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ALTERED PH MODULATES THE INTERACTION OF DIVALENT METALS WITH NA^+/K^+- ATPASE IN BRAIN TISSUE IN VITRO

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ABSTRACT
The active transport of Na^+ and K^+ across the brain cells by the membrane-bound enzyme Na^+/K^+-activated ATPase has a major role in the maintenance of brain water and electrolyte homeostasis. The mechanisms of inhibition of rat brain Na^+/K^+-ATPase by mercuric chloride ($HgCl_2$) were studied in vitro by assessing the effects of pH changes on the activity and interaction of heavy metal with this enzyme. While the pH change positively modulates the activity of the enzyme, the heavy metal significantly inhibited the overall Na^+/K^+-ATPase independent of pH fluctuation. Since mercury exert its toxic effect through interaction with thiol group on the enzyme, susceptibility of the enzyme to heavy metal inhibition (independent of pH) may indicate that sulfhydryl (-SH) group of the enzyme is located in the region not affected by pH fluctuation.

Key words: Na+/K+-ATPase, pH, sulfhydryl (-SH) group, mercury

INTRODUCTION
The activity of Na^+/K^+-ATPase is subjected to various factors including certain divalent metals and organic compound of toxicological interest as well as some drugs [1-4]. Organoselenide has been reported to inhibit the sodium pump via a mechanism that possibly involves the modification of the thiol group at the ATP binding site of the enzyme [5]. It was further observed that Na^+/K^+-ATPase is a sulfhydryl protein with its critical thiol located in the ATP-binding site [4]. Na^+/K^+-ATPase can be observed as SH-donor ligand since the enzyme has 36 SH groups which are held responsible for interactions of this enzyme with various metal ions [7]. Omotayo et al., 2011 further suggested that the inorganic mercury interact at the nucleotide and cationic site of the cerebral Na+/K+ ATPase and that the interaction involves the thiol group. Globally there were discussions of the link between function of an enzyme and its structural conformation (protein folding). There were reports on the identification, the role and conformational coupling in energy transduction of putative Mg^{2+} binding site in P-type ATPases [8-9]. The structural insight from metal catalyzed protein cleavage and short term regulation of the renal Na^+/K^+-ATPase revealed that several reactive states of Na^+/K^+-ATPase could be distinguished by kinetic measurements [10, 11], but these measurements did not provide information about the underlying changes in protein structure. Ligands induce structural differences in the α-subunit (molecular weight, about 100,000) of the enzyme and have been detected by digesting the purified enzyme with trypsin or chymotrypsin [12, 13]. Since different sensitive bonds were exposed and others deformed as the conformation changes, it becomes imperative to unravel the effect of these inducers on the overall activity and the consequent effect on the interactions of some endogenous factors such as pH fluctuation on this protein. That pH changes are associated with certain disease states is known, certain disease conditions, such as diabetes, sickle cell anemia and asthma that are associated with acidic blood in steady state alter the normal pH of the cell [14,15]. However, despite extensive literature on activity of Na+/K+ ATPase in cerebral tissue [16], few studies have investigated the mercury- binding property of this enzyme

under varying pH. Instead most of the studies were carried out under physiological pH (7.4). However pH has been widely postulated as one of the factors influencing conformational state of protein which concomitantly affects their functional and binding properties. So what happens to cerebral Na^+/K^+-ATPase during this time of pH fluctuation?. Mercury has been reported toxic to the living system and exert its toxicity by inhibiting key metabolic enzymes especially the thiol containing enzymes [1]. Can alteration in systemic pH relieve the enzyme of mercury poisoning? This study, therefore, investigate the effect of pH change on cerebral Na^+/K^+-ATPase activity and in the interaction of inorganic mercury with cerebral Na^+/K^+-ATPase.

MATERIALS AND METHODS.

Adenosine triphosphate (ATP), Ouabain and cysteine were obtained from Sigma –Aldrich, All other chemicals used were of analytical grade and obtained from FLUKA, BDH and other standard chemical suppliers.

ANIMALS

Male adults Wistar rats (200-250g) were used. The animals were used according to standard guidelines on the Care and Use of Experimental Animal Resources [17].

