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INHIBITION OF RAT BRAIN PYRUVATE DEHYDROGENASE BY THIAMINE ANALOGS*

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Abstract—The effects of thiamine thiazolone (TT) and thiamine thiazolone pyrophosphate (TTPP) on the *in vitro* and *in vivo* inhibition of pyruvate dehydrogenase complex (PDHC) from rat cortex and hippocampus were characterized. TTPP decreased PDHC activity *in vitro* but had no effect *in vivo* following its direct chronic administration via osmotic mini-pumps into the brains of behaving rats. In contrast, TT had no direct effect *in vitro* following a differential centrifugation purification of the mitochondrial PDHC fraction, but decreased PDHC activity *in vivo*. Additional experiments demonstrated that the cytosolic fraction converted TT to TTPP which, in turn, inhibited PDHC *in vitro*. A mechanism is proposed to explain these effects that is consistent with a non-competitive inhibition of brain PDHC by TTPP.

The mammalian PDHC§ is located in the mitochondria within the inner membrane matrix space [1, 2]. This enzyme complex consists of three enzymes and five coenzymes: pyruvate decarboxylase (EC 1.2.4.1) with coenzyme thiamine pyrophosphate, dihydrolipoyl transacetylase (EC 2.3.1.12) with coenzymes lipoic acid and coenzyme A, and dihydrolipoyl dehydrogenase with coenzymes FAD and NAD. PDHC is responsible for the oxidative decarboxylation of pyruvate to acetyl-coenzyme A [3–5]. The kinetic mechanism of PDHC for mammalian kidney [6] and rat brain [7] has been characterized and is consistent with a multi-site ping–pong mechanism [8]. PDHC is regulated by two enzymes, a kinase and a phosphatase. The kinase, in the presence of ATP, inactivates the PDHC by phosphorylation of seryl residues on the apoenzyme of pyruvate decarboxylase, while the phosphatase, in turn, removes these phosphate groups and reactivates the enzyme complex [3–6]. The above phosphorylation–dephosphorylation cycle has been proposed for bovine kidney and heart [3, 5] and rat brain [9, 10].

The normal adult rat brain uses glucose almost exclusively as an energy source [11]. Because PDHC has a pivotal role in the supply of acetyl-coenzyme A to the Krebs cycle [12], it also plays an important part in the regulation of energy production. Therefore, it is likely that decreases in PDHC activity may affect brain function. Several studies have attempted to inhibit the PDHC by limiting the availability of thiamine for the PDHC coenzyme, thiamine pyrophosphate [13, 14]. This is routinely accomplished by restricting thiamine intake [14], either alone or in combination with pyrithiamine [13, 14]. Pyrithiamine has been demonstrated to decrease brain thiamine pyrophosphate and ultimately PDHC, by inhibiting thiamine pyrophosphokinase [15]. However, because thiamine deficiency produced by pyrithiamine or thiamine-deficient diets also affects other enzymes, as well as protein synthesis, neuronal membrane excitability, and the general nutritional status of the animal [16], it is difficult to attribute any changes in brain function to a specific inhibition of PDHC.

One approach to examine how a decrease in PDHC activity affects central nervous system function is to administer a specific PDHC inhibitor directly into the brain. Thiamine thiazolone pyrophosphate (TTPP) has been shown to be a specific inhibitor of this enzyme complex [17], and exhibits properties of a slow-binding transition-state analog [18] for thiamine pyrophosphate dependent enzymatic reactions [17]. The upper limit of the first order rate constant for the dissociation of TTPP from PDHC corresponds to a half-time of 40 hr [17]. Furthermore, its affinity for PDHC was found to be 20,000 times greater than its prosthetic group, thiamine pyrophosphate [17]. Thus, TTPP can be used as a tool to further characterize the role of PDHC in neurons. If TTPP is to inhibit brain PDHC *in vivo*, it must cross neuronal membranes. However, pyrophosphorylated thiamine derivatives, similar to other phosphorylated compounds, do not actively or passively cross these membranes [19]. Since all studies to date have utilized membrane-free

