz, 1992). The power of the test for
detecting a 25% change at alpha = 0.05 in the
measured parameter among control rats and antide-
presant-treated rats was found to be > 0.91 for all
antidepressant medications examined (Table 1).

3.2 Human studies

PhdLP levels were evaluated in MNL obtained
from a group of patients diagnosed with major
depressive episode, before the initiation of antidepres-
sant treatment and 4 weeks after initiation of the
treatment, and compared with a group of healthy
volunteers. No alteration was found in PhdLP level in
MNL of either untreated patients with major depres-
sive disorder (Ham-D > 20) (99.2%, S.D. = 5.46%,
t = 0.825, df = 42, N.S., Bonferroni’s t-test of multi-
ple comparison against a single control group) or after
4 weeks of the treatment (Ham-D < 8) (99.0%, S.D. =
6.55%, t = 0.824, df = 42, N.S., Bonferroni’s t-test)
in comparison with healthy volunteers (100.0%, S.D. =
5.91%) (Fig. 1). As no statistically significant differ-
ences were observed, we evaluated the possibility of
type II errors by determining the power of the tests,
calculating the non-centrality parameter, and
using power charts (Glantz, 1992). The power of the
test to detect a 10% change at alpha = 0.05 in the
measured parameter among healthy subjects,
untreated patients with depression (\(\phi = 2.27\)),
or antidepressant-treated patients (\(\phi = 2.06\)) was found
to be 0.95 and 0.90, respectively. The power of this test
for detecting a 20% change at alpha = 0.05 was > 0.99
in both cases.

4. Discussion

Growing evidence suggests that G proteins may be
involved in the pathogenesis of mood disorders and
in the mechanism of action of antidepressant and
anti-bipolar treatments (for review, see Avissar
and Schreiber, 2002; Schreiber and Avissar, 2003).
G protein signal transduction is regulated at various
points. A proximal regulatory point is receptor
coupling with G protein. A distal regulatory point
is GTPase activity. Both regulatory points have
been recently found to be of importance to the
mechanism of action of antidepressants and to the
pathophysiology of schizophrenia and mood disor-
ders. Almost all G protein-coupled receptors (GPCR)
are tightly regulated by a common desensitizing
mechanism that involves the activities of two
families of proteins—G protein-coupled receptor
kinases (GRKs), and arrestins. Receptor phosphoryla-
tion by GRKs has been ultimately identified as
the initial and critical step in the uncoupling of
receptor from G protein leading to the attenuation or
desensitization of GPCR signaling. Following phos-
phorylation by GRKs, GPCRs bind to a family of
soluble proteins named arrestins, which “arrest”
GPCR signaling. Arrestins bind to regions of GPCR
that are also primary determinants for G protein
interaction, thus uncoupling GPCR from G proteins.
(Lohse et al., 1990; Luttrell and Lefkowitz, 2002; Stephen and Lefkowitz, 2002). Both GRK and beta-arrestin proteins were found to be involved in the pathophysiology of mood disorders and/or the mechanism of action of antidepressants:

(a) Beta-arrestin-1 levels were recently shown to be significantly elevated by antidepressants of the SSRI, SNRI and NRI types in rat brain. This process became significant within a week and took 2–3 weeks to reach maximal increase. Mononuclear leukocytes of patients with depression presented significantly reduced immunoreactive quantities of beta-arrestin-1, the reduction in beta-arrestin-1 levels being significantly correlated with the severity of depressive symptomatology. These findings suggested a biochemical mechanism for antidepressant-induced receptor down-regulation (Avissar et al., 2004).

(b) A convergent functional genomic approach was used to identify a series of candidate genes involved in the pathogenesis of mood disorders, including G protein-coupled receptor kinase 3 (GRK3), which was also found to be decreased in lymphoblastoid cell lines from a subset of bipolar patients (Niculescu et al., 2000). More recently, a single nucleotide polymorphism (SNP) in the promoter region of GRK3 was found to be associated with bipolar disorder (Barrett et al., 2003). The findings concerning the GRK3 gene are in accord with evidence from a genome-wide linkage survey suggesting that the chromosome 22q12 region contains a susceptibility locus for bipolar disorder.

(c) Specimens of the prefrontal cortex were collected from suicide and non-suicide depressed subjects and control subjects. In drug-free, but not in antidepressant-treated, depressed subjects, an increase in the density of membrane-associated GRK 2/3 was found compared with that in sex-, age-, and PMD-matched controls. (García-Sevilla et al., 1999; Grange-Midroit et al., 2003). Acute treatment (1–6 h) with the tricyclic antidepressant desipramine, but not the SSRI fluoxetine, increased membrane-associated G protein-coupled receptor kinases 2/3 in rat brain. This effect vanished with a prolonged desipramine exposure (24 h). Chronic desipramine for 14 days did not significantly change the immunodensity of GRK 2/3 in the membrane or the cytosol (Miralles et al., 2002). Major depression was found to be associated with reduced platelet GRK 2, while treatment with mirtazapine reversed this abnormality (García-Sevilla et al., 2004).

Regulators of G protein signaling (RGS) proteins form a multifunctional signaling family. A key role of RGS proteins is modulating signaling through G protein pathways by attaching to GTP-bound G alpha proteins and shortening the duration of G protein signaling by acting primarily as GTPase-activating proteins (GAPs). Thus, they shorten the duration of intracellular signaling of many G-protein-coupled receptors (GPCRs) belonging to dopamine, GABA, glutamate and other neurotransmitter systems. At least 20 RGS family members have been identified. The transcript encoding RGS4 was the most consistently and significantly decreased in the postmortem prefrontal cortex of patients with schizophrenia compared with cortices obtained from subjects with no history of mental disorders and subjects with major depressive disorder (Mirmics et al., 2001). RGS4 maps to locus 1q21–22, a chromosome region strongly linked to schizophrenia (Brzustowics et al., 2000), suggesting it as a candidate for a schizophrenia susceptibility gene on this locus. Findings from association and linkage analyses of RGS4 polymorphisms in schizophrenia are in agreement with this suggestion (Chowdari et al., 2002).

The involvement of regulators of G protein function in the pathophysiology of mood disorders and in the mechanism of action of their treatments suggested the study of a third type of G-protein regulatory mechanism, namely, phosducin-like protein. In the present study, we assessed for the first time the possible involvement PhdLP in the pathophysiology and treatment of major depressive disorder. The present data indicate that PhdLP level is not altered in MNL of patients with major depression and that chronic antidepressant treatment with various types of antidepressant medications neither affects PhdLP level in MNL of patients nor in rat brain.
cortex. Thus, it seems that regulators of G protein function of the PhdLP type do not play a role in the molecular mechanisms underlying the pathophysiology of major depression and the therapeutic action of antidepressants.

Acknowledgements

We thank Dr. J. Humrich from the Institute of Pharmacology and Toxicology, University of Wurzburg, Germany for providing the phosducin-like protein antibody.

This study was supported in part by a research grant from the Yadgaroff Family Foundation.

S. Avissar is supported in part by a 2005 NARSAD Independent Investigator Award.

References


