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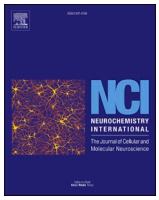

Thiazolium salt mimics the non-coenzyme effects of vitamin B_1 in rat synaptosomes

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ABSTRACT

Long-term studies have confirmed a causal relationship between the development of neurodegenerative processes and vitamin B_1 (thiamine) deficiency. However, the biochemical mechanisms underlying the high neurotropic activity of thiamine are not fully understood. At the same time, there is increasing evidence that vitamin B_1, in addition to its coenzyme functions, may have non-coenzyme activities that are particularly important for neurons. To elucidate which effects of vitamin B_1 in neurons are due to its coenzyme function and which are due to its non-coenzyme activity, we conducted a comparative study of the effects of thiamine and its derivative, 3-decyloxycarbonylmethyl-5-(2-hydroxyethyl)-4-methyl-1,3-thiazolium chloride (DMHT), on selected processes in synaptosomes. The ability of DMHT to effectively compete with thiamine for binding to thiamine-binding sites on the plasma membrane of synaptosomes and to participate as a substrate in the thiamine pyrophosphokinase reaction was demonstrated. In experiments with rat brain synaptosomes, unidirectional effects of DMHT and thiamine on the activity of the pyruvate dehydrogenase complex (PDC) and on the incorporation of radiolabeled [2–^{14}C]pyruvate into acetylcholine were demonstrated. The observed effects of thiamine and DMHT on the modulation of acetylcholine synthesis can be explained by suggesting that both compounds, which interact in cells with enzymes of thiamine metabolism, are phosphorylated and exert an inhibitory/activating effect (concentration-dependent) on PDC activity by affecting the regulatory enzymes of the complex. Such effects were not observed in the presence of structural analogues of thiamine and DMHT without a 2-hydroxyethyl substituent at position 5 of the thiazolium cycle. The effect of DMHT on the plasma membrane Ca-ATPase was similar to that of thiamine. At the same time, DMHT showed high cytostatic activity against neuroblastoma cells.

Abbreviations

ACh	acetylcholine
DMHT	3-decyloxycarbonylmethyl-5-(2-hydroxyethyl)-4-methyl-1,3-thiazolium chloride
DMT	3-decyloxycarbonylmethyl-4-methyl-1,3-thiazolium chloride
dTh	3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazolium chloride
DiOC6 (3)	3,3′-dihexyloxacarbocyanine iodide
DTNB	dithiobis-2-nitrobenzoic acid
OTh	oxythiamine
OThDP	oxythiamine diphosphate
PDH	pyruvate dehydrogenase
PDC	pyruvate dehydrogenase complex
PMS	plasma membrane synaptosomes
PMCA	plasma membrane Ca-ATPase
Th	thiamine
ThDP	thiamine diphosphate
ThTP	thiamine triphosphate
TPK	thiamine pyrophosphokinase

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1. Introduction

Since its discovery, thiamine (vitamin B_1) has been classified as a neuroactive compound because its deficiency primarily leads to nervous system dysfunction (Robinson, 1951). Subsequent studies have confirmed the causal relationship between thiamine deficiency and the development of neurodegenerative processes (Gibson et al., 1982; Plaitakis et al., 1982; Haas, 1988; Bâ, 2008; Parkhomenko et al., 2016; Gibson and Blass, 2007; Sambon et al., 2021).

The role of thiamine in animal cellular metabolism has been quite well studied from a functional biochemistry perspective (Bettendorff et al., 2014; Bunik et al., 2013). Thiamine diphosphate (ThDP), the biologically active form of vitamin B_1, acts as a coenzyme for several crucial enzymes involved in carbohydrate metabolism. The most important of these are transketolase (EC 2.2.1.1) and the primary enzymes of several multienzyme complexes of alpha-ketoacid dehydrogenases, in particular pyruvate dehydrogenase (PDH, EC 1.2.4.1), an enzyme of the pyruvate dehydrogenase complex (PDC), and 2-oxoglutarate dehydrogenase (OGDH, EC 1.2.4.2), an enzyme of the 2-oxoglutarate dehydrogenase complex (OGDC).

However, according to current knowledge, the involvement of vitamin B_1 in metabolic processes is not limited to the coenzymatic role of ThDP. There is increasing evidence that vitamin B_1 has non-coenzymatic functions that are particularly important for neurons (Parkhomenko et al., 1996, 2016, 2019; Polegato et al., 2019; Ostrowsky, 1968; Berman and Fishman, 1975; Bettendorff, 1994; Nghiêm et al., 2000; Mkrtchyan et al., 2015). From the point of view of biochemistry, Y. Ostrovsky in 1968 (Ostrowsky, 1968) clearly expressed the nature of non-coenzyme action of thiamine. Participation of vitamin bound to protein in redox and transphosphorylation reactions determines its non-coenzyme action. To date, thiamine triphosphate (ThTP) has been shown to be a phosphate donor in the phosphorylation reaction of rapsin (Nghiêm et al., 2000), a protein involved in postsynaptic nicotinic acetylcholine receptor (nAChR) clustering. ThDP and ThTP have also been shown to participate in the regulation of acetylcholine synthesis from pyruvate (Parkhomenko et al., 2019), mediated by their participation in the functioning of PDC regulatory enzymes – PDH-phosphatase and PDH-kinase (Parkhomenko et al., 1987). In addition, preliminary data has identified a thiamine binding protein in nerve cell plasma membranes (Parkhomenko et al., 2001) as one of the proteins involved in nAChR function (Mezhenska et al., 2021). Based on these few data, it appears that vitamin B_1, as a precursor of ThDP, a PDH coenzyme, is not only involved in the production of acetyl-CoA, one of the substrates for ACh synthesis, but also controls the metabolism of the latter, possibly acting as ThTP to phosphorylate some cytoskeletal proteins (Resende et al., 2012). Analysis of the work of several research groups (Berman and Fishman, 1975; Bettendorff, 1994; Parkhomenko et al., 1996) suggests the existence of two pools of thiamine derivatives in neurons: a pool of ThDP bound to enzyme proteins (*i*) and a rapidly changing pool of free thiamine derivatives (*ii*). Developing this hypothesis, we proposed (Parkhomenko et al., 2016) that the circulation of the second pool between the intracellular space and the presynaptic cleft may be associated with changes in the membrane potential of the neuron, intracellular thiamine phosphorylation-dephosphorylation, and changes in the activity of the pyruvate dehydrogenase complex (PDC), leading to a modification of acetylcholine (ACh) synthesis from pyruvate. These ideas are partially supported by the literature (Parkhomenko et al., 2016, 2019), but need further clarification.

