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STUDIES ON THE INTERACTION OF CHICK BRAIN MICROSOMAL $(Na^+ + K^+)$-ATPase WITH COPPER

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SUMMARY

Chick brain microsomal ATPase was strongly inhibited by Cu^{2+}. $(Na^+ + K^+)$-ATPase was more susceptible to low levels of Cu^{2+} than Mg^{2+}-ATPase. The inhibition of $(Na^+ + K^+)$-ATPase could be partially protected from Cu^{2+} in the presence of ATP in the preincubation period. When Cu^{2+} (6 μM) was preincubated with the enzyme in the absence of ATP, only sulfhydryl-containing amino acids (D-penicillamine and L-cysteine) could reverse the inhibition. At lower concentrations of Cu^{2+} (< 1.4 μM), in the absence of ATP during preincubation, the inhibition could be completely reversed by the addition of 5 mM L-phenylalanine and L-histidine as well as D-penicillamine and L-cysteine.

Kinetic analysis of action of Cu^{2+} (1.0 μM) on $(Na^+ + K^+)$-ATPase revealed that the inhibition was uncompetitive with respect to ATP. At a low concentration of K^+ (5 mM), V with Na^+ was markedly decreased in the presence of Cu^{2+} and K_m was about twice that of the control. However, at high K^+ concentration (20 mM), the K_m for Na^+ was not affected. At both low (25 mM) and high (100 mM) Na^+, Cu^{2+} displayed non-competitive inhibition of the enzyme with respect to K^+.

On the basis of these data, we suggest that Cu^{2+} at higher concentrations (> 6 μM) inactivates the enzyme irreversibly, but that at lower concentrations (< 1.4 μM), Cu^{2+} interacts reversibly with the enzyme.

INTRODUCTION

The marked inhibition of membrane-bound ATPase activity by exogenous Cu^{2+} was first reported by Peters *et al.*[1,2]. They implicated the inhibition of ATPase activity as the cause of convulsion and death of pigeons after the subarachnoid injection of Cu^{2+}. However, their brain microsomal preparation had relatively low $(Na^+ + K^+)$-ATPase (ouabain-sensitive) activity. Later studies have shown that Cu^{2+} has greater inhibitory effect on $(Na^+ + K^+)$-ATPase than Mg^{2+}-ATPase (ouabain-insensitive)[3,4]. The mechanism of this inhibition remains unclear, although it has been shown by Peters *et al.*[2] that SH-containing compounds such as British Anti-Lewisite and thioctic acid could reverse the inhibition. Interaction of Cu^{2+} with membrane sulfhydryl groups has been reported by other investigators[5,6].

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$(Na^+ + K^+)$-ATPase is one of the most important regulators of brain energy metabolism. Whittam[7] has estimated that it may control 40% of the respiration and metabolic activity of the brain cortex. In view of the regulatory importance of $(Na^+ + K^+)$-ATPase in energy metabolism and ion transport across cell membrane, we felt that a more thorough investigation of the nature of its inhibition by Cu^{2+} might provide a better understanding of the neurotoxicity of Cu^{2+}. In this paper, Cu^{2+} is shown to have two distinct effects on the chick brain microsomal $(Na^+ + K^+)$-ATPase. Cu^{2+} at higher concentrations (> 6 μM) not only denatures the enzyme by interacting with enzyme functional groups, but at lower copper concentrations (< 1.4 μM), it also interacts reversibly with the E_2KATP complex during enzymatic reaction.

MATERIALS AND METHODS

Preparation of microsomes from whole brain

Whole brain microsomes from 1- to 2-day-old male white leghorn chicks were prepared as previously described[8] except for the omission of 1 mM EDTA in the isolation medium for the purpose of avoiding chelation of endogenous metals or those to be added later. The crude microsomal preparation was suspended in 0.25 M sucrose and, for some experiments, used without further purification. More highly purified $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) was obtained by further treatment with deoxycholate and NaI according to Robinson[8] with the exception that the final pellet was washed twice and suspended in 0.25 M sucrose instead of 10 mM Tris–HCl (pH 7.3). This modification resulted in significantly less reduction of activity during storage at −90 °C up to a month. ATPase activity from purified microsomes was predominantly ouabain-sensitive (95%).