OXIDATION OF THIOLS

The rate of thiol oxidation was determined in the presence 50mM Tris HCl, pH 3.4 and 50-150µM of inorganic mercury. The rate of thiol oxidation was evaluated by measuring the disappearance of -SH groups. Free SH groups were determined according to Ellman, 1959. Incubation at 37^0C was initiated by the addition of the thiol compound. Aliquots of the reaction mixture (100µl) were checked for the amount of –SH groups at 412nm after 90-120 min of addition of color reagent 5'5'-dithio-bis (2-nitrobenzoic) acid (DTNB). This procedure was repeated with pHs 5.4, 7.4, 9.4 and 11.4

EFFECT OF Hg^{2+} ON Na^+/K^+ ATPase ACTIVITY

Immediately after the sacrifice, the brain was removed and the homogenate was prepared in 50mM Tris-HCl, pH 3.4. The homogenate was centrifuged at 4000rpm at 4^oC for 7 min and the supernatant was used for assay of Na^+/K^+-ATPase. The reaction mixture for Mg^{2+}-dependent- Na^+/K^+-ATPase assay contained 3mM MgCl, 125mM NaCl, 20mM KCl and 50mM Tris-HCl, pH 3.4 and 100-120µg of protein incubated with 50µM Hg^{2+} in a final volume of 500µl. The reaction was initiated by addition of ATP to a final concentration of 3.0mM. Incubation time was 10min at 37^oC. Controls were carried out under the same condition with addition of 0.1mM Ouabain. Na^+/K^+ ATPase activity was calculated by the difference between the two assays. Released inorganic phosphorus (Pi) was measured by the method of Fiske and Subbarow (1925) [18]. The procedure was repeated using pH 5.4, 7.4, 9.4 and 11.4

RESULTS

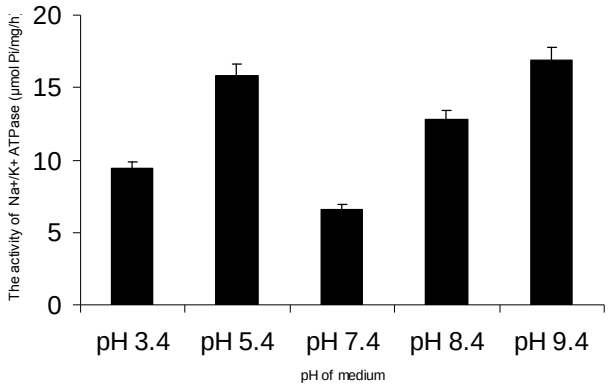

Fig 1. Effect of alterations in pH on the activity of brain Na^+/K^+-ATPase. Data are presented as mean±S.E. *$p < 0.05$ as compared with control (N= 6).

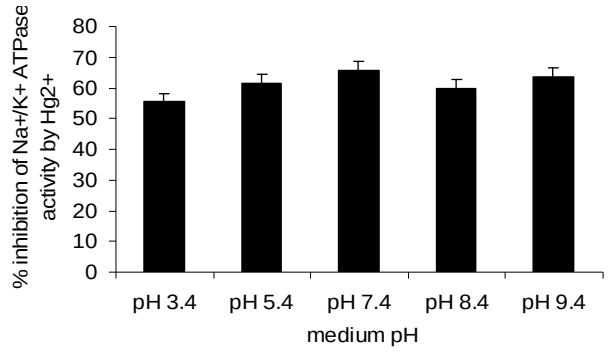

Fig 2. Effect of alterations in medium pH on the interaction of Hg^{2+} with brain Na^+/K^+ ATPase. Data

are presented as mean±S.E. *$p < 0.05$ as compared with control (N= 6).

Assay of Na^+/K^+-ATPase activity

In the enzymatic assay of the Na^+/K^+-ATPase activity of brain tissue under varying pH, it was found that Na^+/K^+-ATPase activity was significantly modulated (Fig. 1). Change in medium pH significantly increased the brain Na^+/K^+-ATPase activity at the pH 3.4, 5.4, 7.4, 8.4 and 9.4. Maximal increase of Na^+/K^+-ATPase activities compare with physiological pH 7.4 was 67.32% and 56.42%, at pH 5.4 and 9.4 respectively. Thus the enzyme has an optimum activity not even at physiological pH as might be expected but at pH 5.4 and 9.4; At pH 9.4 we observed that the enzyme was more active, exhibiting activity that is almost 56% higher than in the physiological pH 7.4 and at pH 5.4 the activity was 67% higher than control (pH 7.4), however the pH change could not reverse the binding/toxicity of mercury on the enzymes at all the pH values tested (fig 2). At all the pH tested the enzyme was significantly inhibited by mercury.