The use of thiamine coenzyme antagonists in experiments is one of the effective approaches to uncover the non-coenzyme functions of vitamin B_1. Three compounds - oxythiamine, piritiamine, and amprolium - are considered classic antagonists of vitamin B_1 among the compounds obtained by modifying its molecule (Tylicki et al., 2018). Of these, only oxythiamine can be phosphorylated in cells and mimic the effect of thiamine on the synthesis of acetylcholine from pyruvate (Parkhomenko et al., 2019). It is still in doubt whether these events are interrelated.

In this context, a thiamine structural analog, 3-decyloxycarbonylmethyl-5-(2-hydroxyethyl)-4-methyl-1,3-thiazolium chloride (DMHT) (Vovk and Romanenko, 1993), has attracted our attention. The ability of DMHT to inhibit neuromuscular transmission was demonstrated in experiments (Romanenko et al., 1995). The affinity sorbent carrying covalently attached DMHT showed high affinity for proteins involved in thiamine metabolism and function (Mkrtchyan et al., 2015). In addition, DMHT was found to be an effective competitive inhibitor of thiamine pyrophosphokinase (TPK, EC 2.7.6.2), the enzyme responsible for the synthesis of ThDP (Pylypchuk et al., 2001). The latter observation suggests that DMHT is an oxythiamine-like antagonist of thiamine, at least at the level of coenzyme function. In the present study, DMHT was used to further elucidate the mechanism of non-coenzyme effect of thiamine on acetylcholine synthesis, as well as to examine some other effects related to non-coenzyme functions of thiamine in neurons.

To make the answer more certain, the comparative experiments were performed with thiamine, DMHT, and their structural analogues without 5-(2-hydroxyethyl) substituent of the thiazolium ring, i.e., the compounds which cannot be phosphorylated in cells (Fig. 1). The effect of DMHT on cell viability was also of interest.

2. Materials and methods

2.1. Reagents and materials

Most biochemical compounds and reagents were purchased from Sigma-Aldrich (USA), unless otherwise noted. [^{14}C]-labeled compounds were purchased from Amersham (UK). 3-Decyloxycarbonylmethyl-5-(2-hydroxyethyl)-4-methyl-1,3-thiazolium chloride (DMHT) and 3-decyloxycarbonylmethyl-4-methyl-1,3-thiazolium chloride (DMT) were synthesized by quaternization of 5-(2-hydroxyethyl)-4-methyl-1,3-thiazole and 4-methyl-1,3-thiazole, respectively, with decyl monochloroacetate as described previously (Vovk and Romanenko, 1993; Shatursky et al., 2021). 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazolium chloride (dTh) in the form of hydrochloride was obtained by reacting 4-methyl-1,3-thiazole with 4-amino-2-methylpyrimidin-5-yl)methyl chloride hydrochloride (Buchman and Sargent, 1945). All chemical reagents used in this work were ultrapure or chemically pure. Double distilled water was used to prepare the solutions.

2.2. Animal and preparation

Ethical statement

All animal experiments were performed in accordance with the Helsinki Declaration on the Guide for the Care and Use of Laboratory Animals, wich defines the conduct of ethical research on animals, and

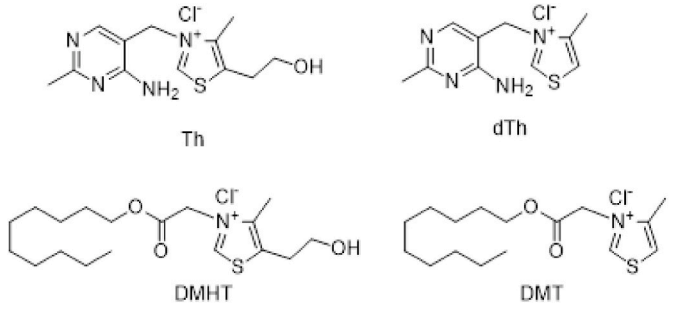

Fig. 1. Thiamine and its synthetic structural analogues used in this work: thiamine (Th); 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazolium chloride (*des*-hydroxyethyl thiamine) (dTh); 3-decyloxycarbonylmethyl-5-(2-hydroxyethyl)-4-methyl-1,3-thiazolium chloride (DMHT); 3-decyloxycarbonylmethyl-4-methyl-1,3-thiazolium chloride (DMT).

the "Rules of work with the experimental animals", approved by the Commission on the Care, Maintenance and Use of Experimental Animals of the Palladin Institute of Biochemistry of the NAS of Ukraine.

2.2.1. Preparation of synaptosomes and synaptosomal plasma membranes

In the experiments, male nonlinear white Wistar rats weighing 120–150 g and maintained on a vivarium diet were used. Rats were decapitated under ether anesthesia. All procedures for isolation of subcellular structures were performed at 0–4 °C with the addition of the protease inhibitor phenylmethylsulfonyl fluoride (FMSF, 0.5 mM) to the buffer. Synaptosomes and neuronal plasma membranes were obtained from rat brain by differential centrifugation in a sucrose density gradient as previously described (Gray and Whittaker, 1962) slightly modified (Protasova et al., 1999) Brains were quickly removed on ice and placed in chilled 0.32 M sucrose solution containing 1 mM EDTA, 1 mM Tris-HCl buffer pH 7.4 and 1 mM FMSF. Homogenization was carried out in the same buffer. The resulting homogenate was centrifuged at 1200g for 20 min to precipitate the nuclei. The supernatant was centrifuged at 23,000g for 20 min. The resulting pellet was resuspended in isolation medium, applied to a 1.2-1.0-0.8 M sucrose gradient, and centrifuged again at 83000g for 60 min. The synaptosome fraction isolated in the 1.0–1.2 M interphase was further diluted with three times the volume of cold water and precipitated by centrifugation at 100,000 g for 30 min.

