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Involvement of sulfhydryl groups in the inhibition of brain ($Na^+ + K^+$)-ATPase by pyrithiamin

Toshio Matsuda [a], Heitaroh Iwata [a, *] and Jack R. Cooper [b]

[a] *Department of Pharmacology, Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565 (Japan) and* [b] *Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510 (U.S.A.)*

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Brain ($Na^+ + K^+$)-ATPase was protected by low concentrations of GSH from the inhibitory effect of pyrithiamin. The possible involvement of sulfhydryl groups in the inhibition was then studied by comparing the effect of pyrithiamin with that of *N*-ethylmaleimide on the enzyme. The treatment of rat brain ($Na^+ + K^+$)-ATPase with thesee inhibitors caused a significant decrease in reactivity of the enzyme to *N*-ethyl[^{3}H]maleimide. *N*-Ethylmaleimide, like pyrithiamin, inhibited the partial reactions of ($Na^+ + K^+$)-ATPase system in parallel with the inhibition of the overall reaction. An SDS-polyacrylamide gel electrophoresis procedure indicated that pyrithiamin and *N*-ethylmaleimide inhibited Na^+-dependent phosphorylation of the $\alpha(+)$ form of rat brain ($Na^+ + K^+$)-ATPase more than that of α, though the selectivity for the $\alpha(+)$ seemed to be higher with the former inhibitor than in the latter. The treatment also decreased sensitivity of the enzyme to ouabain inhibition. However, pyrithiamin- and *N*-ethylmaleimide-induced inactivations of the enzyme differed in the efficacy of GSH for protection and in the effect of the kind of ligands present during the reaction. Furthermore, pyrithiamin did not appear to interact directly with sulfhydryl groups, but caused the formation of disulfide in bovine brain ($Na^+ + K^+$)-ATPase. In contrast to *N*-ethylmaleimide, pyrithiamin did not affect the sulfhydryl-enzymes such as alcohol dehydrogenase and L-alanine dehydrogenase. It is concluded that pyrithiamin modifies the functional sulfhydryl groups of brain ($Na^+ + K^+$)-ATPase in a way different from *N*-ethylmaleimide and causes a structural change and inactivation of the enzyme.

Introduction

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 [1]. We have previously reported that thiamin is an integral part of synaptic plasma membranes where ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) is localized [2] and that pyrithiamin, an antimetabolite of thiamin, exerts a marked inhibition on ($Na^+ + K^+$)-ATPase specifically derived from neuronal tissue [3]. The findings suggest that thiamin is involved in the activity of ($Na^+ + K^+$)-ATPase in nervous tissue but the exact mechanism remains unclear because of the apparent lack of stoichiometry between the thiamin content and the enzyme. On the other hand, on the basis of the finding [4] that there are two molecular forms, termed $\alpha(+)$ and α, of ($Na^+ + K^+$)-ATPase in the brain, we have recently demonstrated that pyrithiamin is a preferential inhibi-

* To whom correspondence should be addressed.
Abbreviation: SDS, sodium dodecyl sulfate.

tor of the $\alpha(+)$ molecular form of $(Na^+ + K^+)$-ATPase [5].

In continuing studies on the mechanism of this inhibition, we have found that GSH antagonizes the inhibitory effect of pyrithiamin on brain $(Na^+ + K^+)$-ATPase, suggesting the possible involvement of sulfhydryl groups in the inhibition. In order to clarify this point, we compared the effect of pyrithiamin with that of *N*-ethylmaleimide, a sulfhydryl-alkylating reagent, on brain $(Na^+ + K^+)$-ATPase. This paper demonstrates that pyrithiamin inactivates the enzyme by decreasing the number of the functional sulfhydryl groups of the enzyme in a fashion different from *N*-ethylmaleimide.

