

Reduced Activity of Red Cell $\text{Na}^+ \text{K}^+$ ATPase and Ca^{2+} ATPase in Patients with Idiopathic Generalized Epilepsy

K. Arundhati¹, S. Mohan Das² and T. Padma³

1. Department of Anatomy (Genetics), AIIMS, New Delhi, India

2. Department of Genetics, Osmania University, Hyderabad, India

3. Department of Neurology, Nizam's Institute of Medical Sciences (NIMS), Hyderabad, India

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ABSTRACT Evidence is now emerging that certain epilepsies may be a family of channelopathies with defects involving mutations in the Na^+ , K^+ , or Ca^{2+} channels whose activities are related to their voltage dependent conditions, or defects in the membrane-bound enzymes $\text{Na}^+ \text{K}^+$ ATPase and Ca^{2+} ATPase that regulate the transport of ions across the cell membrane. In this study we have tried to determine the role of the membrane bound enzymes, sodium-potassium and calcium ATPase in the aetiopathogenesis of idiopathic generalized epilepsies (IGE). We studied 143 cases for sodium-potassium ATPase and 109 cases for calcium ATPase enzymes in comparison to 80 matched controls. Citrated blood samples were collected from these cases and red cells separated from the plasma. Erythrocyte membranes were prepared and tested for activity of both the enzymes. We found significantly lowered activities of both the enzymes in IGE cases as compared to control samples. The familial cases (cases with positive family history) had significantly lower activities of the enzymes than non-familial cases, as did females as compared to males. Our data indicates that genetic defects in the active transport mechanisms like $\text{Na}^+ \text{K}^+$ ATPase and Ca^{2+} ATPase enzymes are associated with human idiopathic epilepsy. These findings add to the growing list of channelopathies in humans and suggest that drugs that directly or indirectly modulate K^+ , Na^+ and Ca^{2+} ion channels will be helpful in the treatment of seizure disorders.

INTRODUCTION

Epilepsy is a heterogeneous disorder characterized by recurrent seizures that affect 3.0% of the world's population (Schewer and Pedley 1990). Seizures may develop as a consequence of structural brain damage or altered metabolic states. When recurrent unprovoked seizures arise without any detectable structural brain lesion, and without any

evident neurological abnormality between seizures, the condition is said to be idiopathic epilepsy. This can be subdivided into idiopathic generalized epilepsy (IGE) and idiopathic partial epilepsy. IGE comprises of several specific syndromes, including generalized tonic-clonic seizures (GTCS), childhood absence epilepsy (CAE) and juvenile myoclonic epilepsy (JME) (Zara et al. 1995).

Most progress in locating epilepsy genes has been made in IGE. They are a heterogeneous group suitable for genetic studies because they are common (accounting for about 40% of epilepsy up to age 40 years) and have a high concordance rate in monozygotic twins (95%), suggesting an almost complete genetic aetiology. The EPMI gene for Unverricht-Lundborg disease maps to 21q (Jain et al. 1997), the gene for northern epilepsy syndrome maps to 8p (Tahvanainen et al. 1994) and the gene for autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) maps to 20q13.2 (Phillips et al. 1995). A claim for linkage of the EJM1 gene for the common generalized syndrome of juvenile myoclonic epilepsy (JME) to 6p is in dispute (Greenberg et al. 1988; Whitehouse et al. 1993). The two genes for benign familial neonatal convulsions (BFNC) with an autosomal dominant mode of inheritance, EBN1 and EBN2, map to chromosomes 20q13 and 8q24 (Leppert et al. 1989; Lewis et al. 1993).

Until recently the molecular basis of the common idiopathic epilepsies has been obscure. It is now emerging that idiopathic epilepsy may be a family of channelopathies. The first genetic defect to be identified in idiopathic epilepsy was in the $\alpha 4$ subunit (CHRNA4) of the neuronal nicotinic acetylcholine receptor, a ligand-gated ion channel. Recently, two voltage-gated potassium channel genes (KCNQ2 and KCNQ3) have been implicated in benign familial neonatal convulsions (Charlier et al. 1998; Singh et al. 1998; Steinlein 2002).

