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EFFECTS OF MONOVALENT CATIONS AND ATP ON BRAIN THIAMINE DIPHOSPHATASE OF NORMAL RATS

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SUMMARY

1. We studied the effects of monovalent cations on brain thiamine diphosphatase from normal rats solubilized by Triton X-100 in the presence and absence of ATP.

2. In the presence of Mg^{2+} and ATP, the enzyme was activated by monovalent cations, especially Na^+ (30–40 mM). However, an antagonism was observed between the actions of Na^+ and K^+.

3. Ca^{2+} also stimulated the enzyme, irrespective of the presence of ATP. Li^+, NH_4^+ or Na^+ inhibited the action of Ca^{2+}. The effects of Ca^{2+} and Mg^{2+} were antagonistic.

4. The optimum pH of the enzyme was 9.3 in the absence of ATP, but on addition of ATP it shifted to 8.6. Further addition of Na^+ or K^+ in the presence of ATP changed the pH optimum to 9.0.

5. The K_m value of the enzyme for thiamine diphosphate was 0.25 mM. Addition of ATP (0.1 mM) decreased the K_m and increased the v_{max} about 2-fold. Similar results were obtained on addition of Na^+.

INTRODUCTION

Several authors[1–3] have observed that electrical stimulation causes release of free thiamine from nerve fibers. Furthermore, ITOKAWA AND COOPER[4,5] recently reported that acetylcholine or tetrodotoxin caused decomposition of thiamine diphosphate and release of thiamine monophosphate or free thiamine from particulate fractions of rat brain and spinal cord into the medium.

We found previously that in thiamine-deficient rat brain the activity of thiamine diphosphatase, which hydrolyses thiamine diphosphate to thiamine monophosphate, increased significantly relative to that in pair-fed animals, while the liver enzyme activities of both types of animal decreased relative to that in control animals. Brain thiamine diphosphatase, like liver enzyme[6], was activated by a low concentration of ATP, but unlike the latter it was bound to insoluble protein[7].

Furthermore, brain enzyme activity of normal rats was markedly increased after injection of physostigmine or ambenonium. After their injection, the levels of total and phosphorylated thiamines in the brain were significantly reduced[8].

So, in view of the hypothesis of VON MURALT[9] that thiamine may participate in nerve transmission or conduction in the central nervous system, we studied the effects of monovalent cations and ATP on brain Mg^{2+}- or Ca^{2+}-dependent thiamine diphosphatase solubilized with Triton X-100 in the presence and absence of ATP.

MATERIALS AND METHODS

Thiamine diphosphate hydrochloride and ATP were gifts from Takeda Chemical Industries Ltd., Osaka and from Kohjin Co., Ltd., Tokyo, respectively. All other reagents were of the best analytical grade available.

Enzyme preparations

Male Sprague–Dawley rats, weighing 200–250 g, were killed by decapitation and their brains were rapidly removed. The brains were homogenized in 10 vol. of acetone for 1.5 min in a Potter–Elvehjem homogenizer at −15°, and then filtered on a glass filter. This procedure was repeated. To inactivate most of the ATPase, the acetone powder was next homogenized in 20 vol. of *n*-butanol, stirred for 20 min at −15°, and filtered.

As an enzyme source, 100 mg of the *n*-butanol powder was extracted with 20 ml of 20 mM $KHCO_3$ for 15 min. The mixture was centrifuged at 10 000 × *g* for 10 min. The supernatant (water-soluble enzymes) was discarded and the pellet was homogenized in distilled water containing 2.0% (w/v) Triton X-100. The homogenate was centrifuged at 10 000 × *g* for 15 min, and the supernatant was used for experiments. The specific activity obtained by this procedure was 10 times that of the brain homogenate.

When necessary, the *n*-butanol powder was treated with chloroform–methanol (2:1, v/v) and a powder was obtained in the same way as the *n*-butanol powder.