Materials and Methods

Enzyme preparations and enzyme assays

$(Na^+ + K^+)$-ATPases were partially purified from rat brain, bovine brain, rat kidney and rabbit kidney, and the activities were measured as previously described [3,5]. In brief, a microsomal preparation (5 mg/ml) was treated with SDS (0.2 mg/mg protein for rat kidney and 0.3 mg/mg protein for the other) and subjected to discontinuous sucrose gradient centrifugation. The specific activities (μmol P_i/mg protein per min) of the enzymes used here were 6.75–9.35 (rat brain), 3.35 (bovine brain), 12.9 (rat kidney) and 8.18 (rabbit kidney). Na^+-dependent phosphorylation and K^+-stimulated phosphatase activity of rat brain $(Na^+ + K^+)$-ATPase were determined as previously described [3]. The activities of alcohol dehydrogenase (EC 1.1.1.1) [6], L-alanine dehydrogenase (EC 1.4.1.1) [7] and thiamin pyrophosphokinase (EC 2.7.6.2) [8] were determined as previously reported. Protein was measured by the method of Lowry et al. [9] using bovine serum albumin as standard.

Phosphorylated intermediate

The enzyme was phosphorylated with [γ-^{32}P]ATP and subjected to SDS-polyacrylamide gel electrophoresis as previously described [5]. The gel was stained, destained, and dried on filter paper. The dried gel was exposed to Kodak X-Omat AR film for 4–7 days. Only the area of the gel around the phosphorylated intermediate was photographed for the clear demonstration of two molecular forms of $(Na^+ + K^+)$-ATPase.

Reaction with N-ethyl[^{3}H]maleimide

N-Ethyl[^{3}H]maleimide (1 mM, 100 mCi/mmol) was reacted with the enzyme in 20 mM imidazole buffer (pH 7.25)/1 mM EDTA for 30 min at 37°C. The reaction was stopped with excess 2-mercaptoethanol. The two large subunits ($\alpha(+)$ and α) were separated by SDS-polyacrylamide gel electrophoresis as previously described [5]. The gels were stained and destained, the bands were cut out with a razor blade, and the radioactivity was determined by liquid scintillation spectrometry after dissolving the polyacrylamide with 30% H_2O_2.

Sulfhydryl and disulfide determinations

GSH at various concentration in the presence and absence of pyrithiamin was determined by the method of Alexander [10]. Sulfhydryl and disulfide contents in the enzyme preparation were assayed according to the producer of Cavallini et al. [11].

Treatment with pyrithiamin, N-ethylmaleimide and diamide

The procedure for the treatment with *N*-ethylmaleimide and diamide was essentially the same as that reported previously for the treatment with pyrithiamin [5]. In brief, the enzyme suspension was incubated with the inhibitors freshly dissolved in 20 mM imidazole-buffer (pH 7.25) at 37°C for 15 min, and centrifuged at $100\,000 \times g$ for 1 h. The pellet was washed once with 0.1 mM EDTA/5 mM imidazole buffer (pH 7.25) and resuspended in 0.16 M sucrose/0.5 mM EDTA/10 mM imidazole buffer (pH 7.25).

Materials

Vanadium-free ATP (disodium and Tris salts), pyrithiamin, ouabain, GSH, dithiothreitol and yeast alcohol dehydrogenase were obtained from Sigma Chemical Co. L-Alanine dehydrogenase was purchased from Boehringer Mannheim. [γ-^{32}P]ATP and *N*-ethyl[^{3}H]maleimide were from Amersham. All other chemicals used here were of the highest purity commercially available. Disodium salts of ATP and *p*-nitrophenyl phosphate

were converted to the Tris salts by passage over an AG 50W-X8 cation exchange resin (Tris form).