To obtain a plasma membrane preparation of synaptosomes (PMS), the brains of four to six rats were pooled. The synaptosomal precipitate was subjected to osmotic shock at 0 °C for 60 min. The resulting suspension was centrifuged at 20,000g for 20 min. The newly obtained precipitate was fractionated in the above three-step sucrose gradient, the plasma membrane fraction of synaptosomes was collected in 0.8–1.0 M sucrose interphase, diluted with 3-fold volume of 5 mM Tris-HCl buffer (pH 7.4) and centrifuged at 100,000g for 30 min. The resulting synaptosomal plasma membrane precipitate was resuspended in isolation medium and stored at −80 °C until use.

2.3. Synaptosomal binding of DMHT

Experiments with [^{14}C]-labeled thiamine were performed as described in (Protasova et al., 1999). Plasma membrane synaptosomes (PMS) were incubated at 37 °C with [2–^{14}C]thiamine (2.5 nmol in 1 mL incubation medium, specific radioactivity 25.7 μCi/mM) in 0. 05 M bicarbonate Ringer's buffer, pH 7.4, including in mM: NaCl, 125; KCl, 4.5; $CaCl_2$, 2.5; KH_2PO_4, 1.3; $MgSO_4 \bullet 7H_2O$, 1.3; $NaHCO_3$, 17.6; glucose, 11. The sample volume was from 0.25 to 0.5 mL. The PMS preparation was added to the sample at a rate of 0.5 mg protein per 1 mL. Background radioactivity (non-specific binding) was determined from a control sample to which unlabeled thiamine was added at 100-fold excess over labeled thiamine. Incubation was terminated after 2.5 min by transferring the samples to an ice bath and adding a 5-fold excess of ice-cold medium containing 0.05% globulin and 25% polyethylene glycol 6000 to precipitate protein. Extravesicular thiamine was removed by rapid filtration through a Whatman GF/C membrane filter and washed with 6 ml cold incubation medium without thiamine. Filters containing preparations were dried in an air stream and transferred to tubes containing SJ-1 scintillation liquid. Radioactivity was measured using an SL-20 "Intertechnique" liquid scintillation counter (France). The specific binding of thiamine to PMS, expressed as radioactivity per mg protein, was determined after subtraction of background radioactivity. Data on the binding of labeled thiamine in the presence of a competing compound are presented as percent of the control sample (incubation with labeled thiamine without addition of effectors).

2.4. Effect of DMHT on the synthesis of Ach from pyruvate

2.4.1. Measurement of label incorporation into ACh from [2–^{14}C]pyruvate

To study the effect of thiamine on ACh synthesis, choline (final concentration 20 μM) and [2–^{14}C]pyruvate (specific activity - 200 GBq/mol, concentration in the sample was 0,25 mM) were added to the medium (Parkhomenko et al., 1991). A final pyruvate concentration of 2.0 mM was achieved by adding cold pyruvate. In most experiments, synaptosomes were incubated in Krebs-Ringer bicarbonate buffer, pH 7.4, and the final protein concentration was 0.5 mg/ml. After all additions, the final volume of the incubation mixture was 0.5 ml. DMHT, thiamine or their derivatives were added to the medium in increasing concentrations in the range of $1 \bullet 10^{-7}$ to $1 \bullet 10^{-4}$ M as indicated in the text. Incubation was performed for 2.5 min at 37 °C, and KCl (60 mM) was added at the end of the incubation to induce depolarization and maximal release of AcCh from the compartments. TCA was immediately added to all samples and the precipitated proteins were separated from the liquid phase by centrifugation. To the clear supernatant containing labeled AcCh, choline was added at a final concentration of 100 mM as ACh co-precipitant and the latter was precipitated as periodate (Häggblad et al., 1983). The final supernatants were carefully removed with a Pasteur pipette and discarded. The radioactivity of [^{14}C]acetylcholine in the precipitate was measured in a liquid scintillation counter (SL-4000 "Intertechnik"). The incorporation of the label into the ACh molecule was calculated as Bq per 1 mg protein in the precipitate. The results in each series were expressed as a percentage of the control sample in that series. The results expressed as a percentage of the control were subjected to statistical treatment.

2.4.2. Phosphorylation of DMHT by TPK

The ability of DMHT and thiamine to undergo phosphorylation was determined using a partially purified thiamine pyrophosphokinase preparation (Pylypchuk et al., 2001), which was isolated by the method of Johnson & Gubler (Johnson and Gubler, 1968), and a conjugated enzyme system (Jaworek et al., 1974) containing Sigma rabbit muscle enzyme preparations of myokinase (EC 2.7.4.3), pyruvate kinase (EC 2.7.1.40), and lactate dehydrogenase (EC 1.1.1.27). The experiment was performed in two steps (Fig. 2). (*i*) The kinase reaction was set up with an incubation mixture consisting of 5 mM MgCl, 5 mM ATP, 400 μM salt or thiamine, 10 mM Tris-HCI buffer pH 8.4, and 1 mg TPK preparation. The reaction mixture was incubated for 17 h at 37 °C. A denatured protein sample was used as a control. The reaction was stopped by heating at 100 °C for 5 min. (*ii*) The amount of produsedAMP using the conjugated enzyme system described above was measured. The sequence of coupling reactions is shown in Fig. 2.