Thiamine diphosphatase assay

The standard incubation mixture contained, in a volume of 2.7 ml, 75 mM of Tris–HCl (pH 9.3), 1.0 mM of thiamine diphosphate, 1.0 mM of $MgCl_2$ or 2.0 mM of $CaCl_2$, and enzyme (700 μg of protein). In some experiments 0.1 mM of ATP, equivalent to one-tenth of the amount of substrate, was also added. Triton X-100 when added during homogenization was present at a concentration of 0.3% in the incubation mixture. Monovalent cations were added as chlorides.

Reactions were started by addition of substrate after 5 min preincubation and carried out for 30 min at 37°. Then 0.3 ml of 60% (v/v) perchloric acid was added with cooling, and the mixture was centrifuged. Perchloric acid was added to the blanks immediately after the substrate. The clear supernatant was extracted with isobutanol and the P_i formed was assayed colorimetrically as phosphomolybdic acid following the method of TAKAHASHI[10]. The enzyme activity was proportional both to the protein concentration and to the incubation time for up to 50 min. Non-specific liberation of P_i from ATP was found to be negligible. At the concentrations used added cations did not influence the determination of P_i under the conditions used.

Specific activity of thiamine diphosphatase is expressed as μmoles P_i formed per mg of protein per h.

The protein content of the enzyme was determined by the method of LOWRY *et al.*[11]. When the mixture contained Triton X-100, turbidity was removed by centrifugation at 4000 × *g* for 10 min after color development.

RESULTS

Effect of Mg^{2+}

Fig. 1 shows the effect of Mg^{2+} on brain thiamine diphosphatase activity in the presence and absence of ATP. The activity was maximal with 0.5 and 1.0 mM of Mg^{2+} in the presence and absence of ATP, respectively. Addition of 25 mM Na^+ *plus*

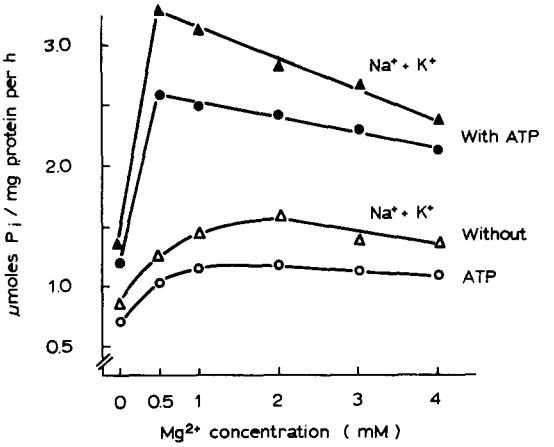

Fig. 1. Effect of Mg^{2+} on brain thiamine diphosphatase activity in the presence and absence of ATP and $Na^+ + K^+$. The standard incubation mixture contained: 75 mM Tris–HCl (pH 9.3), 1.0 mM thiamine diphosphate, 0.3% Triton X-100 and 700 μg of protein. 0.1 mM of ATP or 25 mM $Na^+ + 25$ mM K^+ was added as indicated. Reactions were carried out for 30 min at 37°, after 5 min preincubation. ○, without added ATP and $Na^+ + K^+$; ●, 0.1 mM ATP; △, 25 mM $Na^+ + 25$ mM K^+; ▲, 0.1 mM ATP and 25 mM $Na^+ + 25$ mM K^+.

25 mM K^+ potentiated the activity, but did not change the requirement for Mg^{2+}. It has been reported that rat and bovine liver enzymes require 4.0 mM of Mg^{2+} for maximal activities[12,6], so it seems that the brain enzyme needs less Mg^{2+} than the liver enzymes.

Effects of monovalent cations

The effects of monovalent cations on Mg^{2+}-activated thiamine diphosphatase were examined in the presence and absence of ATP (Fig. 2). Na^+ had the most stimulatory effect and at a concentration of 30–40 mM caused 50 and 35% increase in activity in the presence and absence of ATP, respectively. Other cations did not show clear stimulatory effects both in the presence and absence of ATP.

To see whether the potentiating effects of Na^+ and K^+ on the brain enzyme were synergistic, the effects of various concentrations of Na^+ and K^+ on Mg^{2+}-activated thiamine diphosphatase were tested in the presence and absence of ATP.