Corresponding Author: Dr. Arundhati Sharma, Department of Anatomy (Genetics), All India Institute of Medical Sciences (AIIMS), Ansari Nagar, New Delhi 110 029, India

Phone: 6593489, 6593216

Fax: 91-11-6862663, 6521041

E-mail: arundhati_sharma@yahoo.com

In tottering and lethargic mice, mutations have been identified in the pore forming α and the regulatory β subunits of voltage gated calcium channels (Burgess et al. 1997). Another study (Wallace et al. 1998) shows mutations of the voltage-gated Na^+ channels as candidates for typical febrile seizures, generalized epilepsy with febrile seizures (GEFS+) and related idiopathic generalized epilepsies.

Epilepsy is one of the common diseases found in India. Except for small epidemiological assessments no comparable data exists concerning patients with epilepsy in our country (Jain et al. 1998). In view of the high rate of morbidity, we undertook our study to evaluate the contributions of some genetic, epidemiological and biochemical factors to the aetiopatho-genesis of IGEs. Current evidence indicates that idiopathic epilepsies may be a family with defects involving the K^+ , Na^+ or Ca^{2+} channels or defects in the enzymes $\text{Na}^+ \text{K}^+ \text{ATPase}$ or $\text{Ca}^{2+} \text{ATPase}$ (EC - 3.6.1.3), which are responsible for maintaining the ionic balance in and outside the cell. In this present study we report on the activities of two membrane bound enzymes, $\text{Na}^+ \text{K}^+ \text{ATPase}$ and $\text{Ca}^{2+} \text{ATPase}$ in the red-cell membranes of IGE cases. We have not studied the role of any of the ion channels.

PATIENTS AND METHODS

The subjects were cases with confirmed diagnosis of IGE obtained from the Nizam's Institute of Medical Sciences (NIMS) Hyderabad. Cases presenting with different forms of IGE were studied and epilepsy was defined as a lifetime history of (≥ 2) recurrent seizures. Subjects with epilepsy which was precipitated by head injury, brain lesion, high fever or structural or metabolic disturbances to the CNS were not included.

All the cases were diagnosed and classified according to classification given by International League Against Epilepsy (1989). Diagnosis was based on a detailed clinical history and electroencephalogram (EEG). Information on the clinical, genetic and epidemiological factors was personally collected with the help of a standard questionnaire devised by us. The nature of the work to be carried out was explained to the patients and the required information collected with their consent in agreement with the Helsinki Declaration.

The control group consisted of healthy voluntary donors matched for age and sex with the patient group, who did not have epilepsy or a positive family history for the condition. We studied 143 IGE cases for $\text{Na}^+ \text{K}^+ \text{ATPase}$ and 109 cases for $\text{Ca}^{2+} \text{ATPase}$ activity in comparison to 80 control cases.

2ml of venous blood was drawn from all the patients and control group and collected in 3.1% trisodium citrate. Blood samples, which were haemolysed, were discarded.

The red cells obtained after separation of plasma were washed three times in chilled saline. Erythrocyte membranes were prepared by lysing the red cells in chilled autoclaved distilled water to obtain erythrocyte hemoglobin-poor ghosts in a manner similar to that described by Nakao et al. (1976) for the isolation of red-cell membranes. The haemolysate obtained was centrifuged at 14,000rpm for 10 min. at 4 degrees centigrade. The membranes were washed with chilled double distilled water till a clear suspension of the membranes was obtained and an aliquot from each red-cell membrane or enzyme preparation was tested for activity of both the enzymes.

$\text{Na}^+ \text{K}^+ \text{ATPase}$ activity was estimated by the method of Nakao et al. (1976) in which 0.2ml of the erythrocyte membrane or enzyme preparation was incubated with 1.0ml of the reaction mixture containing NaCl :140 mM, KCl :14 mM, MgCl_2 : 3 mM, Tris-HCl buffer (pH 7.4) $\text{Na}_2 \text{ATP}$:30 mM (Sigma). Two controls were kept of which one was a blank specimen which contained the heat-inactivated enzyme/membrane preparation (boiled for 30 min. in water bath). The second had the inhibitor of $\text{Na}^+ \text{K}^+ \text{ATPase}$, Ouabain at a concentration of 10 μM . The samples were incubated for 2hrs in a 37°C water bath and reaction was terminated by adding 2ml of 10% (w/v) trichloroacetic acid.