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§ Abbreviations: PDHC, pyruvate dehydrogenase complex; R_f, distance traveled from baseline by compound/distance traveled from baseline by solvent; PMSF, phenylmethanesulfonyl fluoride; V_m, maximum velocity obtained when PDHC was saturated with pyruvate; s, pyruvate concentrations in mM; v, specific activity (μmol product/min/mg protein); TT, thiamine thiazolone; and TTPP, thiamine thiazolone pyrophosphate.

in vitro preparations to characterize the regulation and inhibition of PDHC [6, 7, 17], it remains unanswered whether TTPP can inhibit PDHC *in vivo*.

One approach would be to synthesize a nonphosphorylated precursor of TTPP, such as thiamine thiazolone (TT). Imperative to this approach would be the *in vivo* conversion of TT to TTPP by the cytosolic enzyme thiamine pyrophosphokinase similar to the conversion of thiamine to thiamine pyrophosphate [20, 21]. Therefore, the purpose of this study was to determine whether the thiamine analogs, TTPP and TT, would inhibit PDHC *in vivo* in rat cortex and hippocampus. The type of inhibition of brain PDHC by TT and TTPP is characterized *in vitro* and a mechanism is proposed to explain the observed *in vivo* effects of these drugs.

MATERIALS AND METHODS

Sprague–Dawley male rats, specific pathogen free, were purchased from Zivic Miller Laboratories. Alzet osmotic mini-pumps (model 2001) were purchased from the Alza Corp. Phenylmethanesulfonyl fluoride, whole rabbit serum, 2-mercaptoethanol, dithiothreitol, ethylenediaminetetraacetic acid, cocarboxylase, bovine serum albumin, coenzyme A, and Amberlite CG-50 (H^+ form, 100–200 mesh) were purchased from Sigma. Sodium phosphate mono and diabasic, magnesium chloride, isobutanol, *n*-butanol, orthophosphoric acid, sodium hydroxide, phenol reagent (2 N), and Eastman Kodak cellulose TLC plates with fluorescent indicator were purchased from Fisher. Steel tubing (23 gauge) was purchased from Small Parts. Silastic tubing (O.D. = 0.037 in.) was purchased from Dow Corning. Thiamine disulfide was purchased from ICN Biochemicals, and 95% ethanol was purchased from the AAPER Alcohol and Chemical Co.

Homogenation and extraction procedure for isolation of PDHC from rat cortex and hippocampus. The homogenation procedure used to isolate mitochondrial PDHC was a modification of that of Pettit and Reed [22]. Rats were decapitated, and the cortex and hippocampus were excised. The dissected regions were placed in a 10% (w/v) solution of 0.025 M sucrose in 0.025 M sodium phosphate buffer (pH 7.57) with 0.1 mM EDTA And 0.01 M 2-mercaptoethanol. The brain regions were homogenized for 10 min at 2000 *g*. All centrifugations were done at 4°. The supernatant fraction was recentrifuged at 22,000 *g* for 15 min. The pellet was resuspended with 0.025 M sodium phosphate buffer (pH 7.8), 9 μl of 0.05 M PMSF and 9 μl of whole rabbit serum. The suspended pellet was centrifuged at 22,000 *g* for 15 min. The pellet was resuspended in the above buffer system with the addition of 9 μl of 2-mercaptoethanol and recentrifuged at 22,000 *g* for 15 min. The pellet was again resuspended in the same buffer without 2-mercaptoethanol and centrifuged at 22,000 *g* for 15 min. This pellet was then homogenized in sodium phosphate buffer (0.05 M, pH 7.4) containing 0.1% Triton X-100, 2 mM magnesium chloride, 1 mM dithiothreitol, and 0.2 mM EDTA. This 10% (w/v) homogenate was centrifuged at 40,000 *g* for 30 min. The resulting pellet was resuspended in the above buffer and homogenized. This final 10% (w/v) homogenate served as the PDHC enzyme source, and it was stored at −78°. This enzyme was stable for at least a month. Protein determination for the enzyme homogenate was by the method of Lowry *et al.* [23].

