Involvement of Na^+, K^+-ATPase and Endogenous Digitalis-Like Compounds in Depressive Disorders

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Background: *Sodium and potassium-activated adenosine triphosphatase (Na^+, K^+-ATPase) and endogenous digitalis-like compounds (DLC) in the brain have been implicated in the pathogenesis of mood disorders. This hypothesis was examined by the determination of Na^+, K^+-ATPase/DLC system in parietal cortex of patients with different mood disorders and two animal models of depression.*

Methods: *Na^+, K^+-ATPase concentrations in human brain synaptosomal fractions, from patients with mood disorders, schizophrenia, and normal individuals, were determined by ^{3}H-ouabain binding assay. Alpha isoforms were quantified by Western blotting. Brain DLC were measured using sensitive enzyme linked immunosorbant assay (ELISA). The effects of ouabain and ouabain-antibodies on behavior were determined in two animal models of depression.*

Results: *^{3}H-ouabain binding in bipolar patients was significantly lower than in major depressed and schizophrenic patients. Na^+, K^+-ATPase α isoforms in synaptosomal fractions were not different among the groups. DLC levels in the parietal cortex of bipolar patients were significantly higher than in normal individuals and depressed patients. Injection of lipopolysaccharide (intraperitoneally) to rats elicited depression-like symptoms, which were significantly attenuated by pre-injection of ouabain-antibodies. Injection of ouabain and ouabain-antibodies (intracerebroventricular) reduced depression-like symptoms in the forced swimming test in rats.*

Conclusions: *The results support the possibility that Na^+, K^+-ATPase and endogenous DLC participate in the pathogenesis of depressive disorders.*

Key Words: Digitalis-like compounds, ouabain, Na^+, K^+-ATPase, manic depression, mood disorder, LPS, forced swimming

The sodium and potassium-activated adenosine triphosphatase (Na^+, K^+-ATPase) is an enzyme present in the plasma membrane of all mammalian cells. The enzyme hydrolyzes ATP and uses the free energy to drive the transport of potassium into the cell and sodium out of the cell, against their electrochemical gradients (for a review, see Blanco and Mercer 1998; Scheiner-Bobis 2002). Na^+, K^+-ATPase is the major determinant of cytoplasmic Na^+ concentration. As such, it has an important role in regulating the cell volume and the cytoplasmic pH and Ca^{++} levels via the Na^+/H^+ and Na^+/Ca^{++} exchangers, respectively, and in driving a variety of secondary transport processes such as Na^+-dependent glucose and amino acids transport. Na^+, K^+-ATPase is an oligomer composed of stoichiometric amounts of two major polypeptides, namely the α- and the β-subunits (for a review, see Mobasheri et al 2000). A third protein, which belongs to the FXYD family, is associated with the Na^+, K^+-ATPase and is considered to play a regulatory role in its activity (Li et al 2004).

Since Na^+, K^+-ATPase was discovered, it has been established that plant steroids, collectively termed digitalis or cardiac glycosides, bind to a specific site on the enzyme and that this binding results in the inhibition of ATP hydrolysis and ion transport (Kelly and Smith 1996). In the past decade digitalis and digitalis-like compounds (DLC) have been identified in human tissue. Ouabain was identified in human plasma and the adrenal gland (Hamlyn et al 1991; Sich et al 1996), digoxin was shown to be present in human urine (Goto et al 1990), an ouabain isomer was identified in the bovine hypothalamus (Tymiak et al 1993), 19-norbufalin and its peptide derivative were identified in cataractous human lenses (Lichtstein et al 1993), dihydropyrone-substituted bufadienolide was identified in human placenta (Hilton et al 1996), and a marinobufogenin-like compound was identified in human plasma (Fedorova et al 2002). A growing body of evidence strongly supports the notion that the DLC are synthesized in, and released from, the adrenal gland (Laredo et al 1994; Lichtstein et al 1998; Hamlyn 2004). The DLC thus represent a new family of steroidal hormones, the function of which is only starting to emerge (for a review see Lichtstein and Rosen 2001; Schoner et al 2003; Bagrov and Fedorova 2005).

