Inhibition of Neuronal Sodium and Potassium Ion Activated Adenosinetriphosphatase by Pyrithiamin†

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ABSTRACT: Since one of the electrophysiological effects of pyrithiamin, an antimetabolite of thiamin, suggested an interference with sodium pump mechanisms, the effect of pyrithiamin on Na^+,K^+-ATPase was investigated. We found that whereas preincubation of the antimetabolite with nonneuronal preparations of Na^+,K^+-ATPase produced only minimal inhibition, the enzyme derived from brain preparations was markedly inhibited. This inhibition could be prevented by thiamin but not reversed. The kinetic study showed that pyrithiamin acts in a noncompetitive manner with respect to the activation of the enzyme by ATP, Na^+, and K^+. Pyrithiamin inhibited Na^+-dependent phosphorylation and K^+-stimulated phosphatase as well as ouabain binding, and these inhibitions were parallel with that of the overall Na^+,K^+-ATPase reaction. In addition, the antimetabolite caused a significant change in the turbidity of the enzyme suspension. The results suggest that pyrithiamin may induce a structural change of the enzyme complex.

Thiamin is generally thought to have a specific function in nervous tissue independent of its coenzyme role, but the precise mechanism has not yet been elucidated (Cooper & Pincus, 1979). In studies on the role of thiamin, two antimetabolites of the vitamin, pyrithiamin and oxythiamin, have been widely used. Both agents produce weight loss and bradycardia, but only pyrithiamin induces a neurological impairment (Eusebi & Cerecedo, 1949). The difference has been explained by the fact that pyrithiamin, but not oxythiamin, accumulates in the brain and causes a rapid depletion of thiamin (DeCaro et al., 1956; Kordam, 1958; Rindi & Perri, 1961; Gubler, 1976). A number of electrophysiological experiments with these antimetabolites have suggested that thiamin may be involved in excitability in nerve membranes (Kunz, 1956; Petropulos, 1960; Armett & Cooper, 1965; Perri et al., 1970; Goldberg & Cooper, 1975; Goldberg et al., 1975; Barchi, 1976). Armett & Cooper (1965) found that pyrithiamin had two irreversible effects on electrical activity in rabbit vagus nerves: it increased the amplitude of the compound action potential, and it obliterated the posttetanic hyperpolarization. These effects were prevented when the nerve was preincubated with thiamin. The effect of pyrithiamin could not be ascribed to an interference with the ThPP[1]-dependent enzyme systems in the nerve, but it appeared to be related to the displacement of thiamin from the nerve (Cooper, 1968). Since hyperpolarization has been suggested to reflect an increased Na^+,K^+-ATPase activity in excitable cells (Ritchie & Straub, 1957; Gorman & Marmor, 1974), it is conceivable that this effect of pyrithiamin might be referable to an inhibition of this enzyme.

In this paper, we demonstrate that the antimetabolite exerts a marked inhibition only on Na^+,K^+-ATPase derived from neuronal tissue, that the enzyme purified partially from rat brain contains thiamin, and that sodium bisulfite treatment, classically known to destroy thiamin compounds, inhibits the enzyme prepared from brain but not from kidney.

Materials and Methods

Materials. Pyrithiamin and ThPP were obtained from Calbiochem-Behring Corp. ThTP was a gift from Sankyo Chemical Co. Chemicals purchased from Sigma Chemical Co. included ATP (vanadate-free, sodium, and Tris salts), ouabain, thiamin, ThMP, pyrithiamin, and oxythiamin. [^{3}H]ATP, [γ-^{32}P]ATP, and [^{3}H]ouabain were obtained from New England Nuclear, and [^{35}S]thiamin was obtained from Amersham/Searle. All other reagents were of analytical grade.