Spectrophotometric assay procedure to measure PDHC activity. PDHC activity was determined with a Beckman model 25 double-beam spectrophotometer. Absorbance was measured at 340 nm. All assays were performed at room temperature. The assay measures an increase in absorbance at 340 nm as a result of the production of reduced NAD, an end product of the PDHC. The reaction mixture consisted of the following: 1.2 ml of the final homogenate buffer, with Triton X-100 replaced by 1 mg/ml bovine serum albumin, 2.5 mM NAD, 0.18 mM thiamine pyrophosphate, 0.1 mM coenzyme A, and 0.276 ml of final buffer used to make the enzyme homogenate. The reference and sample cuvettes received the above in equal amounts. The final pyruvate concentrations used in the *in vivo* studies were varied from 9.0 μM to 9.0 mM; the final pyruvate concentrations used in the *in vitro* inhibition studies were varied from 0.9 to 9.0 mM. The reaction was initiated by the addition of the enzyme homogenate and enzyme buffer. The final assay mixture was 2.22 ml. Specific activity at each pyruvate concentration was calculated using the molar extinction coefficient for NADH ($E = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$ at 340 nm). A standard PDHC assay was performed using the reaction mixture described in Materials and Methods. The absorbance changes were recorded at 1-min intervals for 20 min at each pyruvate concentration. All pyruvate concentrations examined showed linear absorbance changes between 5 and 20 min for the PDHC assay. Therefore, the difference in absorbance between the 15-min and the 5-min readings was used to calculate the specific activity (micromoles of product per minute per milligram of protein) at each pyruvate concentration.

Synthesis of TT and TTPP. TT and TTPP were prepared according to the procedure of Gutowski [24]. Thin-layer chromatography [using Eastman cellulose plates with fluorescent indicator; ethanol : *n*-butanol : 15 mM citrate, pH 4 (10 : 1 : 6 by vol.)] was used to determine the ultraviolet absorbing component R_f values. The reported R_f values for TT and TTPP are 0.89 and 0.5, respectively, in this solvent system [17, 24]. The ultraviolet spectra of TT in 10 mM potassium phosphate, pH 7.0, exhibited an absorbance maximum at 233 nm, as described in the literature [17, 24].

Inhibition of PDHC by TT and TTPP in vitro. The final concentrations of inhibitor (TT or TTPP) used in this study to characterize the inhibition of PDHC from rat cortex and hippocampus with varied pyruvate concentrations were 0, 12.6, 18.9, 25.2, 37.8 and 56.7 μM. The spectrophotometric assay procedure used to analyze PDHC activity was carried out as previously described with the exception that 0.276 ml of enzyme buffer was reduced to 0.076 ml and the final volume made up to 2.22 ml with 0.2 ml of the inhibitor (TT or TTPP) in concentrations necessary for the final inhibitor concentrations. The

number of assays used per substrate and inhibitor concentrations was 9 for TTPP And 6 for TT. A Hanes plot [25] was constructed for each TTPP concentration used in this study. A linear regression analysis [26] was performed to determine the equation for the line at each inhibitor concentration. A Student's *t*-test [26] determined whether a linear increasing trend existed for the slopes and y-intercepts from the Hanes plot.

The weighted analysis of Wilkinson [27] was used to determine the kinetic parameters of V_m and K_m as a result of inhibition with TT along with their respective standard errors. A Student's *t*-test [26] was used to determine linear decreasing trends for V_m and K_m as a function of inhibitor concentration.

Inhibition of PDHC by TT and TTPP in vivo. The *in vivo* inhibitory effects of TT and TTPP were analyzed as described above using varied pyruvate concentrations. The specific activity values obtained for the control and TT (or TTPP) treated groups were used to determine V_m and K_m values for each of twenty rats. Differences in V_m and K_m values between control and treated groups were analyzed by a *t*-test [26].