Accumulating evidence has suggested that brain Na^+, K^+-ATPase activity may be involved in the etiology of mental disorders. In particular, bipolar mood disorder has consistently been associated with abnormalities in the Na^+, K^+-ATPase activity in erythrocytes (Nurnberger et al 1982; Coppen et al 1966; Shaw 1966; Naylor et al 1971; El-Mallakh and Wyatt 1995; Looney and El-Mallakh 1997). Furthermore, an allelic association between bipolar mood disorder and a specific polymorphism within the gene encoding the α-3 isoform of the α subunit of Na^+, K^+-ATPase has been discovered (Mynett-Johnson et al 1998). It was suggested that the reduced Na^+, K^+-ATPase activity may result from a lower level of α2 isoform of Na^+, K^+-ATPase in the brain (Rose et al 1998). Furthermore, the levels of endogenous digoxin-like immunoreactive compound were found to be lower in manic bipolar patients compared to normal controls (Grider et al 1999). In addition, an intracerebroventricular (intracerebroventricular [ICV]) injection of ouabain induced mania-like symptoms (El-Mallakh et al 2003; Machado-Vieira et al 2004), this effect was prevented by the most common anti manic-depressive drug Lithium (Li et al 1997). Consequently, a comprehensive hypothesis was put forward, linking DLC levels, Na^+, K^+-ATPase activity, and human brain neurotransmission with bipolar mood disorder (Christo and El-Mallakh 1993; Traub and Lichtstein 2000).

In the present study we addressed the hypothesis that Na^+, K^+-ATPase and its hormonal regulators, DLC, are involved in mood disorders. To this end, we measured Na^+, K^+-ATPase

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concentrations and its three α isoforms (α1, α2, and α3) as well as endogenous DLC in samples taken from the parietal cortex of patients with different mood disorders. The involvement of the parietal cortex in depression was attributed to its strong limbic and paralimbic connections, conveyed by input from several thalamic nuclei, such as the central lateral nucleus (Olausson et al 1989) and the nucleus ventralis posterior lateralis pars caudalis (Matsuzaki et al 2004). In addition, the effect of perturbing the Na^+, K^+-ATPase-DLC system, by treatment with ouabain or ouabain-antibodies, on the behavior and plasma and adrenal DLC levels were assessed in rats subjected to either lipopolysaccharide (LPS) treatment or to the forced swimming test, two animal models of depression.

Methods and Materials

Materials

All compounds including LPS, ouabain, and bufalin were purchased from Sigma-Aldrich (St. Louis, Missouri). Sep-pak, C18 columns were purchased from Waters (Milford, Massachusetts). Mouse monoclonal antibodies to the α_1-subunit of Na^+, K^+-ATPase were purchased from Upstate Biotech (Lake Placid, New York), mouse monoclonal antibodies to the α_3-subunit of Na^+, K^+-ATPase were from Sigma Aldrich, and rabbit polyclonal antibodies to the α_2-subunit of Na^+, K^+-ATPase was kindly provided by Thomas Pressley (Texas Tech University, Lubbock, Texas).

Human Tissue Samples

Freshly frozen parietal cortex tissues were obtained from the neuropathology consortium of the Stanley brain collection (Stanley Medical Research Institute, Bethesda, Maryland). The demographic and clinical characteristics of the population, as well as methods of tissue harvest, preparation, and storage have been previously described in detail (Torrey et al 2000). Briefly, subjects were diagnosed as follows: no psychiatric illness, schizophrenia, bipolar illness, or depression (n = 15 per group) and matched for age, gender, and postmortem interval (Table 1). Two senior psychiatrists independently made psychiatric diagnoses (DSM-IV) on the basis of medical records and telephone interviews with family members. The Stanley brain collection is well-matched for age, pH, sex, race, side of the brain, and postmortem interval, the details of which have been previously described (Torrey et al 2000). The ethics approval for the brain collection is held by the Stanley Medical Research Institute. Data in Table 1 outline the postmortem characteristics of the four subject groups.