Results

Fig. 1 shows that GSH decreases the inhibitory effect of pyrithiamin on $(Na^+ + K^+)$-ATPase in a dose-related manner. The inhibitory effect by 1 mM pyrithiamin was almost completely prevented by the simultaneous addition of 0.1 mM GSH, while the protection by GSH against the inactivation by 1 mM *N*-ethylmaleimide required higher concentrations than 1 mM (data not shown). GSH itself did not affect the enzyme activity at the concentration used here. A similar antagonism was obtained with other sulfhydryl reagents such as dithiothreitol and 2-mercaptoethanol (data not shown), indicating the possible involvement of sulfhydryl groups in the inhibitory effect of pyrithiamin. These sulfhydryl reagents also antagonized the inhibitory effect of pyrithiamin on Na^+-dependent phosphorylation of the $\alpha(+)$ molecular form of rat brain $(Na^+ + K^+)$-ATPase as reported previously [5]. When GSH was added to the reaction mixture after the preincubation of the enzyme with pyrithiamin, it did not reverse the inhibitory effect.

In order to clarify the involvement of sulfhydryl groups in the inhibitory effect of pyrithiamin, the effects of pyrithiamin and *N*-ethylmaleimide on sulfhydryl groups of the catalytic subunit of rat brain $(Na^+ + K^+)$-ATPase were studied by determining the reactivity of the enzyme with *N*-ethyl[^{3}H]maleimide (Table I). The enzymes treated with pyrithiamin or *N*-ethylmaleimide in the absence and presence of GSH were reacted with *N*-ethyl[^{3}H]maleimide and then subjected to SDS-polyacrylamide gel electrophoresis to separate the two catalytic subunits of the enzyme. In control enzyme, the $\alpha(+)$ incorporated more *N*-ethyl[^{3}H]maleimide than the α did (Table I). The treatment with 1 mM pyrithiamin or 1 mM *N*-ethylmaleimide inhibited both incorporations of *N*-ethyl[^{3}H]maleimide to the $\alpha(+)$ and the α to the same degree. Low concentrations of GSH almost completely prevented the effect of pyrithiamin, while they produced only minimal protection against the effect of *N*-ethylmaleimide.

It is not known how pyrithiamin decreases the reactivity of sulfhydryl groups of the enzyme. The possibility of a direct interaction between pyrithiamin and sulfhydryl groups was studied by examining the effect of pyrithiamin using a spectrophotometric assay for GSH in a solution without the enzyme. Fig. 2 depicts a standard curve for the determination of GSH in the presence and absence of pyrithiamin. The presence of 1 mM pyrithiamin did not affect the standard curve. We have further examined the possible interaction between pyrithiamin and GSH using thiamin pyrophosphokinase which is known to be inhibited by

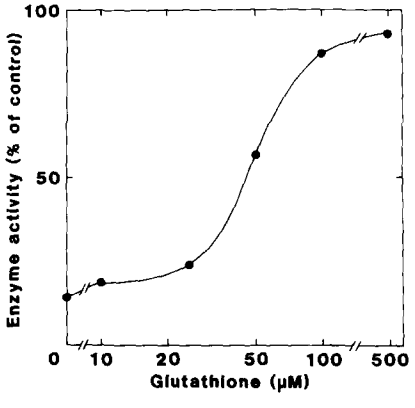

Fig. 1. Effect of GSH on the inhibition of rat brain $(Na^+ + K^+)$-ATPase activity by pyrithiamin. The enzyme was pretreated with 1 mM pyrithiamin in the absence and presence of GSH at the indicated concentrations for 15 min at 37°C. The activity was measured as described under Materials and Methods. Each point is the mean of three experiments.

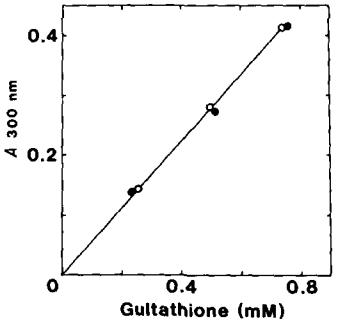

Fig. 2. Effect of pyrithiamin on the determination of GSH. GSH at the indicated concentrations was mixed with excess *N*-ethylmaleimide in the absence (○) and presence (●) of 1 mM pyrithiamin, and the absorbance at 300 nm was measured. A typical experiment is shown.