Enzyme Preparations. Male Sprague-Dawley rats were used throughout. Synaptic plasma membranes were purified from synaptosomes of the cerebral cortex as described previously (Matsuda & Cooper, 1981a). Axolemma and red cell ghosts were prepared by the methods of DeVries et al. (1978) and Hanahan & Ekholm (1974), respectively. Partial purification of brain and kidney Na^+,K^+-ATPases was achieved by gentle extraction with $NaDodSO_4$ by a modification of the method of Jørgensen (1974). The microsomes (5 mg/mL), prepared from cerebral cortex (DeRobertis et al., 1967) and kidney (Jørgensen, 1974), were incubated for 30 min at 22 °C with 1.5 mg/mL $NaDodSO_4$, 3 mM ATP (vanadate free), 1 (brain) or 2 mM (kidney) EDTA, and 50 mM imidazole buffer (pH 7.25). The suspension was then layered over a discontinuous sucrose gradient consisting of successive 8-mL layers of 15, 25, and 30% (w/v) sucrose containing 0.1 mM EDTA–1 mM sodium phosphate (pH 7.4) and centrifuged at 63000*g* for 5–6 h. Fractions at 15–25% sucrose interface (brain) and pellet (kidney) were removed, diluted with 0.1 mM EDTA–1 mM sodium phosphate (pH 7.4) and centrifuged at 78000*g* for 1 h. The resulting pellet was resuspended in 0.32 M sucrose–1 mM EDTA–20 mM imidazole buffer (pH 7.25). The preparations could be stored frozen up to 3 months without loss of enzyme activity. This procedure gave 20–25-fold (brain) and 64–83-fold (kidney) purifications with 4% and 1% protein recovery from the microsomes, respectively. Mg^{2+}-ATPase activity in the brain and kidney enzymes was 5% and 1% of the total ATPase activity, respectively.

Enzyme Assay. Na^+,K^+-ATPase activity was determined in a reaction medium containing 25 mM imidazole buffer (pH

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[1] Abbreviations: ThMP, ThPP, and ThTP, thiamin mono-, pyro-, and triphosphate; ATPase, adenosinetriphosphatase; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; ATP, ADP, and AMP, adenosine tri-, di-, and monophosphate; P_i, inorganic phosphate; Cl_3CCOOH, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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7.25), 3 mM $MgCl_2$, 3 mM ATP, 140 mM NaCl, 20 mM KCl, 0.1 mM EDTA, and enzyme protein as described previously (Matsuda & Cooper, 1981a) with the following modifications: Mg^{2+}-ATPase activity was measured in the presence of 1 mM ouabain for neuronal enzymes or in the absence of Na^+ and K^+ for nonneuronal preparations. Ouabain (1 mM) inhibited Na^+,K^+-ATPase activity in neuronal and nonneuronal preparations by 98% and 85%, respectively. When the reaction mixture in a final volume of 0.1 mL contained thiamin or antimetabolites, 0.1 mL of prewashed charcoal (Sigma, activated) suspension (100 mg/mL) was added to it after termination of the reaction by Cl_3CCOOH to prevent the interference of P_i determination by these compounds. Under these conditions, P_i was completely recovered in the supernatant after centrifugation. For confirmation of our results with this assay, on some occasions the ATPase activity was determined with [^{3}H]ATP (2 mCi/mmol) as substrate. After incubation and deproteinization, the reaction mixture was applied to an AG 1 × 8 column (formate form, 0.75 × 1.13 cm). AMP, ADP, and ATP were successively eluted with 3-mL portions of 2.2 N formic acid, 4 N formic acid–0.2 M sodium formate, and 4 N formic acid–1 M sodium formate, and the radioactivity was determined. Cytochrome *c* oxidase and α-ketoglutarate dehydrogenase was measured as described previously (Matsuda & Cooper, 1981a). The activities of acetylcholinesterase (Ellman et al., 1961), alkaline phosphatase (Cotman & Matthews, 1971), 5′-nucleotidase (Cotman & Matthews, 1971), lactate dehydrogenase (Stolzenbach, 1966), and cyclic-nucleotide phosphodiesterase (Butcher & Sutherland, 1962) were also determined. Calmodulin-deficient phosphodiesterase and calmodulin prepared from bovine brain (Watterson et al., 1976) were used for this latter assay. Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Phosphorylation and Ouabain Binding. The optimum conditions for phosphorylation of the brain Na^+,K^+-ATPase were characterized for $MgCl_2$, NaCl, and KCl concentrations and for time of incubation. The reaction was run in a volume of 0.1 mL at 0 °C with 10 μM [γ-^{32}P]ATP [(5–8) × 10^{14} cpm/mol), 0.2 mM $MgCl_2$, 100 mM NaCl, 25 mM imidazole buffer (pH 7.25) and about 10 μg of enzyme protein. Nonspecific phosphorylation was estimated by an identical reaction mixture except that 20 mM KCl was substituted for the 100 mM NaCl. The reaction was terminated at 20 s by addition of 1 mL (5% w/v) of Cl_3CCOOH containing 0.1 M sodium phosphate, and 0.05 mL of 5 mg/mL bovine serum albumin was added as carrier protein. After 10 min, the precipitate was filtered through a 0.45-μm membrane filter and washed with three 2-mL aliquots of the Cl_3CCOOH solution. The sample filters were dried, and the radioactivity was counted. Nonspecific phosphorylation was less than 5% of that occurring in the presence of 100 mM NaCl. The optimum ouabain assay was done in a volume of 0.1 mL with 1 μM [^{3}H]ouabain (1.8 Ci/mmol), 100 mM NaCl, 3 mM ATP, 3 mM $MgCl_2$, and about 10 μg of enzyme protein. Controls received 5 mM unlabeled ouabain as well. After incubation for 1 h at 37 °C, the samples were filtered through the membrane filter and washed with three 3-mL portions of cold 25 mM imidazole buffer (pH 7.25). The filters were dried, and the radioactivity was counted.