Surgical procedure for in vivo *experiments.* Rats were anesthetized with pentobarbital (50 mg/kg body weight), and cannulae were stereotaxically implanted unilaterally into the hippocampus (A-P = −4.3, lateral = 2.0, D-V = 3.1 cm from the dura mater) [28]. The cannulae were attached to Alza osmotic mini-pumps (Alza Corp., model 2001), which contained either 0.01 M sodium phosphate (pH 7.4) vehicle, TT or TTPP (2 mM). The osmotic mini-pumps were placed subcutaneously at the base of the neck. Silastic tubing (Dow Corning O.D. = 0.037 in.) connected the pumps to the steel cannulae (Small Parts, O.D. = 0.025 in.). Dental cement (Hygenic Corp.) was used to secure the position of the cannulae and the tubing. The cement was attached to the head of each rat with two set-screws into the skull. The flow-rate for the pumps was calibrated by the manufacturer to be 1 μl/hr for 7 days.

Thiamine pyrophosphokinase activity in vitro. The cortex and hippocampus of the rat brain were excised and homogenized as previously described. The homogenate was centrifuged at 2000 *g* for 10 min. The resulting supernatant fraction was recentrifuged at 22,000 *g*. The supernatant fraction of this centrifugation contained thiamine pyrophosphokinase [15, 20, 21] and was used for the *in vitro* conversion of TT to TTPP. The incubation medium consisted of: the supernatant (5 ml), 2 mM TT in 0.01 M sodium phosphate buffer (3 ml, pH 7.4), and 0.492 M ATP (0.2 ml). The TT-treated supernatant fraction was incubated for 3 hr at 37° in a water bath. The incubation medium was then boiled for 1 min and centrifuged at 22,000 *g* for 15 min. The resulting supernatant fraction was applied to an Amberlite CG-50 column (H^+ form, 100–200 mesh, suspended in water) and was eluted with water. The absorbance of the eluant was monitored at 234 nm. The peak fractions were analyzed by TLC using the same solvent system as described for the synthesis of TT and TTPP. The samples that contained a single ultraviolet-absorbing component with R_f values between 0.44 and 0.51 were combined and rotary evaporated to dryness to yield TTPP. This TTPP synthesized from the cytosolic enzyme thiamine pyrophosphokinase was reconstituted in 0.01 M sodium phosphate buffer (pH 7.4) to make a final concentration of 56.7 μM. An equal volume of TTPP or 0.01 M sodium phosphate (pH 7.4) control replaced 0.02 ml of enzyme buffer. The final volume was 2.22 ml. PDHC activity from the control and TTPP-treated assays was analyzed by Student's paired *t*-test [26].