TABLE I

THE EFFECT OF PYRITHIAMIN AND *N*-ETHYLMALEIMIDE ON THE REACTIVITY OF THE CATALYTIC SUBUNITS OF RAT BRAIN ($Na^+ + K^+$)-ATPase WITH *N*-ETHYL[^{3}H]MALEIMIDE

The enzyme (50 μg/ml) was treated with the reagents specified in the Table at 37°C for 15 min, and centrifuged. The treated enzyme was then reacted with *N*-ethyl[^{3}H]-maleimide and an aliquot (20 μg protein) was subjected to SDS-polyacrylamide gel electrophoresis as described under Materials and Methods. Results, shown as % of control, represent the means ± S.E. of 5–12 determinations. Under the conditions, the control incorporations to the α(+) and the α form were 220 ± 9 and 67 ± 9 pmol per band, respectively ($n = 13$).

Reagents	*N*-Ethyl[^{3}H]maleimide reactivity (% of control)	
	α(+) form	α form
1 mM pyrithiamin	37.5 ± 1.7	38.5 ± 3.8
1 mM pyrithiamin + 0.1 mM GSH	74.7 ± 2.9	59.0 ± 3.5
1 mM pyrithiamin + 0.5 mM GSH	79.5 ± 4.9	72.1 ± 10.7
1 mM *N*-ethylmaleimide	39.8 ± 1.0	31.4 ± 3.4
1 mM *N*-ethylmaleimide + 0.1 mM GSH	42.7 ± 1.1	35.1 ± 4.9
1 mM *N*-ethylmaleimide + 0.1 mM GSH	52.9 ± 1.6	42.6 ± 5.6

pyrithiamin [1]. GSH (200 μM) did not affect the inhibitory effect of pyrithiamin (10–100 μM) on the enzyme in a rat brain soluble fraction (data not shown). These findings suggest that pyrithiamin does not interact directly with sulfhydryl groups of the enzyme as *N*-ethylmaleimide does. Then, we examined the effect of pyrithiamin treatment (0.67 mM, for 15 min at 37°C) on disulfide and sulfhydryl contents in bovine brain ($Na^+ + K^+$)-ATPase preparation. The bovine brain enzyme showed two molecular forms as described previously [4] and pyrithiamin inhibited the activity as the case of rat brain ($Na^+ + K^+$)-ATPase (data not shown). The sulfhydryl contents were determined to be 89 ± 9 (control) and 24 ± 3 (treated) nmol/mg protein and the disulfide contents were 59 ± 5 (control) and 82 ± 9 (treated nmol/mg protein (means ± S.E. of four or five determinations). The decrease in sulfhydryl content ($p < 0.01$) and the increase in disulfide content ($p < 0.05$) by the treatment were statistically significant.

We have previously reported that pyrithiamin inhibited Na^+-dependent phosphorylation and K^+-stimulated phosphatase activity in parallel with the inhibition of the overall ($Na^+ + K^+$)-ATPase reaction [3]. Since there are conflicting reports [12–17] about the effect of *N*-ethylmaleimide on the overall reaction and the partial reactions of ($Na^+ + K^+$)-ATPase system, in order to compare the effect of pyrithiamin with that of *N*-ethylmaleimide, we studied the effect of *N*-ethylmaleimide on these reactions of rat brain ($Na^+ + K^+$)-ATPase (Fig. 3). *N*-Ethylmaleimide inhibited these activities in parallel as pyrithiamin did. Sweadner [4] has shown that the α(+) form of ($Na^+ + K^+$)-ATPase is more sensitive to *N*-ethylmaleimide than the α form and we [5] have recently demonstrated that pyrithiamin inactivates preferentially the α(+) form. To compare the selectivity of pyrithiamin for the α(+) form with that of *N*-ethylmaleimide or diamide, a