Thiamin Assay. Thiamin in the partially purified enzyme was determined as follows: The enzyme suspension was first washed with water, and then the pellet was suspended in water; 0.9 mL of the suspension was incubated with 0.72 g of urea for 2 h at 37 °C, and then the mixture was extracted 3 times with chloroform. The resulting clear aqueous solution was used for thiamin determination as described previously with alkaline cyanogen bromide (Matsuda & Cooper, 1981b). ThPP solution as the standard was treated similarly.

$NaDodSO_4$–Polyacrylamide Slab Gel Electrophoresis. The enzyme suspension was centrifuged, and the pellet was dissolved in 62.5 mM Tris-HCl (pH 6.8)–2% $NaDodSO_4$–5% 2-mercaptoethanol. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970), and the gel was scanned at 560 nm with a Shimazu CS-900 TLC scanner.

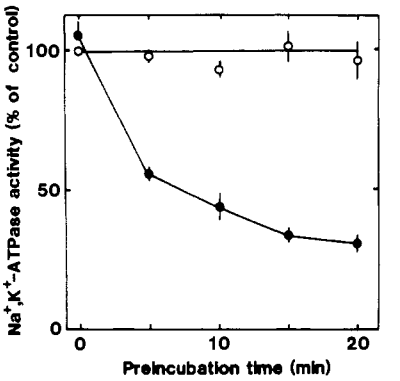

FIGURE 1: Inhibition of synaptic plasma membrane Na^+,K^+-ATPase activity by pyrithiamin. The synaptic plasma membranes, suspended in 15 mM imidazole buffer (pH 7.25) and 0.25 mM EDTA, were preincubated without (○) and with 1 mM pyrithiamin (●) at 37 °C in a volume of 0.04 mL. The reaction was carried out for 2 min by the addition of 0.06 mL of reaction mixture containg 25 mM imidazole buffer (pH 7.25), 3 mM $MgCl_2$, 3 mM ATP, 140 mM NaCl, 20 mM KCl, and ±1 mM ouabain. Each point is the mean ± SE of three to four experiments using three different enzyme preparations. The control activity (mean ± SE) was 2.41 ± 0.11 μmol of P_i (mg of protein)$^{-1}$ min^{-1}.

Results

Figure 1 shows that Na^+,K^+-ATPase activity in synaptic plasma membranes is significantly decreased by pretreatment of the membranes with pyrithiamin. This inhibition required preincubation of the enzyme with the antimetabolite. Maximum inhibition was observed by treatment for about 15 min. Unless otherwise indicated, all experiments reported used this preincubation procedure, and the concentration of the drugs was expressed as that during the preincubation in this study. Pretreatment with oxythiamin, thiamin, ThMP, ThPP, and ThTP at 0.025–2.5 mM did not affect the enzyme activity (data not shown). Since pyrithiamin was purchased as the hydrobromide salt, we checked hydrobromide for an effect, but at a concentration of 1.25 mM it did not influence the activity. We also examined the effect of tregonelline (3-carboxy-1-methylpyridinium hydroxide) on the enzyme, since, among the thiamin compounds tested, only pyrithiamin consists of a pyridinium salt: this compund had no effect on activity.