The method involved phosphorylating AMP to ADP by adenylate kinase (myokinase), and then with the participation of pyruvate kinase ADP was phosphorylated by phosphoenolpyruvate (PEP) to produce pyruvate. In the presence of excess NADH, pyruvate was converted to lactate under the action of lactate dehydrogenase. The reaction was monitored by the formation of NADH at 340 nm and expressed as μmol of NADH formed per minute per mg of protein. The change in the amount of used NADH was measured by the change in absorbance at 340 nm. Each mole of AMP produced by the thiamine pyrophosphokinase reaction resulted in the oxidation of 2 mol of NADH.

2.4.3. Assay for the activity of pyruvate dehydrogenase complex

To determine the effect of DMHT and thiamine on the activity of the pyruvate dehydrogenase complex, an experimental setup similar to that

(i) The thiamine pyrophosphokinase reaction:

$$ATP + Thiamine \longrightarrow AMP + ThDP$$

(ii) Determining the amount of AMP produced.

Adenylate kinase: $AMP + ATP \longrightarrow ADP + ADP$

Pyruvate kinase: $ADP + PEP + H^+ \longrightarrow Pyruvate + ATP$

Lactate dehydrogenase: $Pyruvate + H^+ + NADH \longrightarrow Lactate + NAD^+$

Fig. 2. Analysis of thiamine pyrophosphokinase activity through step reactions.

described in section 2.4.1 was used, but without the use of radiolabeled pyruvate. After 2.5 min incubation of synaptosomes with different concentrations of thiamine or DMHT (2.4.1), aliquots (usually 0.08 ml) were immediately taken and placed in the prepared 2 ml medium for determination of enzymatic activity. The reaction medium consisted of 0.1 M potassium phosphate buffer with sodium desoxycholate 0.3 % (w/v), pH 8.0; 0.2 mM ThDP, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM CoA, 0.4 mM NAD^+, 2 mM 2-pyruvate, and 5 mM sodium oxamate (Sigma-Aldrich). The reaction was initiated by the addition of pyruvate and monitored by the formation of NADH at 340 nm as described (Parkhomenko et al., 2011) and expressed as μmol of NADH formed per minute per mg of protein. The initial reaction rate was measured.

2.5. Assay of Ca^{2+}-ATPase (PMCA) activity

The synaptosomal plasma membrane Ca^{2+}-ATPase (PMCA) activity was measured using the colorimetric method outlined by Chan et al. (1986). This method is based on the conversion of ATP to ADP and Pi, with the latter being detected as the final product of the reaction. The formation of a colored complex between inorganic phosphate, molybdate, and malachite green was then measured. The reaction mixture was prepared in a final volume of 0.5 mL containing 50 mM Hepes/KOH pH 7.4, 100 mM KCl, 200 μM $CaCl_2$, 5 mM NaN_3, 200 μM EGTA (2.7 μM free Ca^{2+}), 20–30 μg of membrane preparation protein, and 1 mM ATP. The reaction was initiated by adding ATP and continued for 5 min at 25 °C. It was then terminated by adding 2 mL of malachite green/-molybdate/polyvinyl alcohol reagent. PMCA activity was calculated by recording the formation of the phosphomolybdate-malachite green complex spectrophotometrically at 630 nm and taking into account the molar extinction coefficient of the complex at 630 nm which was 19615 $M^{-1}cm^{-1}$.

2.6. Measurement of the membrane potential of isolated nerve terminals

We studied the effects of thiamine derivatives on the membrane potential of isolated synaptosomes using the potential-sensitive fluorescent probe DiOC6(3) (3,3′-dihexyloxacarbocyanine iodide) as described previously (Danylovich et al., 2011). Fluorescence changes of DiOC6(3) at an optimal concentration of 100 nM were studied using a SIGNE-4M spectrofluorometer (Latvia), emission at 505 nm; excitation at 485 nm.

2.7. Cell-based studies

2.7.1. Neuroblastoma cell line N1E11

Neuroblastoma cell line N1E11 obtained from the biobank of the Bogomolets Institute of Physiology of the National Academy of Sciences of Ukraine, is a well-known and well-characterized neuronal cell line isolated from mouse neuroblastoma. The cells were cultured in Dulbeco modified Eagle medium by (DMEM) supplemented with 10 % FCS serum and 0.05 % gentamicin antibiotic. The incubator was maintained at 37 °C with high humidity conditions and an atmosphere with 5 % CO_2 and 95 % air. Medium was changed every two days and cells were subcultured every four days. For experiments, cells were seeded in 96-well plates, at approximately 10^5 cells per well. DMHT was added to the medium 24 h after the start of cell culture to allow the cells to acclimate. The effect of DMHT on the cells was evaluated after 3 days of culture.

2.7.2. MTT assay

The level of cell viability (relative number of living cells remaining in the experiment) was determined using the MTT reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, according to the standard technique (Chornyy and Parkhomenko, 2008). After measuring the absorbance of the MTT-stained cell suspension at 570 nm on a plate reader, their relative amount in the sample was calculated.

2.8. IC50 value

The IC50 value (inhibitor concentration at which the inhibition is 50 % of the maximum) was determined from the graph of the inhibition value versus concentration of the tested compound (Sebaugh, 2011).

2.9. Statistical analysis

Experimental data were processed using generally accepted statistical methods. Unless otherwise stated, data are expressed as the mean ± standard error of the mean (SEM). Data from 3 independent experimental series were pooled. A value of $P < 0.05$ was considered statistically significant. Student's t-test was used to perform the statistical analysis. Microsoft Excel computer programs (Office 365 package) with BioStat add-on from AnalystSoft and GraphPad Prism, version 8.0 (USA) were used for calculations and graphical presentation of the obtained results.

3. Results

3.1. Effect of DMHT on Ach synthesis from pyruvate

3.1.1. The dose-dependent effect of DMHT, thiamine and its derivatives on the incorporation of the [2–^{14}C]pyruvate label into ACh and on the activity of PDC

These experiments were performed on synaptosomes (isolated nerve endings). The use of synaptosomes to study molecular events at the synapse is now well established. They can be regarded as model nerve endings that can be electrically or ionically depolarized, and the subsequent biochemical events, such as neurotransmitter release, inactivation, and resynthesis, can be studied (Doltchinkova et al., 2021).