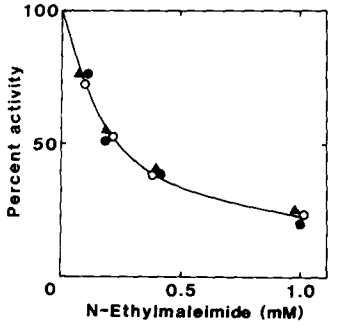

Fig. 3. Effect of *N*-ethylmaleimide on ($Na^+ + K^+$)-ATPase activity, Na^+-dependent phosphorylation and K^+-stimulated phosphatase activity. Rat brain ($Na^+ + K^+$)-ATPase was treated with *N*-ethylmaleimide at the indicated concentrations for 15 min at 37°C and washed as described under Materials and Methods. The treated enzyme was used for the assays for ($Na^+ + K^+$)-ATPase activity (○), Na^+-dependent phosphorylation (●) and K^+-stimulated phosphatase activity (▲). Each point represent the mean of three to six experiments with two different enzyme preparations. The standard error was within 10% of the mean.

sulfhydryl-oxidizing reagent, the effect of these inhibitors on the two molecular forms of rat brain ($Na^+ + K^+$)-ATPase was examined. The treatment with 1 mM *N*-ethylmaleimide or 5 mM diamide for 15 min at 37°C caused a marked inhibition (by 80–85%) of rat brain ($Na^+ + K^+$)-ATPase, while it produced less inhibition (by 20–35%) of the rat kidney and rabbit kidney enzymes. SDS-polyacrylamide gel electrophoresis of rat brain ($Na^+ + K^+$)-ATPase showed that the sulfhydryl-blocking reagents decreased Na^+-dependent phosphorylation of the $\alpha(+)$ form much more than that of the α form, though the selectivity for the $\alpha(+)$ form appeared to be higher in the case of pyrithiamin than with the sulfhydryl reagents (Fig. 4). Fig. 5 shows the effect of *N*-ethylmaleimide and diamide on the sensitivity of brain and kidney ($Na^+ + K^+$)-ATPase to ouabain inhibition. The untreated rat brain enzyme showed two components of ouabain inhibition which reflected the presence of two molecular forms, whereas only one component is present in the kidney enzymes, in agreement with the previous reports [4,5]. Though there was a marked difference in the ouabain sensitivity between rabbit and rat kidney enzymes, both enzymes corresponded to the α form [5]. *N*-Ethylmaleimide or diamide both markedly decreased the sensitivity of the brain enzyme to ouabain inhibition: the low affinity component of ouabain inhibition was the major one in the treated en-

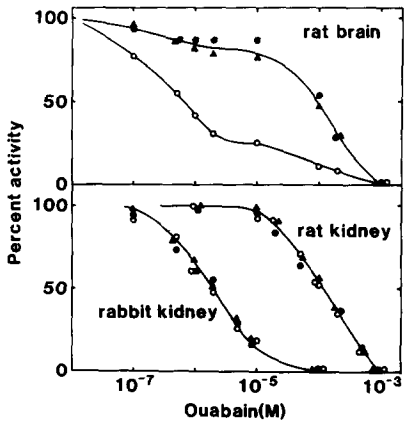

Fig. 5. Effect of *N*-ethylmaleimide and diamide on dose-response curves of the activities of ($Na^+ + K^+$)-ATPases from rat brain, rat kidney and rabbit kidney versus ouabain concentrations. The enzyme was treated without (○) and with 1 mM *N*-ethylmaleimide (▲) or 5 mM diamide (●) as described under Materials and Methods. The ATPase reaction in the presence of various concentrations of ouabain was carried out for 30 min and protein concentration in the medium was adjusted to 2 μg/tube by the addition of bovine serum albumin as previously reported [5]. Points are values from a representative experiment which has been repeated three times.