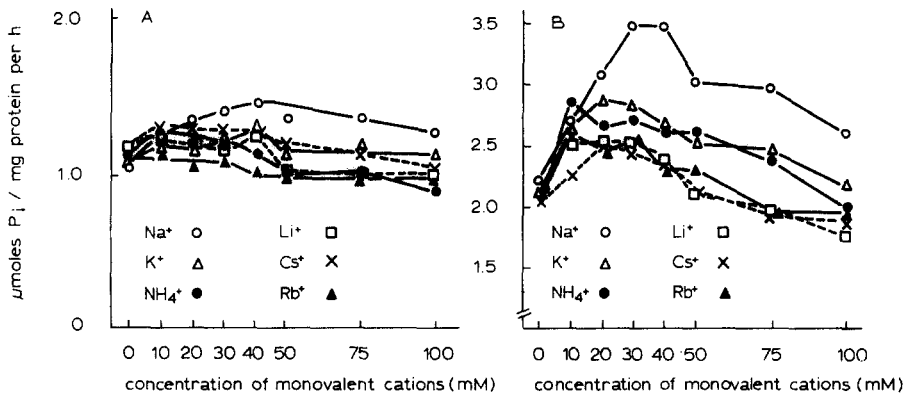

Fig. 2. Effects of monovalent cations on brain thiamine diphosphatase activity in the presence (B) or absence (A) of ATP. The standard incubation mixture contained: 75 mM Tris–HCl (pH 9.3), 1.0 mM thiamine diphosphate, 1.0 mM $MgCl_2$, 0.3% Triton X-100 and 700 μg of protein. 0.1 mM of ATP was added as indicated. After 5 min preincubation, reactions were carried out for 30 min at 37°. Monovalent cations were added as chlorides.

As shown in Fig. 3, the values in the presence of K^+ and absence of Na^+ were elevated and the activities in the presence of both Na^+ and K^+ were lower than those with Na^+ only in the presence of ATP and not in the absence of ATP. The significance of this inhibitory effect of K^+ on Na^+-induced stimulation is unknown. It is of interest that the actions of Na^+ and K^+ on brain phosphatase were antagonistic, not synergistic. We also studied the effect of various concentrations of Na^+ and K^+ in a total 100 mM on the enzyme activity. There was no characteristic activation peak caused by a specific ratio of Na^+ and K^+ concentrations, as seen with (Na^+–K^+)-dependent ATPase.

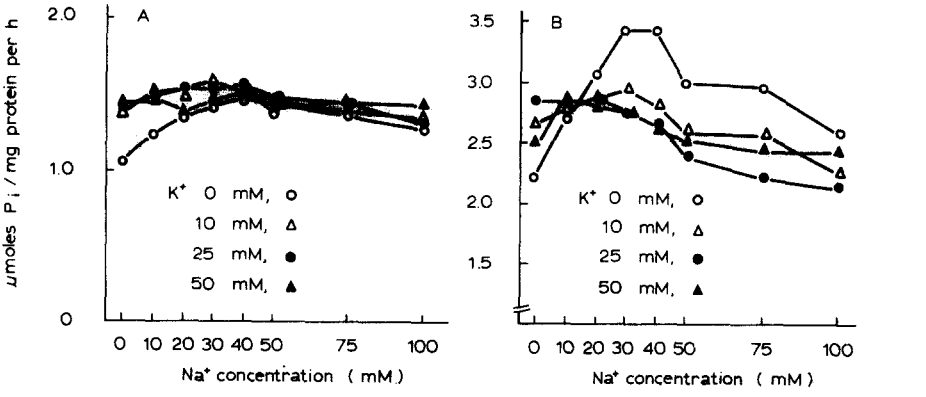

Fig. 3. Effect of K^+ concentration on brain thiamine diphosphatase activity in the presence (B) or absence (A) of ATP. The standard incubation mixture contained: 75 mM Tris–HCl (pH 9.3), 1.0 mM thiamine diphosphate, 1.0 mM $MgCl_2$, 0.3% Triton X-100 and 700 μg of protein. 0.1 mM ATP was added as indicated. After 5 min preincubation, reactions were carried out for 30 min at 37°.