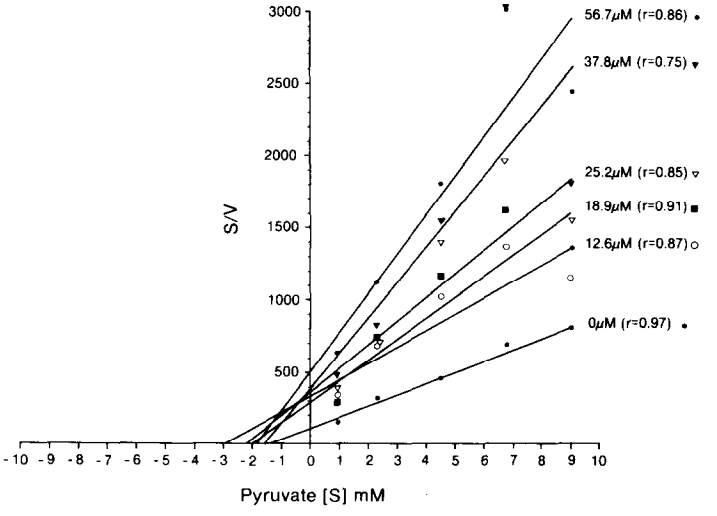

Fig. 1. Hanes plot of the *in vitro* inhibitory effects of TTPP on PDHC activity. The concentrations listed for each line represent the TTPP concentrations used with the various pyruvate concentrations. The equation of the line at each TTPP concentration was determined by linear regression analysis. The correlation coefficients for each line are shown in parentheses. A linear trend analysis for increasing slope was significant ($P < 0.05$, Student's *t*-test). The PDHC assays were performed as described in the text. The symbols represent the observed s/v values obtained for the TTPP concentrations: 0 (●), 12.6 (○), 18.9 (■), 25.2 (▽), 37.8 (▼), and 56.7 (●) μM.

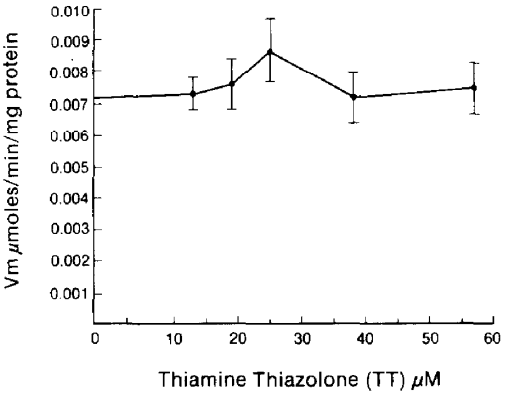

Fig. 2. *In vitro* effects of TT on PDHC activity. The points on the graph represent the mean values ± SEM (N = 9) at those TT concentrations. Enzyme assays were performed as described in the text. Key: μmoles, μmoles of product.

RESULTS

The *in vitro* analysis of the inhibitory effect of TTPP on PDHC activity from rat cortex and hippocampus is shown by a Hanes plot in Fig. 1. The lines at different inhibitor concentrations were calculated by linear regression analysis [26] based on the mean of nine specific activity values at each substrate (pyruvate) and inhibitor (TTPP) concentration. The correlation coefficients were greater than 0.74 for each line. A linear trend analysis for increasing slope and y-intercept was determined by a *t*-test. The slopes showed a significantly increasing trend ($P < 0.05$) as a function of inhibitor concentration. The y-intercepts were not significantly different.

The effect of TT on the *in vitro* PDHC activity from rat cortex and hippocampus is shown in Fig. 2. The method of Wilkinson [27] was used to determine V_m values. At the concentrations of TT tested, no significant effects were observed *in vitro* when TT was added to the mitochondrial PDHC fraction. When the V_m for each inhibitor at a given concentration was examined, TTPP produced a greater inhibition of PDHC than that seen with TT ($P < 0.05$). No changes in K_m values were observed (data not shown).

Table 1. *In vivo* effects of TTPP on PDHC activity*

	V_m (μmol product/min/mg protein)	K_m (μM)
Control	0.0060 ± 0.0006	16.3 ± 3.0
TTPP	0.0054 ± 0.0005	13.4 ± 2.5

* Each value represents the mean ± SEM. N = 9. Analysis by Student's *t*-test indicated that the difference between control and TTPP-treated values was not significant for either V_m or K_m.

The results of the *in vivo* inhibitory effect of TT on PDHC activity are shown in Fig. 3. The V_m of the TT-treated group was significantly less than the V_m of the control group ($P < 0.05$), whereas no significant difference was found between the K_m values. The results of the *in vivo* inhibitory effects of TTPP on PDHC activity are shown in Table 1. Neither V_m nor K_m values were significantly different from controls. Therefore, TT was effective in inhibiting PDHC only when administered *in vivo*.