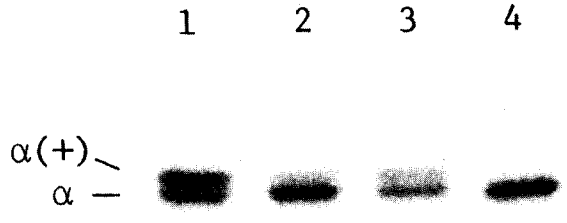

Fig. 4. Effect of *N*-ethylmaleimide, diamide and pyrithiamin on the $\alpha(+)$ and α forms of rat brain ($Na^+ + K^+$)-ATPase. The enzyme was treated with the inhibitors for 15 min at 37°C, and centrifuged. The treated enzymes were phosphorylated by [γ-^{32}P]ATP and subjected to SDS-polyacrylamide gel electrophoresis as described under Materials and Methods. The amount of the treated enzymes (lane 2, *N*-ethylmaleimide; lane 3, diamide; lane 4, pyrithiamin) subjected to SDS-polyacrylamide gel electrophoresis was 20 μg protein, but that of the control enzyme (lane 1) was reduced to 10 μg protein for a clearer demonstration of the two bands in the gel. An autoradiograph of the gel is shown. The concentration of acrylamide was 5%.

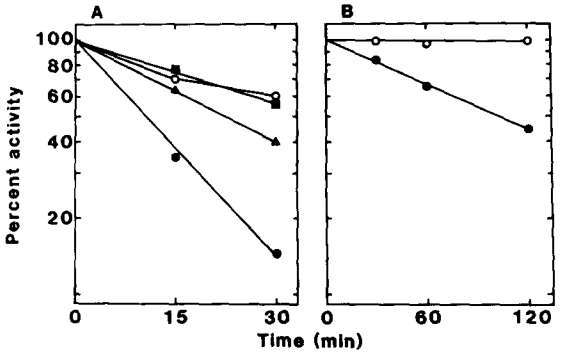

Fig. 6. Effect of pyrithiamin and *N*-ethylmaleimide on the activities of alcohol dehydrogenase (A) and L-alanine dehydrogenase (B). The enzymes, diluted in Tris-HCl (pH 7.5), were treated with 1 mM pyrithiamin (○) and *N*-ethylmaleimide at 0.2 (■), 0.4 (▲) and 1.0 mM (●) for the indicated time at 25°C, and the activities were determined as described under Materials and Methods. Each point represents the mean of three to four experiments. The standard error was within 10% of the mean.

zyme. In contrast, ouabain sensitivity of kidney ($Na^+ + K^+$)-ATPases was not changed by the sulfhydryl reagents.

The previous paper [3] indicated the specificity of pyrithiamin-inhibition for ($Na^+ + K^+$)-ATPase. This was further examined here using sulfhydryl-enzymes (Fig. 6). *N*-Ethylmaleimide significantly inhibited alcohol dehydrogenase and L-alanine dehydrogenase, with a higher degree of inhibition observed with the former enzyme than in the latter. On the other hand, treatment with 1 mM pyrithiamin produced only minimal inhibition of alcohol dehydrogenase and it did not affect L-alanine dehydrogenase.

Since it was reported that the degree of inhibition of ($Na^+ + K^+$)-ATPase by *N*-ethylmaleimide depended on the kind of ligands (Na^+, K^+, Mg^{2+}, ATP) present during the reaction [18–21], we studied the effect of the ligands on the inhibitory effect of pyrithiamin on the enzyme. The inhibition was partially prevented by high concentrations of Na^+ and K^+, independent of the ratio (Fig. 7A). A similar prevention was also observed by increasing the concentration of Tris-buffer (Fig. 7B), indicating that the inhibition of the enzyme by pyrithiamin might depend on the ionic strength of the medium. The effect of various ligands on inhibition of rat brain ($Na^+ + K^+$)-ATPase by pyrithiamin was then examined under conditions