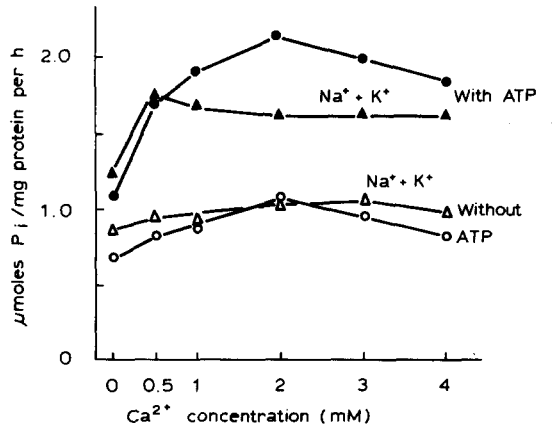

Fig. 4. Effect of Ca^{2+} on brain thiamine diphosphatase activity in the presence or absence of ATP and $Na^+ + K^+$. The standard incubation mixture contained: 75 mM Tris–HCl (pH 9.3), 1.0 mM thiamine diphosphate, 0.3% Triton X-100 and 700 μg of protein. 0.1 mM of ATP or 25 mM $Na^+ + 25$ mM K^+ was added as indicated. Reactions were carried out for 30 min at 37°, after 5 min preincubation. ○, without added ATP and $Na^+ + K^+$; ●, 0.1 mM ATP; △, 25 mM $Na^+ + 25$ mM K^+; ▲, 0.1 mM ATP and 25 mM $Na^+ + 25$ mM K^+.

From these findings, it seems that brain thiamine diphosphatase is an Mg^{2+}–Na^+–ATP-activated pyrophosphatase.

Effect of Ca^{2+}

Ca^{2+}, as well as Mg^{2+} and Mn^{2+}, stimulate the activity of purified liver thiamine diphosphatase[6], so the effect of Ca^{2+} on the brain enzyme in the presence and absence of ATP, Na^+ and K^+ were examined (Fig. 4). Both in the presence and absence of ATP, enzyme activity reached a maximum with 2.0 mM of Ca^{2+} and decreased at higher concentration of Ca^{2+}. However, the coexistence of Na^+ and K^+

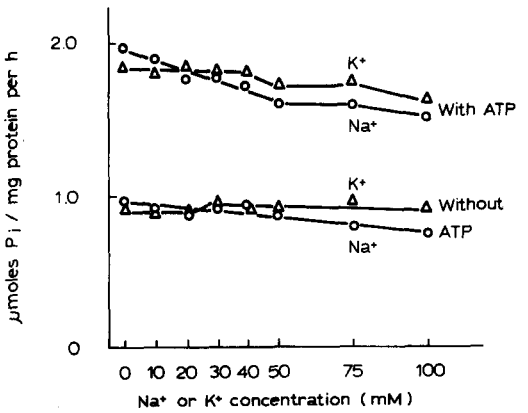

Fig. 5. Effect of Na^+ or K^+ on brain thiamine diphosphatase activity in the presence of Ca^{2+}. The standard incubation mixture contained; 75 mM Tris–HCl (pH 9.3), 1.0 mM thiamine diphosphate, 2.0 mM $CaCl_2$, 0.3% Triton X-100 and 700 μg of protein. 0.1 mM of ATP was added as indicated. After 5 min preincubation, reactions were carried out for 30 min at 37°.

TABLE I

EFFECTS OF MONOVALENT CATIONS ON BRAIN THIAMINE DIPHOSPHATASE ACTIVITY IN THE PRESENCE OF Ca^{2+}

The standard incubation mixture contained: 75 mM Tris–HCl (pH 9.3), 1.0 mM thiamine diphosphate, 2.0 mM $CaCl_2$, 0.3% Triton X-100 and 700 μg of protein. 0.1 mM of ATP was added as indicated. After 5 min preincubation, reactions were carried out for 30 min at 37°.