Adenosine triphosphatase assay

Total ATPase activity was determined in 1 ml in the presence of various amounts of enzyme protein, 20 mM Tris–HCl buffer (pH 7.4), 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 3 mM Tris–ATP or disodium ATP (Boehringer-Mannheim Co.), and various concentrations of $CuSO_4$. Mg^{2+}-ATPase activity was determined under the same experimental conditions except 0.25 mM ouabain (Calbiochem) was added or K^+ was omitted. The difference between the total ATPase and the Mg^{2+}-ATPase activity was taken to be the $(Na^+ + K^+)$-ATPase activity. The reaction was initiated either by adding the enzyme or by adding ATP after the enzyme had been preincubated with $CuSO_4$ for 10 min at 37 °C. The reaction was normally carried out for 15 min at 37 °C. ATPase activity was assayed by measuring the rate of liberation of inorganic phosphate from ATP by the method of Berenblum and Chain[9]. Protein concentration was determined according to the procedure of Lowry *et al.*[10].

Calculation of kinetic parameters

K_m and V for ATP were obtained from Lineweaver–Burk plots by least-squares lines. In the determination of K_m and V for Na^+ and K^+, double reciprocal plots were usually not linear, a finding previously reported by Robinson[8] suggesting the allosteric nature of the enzyme. In order to obtain these kinetic parameters, a computer program, reported by Dunne *et al.*[11], was used to determine the value

of n in the following two equations which gave the highest correlation for a least-squares straight line for the data points:

$$1/v = 1/V + K_m^n/V[1/(S)^n] \quad (1)$$

$$(S)^n/v = K_m^n/V + [1/V](S)^n \quad (2)$$

The n value determined analytically above is equivalent to the n_H of the Hill equation; and, unlike the graphically determined Hill coefficient, n_H, n is independent of an accurate prior determination of V. The degree of fit to a straight line was expressed in the correlation coefficient (maximum of 1.0). The correlation coefficient for all calculations reported in this paper was above 0.95. The n values were calculated by the computer routine to $\pm$ 0.05. The n values calculated from either of the two equations above were within 5 %. After the determination of n, the computer program used the previously calculated n value to calculate the K_m and V values from the slope and intercept of the least-squares line.

RESULTS

Ionic activation of ATPase

In order to determine the most effective concentrations of Na^+ and K^+ on ATPase activity, measurements were made over a range of concentration of these ions. The total concentration of these ions was held at a constant level of 0.12 M to eliminate effects of osmolarity. A molar ratio of 5 for Na^+: K^+ was found to give the maximal $(Na^+ + K^+)$-ATPase activation. This observation closely resembles those previously reported for enzyme preparations from rabbit kidney[12], beef brain[13], and rat brain synaptosomes[14]. The Mg^{2+}-ATPase in our chick enzyme preparation was also independent of Na^+ or K^+ concentrations. In the absence of either of these ions, essentially no effect on Mg^{2+}-ATPase activity was detected.

Inhibition of chick brain microsomal ATPase by Cu^{2+}

Both $(Na^+ + K^+)$-ATPase (ouabain-sensitive) and Mg^{2+}-ATPase (ouabain-insensitive) were inhibited by Cu^{2+}. However, the $(Na^+ + K^+)$-ATPase activity was far more susceptible to low levels of Cu^{2+} than Mg^{2+}-ATPase (Fig. 1). It can be seen from Fig. 1 that there was a sharp decrease in the activity of the ouabain-sensitive component of the ATPase between 5–7 μM Cu^{2+}. Complete inhibition occurred when Cu^{2+} was 50 μM. The ouabain-insensitive enzyme showed a much more gradual decrease of hydrolytic activity over the same range of Cu^{2+} concentration and was still detectable (34 % of control) at a Cu^{2+} concentration of 100 μM. Half-maximal inhibition of the $(Na^+ + K^+)$-ATPase occurred at approximately 6.0 μM Cu^{2+} for the purified enzyme preparation with a protein concentration of 77 μg/ml (Fig. 2). The degree of Cu^{2+} inhibition of $(Na^+ + K^+)$-ATPase depended upon the concentration of the enzyme protein. An approximate doubling of the enzyme concentration shifted the inhibition curve to the right, suggesting nonspecific complexing of Cu^{2+} by added protein. The sigmoidal nature of the Cu^{2+}-inhibition curves suggests binding of Cu^{2+} to nonspecific sites, producing the initial lag in Cu^{2+} inhibition.