Pyrithiamin inhibited other enzymes localized in the synaptic plasma membranes, but the inhibitions were considerably less than that of Na^+,K^+-ATPase (Table I, part A). The effect of pyrithiamin on the enzymes localized in other subcellular fractions was also examined (Table I, parts B and C). Among the enzymes tested, only α-ketoglutarate dehydrogenase, which tightly binds ThPP as a coenzyme, was inhibited by the antimetabolite. With this enzyme the pyrithiamin treatment was performed at 25 °C for 30 min because of stability problems. The effect of pyrithiamin on Na^+,K^+-ATPase in various rat tissue preparations is shown in Figure 2. The inhibition of Na^+,K^+-ATPase in neuronal preparations was considerably

Table I: Inhibitory Effect of Pyrithiamin on Several Membrane Enzymes[a]

enzymes	inhibition (%)
(A) synaptic plasma membranes	
Na^+, K^+-ATPase	75 ± 4
Mg^{2+}-ATPase	23 ± 4
acetylcholinesterase	29 ± 2
alkaline phosphatase	28 ± 2
5'-nucleotidase	0
(B) mitochondria	
cytochrome *c* oxidase	7 ± 3
α-ketoglutarate dehydrogenase	49 ± 6
(C) soluble	
lactate dehydrogenase	0
cyclic-AMP phosphodiesterase	
without calmodulin	0
with calmodulin	0

[a] Each enzyme preparation was preincubated with 1.25 mM pyrithiamin for 15 min at 37 °C except for the case of α-ketoglutarate dehydrogenase in which the preincubation was for 30 min at 25 °C. The crude mitochondrial fraction prepared as described previously (Matsuda & Cooper, 1981a) was lysed with water, and the residue was used as the mitochondrial fraction. The specific activities (μmol mg^{-1} h^{-1}) were as follows: Na^+, K^+-ATPase, 131; Mg^{2+}-ATPase, 43; acetylcholinesterase, 7.2; alkaline phosphatase, 0.22; 5'-nucleotidase, 0.15; cytochrome *c* oxidase, 43.8; α-ketoglutarate dehydrogenase, 2.45; lactate dehydrogenase, 139; cyclic-AMP phosphodiesterase without and with calmodulin plus Ca^{2+}, 9.3 and 38.8, respectively. The value is the mean ± SE of three to four experiments.

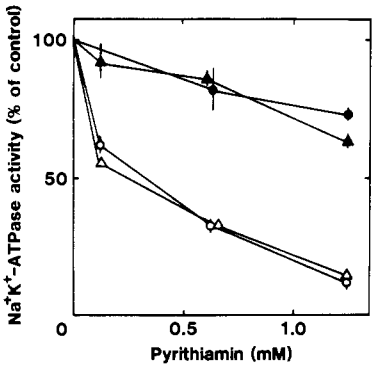

FIGURE 2: Effect of pyrithiamin on Na^+,K^+-ATPase activity in partially purified kidney (●), red cell ghost (▲), axolemma (○), and partially purified brain enzyme preparations (△). Each preparation was preincubated with pyrithiamin for 15 min at 37 °C. Each point is the mean ± SE of three to four experiments with two to three different enzyme preparations. The control activities for partially purified kidney, red cell ghost, axolemma, and partially purified brain enzyme preparations were 10.2 ± 1.1, 0.025 ± 0.005, 1.93 ± 0.28, and 7.98 ± 0.53 μmol of P_i (mg of protein)$^{-1}$ min^{-1}, respectively (mean ± SE).

more significant than in nonneuronal tissues. The inhibition of the partially purified brain enzyme was also dependent on the preincubation time, like that of synaptic plasma membranes (data not shown). The simultaneous addition of thiamin in the preincubation medium antagonized the inhibitory action of pyrithiamin in the membrane preparation (Table II) and in the purified brain enzyme (data not shown). At a low concentration of pyrithiamin, inhibition was completely prevented by thiamin, while the antagonism was only partial at a high concentration of the antimetabolite. Similar antagonism was observed with ThMP, ThPP, ThTP, and oxythiamin (data not shown).