To clarify the apparent differences between the *in vivo* and the *in vitro* effects of TT, an *in vitro* study was conducted to determine whether the 22,000 *g* cytosolic supernatant fraction from rat cortex and hippocampus was capable of converting TT to a product that inhibits PDHC. Following a 3-hr incubation period and precipitation of proteins, the TT-treated cytosolic fractions were applied to an Amberlite CG-50 column. The peak fractions, determined by absorbance of the eluant at 234 nm, showed R_f values between 0.44 and 0.51. These values correspond to those reported for TTPP [17, 24]. These fractions were rotary evaporated and reconstituted in 0.01 M sodium phosphate buffer to approximately 56.7 μM. This cytosolic synthesized product was bioassayed for its ability to inhibit PDHC activity. Figure 4 shows that the specific activity of PDHC was decreased significantly ($P < 0.05$) from control values when in the presence of this product. A thin-layer chromatogram of tentatively indicated that this product was TTPP. TLC has been used previously

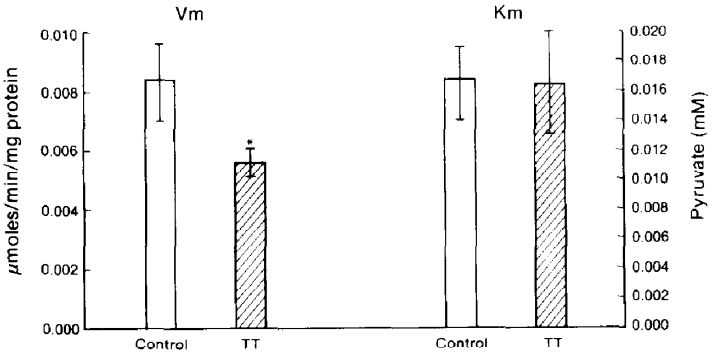

Fig. 3. *In vivo* inhibitory effect of TT on PDHC activity. Each bar graph shows the mean value of the group ± the SEM, N = 9. Enzyme assays were performed as described in the text. Key: μmoles, μmoles of product; and (*) $P < 0.05$, significantly less than control value.

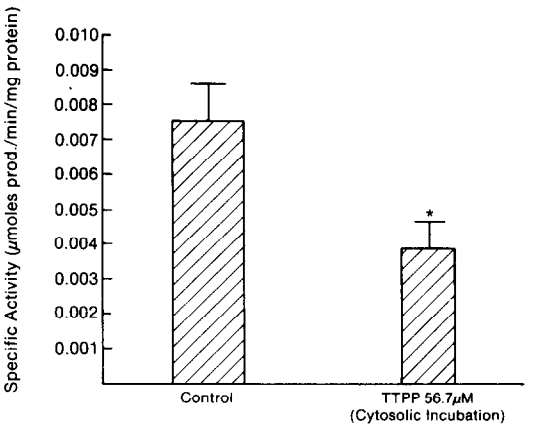

Fig. 4. Reconstituted product (TTPP) from the incubation of TT in the cytosolic fraction from rat cortex and hippocampus and its ability to inhibit PDHC activity. Enzyme assays were performed as described in Materials and Methods. Bar graphs represent the mean specific activity values ± SEM, N = 9. Key: (*) $P < 0.05$, significantly decreased PDHC activity compared to control value (paired *t*-test).

to identify TTPP [29, 30]. The purified product was simultaneously chromatogramed with synthesized TT and TTPP. The R_f value of the product was identical to the R_f value of the synthesized TTPP.

DISCUSSION

The results of the present study are the first to demonstrate that two thiamine analogs, TT and TTPP, inhibit PDHC from brain tissue. These effects, however, were confined to decreases in V_m with no changes in K_m. TTPP was only effective when added to the purified brain mitochondrial fraction (Fig. 1) containing PDHC, but was without effect when administered *in vivo*. In direct contrast, TT inhibited PDHC *in vivo* (Fig. 3) but had no effect when examined *in vitro* (Fig. 2). Upon further investigation, TT was capable of inhibiting PDHC *in vitro*, but only when incubated in the cytosolic fraction prior to its addition to the PDHC-containing mitochondrial fraction.