Current evidence suggests that acetyl-CoA, which is formed from pyruvate when it is oxidized by the pyruvate dehydrogenase complex (PDC), is predominantly used for the synthesis of ACh in neurons. Studies by Lefresne et al., 1977, 1978a, 1978b have shown that when synaptosomes were incubated with [2–^{14}C]pyruvate, a significant portion of the label was transferred to acetylcholine. We have previously used this information to design experiments to investigate the effect of thiamine on ACh synthesis from pyruvate (Parkhomenko et al., 1991). In the experiments, freshly isolated synaptosomes were incubated in Krebs-Ringer medium with the addition of labeled pyruvate and different concentrations of thiamine. At the end of the incubation, PDC activity in synaptosomes and the incorporation of the label from [2–^{14}C] pyruvate into ACh were determined as described in the Methods section (2.4). In this study, a similar experimental design was used to evaluate the effect of the thiamine derivative DMHT on ACh synthesis. For comparison, the same experiments were performed with the addition to the incubation medium of each of the following compounds: thiamine, DMT (DMHT analog without the hydroxyethyl radical) or dTh (thiamine analog without the hydroxyethyl radical) (Fig. 1).

The latter two compounds do not have a hydroxyethyl substituent in their structure and therefore cannot be acceptors of the pyrophosphate group with ATP when interacting with thiamin pyrophosphokinase. We took advantage of this fact to test whether the effect of thiamine (Parkhomenko et al., 1991, 2019) and possibly DMHT on ACh synthesis could indeed be due to the action of their phosphorylated derivatives on the formation of acetyl-CoA from pyruvate. The concentration of the derivatives in the experiment ranged from 0.1 to 10 μM. These concentrations correspond to the range of concentrations of thiamine derivatives in brain. The results are summarized in Fig. 3.

As can be seen from the above data, the effect of DMHT and thiamine on the incorporation of labeled carbon from [2–^{14}C]pyruvate into ACh, as well as on PDC activity, has a similar trend. Namely, both processes decreased as the concentration of effectors (thiamine or DMHT) in the incubation medium increased from 10^{-7} to 10^{-5} M (Fig. 3A). At the highest concentrations of this range ($1 \bullet 10^{-5}$ M), the inhibition

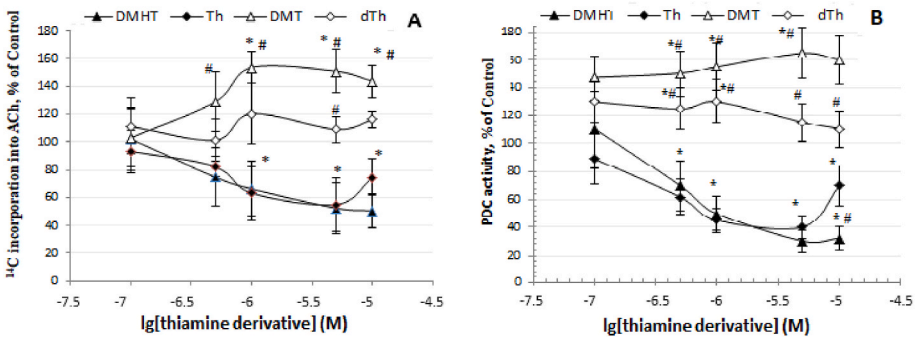

Fig. 3. Effects of DMHT, thiamine, DMT, and dTh on the incorporation of radiocarbon from [2–^{14}C]pyruvate into ACh (A) and on PDC activity (B) in synaptosomes. Abbreviations are as in Fig. 1 (A) The sample volume was 0.5 mL. Labeled pyruvate was added at ~25000 bq per sample (corresponding to 0.25 mM concentration). The actual result was 23074 ± 2445 Bq. A final pyruvate concentration of 2.0 mM was achieved by adding cold pyruvate. The reaction was initiated by adding freshly isolated synaptosomes at a rate of 0.5 mg protein per 1 mL. The incorporation of labeled carbon into the ACh molecule after precipitation as periodate (2.3.1) was first recorded in Bq per 1 mg protein in the sample. Data expressed as % of control were subjected to statistical processing. In the control sample, the synaptosomes were incubated in Krebs-Ringer's solution with the isotope without addition of effectors. Radioactivity in the control sample was taken as 100 %, corresponding to 1054 ± 98 Bq per the sample. The mean ± SEM of three determinations in three independent experiments represents each data point. $^{*}p < 0.05$ compared to control samples (without effector), $^{\#}p < 0.05$ compared to the same thiamine concentration. (B) The initial reaction rate was used to evaluate PDC activity in separate non-radiometric experiment (2.4.1). The sample volume was 2.0 mL. Synaptosomes were added to the sample at 0.100 mg per 1 ml. Each data point represents the mean ± SEM of three independent experiments. A value of 100% PDC activity corresponds to 90 ± 16 nmol NADH/min per 1 mg protein. $^{*}p < 0.05$ compared to control samples (without effector), $^{\#}p < 0,05$ compared to the same thiamine concentration.

decreased in the case of DMHT and increased slightly, especially for PDC, in the case of thiamine (Fig. 3 B).

Previously, we had observed the same effect of thiamine in experiments on synaptosomes (Parkhomenko et al., 1991, 2019) and explained it by a change in the ThTP/ThDP ratio in the cells with increasing concentration of thiamine in the incubation medium. The non-coenzymatic effect of these thiamine phosphates on the activity of PDC regulatory enzymes was previously demonstrated in experiments with partially purified enzymes. It was shown that ThTP at concentrations below 1 μM nonlinearly reduced PDH phosphatase activity by more than 70% (Parkhomenko et al., 1987, 2019), while ThDP inhibited PDH kinase activity starting at concentrations of 10^{-6} M. Since the effect of DMHT in this study is similar to that of thiamine, it suggests that DMHT is able to be metabolized in the cell with the participation of the same proteins as thiamine.