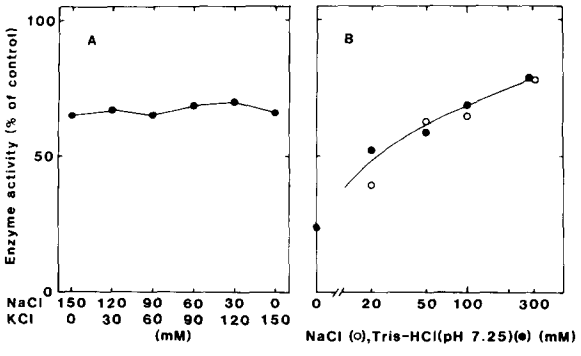

Fig. 7. Effect of monovalent cations and Tris-HCl buffer on the inhibition of rat brain ($Na^+ + K^+$)-ATPase activity by pyrithiamin. The enzyme was pretreated for 15 min with 1 mM pyrithiamin in the presence of various concentrations of Na^+ and K^+, keeping the total cation concentration constant at 150 mM (A), or in the presence of various concentrations of Na^+ or Tris-HCl buffer (pH 7.25) (B). Points are values from a representative experiment which has been repeated three times.

TABLE II

EFFECT OF VARIOUS LIGANDS ON THE INHIBITION OF RAT BRAIN ($Na^+ + K^+$)-ATPase BY PYRITHIAMIN

The enzyme was preincubated with 1 mM pyrithiamin in the presence of the indicated ligands for 15 min at 37°C, and the enzyme activity was determined. The concentrations of the ligands were kept constant at 3 mM ATP, 3 mM $MgCl_2$, 140 mM NaCl and 20 mM KCl during the enzyme reaction. Results, shown as % of control, represent the means ± S.E. of four or five experiments.

Ligands added during the treatment	($Na^+ + K^+$)-ATPase activity
None	13.9 ± 0.9
1 mM ATP	43.8 ± 3.4
1 mM $MgCl_2$ + 10 mM NaCl	25.3 ± 2.5
1 mM $MgCl_2$ + 10 mM KCl	32.9 ± 5.2
1 mM ATP + 1 mM $MgCl_2$ + 10 mM NaCl	52.9 ± 5.4
1 mM ATP + 1 mM $MgCl_2$ + 10 mM KCl	27.7 ± 5.8

to eliminate the effect of their ionic strength (Table II). ATP partially antagonized the inhibitory effect of pyrithiamin, while other nucleotides such as ADP, AMP, ITP and UTP did not (data not shown). The effect of ATP was not changed by the addition of Mg^{2+} and Na^+, but was decreased by addition of Mg^{2+} and K^+. In the absence of ATP Mg^{2+} with Na^+ or K^+ had less effect on the inhibition.

Discussion

The present study demonstrates that the inhibitory effect of pyrithiamin on brain ($Na^+ + K^+$)-ATPase may be mediated by a modification of functional sulfhydryl groups of the enzyme. The inhibition of the enzyme activity by pyrithiamin was accompanied by a decrease in sulfhydryl groups of the enzyme as determined by the reactivity to *N*-ethyl[^{3}H]maleimide (Table I). This might be supported by the determination of sulfhydryl content in the enzyme, though the preparation used here was not pure. The effects of pyrithiamin on sulfhydryl groups of the enzyme and the enzyme activity were prevented by addition of GSH in parallel. These findings suggest the involvement of sulfhydryl groups in the inhibition by pyrithiamin. We have recently reported that the

