Biochimica et Biophysica Acta, 482 (1977) 71—78

BBA 68140

POSSIBLE REGULATION OF THIAMINE DIPHOSPHATASE ACTIVITY IN RAT BRAIN MICROSOMES BY LIPIDS

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(Received November 23rd, 1976)

Summary

The effects of various treatments, which affect membrane structure, on microsomal thiamine diphosphatase and thiamine triphosphatase activities of rat brain, were examined.

The treatment of microsomes at alkaline pH caused a 2-fold activation of the thiamine diphosphatase, this being related to a change in membrane structure which was evidenced by a decrease of the turbidity of the microsomal suspension. Repeated freezing and thawing after hypo-osmotic treatment also increased the activity of microsomal thiamine diphosphatase. In addition, the thiamine diphosphatase activity was enhanced by treatment of the microsomes with phospholipase C or acetone. This lipid depletion resulted in a marked reduction in the apparent K_m value of the thiamine diphosphatase with a corresponding loss in heat stability of the enzyme. We found further that brain thiamine diphosphatase was solubilized by Triton X-100. This decreased the phospholipid content in the preparation, but did not affect the apparent K_m value and heat stability of the enzyme.

In contrast with thiamine diphosphatase, thiamine triphosphatase was inactivated by treatment at alkaline pH or with acetone. However, treatment with phospholipase C did not affect the activity of thiamine triphosphatase.

Introduction

Since it was reported that neuroactive drugs such as acetylcholine and tetrodotoxin caused the release of thiamine and thiamine monophosphate from nerve membrane fractions through the hydrolysis of thiamine esters in the membrane [1,2], some properties of the enzymes involved in thiamine metabolism in the central nervous system have been investigated [3—15]. However, no drugs were found to influence the activities of these enzymes, and so far no physiological role of these enzymes has been established.

Recently, we found that chlorpromazine causes an inhibition of microsomal and soluble thiamine triphosphatases and a marked activation of thiamine diphosphatase in rat brain [16—19]. Furthermore, from studies on the mechanism of the action of chlorpromazine on thiamine phosphatases, we suggested that these opposite effects are due to a different organization of the enzymes in the membrane [18,19].

These observations lead us to consider that protein-lipid interactions which induce a conformational change in the membrane may be important for the regulation of thiamine metabolism in the central nervous system. In order to explore this point further, we studied the relationship between the enzymes degrading phosphorylated thiamine and the lipid environment of the enzymes in the membrane. This paper describes a possible regulation by lipids of the activity of microsomal thiamine phosphatases, mainly thiamine diphosphatase, of rat brain.

Materials and Methods

Chemicals. Thiamine diphosphate, Tris/ATP and phospholipase C (*Clostridium welchii*, Type I, Lot 53C-6870) were purchased from the Sigma Chemical Co. Thiamine triphosphate was a gift from Takeda Chemical Industries Ltd., Osaka. Thiamine diphosphate was purified by chromatography on a colum of Amberlite IRC-50 (H^+) [20]. Purities of thiamine triphosphate and thiamine diphosphate were determined by paper electrophoresis to be greater than 97% and 99.8%, respectively. All other reagents were of the best analytical grade available.

Preparation of microsomes. The brains of male Sprague-Dawley rats, weighing 200—250 g, were homogenized in 10 vols. of 0.25 M sucrose and microsomes were prepared from the homogenate as described previously [18]. Freshly prepared microsomes were always used. Alkaline pH treatment of microsomes was performed as described previously by Yamazaki and Hayaishi [21] except that the treatment was carried out for 30 min.

Phospholipase C treatment. The microsomal suspension (1 mg protein/ml) was pretreated with phospholipase C (0.5 mg/ml) for 30 min at 37°C according to the procedure of Taniguchi and Tonomura [22]. Control samples were subjected to the same treatment in the presence of 1 mM ethyleneglycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid instead of 11.4 mM $CaCl_2$. After treatment, the mixture was centrifuged at 105 000 × *g* for 1 h and washed with ice-cold 0.25 M sucrose once. The resulting sediment was resuspended in the sucrose solution for enzyme activity and phospholipid determinations.