Added cation	*Atomic or mol. wt.*	*μmoles P_i/mg protein per h*			
		Cation concn. 50 mM		*Cation concn. 100 mM*	
		− ATP	*+ ATP*	*− ATP*	*+ ATP*
None		0.95	1.87		
Li^+	6.9	0.80	1.50	0.72	1.41
NH_4^+	18	0.81	1.54	0.68	1.26
Na^+	23	0.87	1.60	0.75	1.51
K^+	39	0.94	1.73	0.91	1.63
Rb^+	85.5	0.99	1.63	0.99	1.62
Cs^+	132.9	0.99	1.63	0.99	1.62

reduced the stimulatory effect of Ca^{2+} on the enzyme activity in the presence of ATP, though this was not obvious in the absence of ATP.

The effect of Na^+ or K^+ alone on the enzyme activity was weaker than that of both Na^+ and K^+ in the presence of Ca^{2+} and ATP (Fig. 5). Table I shows that Li^+ and NH_4^+ were the most inhibitory of the monovalent cations tested, irrespective of the presence of ATP. Thus monovalent cations which are smaller in atomic or molecular weight than K^+ suppress the activation induced by Ca^{2+} or Ca^{2+} *plus* ATP.

Antagonism between Ca^{2+} and Mg^{2+}

The effects of various activators on the enzyme activity were examined in the presence of Ca^{2+} and Mg^{2+} (Fig. 6). In the absence of ATP, the stimulatory effect of Mg^{2+} was antagonized at a concentration of 0–1.0 mM Ca^{2+}, either in the presence

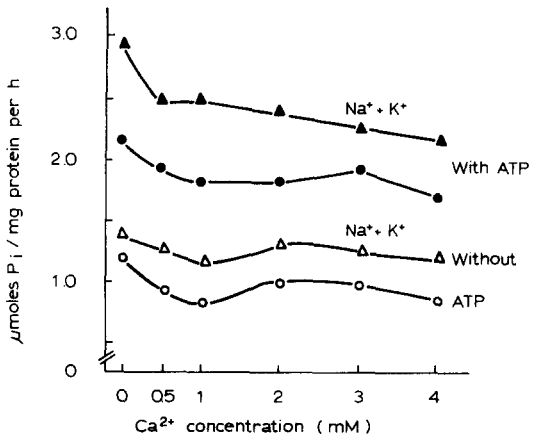

Fig. 6. Effect of Ca^{2+} on Mg^{2+}-activated brain thiamine diphosphatase activity in the presence or absence of ATP and $Na^+ + K^+$. The standard incubation mixture contained: 75 mM Tris–HCl (pH 9.3), 1.0 mM thiamine diphosphate, 1.0 mM $MgCl_2$, 0.3% Triton X-100 and 700 μg of protein. 0.1 mM of ATP or 25 mM $Na^+ + 25$ mM K^+ was added as indicated. After 5 min preincubation, reactions were carried out for 30 min at 37°. ○, without added ATP and $Na^+ + K^+$; ●, 0.1 mM ATP; △, 25 mM $Na^+ + 25$ mM K^+; ▲, 0.1 mM ATP and 25 mM $Na^+ + 25$ mM K^+.

TABLE II

EFFECTS OF DIVALENT CATIONS ON BRAIN THIAMINE DIPHOSPHATASE ACTIVITY IN THE PRESENCE AND ABSENCE OF ATP

The standard incubation mixture contained: 75 mM Tris–HCl (pH 9.3), 1.0 mM thiamine diphosphate, 0.3% Triton X-100 and 500 μg of protein. 0.1 mM of ATP was added as indicated. After preincubation for 5 min, reactions were carried out for 20 min at 37°. Each figure represents the average of 3 determinations.