Attempts were made to analyze the effect of pyrithiamin on the kinetic behavior of Na^+,K^+-ATPase, using the partially purified enzyme (Figure 3). Activation of Na^+,K^+-ATPase by ATP and K^+ are plotted according to the method of

Table II: Antagonism by Thiamin of the Pyrithiamin Inhibition of Na^+,K^+-ATPase Activity[a]

[pyrithiamin] (mM)	[thiamin] (mM)	Na^+,K^+-ATPase activity (% of control)
0.25	0	73 ± 3
0.25	2.5	96 ± 6[b]
0.25	12.5	95 ± 2[c]
1.25	0	30 ± 4
1.25	2.5	51 ± 9
1.25	12.5	73 ± 10[b]

[a] The synaptic plasma membranes were pretreated with pyrithiamin in the absence or presence of thiamin for 15 min at 37 °C, and then the enzyme assay was carried out as described under Materials and Methods. The value is the mean ± SE of four to five experiments using three different enzyme preparations. [b] $P < 0.025$, compared to the corresponding value without thiamin. [c] $P < 0.005$, compared to the corresponding value without thiamin.

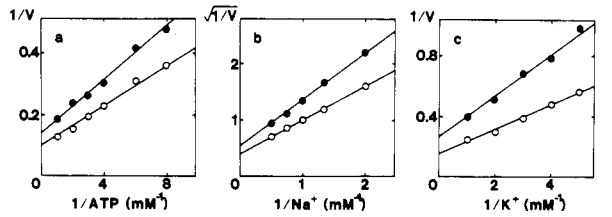

FIGURE 3: Effect of pyrithiamin on the kinetics of activation of Na^+,K^+-ATPase by ATP, Na^+, and K^+. The partially purified brain enzyme was preincubated with (●) and without (○) 0.3 mM pyrithiamin for 15 min at 37 °C, and the activity was determined as described under Materials and Methods. The concentrations of ATP, Na^+, and K^+ were varied as shown in (a), (b), and (c), respectively, while the final concentrations of K^+ (b) and Na^+ (c) were kept at 1 and 10 mM, respectively. Results are representative of three separate experiments. *V* is expressed as μmol of P_i (mg of protein)$^{-1}$ min^{-1}. The specific activity of the enzyme was 7.95 μmol of P_2 (mg of protein)$^{-1}$ min^{-1} under the standard assay conditions.

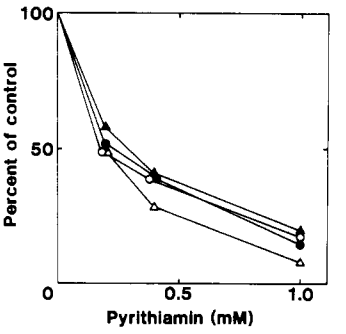

FIGURE 4: Effect of pyrithiamin treatment on Na^+-dependent phosphorylation (○), K^+-stimulated phosphatase activity (▲), Na^+,K^+-ATPase activity (●), and ouabain binding (△). The partially purified brain enzyme was pretreated with pyrithiamin at the concentrations indicated for 15 min at 37 °C and washed. The resulting preparation was used for the assay. Each point represents the means of three to four experiments with two to four different enzyme preparations. The SE is omitted because of spacial limitations, but it was within 10% of the mean. The control activities for Na^+-dependent phosphorylation, ouabain binding, K^+-stimulated phosphatase, and Na^+,K^+-ATPase were 0.75 ± 0.06 nmol/mg of protein, 0.81 ± 0.02 nmol/mg of protein, 0.78 ± 0.03 μmol of *p*-nitrophenol (mg of protein)$^{-1}$ min^{-1}, and 8.56 ± 0.87 μmol of P_i (mg of protein)$^{-1}$ min^{-1}, respectively (mean ± SE).

Lineweaver & Burk (1934), while the square root of the reciprocal of velocity is plotted against the reciprocal of Na^+ concentration for the Na^+ activation in order to obtain a straight line (Ahmed et al., 1966; Ahmed & Thomas, 1971). In all cases, pyrithiamin inhibited mainly by lowering the V_{max} without a significant change of the apparent K_m, suggesting that it interacts at the site other than activation sites of the

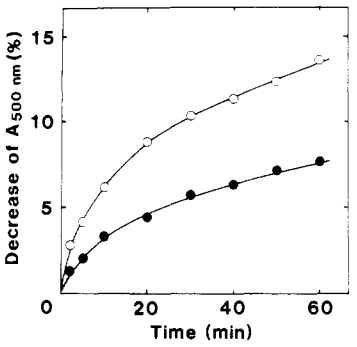

FIGURE 5: Changes in turbidity of the partially purified brain Na^+,K^+-ATPase preparation. The enzyme suspension containing 20 mM imidazole buffer (pH 7.25), 0.25 mM EDTA, and 80 mM sucrose was incubated in the absence (○) or presence (●) of 1.25 mM pyrithiamin at room temperature, and the optical density was recorded at 500 nm. The result is shown as percent decrease. The optical density at zero time was 0.200.

enzyme by ATP, Na^+, and K^+.