Analogues of thiamine and DMHT that do not contain a hydroxyethyl substituent, namely DMT and dTh, increased radiolabel incorporation into ACh as well as PDC activity to varying degrees at all concentrations studied (Fig. 3A and B). This effect is different from that of vitamin B_1 and suggests that DMT and dTh may cause a significant imbalance in ion fluxes. This result prompted us to investigate the effect of all thiamine derivatives on the membrane potential of synaptosomes (*3.3.1)*.

To better understand the mechanisms underlying the effect of DMHT on PDC activity, we next sought to answer two questions: 1) whether DMHT can compete with thiamine for binding to PMS, and 2) whether DMHT can act as a substrate for TPK in a manner similar to that of thiamine.

3.1.2. Ability of DMHT to compete with [^{14}C]thiamine for binding to synaptosomal plasma membranes

To determine the ability of DMHT to interact with synaptosomal plasma membranes, we used a competition assay with labeled thiamine (Protasova et al., 1999). Cold thiamine was also used for comparison in parallel assays. We analyzed the ability of the compounds tested to inhibit [^{14}C]thiamine binding to PMS. A sample containing only labeled thiamine was used as a control. The results, expressed as a percentage of the control, are shown in Fig. 4A.

The analysis indicated that DMHT, like cold thiamine, significantly

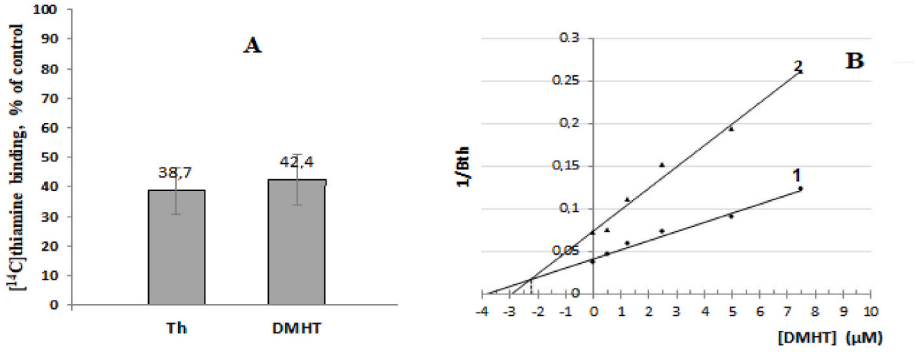

Fig. 4. Interaction of DMHT with plasma membranes of synaptosomes (PMS). (**A**) Effect of DMHT and thiamine (both at 25 μM) on the binding of [^{14}C]thiamine (sample concentration ~2.5 μM) to PMS. 0.25 mg of PMS protein was added to 0.5 ml of the incubation mixture. Specific binding was determined as the difference between total and nonspecific binding. Additional assays were performed with the addition of 100 μM unlabeled thiamine to determine non-specific binding. The results, expressed as a percentage of the control sample (PMS incubated with the isotope in Krebs-Ringer's solution without the addition of effectors) in each series, were subjected to statistical analysis. The 100 % binding value in the control corresponds to 380 ± 39 Bq in the sample or 26.71 ± 2.73 pmol per 1 mg protein (1 pmol corresponds to 57.05 Bq). Each data point represents the mean ± SEM of three independent experiments (three replicates each), $p > 0,05$ when comparing the value for DMHT with the value for thiamine. (**B**) Dose-dependent inhibition of [^{14}C]thiamine binding to PMS in the presence of DMHT expressed in Dixon coordinates. Denotes: "B_{th}" is the amount of labeled thiamine bound to the membranes. For lines 1 and 2, the concentrations of ^{14}C-thiamine in the incubation medium were of 1.24 μM and 2.45 μM, respectively. Each data point represents the average of three experiments.

inhibited [^{14}C]thiamine binding to PMS, confirming the ability of DMHT to interact with the same regions of the neuronal membrane as thiamine. Fig. 4B shows the results of a dose-dependent study of DMHT inhibition of labeled thiamine binding to PMS in Dixon coordinates. From these data, the K_i value for this process is approximately 2.4 μM. In determining the IC50 for this process, we also obtained an approximate IC50 value of 2.0 ± 0.3 μM. Together with previous observations, this confirms the high affinity of DMHT for thiamine binding sites on proteins.

3.1.3. DMHT as a substrate for thiamine pyrophosphokinase

Previously, using a partially purified preparation of rat brain thiamine pyrophosphokinase (TPK), DMHT was shown to be a competitive inhibitor of this enzyme (Pylypchuk et al., 2001). This observation, together with the presence of a hydroxyethyl group in the DMHT molecule, suggests that DMHT can be phosphorylated by thiamine pyrophosphokinase. In this case, instead of thiamine, DMHT would become the substrate for TPK and the reaction would proceed with the formation of pyrophosphorylated DMHT (DMHT-PP) and AMP.

To answer the second question, i.e. to evaluate the ability of DMHT to participate in the thiamine pyrophosphokinase reaction as an acceptor of the pyrophosphate group with ATP, a conjugated enzyme system was used (Jaworek et al., 1974). The formation of AMP as the product of this reaction should indicate the transfer of the pyrophosphate group to the hydroxyethyl residue of DMHT. A similar reaction with thiamine, the actual substrate of TPK, was performed in parallel. The results are given in Table 1.

A preparation of thiamine pyrophosphokinase (TPK) isolated from rat brain (Pylypchuk et al., 2001) and a conjugated enzyme system consisting of myokinase, pyruvate kinase, and lactate dehydrogenase were used in the experiment. The principle of the method involves phosphorylation of AMP formed in the TPK reaction to ADP by myokinase, phosphorylation of ADP by phosphoenolpyruvate with formation of pyruvate, which was further reduced by lactate dehydrogenase and NADH. The incubation mixture contained 5 mM MgCl, 5 mM ATP, 400 μM DMHT or thiamine, 10 mM Tris-HCl buffer pH 8.4, and 1 mg TPC preparation. The amount of NADH used in the reaction was estimated from the decrease in absorbance at 340 nm. The formation of 1 mol of AMP was accompanied by the oxidation of 2 mol of NADH. Values are expressed as the mean ± standard deviation of three independent experiments, $p > 0.05$ when comparing the value for DMHT with the value for thiamine.