Solubilization of thiamine diphosphatase. To microsomal suspensions (about 4 mg protein/ml), an equal volume of 2% (w/v) Triton X-100 in 0.25 M sucrose was added. The mixture was incubated at 4°C for 30 min and centrifuged at 105 000 × *g* for 1 h to remove the non-solubilized part. The clear supernatant was fractionated with solid $(NH_4)_2SO_4$ to remove most of the detergent. The fraction that precipitated between 40 and 70% $(NH_4)_2SO_4$ was suspended in 0.25 M sucrose. The suspension was dialysed overnight and used as a solubilized preparation.

Enzyme assays. Thiamine triphosphatase and thiamine diphosphatase activi-

ties were determined as described previously [18].

ATPase activity was measured in terms of production of P_i [23] during the incubation for 10 min at 37°C. The standard medium for total ATPase contained 40 mM Tris · HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM ATP, 140 mM NaCl, 14 mM KCl and about 50 μg/ml of microsomal protein. Mg^{2+}-ATPase activity was measured in the same reaction mixture in which Na^+ and K^+ were omitted. ($Na^+ + K^+$)-ATPase activity was determined by subtracting Mg^{2+}-ATPase from total ATPase activity. Appropriate controls were carried out minus enzyme or substrate.

Protein was determined according to the method of Lowry et al. [24] with bovine serum albumin as a standard.

Phospholipid determination. Lipid extracts of enzyme preparations were prepared by the method of Kates [25]. The extract was ashed and the P_i determined [23].

Results

Alkaline pH treatment

Fig. 1 shows that incubation of microsomes in the pH range of 6.5—10 had little effect on thiamine diphosphatase activity. Above pH 10.5 there was a large increase in thiamine diphosphatase activity with a peak effect at pH 11.5. An instantaneous clarification of the microsomal suspension, an observation which suggests that lipid-protein interactions in the membrane are being altered [26], was seen at alkaline pH. Centrifugation of either the untreated or treated microsomes at 105 000 × *g* for 1 h produced a clear supernatant free from the enzyme activity and a precipitate containing all of the activity.

On the other hand, thiamine triphosphatase activity was decreased by treatment at alkaline pH.

When the same high pH levels were reached with NaOH or KOH instead of NH_4OH, similar results were obtained.

Treatment with phospholipase C

When the microsomes were pretreated with phospholipase C in the presence

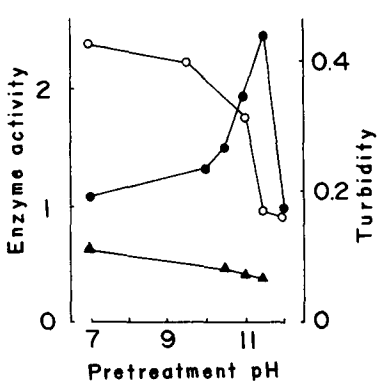

Fig. 1. Effect of treatment of microsomes at indicated pH on thiamine diphosphatase (●——●) and thiamine triphosophatase (▲——▲) activities. The turbidity of microsomal suspension was monitored at 520 nm (○——○). Enzyme activity is expressed as μmol P_i/h per mg protein.

or absence of Ca^{2+}, an activator of phospholipase C, thiamine diphosphatase activity increased to about twice that of the control but thiamine triphosphatase activity was unaffected (Table I).

Thiamine triphosphatase and Mg^{2+}-ATPase activities were unaffected by treatment with phospholipase C in the absence of Ca^{2+} (Table I, Control), but thiamine diphosphatase and $(Na^+ + K^+)$-ATPase activities in control samples were about 30% and 8 times higher than those in untreated microsomes, respectively. In this experiment, phospholipase C-treated microsomes was centrifuged and washed once as mentioned in Materials and Methods. But a little contamination by phospholipase C of the reaction mixture including Ca^{2+} is possible, so this contamination might contribute to the slight increase of thiamine diphosphatase activity. As another explanation, it is considered that phospholipase C preparation contains some factor which induces an increase of $(Na^+ + K^+)$-ATPase activity, but the details are unknown.

The degree of increase in thiamine diphosphatase activity and of decrease in phospholipid content were unchanged even after treatment with phospholipase C for a more long time.

Since phospholipase C of *Clostridium welchii* is known to have moderate collagenase and neuraminidase activities [27], experiments were performed in which phospholipase C was substituted with purified collagenase (Boehringer Mannheim) or neuraminidase (Sigma, Type V). However, pretreatment of microsomes with these enzymes (0.001—0.5 mg/ml) did not cause any effect on the thiamine diphosphatase and the ATPase activities (data not shown).