It has been proposed that Na^+,K^+-ATPase hydrolyzes ATP in a stepwise fashion involving Na^+-dependent phosphorylation of the enzyme and K^+-dependent hydrolysis of the phosphoenzyme. We examined the effect of pyrithiamin treatment on these reactions in the partially purified brain enzyme (Figure 4). The decreased activity of Na^+,K^+-ATPase was still observed after the preparation pretreated with pyrithiamin was washed, indicating the irreversibility of the action. In the same preparation, Na^+-dependent phosphorylation and K^+-stimulated phosphatase activity were decreased to the same degree as that of Na^+,K^+-ATPase activity. In addition, pyrithiamin decreased the binding of ouabain to the enzyme.

The occurrence of thiamin in the Na^+,K^+-ATPase preparation was examined to investigate the possibility that endogenous thiamin might be related to pyrithiamin's action as was the case with a previous study using the rabbit vagus nerve (Cooper, 1968). Thiamin was found to be 33 ± 5 pmol/mg of protein (n = 4) in the partially purified brain enzyme. In the preparation, the catalytic subunit phosphorylated by [γ-^{32}P]ATP accounted for approximately 17% of the total protein, determined by $NaDodSO_4$–polyacrylamide gel electrophoresis and the planimetry of the area, so that the preparation contained the catalytic subunit (M_r 98 000) at 1.7 nmol/mg of protein. Thus, the molar ratio between thiamin and the catalytic subunit is about 1/50. On the other hand, the ratio among the phosphorylation site, ouabain binding site, and catalytic subunit is about 1/1/2, since maximum phosphorylation and ouabain binding sites in the preparation were 0.75 and 0.81 nmol/mg of protein, respectively (Figure 4, legend). The presence of thiamin in the enzyme preparation was also examined with thiamin-deficient rats. In this experiment, endogenous thiamin was labeled by injecting [^{35}S]thiamin into the thiamin-deficient rats: subsequently the radioactivity in the purified enzyme preparation was assayed. The radiolabeled thiamin was detected in the partially purified brain enzyme at 5.5 pmol/mg of protein.

Figure 5 shows the effect of pyrithiamin on turbidity of the brain Na^+,K^+-ATPase suspension as room temperature. The turbidity of the enzyme suspension decreased with time, and this decrease was significantly depressed by the presence of pyrithiamin. A similar observation with pyrithiamin was noted under the same conditions used for the ATPase assay. Oxythiamin and thiamin had no effect on the turbidity.

In his original determination of the structure of thiamin, Williams (1935) discovered that the vitamin could be cleaved by sodium bisulfite. Accordingly, we examined the effect of sodium bisulfite on the enzyme activity. The partially purified enzyme suspensions (1.5 mg/mL) containing 50 mM imidazole buffer (pH 6.5) of brain and kidney were incubated at 37 °C for 30 and 60 min in the absence or presence of 100 mM sodium bisulfite; we determined the effect of the agent on activity using both the regular ATPase assay and also the [^{3}H]ATP assay as described under Materials and Methods. The bisulfite treatment inactivated Na^+,K^+-ATPase activity in the brain preparation by 89% and 98% in 30- and 60-min incubation, respectively (n = 3), but had an insignificant effect on the kidney enzyme. Both enzyme activities were not changed by the incubation in the absence of sodium bisulfite. In parallel experiments, authentic ThPP (0.02 mM) was destroyed by 61% and 71% in 30- and 60-min incubation, respectively. Further, in preliminary experiments, we found that the bisulfite treatment destroyed the endogenous thiamin of the brain preparation as it concurrently decreased the activity of α-ketoglutarate dehydrogenase.

Discussion

In this paper, we have shown that neuronal Na^+,K^+-ATPase activity is inhibited by pyrithiamin at the same concentration as used in the electrophysiological experiments on the vagus nerve mentioned in the introduction. The effect of the enzyme seemed to be irreversible because the decreased activity was not recovered by washing the preparation and by further treatment with thiamin or sulfhydryl reagents (data not shown). In addition, since this inhibition was antagonized in the presence of thiamin, these observations are in agreement with the results obtained in the electrophysiological experiments (Armett & Cooper, 1965). This study thus offers a mechanism for part of the electrophysiological action of pyrithiamin. The physiological function of thiamin in conduction which was suggested from the previous study thus might be related to the regulation of Na^+,K^+-ATPase activity.