effect of pyrithiamin is different from that of *N*-ethylmaleimide on rat brain $(Na^+ + K^+)$-ATPase [5]. The present study provides additional evidence on the difference. The inhibitory effect of pyrithiamin was partially prevented by high concentrations of ions and by ATP, but not by ADP. The protective effect of ATP was not decreased by the addition of Mg^{2+} and Na^+, which stimulates the phosphorylation. On the other hand, many investigators [17–21] reported that the inhibition of the enzyme by *N*-ethylmaleimide depended on the presence of Na^+ and K^+, independent of the ionic strength. They also showed that ADP as well as ATP protected against the inhibitory effect of *N*-ethylmaleimide and that the effect of ATP is decreased by Mg^{2+} and Na^+. The effect of 1 mM pyrithiamin on the enzyme, in contrast to that of *N*-ethylmaleimide, was almost completely protected by one tenth the concentration of GSH. The idea that pyrithiamin interacts directly with sulfhydryl groups of the enzyme might be ruled out by the following findings. (1) Pyrithiamin did not interfere with the assay method for the determination of GSH. (2) GSH did not affect the inhibition of thiamin pyrophosphokinase by pyrithiamin. (3) Pyrithiamin did not inhibit the activities of sulfhydryl-enzymes as *N*-ethylmaleimide did. Since we found that pyrithiamin caused the formation of disulfide bonds in the enzyme preparation, this effect may account for the decrease in sulfhydryl groups of the enzyme preparation. In view of these findings, it is concluded that pyrithiamin acts on brain $(Na^+ + K^+)$-ATPase in a fashion different from *N*-ethylmaleimide.

There are two molecular forms of $(Na^+ + K^+)$-ATPase in the brain [4,5,22–24]. The original study on the molecular forms showed that the $\alpha(+)$ form was more reactive than the α form towards Cu^{2+}-*o*-phenanthroline which catalyzed the oxidation of sulfhydryl groups [4]. This finding suggested that the $\alpha(+)$ form might have more exposed sulfhydryl groups than the α form. In this study, we have unexpectedly found that pyrithiamin decreases not only the reactivity of sulfhydryl groups of the $\alpha(+)$ form but also that of sulfhydryl groups of the α form, though it inactivates only the $\alpha(+)$ form. This suggests that the α form, unlike the $\alpha(+)$ form, has few sulfhydryl groups essential for the activity. Another possibility is that there is a difference in reactivity of the essential sulfhydryl groups to pyrithiamin between the $\alpha(+)$ and the α forms: the essential sulfhydryl groups of the α form are less reactive to pyrithiamin than those of the $\alpha(+)$ form. The former might be supported by the finding that the treatment of rat brain $(Na^+ + K^+)$-ATPase with *N*-ethylmaleimide or diamide inhibited Na^+-dependent phosphorylation of the $\alpha(+)$ form (Fig. 4) and caused a marked decrease in the sensitivity of the enzyme to ouabain inhibition (Fig. 5).

The treatment with pyrithiamin and *N*-ethylmaleimide decreased sulfhydryl groups of the enzyme to the same degree (Table I), but the selectivity of these inhibitors for the $\alpha(+)$ form was not the same (Fig. 4). Furthermore, there was a marked difference in the concentration of GSH required for the protection between pyrithiamin- and *N*-ethylmaleimide-induced inactivations of the enzyme. These observations suggest that the sulfhydryl groups of the enzyme that react with *N*-ethylmaleimide are not entirely the same as those influenced by pyrithiamin. This appears to be the case in view of the difference in mechanism for decreasing the number of sulfhydryl groups of the enzyme between pyrithiamin and *N*-ethylmaleimide. In this connection, it should be noted that the effect of pyrithiamin on the sulfhydryl-enzymes contrasted sharply with that of *N*-ethylmaleimide (Fig. 6).

We previously reported that pyrithiamin caused a change in the turbidity of the enzyme suspension [3]. This effect was also protected against by low concentrations of GSH (data not shown). These observations, along with the current demonstration that the effect of pyrithiamin on the enzyme activity is accompanied by a change in the content of sulfhydryl groups of the enzyme, suggest that pyrithiamin causes a structural change with a subsequent inactivation of the enzyme by an oxidation of functional sulfhydryl groups of the enzyme to the disulfide form. Though the mechanism is not known in detail, the formation of disulfide may account for the pyrithiamin-induced turbidity change which probably reflects changes in membrane aggregation as previously observed [3].

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