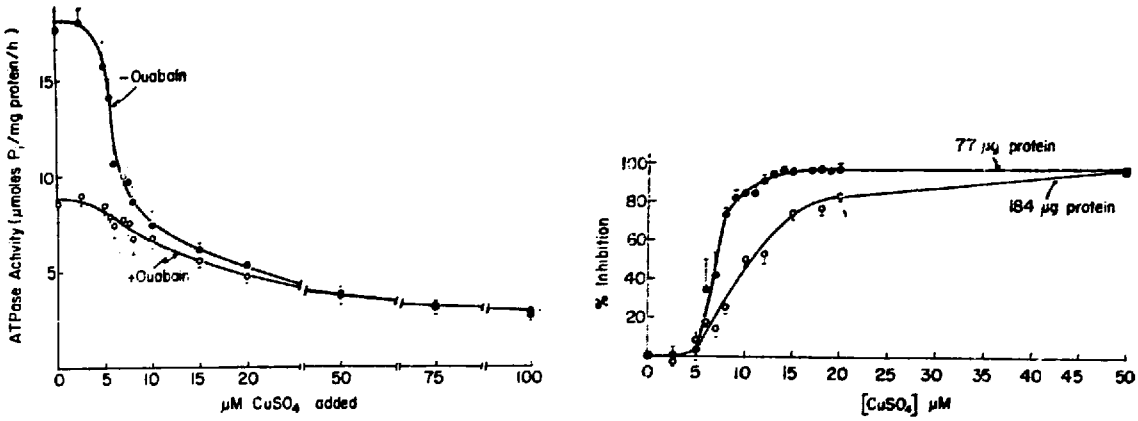


Fig. 1. Inhibition of crude chick brain microsomal ATPase activity by Cu^{2+} in the presence or absence of 250 μM ouabain. Samples of microsomes (109 μg protein) were incubated for 15 min at 37 °C in a medium containing 20 mM Tris–HCl buffer (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 3 mM ATP, and various concentrations of $CuSO_4$. Each point represents the mean ± S.D. of 4–8 individual determinations.

Fig. 2. Inhibition of purified brain microsomal ($Na^+ + K^+$)-ATPase by Cu^{2+} at two protein concentrations. Incubation conditions were the same as described in Fig. 1, and the reaction was initiated with the addition of the enzyme. Each point represents the mean ± S.D. of 4 experiments.

Effect of sequence of addition of incubation components on Cu^{2+} inibition of ($Na^+ + K^+$) ATPase

The presence of ATP during the preincubation period has been shown to protect the enzyme from SH-blocking reagents such as *N*-ethylmaleimide[15] and *p*-mercuribenzoate[16]. As seen in Table I, Cu^{2+} inhibition of the enzyme was greatest

TABLE I

EFFECT OF SEQUENCE OF ADDITION OF INCUBATION COMPONENTS ON Cu^{2+} INHIBITION OF ($Na^+ + K^+$)-ATPase

The reaction medium contained 20 mM Tris–HCl (pH 7.4); 100 mM Na^+; 20 mM K^+; 5 mM Mg^{2+}; 3 mM ATP (sodium salt); 100 μg enzyme protein in a final volume of 1 ml. Each value given is the average of 3–7 individual determinations.

Conditions	*($Na^+ + K^+$)-ATPase (% of control)*	*Comment*
Preincubation: Tris, Na^+, K^+, Mg^{2+}, and ATP Initiate: enzyme	100	
Preincubation: Tris, Na^+, K^+, Mg^{2+}, ATP, and Cu^{2+} (6 μM) Initiate: enzyme	50.4	Inhibition could be completely reversed by 5 mM L-histidine, L-phenylalanine, and D-penicillamine
Preincubation: Tris, Na^+, Cu^{2+} (6 μM), ATP, Mg^{2+}, and enzyme Initiate: K^+	36.0	Inhibition could be completely reversed by 5 mM L-histidine, L-phenylalanine, and D-penicillamine
Preincubation: Tris, Na^+, K^+, Mg^{2+}, Cu^{2+} (6 μM), and enzyme Initiate: ATP	8.8	Could not be reversed by 5 mM L-histidine, L-phenylalanine; can be reversed to 50% and 25% by 5 mM D-penicillamine and L-cysteine, respectively.
Preincubation: Tris, Na^+, K^+, Mg^{2+}, Cu^{2+} (1.4 μM), and enzyme Initiate: ATP	59.3	Inhibition could be completely reversed by 5 mM L-histidine, L-phenylalanine, and D-penicillamine.