It is well-known that Na^+,K^+-ATPase consists of two different subunits: a large polypeptide (designated α) and a small polypeptide (designated β). The α subunit is often referred to as the catalytic subunit since it is specifically phosphorylated from ATP (Uesugi et al., 1971; Kyte, 1971) and contains the binding site for ouabain (Ruoho & Kyte, 1974; Hegyvary, 1975). If thiamin is an essential cofactor for the enzyme, the stoichiometry would be important. In this study, we examined it by determining the content of thiamin vs. the catalytic subunit, phosphorylation site, and ouabain binding site. The content was markedly low compared to phosphorylation and ouabain binding sites. The molar ratio between thiamin and the enzyme would be 1/50, if the Na^+,K^+-ATPase holoenzyme is an $\alpha_2\beta_2$ tetramer (Peterson & Hokin, 1981) and only one of the two subunits can be phosphorylated at any one time (Stein et al., 1973; Perrone et al., 1975). This suggests that thiamin is not coupled directly to the minimum functional unit of the enzyme, though there is so far little information about the stoichiometric arrangement of subunits of the brain enzyme, and higher order aggregates of the $\alpha_2\beta_2$ subunit may exit in the native membrane state as reported by Hansen et al. (1979).

In the previous study (Matsuda & Cooper, 1981a), showing that thiamin is localized in the synaptic plasma membranes at a concentration of 10 pmol/mg of protein, thiamin was determined by a chemical method using alkaline cyanogen bromide and ensured by using thiaminase I which specifically destroys thiamin compounds. In this study, we did not certify the content determined by the chemical method, because it was too low to account for its role as a coenzyme or cofactor of the Na^+,K^+-ATPase. However, the presence of thiamin

in the preparation was confirmed by the experiment using rats injected with [^{35}S]thiamin. The amount of [^{35}S]thiamin was lower than that determined by the chemical method. This difference is likely due to the incomplete replacement of endogenous thiamin with labeled vitamin. Though the amount determined by the chemical method may be overestimated, these results at least indicate the presence of thiamin in the preparation.

The inhibition of Na^+,K^+-ATPase by pyrithiamin was antagonized with thiamin phosphate esters and oxythiamin as well as thiamin. This finding suggests that the thiamin structure is essential for the interaction of pyrithiamin with the enzyme. The presence of thiamin in the preparation, together with the previous findings (Decaro et al., 1956; Kordam, 1958; Rindi & Perri, 1961; Gubler, 1976; Cooper, 1968), suggests that pyrithiamin may have inhibited the activity of the enzyme by interacting with endogenous thiamin which is possibly localized in the vicinity of the enzyme complex. Further support for this contention and a specificity of the antimetabolite for neuronal Na^+,K^+-ATPase arise from our observation that the activity of Na^+,K^+-ATPase from brain (but not from kidney) was decreased by treatment of the enzyme with sodium bisulfite. These findings, in conjunction with our recent report (Matsuda & Cooper, 1981a) that thiamin is an integral component of nerve membranes, suggest that the vitamin may be involved in the activity of Na^+,K^+-ATPase in neuronal tissues. However, as noted earlier the apparent lack of stoichiometry between the vitamin content and the enzyme makes it uncertain how thiamin may be involved with the enzyme complex.

In this study, we also examined the effect of pyrithiamin on the partial reactions of Na^+,K^+-ATPase. The treatment with pyrithiamin leads to inhibition of Na^+-dependent phosphorylation and K^+-stimulated phosphatase activity as well as ouabain binding. Their inhibitions run entirely parallel with that of the overall Na^+,K^+-ATPase reaction. Thus, the effect of pyrithiamin may be explained as a complete loss of active enzyme by alterations in the quaternary structure of the enzyme complex. This is in agreement with our finding that pyrithiamin, but not other thiamin compounds, shows a significant effect on turbidity changes of the enzyme suspension, suggesting a structural change of the enzyme complex.

Acknowledgments

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Registry No. ATPase, 9000-83-3; pyrithiamin, 534-64-5; thiamin, 59-43-8.

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