The kinetic studies of TTPP on PDHC shown by the Hanes plots (Fig. 1) illustrate that V_m decreased as the concentrations of TTPP were increased. Further analysis of the relationship between V_m and TTPP concentrations, using linear regression analysis [26], showed a correlation coefficient of $r = -0.90$ (figure not shown). By contrast, the K_m/V_m values as illustrated by the Hanes plots did not change as a function of TTPP concentrations. Therefore, the effects of TTPP on PDHC appear to be confined to V_m, and the decreases appear to fit a linear decrease over increasing inhibitor concentrations (TTPP). These results suggest that the nature of the inhibition is non-competitive [25]. The concept of a non-competitive inhibitor was originally proposed to account for an alteration in the catalytic active-site on the enzyme without altering the binding affinity of the enzyme for its substrate [25]. Since TTPP appears to behave according to this definition, it most likely alters the active-site of brain PDHC while not affecting the binding of pyruvate.

The present *in vitro* effects of both TT and TTPP on PDHC from rat cortex and hippocampus corroborate the earlier work of Gutowski and Lienhard [17] reporting inhibition of PDHC from *Escherichia coli*. PDHC activity decreased with TTPP but not with TT. Our *in vitro* and *in vivo* kinetic studies of brain PDHC have extended these findings. The decrease in V_m without changes in K_m were observed not only following the *in vitro* administration of TTPP but also following the *in vivo* infusion of TT into the brain.

The apparent contradictory results of the *in vivo* and *in vitro* effects of TT and TTPP can be explained by the fact that TT must be converted to TTPP prior to its inhibition of PDHC. TT is similar in structure to the endogenous compound thiamine. The only structural difference between thiamine and TT is at the 2-position of the thiazole ring where TT has a carbonyl group instead of a hydrogen [17, 24, 29]. It is known that thiamine is transported into cells via a protein carrier [31]. The intracellular thiamine is converted to its pyrophosphorylated form by the cytosolic enzyme thiamine pyrophosphokinase [15, 20, 21]. The thiamine pyrophosphate then enters the mitochondria and serves as a coenzyme for pyruvate decarboxylase. The close structural resemblance of TT to thiamine suggests that this compound undergoes the same transport into the cell, becomes pyrophosphorylated by thiamine pyrophosphokinase, and enters the mitochondria via the same mechanism as thiamine. It is at this proposed pyrophosphorylation step in the cytosol that TT may be converted to TTPP. The newly synthesized TTPP could then bind at the coenzyme site on pyruvate carboxylase and inhibit the PDHC. This proposed conversion of TT to TTPP in the cytoplasm of brain cells is supported by our cytosolic incubation studies with TT. The purified product from these studies was found to inhibit PDHC activity. The thin-layer chromatogram of this product suggests that this compound is TTPP. If TT must be pyrophosphorylated to TTPP in the cytosol to inhibit PDHC effectively, then direct addition of TT to the purified mitochondrial fraction would not inhibit PDHC. This was, in fact, the case. TT did not inhibit PDHC activity *in vitro*. The findings that TT inhibited PDHC activity *in vivo* and also following prior *in vitro* incubation in the cytosolic fraction containing thiamine pyrophosphokinase [15, 20, 21] are further evidence that TT must be converted to TTPP prior to its inhibitory effects on the PDHC.

The *in vivo* TTPP experiments showed no change in K_m or V_m values from their control counterparts. These results are what would be expected if the pyrophosphorylated compound (TTPP) were unable to cross the cell membrane. Previously, Yoshioka *et al.* [19] demonstrated that thiamine phosphates are not transported across cell membranes. Based on the above results for TTPP *in vivo*, it appears that this is also the case for rat cortical and hippocampal cells. Thus, only the non-pyrophosphorylated form, TT, is capable of crossing the cell membrane and inhibiting the PDHC *in vivo*.

In conclusion the present findings have further characterized the mechanism and nature of the inhibition of PDHC by these thiamine analogs and provide initial support for the utility of TT as a specific tool to study, *in vivo*, the role PDHC plays in neurodegenerative disease states and brain energy metabolism.

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