when the enzyme was preincubated with 6 μM Cu^{2+} for 10 min in the absence of ATP; the inhibition could not be reversed by the addition of 5 mM L-histidine or L-phenylalanine. However, the inhibition could be reversed to 50% and 25% of the control activity by the addition of 5 mM D penicillamine and L-cysteine, respectively. At low concentration of Cu^{2+} (1.4 μM), in the absence of ATP during preincubation, the inhibition could be completely reversed by the addition of amino acids. When the enzyme was preincubated with Cu^{2+} in the absence of ATP, the half-maximal inhibition of the $(Na^+ + K^+)$-ATPase occurred at 1.4 μM (Fig. 3).

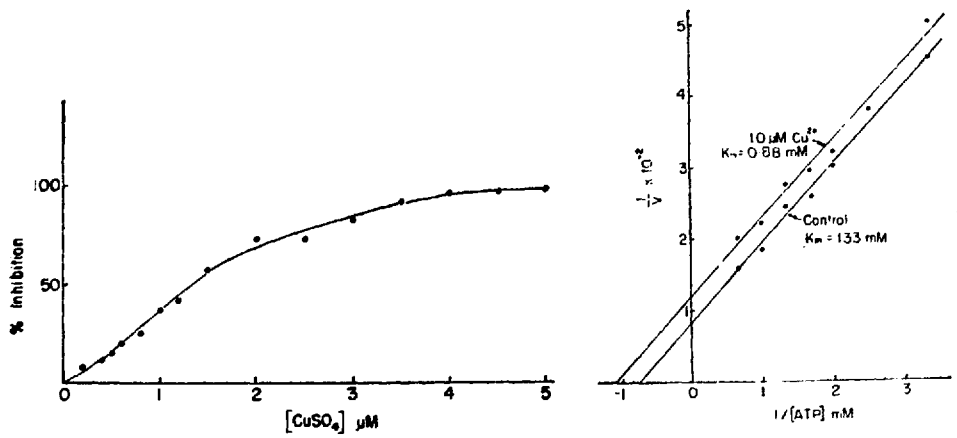


Fig. 3. Inhibition of purified brain microsomal $(Na^+ + K^+)$-ATPase by Cu^{2+}. The enzyme (28 μg protein) was preincubated for 10 min at 37 °C with the standard incubation assay media except in the absence of ATP. The reaction was initiated with 3 mM ATP. The points represent the average values of two individual determinations.

Fig. 4. Lineweaver–Burk plot of $(Na^+ + K^+)$-ATPase activity with respect to ATP concentrations. Experiments were performed at 37 °C in preincubation media containing 28 μg enzyme protein, 100 mM NaCl, 20 mM KCl, 20 mM Tris–HCl (pH 7.4), in the absence (●—●) and presence (○—○) of 1 μM $CuSO_4$. The reaction was initiated with equimolar concentrations of $MgCl_2$ and Tris–ATP. Each point represents the average value of two inidvidual determinations, and the lines were drawn according to the least-squares fit.

Kinetic studies

Various kinetic parameters of the purified enzyme were determined in the presence and absence of low (1.0 μM) amounts of Cu^{2+}. In order to avoid the complication due to chelation of Cu^{2+} by ATP, all the enzymatic reactions were initiated with ATP after the enzyme had been preincubated with Cu^{2+} for 10 min. The two parallel straight lines (slopes for both lines are 0.01 as calculated from the least-squares) for Lineweaver–Burk plots with respect to ATP are characteristic of uncompetitive inhibition, suggesting that there is no connection between the binding of Cu^{2+} and ATP in the reaction sequence.

At low concentration of K^+ (5 mM), V for Na^+ was markedly decreased (Fig. 5A). Km in the presence of Cu^{2+} was about twice that of the control. However, at high K^+ concentration (20 mM), the modified Lineweaver–Burk plots showed that Cu^{2+} decreased the apparent V without changing the K_m (Fig. 5B), a non-competitive inhibition by copper. At both low (25 mM) and high (100 mM) Na^+, Cu^{2+} displays non-competitive inhibition of the enzyme with respect to V^+ (Figs 6A and 6B).