Divalent cation added	*Concn. (mM)*	*μmoles P_i/mg protein per h*		
		−ATP	*+ATP*	$\frac{+ATP}{-ATP}$
None	—	0.43	0.93	2.2
Mg^{2+}	1.0	1.40	2.70	1.9
Ca^{2+}	1.0	1.13	2.33	2.1
Mn^{2+}	1.0	0.84	2.21	2.6
Zn^{2+}	1.0	0.43	0.86	2.0
Co^{2+}	1.0	0.43	0.77	1.8

or absence of Na^+ *plus* K^+, but, higher concentrations of Ca^{2+} were less inhibitory. Since 1.0 mM of Ca^{2+} is equivalent to the amount of Mg^{2+} added, it seems that the stimulatory effect of Mg^{2+} is counteracted by an equimolar amount of Ca^{2+}. However, Ca^{2+} did not exhibit the bi-phasic action on the enzyme, nearly decreasing the stimulatory effect of Mg^{2+} in the presence of ATP. When added separately Mg^{2+} and Ca^{2+} both stimulate the enzyme activity, but when added together their actions are antagonistic. Further studies are required on the details of the mechanism of this antagonism.

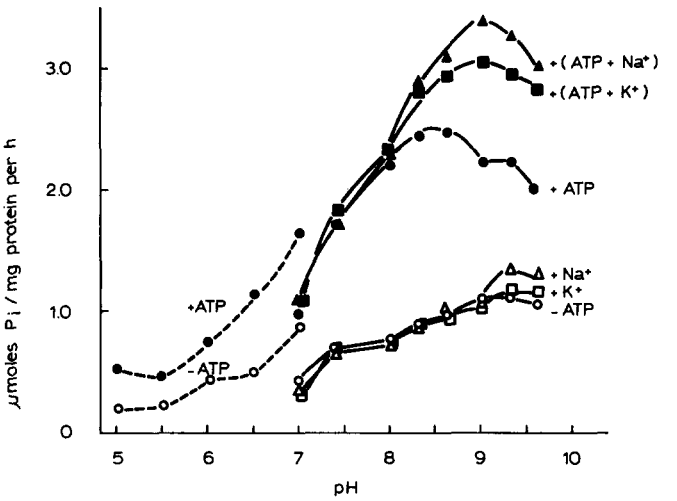

Fig. 7. Effect of pH on brain thiamine diphosphatase activity in the presence or absence of ATP, Na^+ or K^+. The standard incubation mixture contained: 75 mM of Tris–maleate–KOH (pH 5–7, dotted lines) or Tris–HCl (pH 7–9.6, solid lines), 1.0 mM thiamine diphosphate, 1.0 mM $MgCl_2$, 0.3% Triton X-100 and 700 μg of protein. 0.1 mM of ATP, 30 mM Na^+ and 20 mM K^+ were added as indicated. Reactions were carried out for 30 min, after preincubation for 5 min.

Effects of divalent cations

We examined the effects of metal cations on the enzyme activity (Table II). Mg^{2+} caused most stimulation, either in the presence or absence of ATP. But, activation by ATP was more marked with Mn^{2+} than with Mg^{2+} or Ca^{2+}. Zn^{2+} and Co^{2+} had no effect (cf. YAMAZAKI AND HAYAISHI[6]).

Effect of pH

The pH–activity curves of the enzyme in the presence or absence of various activators are shown in Fig. 7. KIESSLING[13] reported that an acetone powder of the brain enzyme has pH optima at 6.0 and 9.3, but we could detect no peak between pH 5 and 7. However, the activity increased with pH up to about pH 7.0. This seems

TABLE III

INFLUENCES OF STEPWISE TREATMENTS WITH VARIOUS ORGANIC SOLVENTS ON BRAIN THIAMINE DIPHOSPHATASE ACTIVITY

The standard incubation mixture contained: 75 mM Tris–HCl (pH 9.3), 1.0 mM thiamine diphosphate, 1.0 mM $MgCl_2$ and 700 μg of protein. 0.1 mM of ATP was added as indicated. After preincubation for 5 min, reactions were carried out for 30 min at 37°.

Preparation	*μmoles P_i/mg protein per h*			
	*Original activity**		*Activity after extraction***	
	−ATP	*+ATP*	*−ATP*	*+ATP*
Acetone powder	0.17	0.78	1.11	2.30
n-Butanol powder	0.22	0.69	1.17	2.41
Chloroform–methanol (2:1, v/v) powder	0.25	0.26	2.49	2.53

* Each powder (100 mg) was homogenized in distilled water and this homogenate was used as enzyme source.