Other treatment of microsomes

When the microsomal suspension was dialysed against distilled water overnight at 4°C, thiamine diphosphatase activity was slighly stimulated. Furthermore, the repeated freezing and thawing of the dialysed preparation induced a

TABLE I

EFFECT OF TREATMENT OF MICROSOMES WITH PHOSPHOLIPASE C ON THIAMINE DIPHOSPHATASE, THIAMINE TRIPHOSPHATASE AND ATPase ACTIVITIES

Microsomal suspension (1 mg/ml) was pretreated with phospholipase C (0.5 mg/ml) for 30 min at 37°C in the presence of $CaCl_2$ (treated) or ethyleneglycol bis(β-aminoethylether)-*N,N'*-tetraacetic acid (control). Phospholipid content is expressed as μmol P_i/h per mg protein and ATPases are in μmol P_i/min per mg protein. The values are the means ± S.E. of four experiments.

Treatment	Phospholipid (% of control)	Enzyme activity (% of control)			
		Thiamine diphosphatase	Thiamine triphosphatase	Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase
Before	0.75 ± 0.02	1.23 ± 0.04	0.51 ± 0.02	0.32 ± 0.01	0.20 ± 0.01
After					
control	1.06 ± 0.01 (100)	1.60 ± 0.06 (100)	0.46 ± 0.04 (100)	0.39 ± 0.03 (100)	1.66 ± 0.17 (100)
treated	0.48 ± 0.03 * (45)	3.07 ± 0.08 ** (194)	0.41 ± 0.01 (89)	0.28 ± 0.01 * (70)	1.01 ± 0.02 * (61)

* Significantly lower than control at $P < 0.025$.
** Significantly higher than control at $P < 0.005$.

TABLE II

CHANGES OF MICHAELIS CONSTANTS OF THIAMINE DIPHOSPHATASE AND PHOSPHOLIPID CONTENT BY VARIOUS TREATMENTS

K_m, V and phospholipid content are expressed as mM thiamine diphosphate, μmol P_i/h per mg protein and μmol P_i/mg protein, respectively. The values are means ± S.E. The number of experiments is shown in parentheses.

	K_m	V	Phospholipid content
Untreated	1.1	1.34	0.75 ± 0.03 (7)
Freezing and thawing after dialysis	1.1	4.00	1.00 ± 0.04 (3)
Treated with NH_4OH	1.1	3.13	0.73 (2)
Solubilized with Triton X-100	0.9	3.70	0.07 ± 0.03 (3)
Treated with acetone	0.4	4.59	0.32 ± 0.01 (4)
Treated with phospholipase C	0.4	3.39	0.48 ± 0.03 (4)

3-fold activation of thiamine diphosphatase activity. The dialysis procedure was considered a hypo-osmotic effect because the repeated freezing and thawing after dialysis against isotonic KCl, NaCl or sucrose instead of distilled water showed little effect on the enzyme activity.

Thiamine diphosphatase activity was also stimulated by Triton X-100. Subsequently, we found that the detergent was effective for solubilization of the enzyme. These results are summarized in Table II.

Michaelis constant, heat stability of thiamine diphosphatase and phospholipid content

Changes of the apparent K_m values of thiamine diphosphatase and phospholipid content by various treatments are given in Table II. Fig. 2 represents the effect of heating the microsomal suspension in 0.25 M sucrose (pH 7.4) on thiamine diphosphatase activity. The treatment of microsomes with acetone or phospholipase C resulted in a marked reduction in the apparent K_m value and a decrease of phospholipid content in the preparation. Furthermore, thiamine diphosphatase in the microsomes was found to be more labile to heat. Other treatments except solubilization with Triton X-100 have little effect on the apparent K_m value and phospholipid content. The kinetic parameter and heat

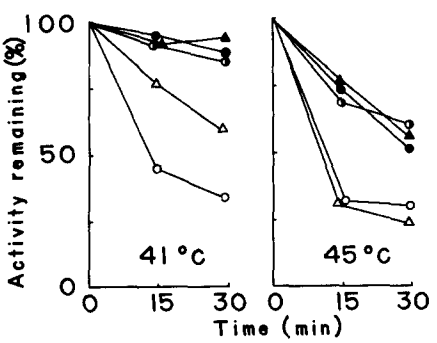

Fig. 2. Heat stability of thiamine diphosphatase in microsomes untreated (●——●) and treated with NH_4OH (◑——◑), Triton X-100 (▲——▲), acetone (△——△) or phospholipase C (○——○).

stability of the solubilized preparation may be complicated because the preparation contains a small amount of the detergent.