At low concentrations of K^+ (5 mM), both V and K_m were affected at infinite Na^+ concentration. When both the K^+ and Na^+ were increased to saturating

levels (Figs 5B and 6B), Cu^{2+} affected only the V and not the K_m values for either Na^+ or K^+.

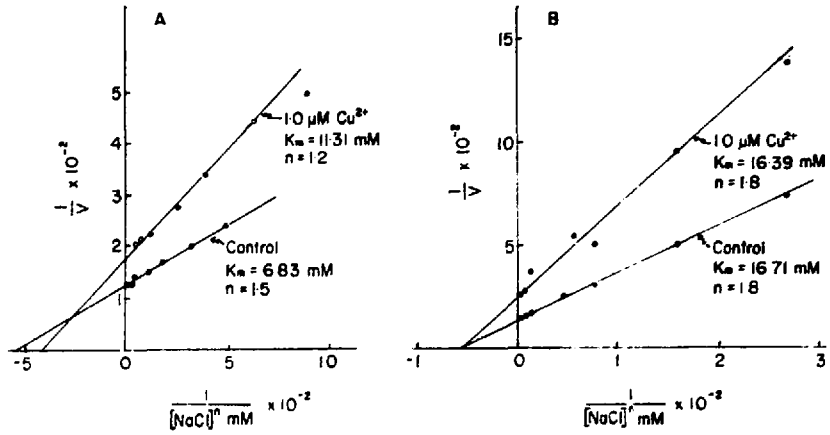


Fig. 5. Effect of Cu^{2+} on activation of (Na^+ + K^+)-ATPase by NaCl at (A) low KCl concentration (5 mM), and (B) high KCl concentration (20 mM). Experiments were carried out as in Fig. 4 in media containing 28 μg enzyme protein, 3 mM Tris–ATP, 5 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.4), in two concentrations of KCl and various concentrations of NaCl, in the absence (●—●) and presence (○—○) of $CuSO_4$. The points represent the average value of three determinations, and they were calculated from a computer routine described in Methods. The lines were drawn according to the least-squares fit.

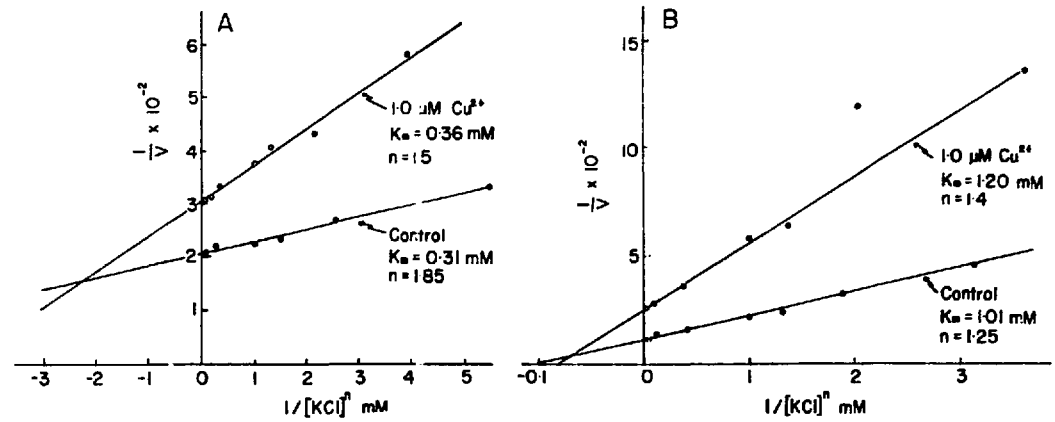


Fig. 6. Effect of Cu^{2+} on activation of (Na^+ + K^+)-ATPase by KCl at (A) low NaCl concentration (25 mM), and (B) high NaCl concentration (100 mM). Experimental conditions were the same as in Fig. 5. The points represent the average values of three determinations, and they were calculated from a computer routine described in Methods. The lines were drawn according to the least-squares fit.