** Each powder (100 mg) was extracted with 20 ml of 20 mM $KHCO_3$ with stirring at 0° for 15 min and centrifuged at 10 000 × *g* for 10 min. The precipitates were homogenized in distilled water contained 2.0% Triton X-100 and centrifuged at 10 000 × *g* for 15 min. The supernatants were used as enzyme sources. Each figure represents the mean of 3–6 determinations.

to be due to the stimulatory effect of K^+ in Tris–maleate–KOH buffer used in this pH range. The pH optimum was 9.3 in the absence of activator. Almost the same results were obtained in the presence of Na^+ or K^+ ion without ATP. But, activation by ATP shifted the pH optimum to 8.6 and on addition of Na^+ or K^+ as well it changed further to 9.0.

Effect of treatments with various organic solvents

Table III shows the effects on the enzyme activity of stepwise treatments with various organic solvents. ATP stimulated the activities of acetone and *n*-butanol powders. But, it had no effect on the chloroform–methanol powder, the activity of this powder being the same or more in the absence of ATP. This suggests that some lipid of the enzyme, which is involved in the stimulatory effect of ATP and present at a site differing from the substrate site, was removed by chloroform–methanol.

Influence of substrate concentration

To determine the K_m and v_{max} values of brain thiamine diphosphatase for

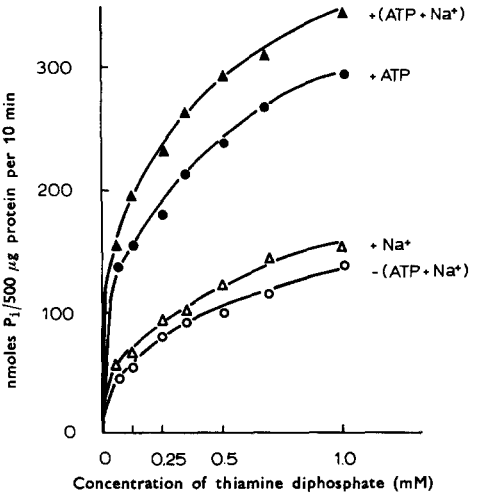

Fig. 8. Effect of substrate concentration on the rate of brain thiamine diphosphatase activity in the presence or absence of ATP or Na^+. The incubation mixture contained: 75 mM Tris–HCl (pH 9.3), 1.0 mM $MgCl_2$, 0.3% Triton X-100 and 500 μg of protein. 0.1 mM of ATP and 30 mM Na^+ were added as indicated. Reactions were carried out for 10 min at 37°. ○, without added ATP and Na^+; ●, 0.1 mM ATP; △, 30 mM Na^+; ▲, 0.1 mM ATP and 30 mM Na^+.

thiamine diphosphate, we examined the influence of substrate concentration on the reaction rate of the enzyme in the presence or absence of ATP and/or Na^+. Fig. 8 shows that additions of Na^+ alone, ATP alone and ATP *plus* Na^+ in this order caused progressive increase in the velocity of the enzyme activity at each concentration of the substrate. Quantitative recovery of ATP (97%) added to the standard incubation mixture was obtained after reaction for 10 min at 37° when the reaction components were chromatographed on a Dowex 1 column (chloride form, 200–400 mesh) following the method by COHN AND CATER[14].

Lineweaver–Burk plots were linear in the presence or absence of ATP and/

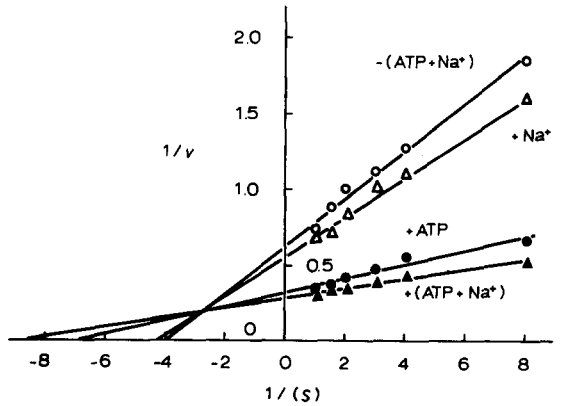

Fig. 9. Double reciprocal plots of velocity of brain thiamine diphosphatase reaction in the presence or absence of ATP or Na^+.