$\text{Ca}^{2+} \text{ATPase}$ activity in red cells was determined by the method of Shami and Radde (1974). The procedure followed was essentially the same except that the enzyme preparation was incubated in 1.0 ml of reaction mix containing CaCl_2 :5 mM, Tris-HCl:20 mM (pH 8.2), NaCl :10 mM and $\text{Na}_2 \text{ATP}$: 5 mM. Ethacrynic acid, the inhibitor of $\text{Ca}^{2+} \text{ATPase}$ was added at a concentration of 5 mM (Sigma).

The rate of ATP hydrolysis was determined by measuring the amount of Pi released from the samples per unit time. Inorganic phosphate

content was measured by the method of Fiske and Subba Row (1965) and total protein by the method of Lowry et al. (1951). The enzyme activity was calculated from a standard curve of potassium dihydrogen phosphate using the formula,

$$\frac{\text{Concentration of Pi standard}}{\text{OD value of standard}} \times \frac{\text{OD of test sample}}{\text{concentration of protein}}$$

Difference in the activity of enzyme in the absence and presence of their respective inhibitors was taken as the enzyme activity and results expressed as moles of Pi liberated per hour per mg of protein.

Mean enzyme activity was calculated for the different groups and tested for its significance using 't-test'

RESULTS

IGE cases in general had significantly lower activity of $\text{Na}^+ \text{K}^+$ ATPase (0.114 ± 0.01 ; Mean $\pm$ SE) than control samples (0.233 ± 0.04 ; $P < 0.01$; Table 1). Females showed lowered activity of the enzyme (0.082 ± 0.01) than males (0.125 ± 0.02), which was significant at 10% level. Familial cases showed slightly decreased levels of the enzyme when compared to the non-familial cases, which however was not statistically significant, but it may be noted that reduced activity of the enzyme in the familial females was significant (0.059 ± 0.003) as compared to males (0.126 ± 0.02 ; $P < 0.05$).

In general reduced activity of Ca^{2+} ATPase

was seen in IGE cases (0.055 ± 0.004) as compared to controls (0.072 ± 0.01).

Familial cases showed a decrease in the activity of the enzyme (0.047 ± 0.01) than non-familial cases (0.063 ± 0.01 ; Table). Females showed lowered activity (0.046 ± 0.01) than males (0.062 ± 0.01).

The results obtained show a trend of lowered activities of both the enzymes in epilepsy, particularly in the familial cases and in females of both familial and non-familial cases. However, the differences observed were significant only in some of the groups suggesting the need to study larger number of cases, thereby increasing the sample size, which would confirm the differences yielding statistically significant results.

DISCUSSION

Sodium potassium ATPase and Calcium ATPase are membrane bound enzymes and play a pivotal role in the homeostasis of $\text{Na}^+ \text{K}^+$ and Ca^{2+} in cells. Apart from these active transport mechanisms, ionic balance is also affected by the activities of sodium and potassium channels whose activity (open or closed condition) is voltage dependent.

Among the idiopathic generalized epilepsies BFNC is the first human IGE in which mutations in the voltage gated $\beta 1$ subunit gene of the K^+ and Na^+ channels has been implicated (Keating and Sanguinetti 1996). Therefore an alteration in a gene that directly regulates neuronal excitability

Table 1: Activity of red-cell $\text{Na}^+ \text{K}^+$ ATPase and Ca^{2+} ATPase enzyme in patients with IGE

	N	$\text{Na}^+ \text{K}^+$ ATPase		N	Ca^{2+} ATPase	
		Mean	Standard Error (SE)		Mean	Standard Error (SE)
<i>Control Sample</i>	80	0.233	0.04	80	0.072	0.01
<i>IGE</i>						
Males	91	0.125	0.02	63	0.062	0.01
Females	52	0.082 ⁺	0.01	46	0.046	0.01
Total	143	0.114**	0.01	109	0.055	0.004
<i>Familial IGE</i>						
Males	44	0.126	0.02	34	0.046	0.01
Females	22	0.059*	0.003	18	0.048	0.01
Total	66	0.082	0.01	52	0.047 ⁺	0.01
<i>Non-familial IGE</i>						
Males	47	0.119	0.02	29	0.081	0.01
Females	30	0.106	0.02	28	0.044	0.01
Total	77	0.113	0.02	57	0.063	0.01