The four subject groups were matched for several clinical variables. According to analysis of variance (ANOVA), the groups did not differ in terms of age or postmortem interval. Chi square analysis indicated that the groups did not differ in terms of gender (Table 1). Determination of DLC levels was performed on all the samples (15 in each group) and their identity was revealed by the Stanley Institute after the results were analyzed. The ^{3}H-ouabain binding assays and Western blot analyses were performed (8 patients from each group) after the identity of the patients was known to us, but in all the assays, the tissue samples were analyzed in parallel by a researcher who was blind to the subjects' diagnosis.

Preparation of Human Brain Synaptosomes

Crude synaptosomal membrane was prepared from the human parietal cortex samples by homogenizing the tissue in 10 volumes (w/v) of ice-cold .32 M sucrose using a Teflon-glass homogenizer (Zivan, Haifa, Israel). The homogenate was centrifuged for 10 min at 1000 g at 4°C. The supernatant was decanted and centrifuged at 28500 g for 10 min at 4°C. The pellet was re-suspended in the sucrose solution and centrifuged again under the same conditions. The resulting pellet was re-suspended in 50 mM Tris buffer, pH 7.4, and dispersed using the glass-glass homogenizer. The preparation was diluted to a final protein concentration of 5–10 mg/ml and was stored at −70 °C in aliquots of 1 ml.

^{3}H-Ouabain Binding to Synaptosomal Fraction. Two-hundred μl of synaptosomes (10 μg protein) were incubated for 1 hour at 37°C with 300 μl of a solution containing final concentrations of 30 mM Tris-HCl buffer, pH 7.4, .2 mM EDTA, 80 mM NaCl, 4 mM $MgSO_4$, 2 mM ATP (Tris salt, vanadium-free), and 2.6 nM ^{3}H-ouabain in the presence or absence of 100 μM nonradioactive ouabain. The reactions were terminated by the addition of 3 ml ice-cold 50 mM Tris-HCI, pH 7.4, followed by passage over Whatman GF/B filters (Whatman International, Ltd. Maidstone, United Kingdom). The filters were washed twice with 3 ml Tris buffer, dried, and counted in a liquid scintillation counter. Specific binding was calculated by subtracting the binding observed in the presence of 100 μM unlabeled ouabain from that observed in the absence of unlabeled ouabain, which represented 94% of the total binding.

Western Blot Analysis

Protein samples were solubilized in loading buffer (sodium dodecyl sulfate [SDS] ×6) containing 375 mM Tris, pH 6.8, 12% (w/v) SDS, 60% (v/v) glycerol, 1% 2-β-mercaptoethanol (v/v), .1% (w/v) and bromphenol blue, and incubated at 65° C for 3 min. SDS-PAGE was used to separate proteins on 7.5% gel. Proteins were loaded onto the gel (8 μg/20 μl) and subjected to electrophoresis for 1–1.5 h at 100 V with protein standards (Color Marker, Sigma Aldrich) and then transferred to polyvinylidene fluoride membrane (PVDF Immubilon P, .45 μm, Millipore, Billerica Massachusetts). The presence of transferred proteins on the blot and their equal quantity in all lines was confirmed by staining with Ponceau S (Sigma Diagnostics, St. Louis, Missouri). After

Table 1. Postmortem Characteristics of 60 Brain Specimens from Subjects with Major Depressive Disorder, Bipolar Disorder, Schizophrenia, and No Psychopathology

	Control n = 15	Schizophrenia n = 15	Bipolar n = 15	Depression n = 15
Age of Death (years, Mean ± SD)	48.1 ± 10.7	44.5 ± 13.1	42.3 ± 11.7	46.5 ± 9.3
Age of Onset of the Disease (years, Mean ± SD)	—	23.2 ± 8	21.5 ± 8.3	33.9 ± 13.3
Gender (male/female)	9/6	9/6	9/6	9/6
Ethnicity	14C, 1AA	13C, 2AA	14C, 1AA	15C
PostMortem Interval (hours, Mean ± SD)	23.7 (9.9)	33.7 (14.6)	32.4 (15.9)	27.5 (10.7)

A, Asian; AA, African-American; C, Caucasian.

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