Discussion

The data in this paper indicate that a variety of treatments, affecting the lipoprotein membrane structure of microsomes, greatly increase the activity of thiamine diphosphatase.

The effect of high pH on thiamine diphosphatase activity was found not to be related to solubilization of the enzyme as with liver thiamine diphosphatase [21]. From the evidence that the increase of thiamine diphosphatase activity was accompanied by extensive clarification of the microsomal suspension, this finding may result from a high pH-induced modification of the enzyme's environment in such a way that the normal constraint on the activity is relaxed. This result is similar to the effect seen by Stetten and Burnett [26] with microsomal glucose-6-phosphatase from rat liver and by Vessey and Zakim [28] with microsomal uridine diphosphate-glucuronyltransferase from bovine liver.

Since freezing and thawing or hypo-osmotic treatment is said to destroy the membrane structure, the activation of thiamine diphosphatase activity by repeated freezing and thawing after dialysis is probably related to the change of the membrane structure.

It is well known that treatment with phospholipase C, which specifically hydrolyzes phosphatidylcholine in microsomal membrane [29], decreases the ATPase activity and Ca^{2+} transport. In the present investigation, the ATPase activity was also decreased by the treatment with phospholipase C. Furthermore, the turbidity of the reaction medium increased with the time of the treatment (data not shown) as reported previously by Taniguchi and Tonomura [22] and Finean and Martonosi [30].

Martonosi et al. [27] reported that phospholipase C of *Clo. welchii* contained moderate amounts of contaminating collagenase and neuraminidase activities. But, it is unlikely that the effects observed could be attributed to these contaminating enzymes since collagenase and neuraminidase had no effect on the thiamine diphosphatase and the ATPase activities under conditions used in the treatment with phospholipase C. It is of course possible that there are other unknown contaminants in phospholipase C preparation used which cause an increase of thiamine diphosphatase activity. Furthermore, the possibility that the activation of thiamine diphosphatase activity is due to soluble end-products of phospholipase C is excluded since these factors are readily removed by washing of the membrane with 0.25 M sucrose following phospholipase C treatment. These results indicate that the thiamine diphosphatase activity is stimulated by a breakdown of the microsomal phospholipids. Treatment with phospholipase C under our conditions also decreased ATPase activity as reported by Taniguchi and Tonomura [22]. The finding that microsomal thiamine triphosphatase does not require phospholipids for its maximal activity supports the idea [10,11] that membrane thiamine triphosphatase is different from ATPase.

It is of interest that the treatment with phospholipase C in the absence of Ca^{2+} induced a marked activation of $(Na^+ + K^+)$-ATPase, but not Mg^{2+}-ATPase.

This unexpected result could be due to heat-labile factor(s) in the phospholipase C preparation used since the same treatment without phospholipase C or with boild phospholipase C (for 5 min) did not affect the enzyme activity.

Microsomal phospholipid content was significantly decreased by treatment with phospholipase C or acetone. This lipid depletion caused a marked activation of thiamine diphosphatase activity and was accompanied by a significant reduction of the apparent K_m value of thiamine diphosphatase. The addition of phospholipid, such as phosphatidylcholine, to phospholipase C-treated thiamine diphosphatase did not cause any inhibition of the enzyme activity. In addition, acetone extracts isolated from brain microsomes also did not impair the activity of the lipid-depleted thiamine diphosphatase. Thus, phospholipids may not act as specific inhibitors of thiamine diphosphatase, but may serve as a barrier which makes the enzyme inaccessible to the substrate, although further studies are required on this point. Furthermore, the lipid-depleted enzymes were susceptible to heat inactivation. The result suggests that the phospholipids in microsomes provide effective protection for thiamine diphosphatase against heat inactivation. This speculation is supported by a preliminary experiment which showed that the addition of phosphatidylcholine partially protected against heat inactivation of thiamine diphosphatase in microsomes treated with phospholipase C.

Overall, the results support our previous idea that thiamine diphosphatase exists in a "latent form" and is influenced by microenvironmental changes within the membrane [18,19].

Acknowledgments

We thank Dr. J.R. Cooper for his advice and criticism.

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