** p < 0.01; * p < 0.05; + p < 0.1

could produce an epileptic disorder of the kind of BFNC. Voltage - gated potassium channels repolarize neuronal membranes that have been depolarized by Na^+ and Ca^{2+} voltage gated ion channels. K^+ channels are also thought to repolarize neuronal membranes after activation of excitatory neurotransmitter ion channels, including glutamate and acetylcholine. In the presence of voltage-gated potassium channel gene (KCNQ2) channels with reduced function excitatory ligand and voltage gated channels that are activated would remain open longer (Keating and Sanguinetti 1996). Such unchecked activity of excitatory systems could lead to an epileptic phenotype. Additional studies using brain slices and whole animal models have implicated altered K^+ regulation as having a causal role in epilepsy.

The decrease in the activity of the enzymes $\text{Na}^+ \text{K}^+ \text{ATPase}$ and $\text{Ca}^{2+} \text{ATPase}$ observed in this study is similar to the results seen in the case of animal models also. Rapport et al. (1981) have found reduced activity of $\text{Na}^+ \text{K}^+ \text{ATPase}$ in human and monkey epileptic brain. Trams and Lauter (1978) found a deficiency of ecto - $\text{Ca}^{2+} \text{ATPase}$ in seizure prone mice. Defects in transport of Na^+ and K^+ have been implicated in epilepsy and this may be due to reduced clearance of extracellular K^+ released from depolarized neurons. The defect could be in the passive mechanisms or active transport mechanisms like $\text{Na}^+ \text{K}^+ \text{ATPase}$ enzyme itself which could modify the excitability levels of cells by affecting Ca^{2+} transport and thereby the $\text{Ca}^{2+} \text{ATPase}$ activity. Lowered enzyme activity of $\text{Na}^+ \text{K}^+ \text{ATPase}$ and $\text{Ca}^{2+} \text{ATPase}$ in epilepsy might be due to the above-mentioned defects apart from a possible defect in the cell membrane. In the present study we have correlated the reduced activity of red cell $\text{Na}^+ \text{K}^+ \text{ATPase}$ and $\text{Ca}^{2+} \text{ATPase}$ to IGE. Such a correlation explains the pathological condition but not the genetic defects. The phenotypic appearance of any enzymatic activity may be due to underlying genetic defects or related to its enhancers and inhibitors. Recent studies are focussing on the genetic aspects of these defects. From a study by Buono et al. (2000) on murine and human epilepsy emerged a prominent candidate gene, the ATP1A2 which encodes an alpha subunit of the $\text{Na}^+ \text{K}^+$ pump that documents a key role for genes coding for proteins involved in ion homeostasis.

The familial cases (cases with a positive family history) having a greater genetic component may be inheriting these defects along with epilepsy and hence may be showing reduced activity of the enzymes as compared to the non-familial cases. Reasons for differences observed in the activity of males and females are not clear. Our study of IGE cases has revealed a significantly high frequency of affected males as compared to females (Arundhati et al. 1994). One of the reasons for this difference could be due to the segregation of some sex-linked lethal or semi-lethal in females along with the disease condition because of which the number of affected females is low, but once the females are affected the condition may turn more severe, and hence the lowered activity of the enzymes in them as compared to males. Possibility of genomic imprinting cannot be ruled out in epilepsy as suggested earlier (Arundhati et al. 1994).

In conclusion, it may be said that genetic defects in the active transport mechanisms like $\text{Na}^+ \text{K}^+ \text{ATPase}$ and $\text{Ca}^{2+} \text{ATPase}$ enzymes themselves are associated with human idiopathic epilepsy. These findings add to the growing list of channelopathies in humans (Ptacek, 1997) and suggest that drugs that directly or indirectly modulate the activities of the ATPases will be helpful in the treatment of seizure disorders.

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