DISCUSSION

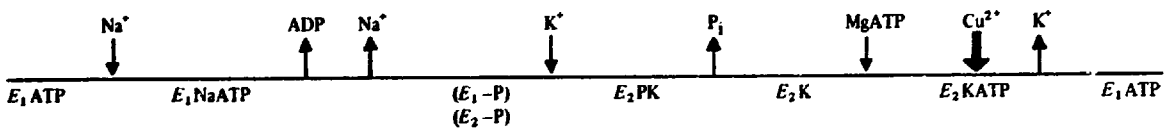
In agreement with the findings of Bowler and Duncan[3] and Donaldson *et al.*[4] for rat brain microsomal ATPase, we have shown that the chick brain microsomal (Na^+ + K^+)-ATPase is more susceptible than Mg^{2+}-ATPase to Cu^{2+} inhibition (Fig. 1). While not examined in these studies, Donaldson *et al.*[4] noted that the rat hippocampal and hypothalamal microsomal (Na^+ +K^+)-ATPase is more susceptible to Cu^{2+} inhibition than the enzyme from other regions of the brain.

The copper content of the whole rabbit brain is 50 nmole/g[17] which, if free, is sufficient to cause significant inhibition of the brain ATPase. It is clear, therefore,

that the concentration of free copper must be much lower being complexed with normal copper-containing proteins[18] and small molecular weight ligands within nerve cells. Many other copper binding sites are also indicated by the reduced inhibition observed as the concentration of protein is increased in the $(Na^+ + K^+)$-ATPase assay (Fig. 2).

The protection of ATP against copper inhibition is reminiscent of similar protective effects against inhibition by sulfhydryl reagents such as *p*-mercuribenzoate and *N*-ethylmaleimide. The protection that ATP affords against Cu^{2+} inhibition can be partly explained on the basis of Cu^{2+} chelation by ATP[19]. It should be noted, however, that reversal of inhibition by Cu^{2+} at 1.4 μM can be accomplished by L-histidine and L-phenylalanine. As a result of this observation, the kinetics of Cu^{2+} inhibition were examined at a lower concentration (1 μM).

In order to describe kinetic mechanisms, the shorthand notation of Cleland[20] is used to express the operation of $(Na^+ + K^+)$-ATPase. The enzyme is denoted by a horizontal line, and successive additions of substrates and release of products are designated by vertical arrows, as shown below. Enzyme forms are shown under the line. According to this scheme, ATP is bound to the K^+ form of the enzyme as suggested by Hegyvary and Post[21]. Dissociation of K^+ is a rate-limiting step in $(Na^+ + K^+)$-ATPase and K^+ must dissociate before Na^+ can bind with the enzyme and catalyze transphosphorylation[21].



The question of most interest from a mechanistic point of view is the point in the reaction sequence at which the dead end inhibitor, Cu^{2+}, might interact. A tentative solution to this question was devised using the procedures suggested by Cleland[20]. In the first analysis, inhibition by Cu^{2+} (< 1.4 μM) was not competitive with respect to either Na^+, K^+, or ATP, ruling out interaction with either E_1ATP, (E_1~P) (E_2~P) or E_2K. Interaction with E_2PK does not seem likely since inhibition with respect to variable Na^+ or K^+ would both be expected to be uncompetitive in contrast to the observed non-competitive inhibition. Of the two remaining forms — E_1NaATP and E_2KATP — a unique choice cannot be made. However, interaction with E_2NaATP is the least preferred as uncompetitive inhibition should be observed with variable Na^+, K^+, or MgATP. On the other hand, if Cu^{2+} interacts with E_2KATP, inhibition with respect to MgATP should be uncompetitive, with respect to Na^+, non-competitive, and with respect to K^+, uncompetitive. Since the predicted inhibition with respect to K^+ in the last case is at variance with observation, it is necessary to argue that sufficient contaminating ADP is present in the assay to provide a reversible connection between interaction of K^+ and E_2KATP or that the mechanism for the reaction as defined is oversimplified. Therefore, this analysis must be considered tentative until (1) the mechanism can be more clearly defined, and (2) much more extensive data showing effects of ADP and a greater range of Na^+, K^+, and ATP concentrations are obtained.

Upon consideration of the results reported in this paper, it is possible that in certain pathological conditions leading to devastating neurological disturbances (such as the accumulation of cerebral Cu^{2+} in Wilson's disease) can be due to the

marked effect of Cu^{2+} on $(Na^+ + K^+)$-ATPase. Inhibition of this enzyme system may be the mechanism by which Cu^{2+} induces swelling in mitochondria[22] and inhibits the oxygen consumption of brain homogenates[23] through the disruption of ionic balance and energy metabolism.

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