The results confirm the possibility of DMHT-PP formation when synaptosomes are incubated under our conditions. Furthermore, the amount of AMP obtained in the thiamine substrate reaction was not significantly different from that obtained with DMHT.

3.2. Effect of DMHT, thiamine and their analogues on plasma membrane Ca^{2+}-ATPase (PMCA) activity

Since most intracellular reactions are calcium mediated, calcium ions are important mediators of signal transduction in cells. Changes in the concentration of calcium ions in specialized cells lead to a variety of biological effects at the level of organs and tissues (Zhang et al., 2023; Sukumaran et al., 2021; Díaz-Soto et al., 2016). According to modern concepts, the concentration of calcium ions in the cytoplasm of the cell is nanomolar, while in the extracellular environment it is much higher, reaching millimolar concentrations (Díaz-Soto et al., 2016). An adequate and nearly constant concentration of extracellular calcium (~1.0 mM) must be maintained to ensure proper muscle and nerve function. An active calcium ion transport system maintains such a concentration difference. Among all the systems involved in this process, cells use Ca^{2+}-ATPases as active high-affinity transporters to pump Ca^{2+} ions across the plasma membrane Ca^{2+}-ATPase or sarcoendoplasmic reticulum organelle membrane (Berrocal and Mata, 2023). An increase in calcium concentration in neurons during thiamine deficiency has been described in the literature (Kimura and Itokawa, 1977; Huang et al., 2010a). We considered that it was appropriate and feasible to test the effect of DMHT, thiamine and their analogues on PMCA activity. The effector concentrations in this study ranged from 0.1 to 50 μM, covering the range of physiological concentrations of thiamine and its bioactive derivatives in cells (10^{-6}-10^{-5} M). The results obtained are summarized in Fig. 5.

The data above shows that DMHT and thiamine activate PMCA *in vitro* at concentrations range of 10^{-6} - 10^{-4} M. However, the analogues without hydroxyethyl group have a different effect. Specifically, the activating effect of DMT and dTh becomes inhibitory when the concentration of these compounds exceeds 10^{-5} M. The data obtained may indicate that the presence of the hydroxyethyl substituent is not essential in the interaction of the thiamine molecule with the PMCA structure. The data obtained suggests that the presence of the hydroxyethyl group (and its phosphorylation) is not necessary for the interaction of the thiamine molecule with the PMCA structure.

Table 1
Amount of AMP formed in the reaction when TPK was incubated with DMHT or thiamine.

Substrate	AMP, nmol/hour• mg protein
DMHT	6.5 ± 0.86
Thiamine	5.6 ± 0.49

3.3. Effects of DMHT on nerve cell viability

3.3.1. Effect of DMHT, thiamine and their analogues on the membrane potential of synaptosomes

There are several ion channels in the plasma membranes of neurons, which ensure the distribution of ions between the intracellular and extracellular space, and the result of their function is the formation of the cell membrane potential. The intracellular concentration of ions, especially calcium ions, can be crucial for the regulation of metabolic processes and in the development of pathologies (Zhang et al., 2023; Sukumaran et al., 2021; Díaz-Soto et al., 2016).

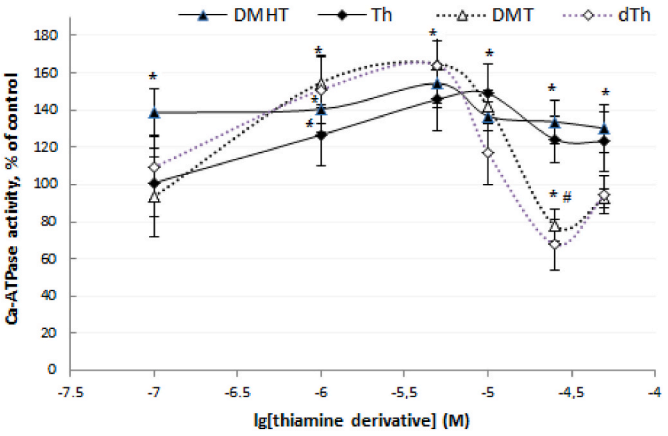

Fig. 5. Effect of DMHT, thiamine, and their structural analogues on PMCA activity in synaptosomal plasma membrane preparations. The volume of the incubation mixture in the sample was 0.5 ml. The reaction was started by adding PMCA preparation at the rate of 0.02–0.03 mg protein and stopped by adding 2 ml of mixed reagent for colorimetric determination of Pi (Chan et al., 1986). The amount of P_i formed was calculated from the molar extinction coefficient of the malachite green-phosphomolybdenum complex at 630 nm, which was 19615 $M^{-1}{\cdot}cm^{-1}$. Results are expressed as percent of control (PMCA activity in a sample incubated without addition of thiamine derivatives). Value for 100 % activity corresponds to 0.229 ± 0.042 μmol/min P_i per 1 mg of protein. Each data point represents the mean ± SEM of 5 determination. $^{*}p < 0.05$ compared to control samples (without effector), $^{\#}p < 0.05$ compared to the same thiamine concentration.

The membrane potential of an excitable cell in a non-excited state is known as the resting potential. When a cell is exposed to a stimulus, i.e. a chemically active substance that interacts with the cell membrane by changing its permeability to certain ions, a depolarization phase occurs. Such stimuli are neurotransmitters. Measuring the membrane potential of nerve cells gives a general idea of their functional state. We investigated the effect of DMHT, thiamine and their derivatives on the MP of isolated synaptosomes using the fluorescent probe DiOC6(3).