or Na^+ (Fig. 9). The K_m values were 0.25 and 0.145 mM in the absence and presence of ATP. ATP increased the v_{max} of the enzyme about 2-fold with a corresponding decrease in the K_m value. The effect of Na^+ was similar to that of ATP, but slightly less. The coexistence of ATP and Na^+ decreased the K_m most, to a value of 0.119 mM.

DISCUSSION

In this work we found that Na^+-activated brain thiamine diphosphatase solubilized with Triton X-100 in the presence of Mg^{2+} or Mg^{2+} *plus* ATP. From the results in Fig. 2, the concentration of Na^+ necessary to produce half maximal activity in the presence of Mg^{2+} *plus* ATP were calculated as 13 mM. K^+ antagonized the stimulatory effect of Na^+, so brain thiamine diphosphatase is an Mg^{2+}–Na^+–ATP-activated pyrophosphatase.

When added separately Mg^{2+} and Ca^{2+} potentiated activity either in the presence or absence of ATP, but when added together their effects were antagonistic. It is very interesting that Na^+, NH_4^+ and Li^+ inhibited activity in the presence of Ca^{2+} or Ca^{2+} *plus* ATP, because these phenomena might be related to alteration of enzyme properties due to conformational changes resulting from binding of Ca^{2+} at the substrate site.

ATP stimulates the enzyme due to its binding to a site other than the substrate site. ATP, Na^+ and K^+ shifted the pH optimum. Fig. 9 shows that ATP and/or Na^+ decreased the K_m of the enzyme for thiamine diphosphate, and increased the v_{max}. YAMAZAKI AND HAYAISHI[6] showed that purified bovine liver thiamine diphosphatase and inosine diphosphatase were the same enzyme and were activated allosterically by both ATP and other triphosphonucleosides. Further studies on the mechanism of the activation by ATP and Na^+ are required to elucidate whether brain thiamine diphosphatase is an allosteric enzyme or not.

The K_m value of purified liver thiamine diphosphatase for thiamine diphosphate was reported to be 20.7 mM[6]. The K_m value of the brain enzyme is 83 times less. The brain of rats contains only 3 μg of total thiamine per g tissue, while liver contains more than 9 μg[15], most of the thiamine in those organs being in the form of thiamine diphosphate[16]. So, it is natural that brain thiamine diphosphatase has a smaller K_m value than the liver enzyme since it decomposes considerably smaller concentration of substrate.

MATSUDA *et al.*[17] observed that Na^+ or Mg^{2+} *plus* ATP stimulates the release of acetylcholine from synaptic vesicles of rat brain. We have previously reported that brain thiamine diphosphatase activity of normal rats was markedly increased after injection of physostigmine or ambenonium[8]. Furthermore, many authors have observed that thiamine or thiamine monophosphate was released by electrical stimuli[1-3] and acetylcholine[4] from nerve fibers. Therefore, the release of thiamine or thiamine monophosphate from the nerve tissue is presumably related to the stimulation of Mg^{2+}-dependent thiamine diphosphatase by Na^+ and ATP under those excitatory conditions.

Recently, it was reported that thiamine diphosphatase of rat cerebral cortex is localized in the nerve ending fraction, especially in Golgi membrane fragments but not in synaptic vesicles[18]. However, the same author found that the synaptic vesicle fraction of the sciatic nerve from cats contains much thiamine diphosphatase[19].

As suggested in a previous report from this laboratory[8], brain thiamine diphosphatase may participate in the blood-brain barrier for thiamine. To examine the significance of thiamine release from nervous tissues, further studies on the nature and role of this enzyme in the brain are required and especially on changes of the enzyme activity on induction of definite behavioral effects by various neuroactive drugs.

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