DISCUSSION

An interesting study is the alteration of pH to study the underlying functional implication of the transitions in the protein structure. It has also become feasible to examine whether the protein conformations induced by change in medium pH concomitantly affect the biological activity and binding property of enzymes. The modulation of Na^+/K^+-ATPase activity has been implicated in several pathophysiologic processes, including cardiovascular, renal, neuronal, and metabolic disorders; having in common a dysfunction in salt and water homeostasis [19], and this enzyme is very well expressed in these organs [20]. Figure 1 shows the effect of varying pH on the activity of the protein. In acidic medium, it would be expected for the enzyme's thiol group to get oxidized by the electrophilic medium resulting to loss of activity. In contrary the enzyme exhibited a high positive sensitivity to variation in both acidic and alkaline pH which suggests its significant presence in cells active in ionic regulation. It could be argued that the medium has limited access to the thiol group which could explain the stability of the SH group independent of the acidity of the medium. And this could possibly happen when the thiol group is located in the hydrophobic portion of the enzyme fully insulated from the aqueous medium. Nevertheless, considering that chemicals which have pH-dependent solubility will distribute throughout body fluids during certain condition. Patients with these conditions have been found to commonly have abnormal enzymatic processes that affect the sodium-potassium ATPase energy channels [22], which appears to be a major factor in the condition and for which mercury is a known cause [23,24]. This also has been found to result in inflammatory processes that cause muscle tissue damage and result in higher levels of urinary excretion of creatine, choline, and glycine [25,26]. Results of our studies do not permit the elucidation of the precise molecular mechanism through which pH increased the Na^+/K^+-ATPase activity however, we can suggest due to observations stated above that the sensitivity of the enzyme to pH may not involve the thiol group. Considering our results we may consider that pH -change favours the decrease of individual blood pressure level. Blood pressure levels are

controlled by complex combination of processes that influence cardiac output and peripheral vascular resistance [27] by cerebral cells. Since Na^+/K^+-ATPase contributes to myocardial contractility and vascular smooth muscle flexibility by modulation of Na+ intracellular level, changes on Na^+/K^+-ATPase activity may be associated with the regulation of individual blood pressure level [28,29]. Several studies have suggested and supported the involvement of Na^+/K^+-ATPase on the pathogenesis of hypertension, showing a suppression of the enzyme expression in different animal models of experimental hypertension, as compared to the normotensive controls. Moreover, the treatment with certain antihypertensive drugs that reduce the blood pressure to normal level reversed the referred suppression of its expression, which seems to be pressure-sensitive [30]. Alteration in pH had no effect on interaction of mercury with cerebral Na^+/K^+-ATPase activity but produced an increase of *in vitro* Na^+/K^+-ATPase activity of brain, these findings suggest as earlier stated that pH may be inductor of Na^+/K^+-ATPase.

The amount of chemical that reaches the target cell has been reported to depend on its physicochemical and pharmacokinetic proprieties affecting rate of penetration, transport mechanism from the administration site to the affected cells, and biotransformation in metabolites more active, less active, inactive or with different kind of activity [12,31]. Mercury (especially mercury vapor) rapidly crosses the blood-brain barrier and is stored preferentially in the pituitary gland, hypothalamus, thyroid gland, adrenal gland, and occipital cortex in direct proportion to the number and extent of amalgam surfaces. The observation that chronic [32,10,5] or nanomolar [33] administration of cardiac glycosides reversed the inhibition of Na^+/K^+-ATPase and increases its *in vivo* activity has already been reported by several authors, as well, this effect is associated with an increase in enzyme expression at certain experimental conditions. However, considering that Na^+/K^+-ATPase activity in the cerebral tissue was stimulated by altering the pH when compared with control group it is possible that altered pH may cause an isoform-specific induction of protein expression of brain Na^+/K^+-ATPase and this effect may be the main reason for increase in such activity. On the other hand the inability of the pH change to affect the interaction of mercury with the protein may be due to lipophilic nature of the binding site which selectively exclude contact with the aqueous medium This observation further support the finding that the thiol group (-SH) Na^+/K^+-ATPase of are located in the hydrophobic portion of the protein. The Na^+/K^+-ATPase from skeletal, cardiac and vascular smooth muscles, and that from brain are similar. Thus, it is possible that any effect observed on brain Na^+/K^+-ATPase could also take place in some degree in referred muscular tissues. Stimulation of Na^+/K^+-ATPase activity or its synthesis increase the net cellular uptake of K^+ and reduces the retention of cellular Na^+. Furthermore, this effect decreases the muscle Ca^{2+} via the Na^+/Ca^{2+} exchanger and leads to the decrease of muscle contractility. As a consequence, hypokalaemic relaxion of skeletal muscle or decrease in myocardial contractility or vasoconstriction could be produced. On the contrary, deactivation or decrease in the concentration of Na^+/K^+-ATPase lead to the decrease of cellular K^+, influx of

cellular Na^+ and increase of the muscle Ca^{2+} concentration, reducing the muscular contractile activity or the vasodilatation [34-36]. Conclusively, the results showed that though pH modulated the activity of Na^+/K^+-ATPase it did not ameliorate the damage by mercury to neuronal cells. This also supports the finding that tends to explain the localization of the thiol group in the lipophilic region of the electrogenic pump. The study may suggest that alteration in pH would result in an increase in ATP production, ionic transport and activity of Na^+/K^+-ATPase, which may affect the action potential, leading to modulation of neuronal activities.

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