The probe DiOC6(3) is a lipophilic cation that accumulates within a confined membrane space in a potential-dependent manner. An increase in the negative potential at the cell membrane leads to an increase in its accumulation. This is accompanied by increased fluorescence. In our experiments, synaptosomes were incubated in Krebs-Ringer-Tris buffer with one of the tested compounds added at 50 and 100 μM for 2.5 min. Fluorescence was measured as described in the Methods section (2.6). The results are shown in Fig. 6.

As can be seen from the data presented, all the compounds studied can induce plasma membrane depolarization in isolated nerve cells. The action of DMHT at a concentration of 50 μM on membrane potential (MP) is like to that of thiamine, but DMHT causes a more pronounced depolarization when the concentration is increased to 100 μM. The hydroxyethyl-free analogues were found to be significantly more effective than the parent compounds in reducing MP. Under the same conditions, only DMHT and thiamine caused a dose-dependent decrease in MP. The findings suggest that concentrations of DMHT above physiologic thiamine levels, as well as DMT and dTh can impair cellular metabolism.

3.3.2. *Effect of DMHT on nerve cells viability*

Our results indicate that under *in vitro* conditions at physiological concentrations, DMHT, whose molecule contains an unchanged thiazolium fragment of thiamine, mimics the effect of thiamine on PDC activity and, consequently, on ACh synthesis (3.1). The effect of DMHT on PMCA activity (3.2) as well as on the membrane potential of nerve terminals (3.3.1) under the same conditions is also similar to that of thiamine. At the same time, as a competitive inhibitor of TPK (Pylypchuk et al., 2001), this compound has been shown to be capable of undergoing pyrophosphorylation (3.1.3) and therefore may form an anticoenzyme in cells. We tested how these properties of DMHT would affect the vital activity of cells *ex vivo*. Since vitamin B_1 is a neurotropic compound, we tested the effect of DMHT on the viability of N1-E115 neuroblastoma cells (3-day culture). The cells in this line, although cancerous, express neuronal marker proteins and have a neuron-like morphology.

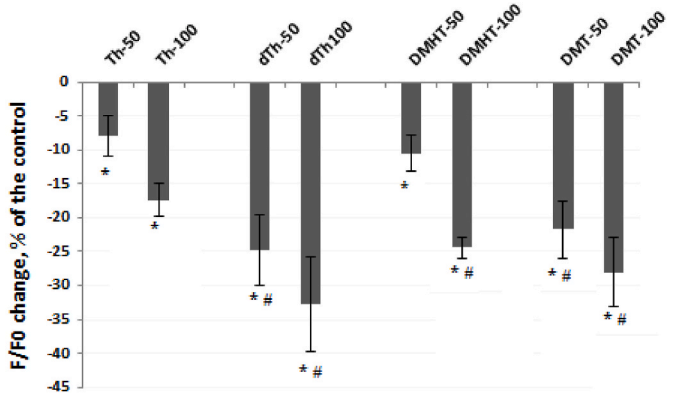

Fig. 6. Effect of DMHT, thiamine, DMT and dTh on the membrane potential of synaptosomes. The concentration of the test substance is indicated by the index 50 (50 μM) and 100 (100 μM), respectively. Data are expressed as percentage decrease in membrane potential compared to control (synaptosomes were incubated in Krebs-Ringer's solution without addition of thiamine derivatives). Each index represents the mean ± SEM of three independent experiments performed in triplicate, $^{*}p < 0.05$ compared to control samples (without effector), $^{\#}p < 0.05$ compared to the same thiamine concentration.

As can be seen from the data in Fig. 7A, the inhibitory effect of DMHT on the growth of N1-E115 neuroblastoma cells is dose-dependent at a concentration of 10–500 μM. From these data, an approximate IC50 value of 21 μM for thiazolium salt inhibition of cell growth was determined (Fig. 7 B). Apparently, the ability of DMHT to mimic the non-coenzyme effects of thiamine determines its rather low toxicity to cells.

4. Discussion

The structure of each vitamin molecule is unique. Even small changes in this structure lead to significant changes in the properties of the resulting compound, which may be completely opposite to those of the original vitamin. Such compounds are called 'antivitamins' (Tylicki et al., 2018). In most cases, the cellular molecules targeted by antivitamins are the same as those targeted by natural vitamins. This means that some antivitamins can be used to study the molecular mechanisms of action of the original vitamin. The structural features of thiamine and TDP determine their natural affinity for many enzymes and other proteins (Mkrtchyan et al., 2015; Mezhenska et al., 2021). These functions can be reduced by antivitamins formed in cells or ingested with food. At the same time, there is increasing interest in the potential use of known antivitamins and new synthetic compounds structurally similar to thiamine, for example in the treatment of cancer and bacterial and fungal diseases (Tylicki et al., 2018). Along with this the studies of antivitamins shapes the understanding of the mechanism of vitamin B_1 activity in vivo. We exploited this opportunity in the case of DMHT, which has been shown previously to be a substrate for thiamin pyrophosphokinase, competing with thiamine (Pylypchuk et al., 2001), as well as interacts with proteins involved in vitamin B_1 metabolism (Mkrtchyan et al., 2015).

To find an answer to the question whether the high neurotropic activity of thiamine could really be related to some biochemical non-coenzyme effects reactions of thiamine, unique to nerve cells, we have analyzed the literature data and the results of our own studies related to the peculiarities of vitamin B_1 metabolism in nerve cells and its role in their functioning (Parkhomenko et al., 1996, 2016). The main conclusion was that thiamine metabolism in the nerve cell is not fundamentally different from that in cells of other tissues. Another conclusion is quite probable: it is the peculiarities of the structural and functional organization of nerve cells that determine the exclusive importance of certain biochemical reactions involving thiamine and having a universal distribution. Nerve cells have special properties of excitability and conductivity that distinguish them from other cells of the organism. These properties of nerve cells are determined by the peculiarities of their structure and cause high dynamism of intracellular metabolism. Thus, it has been shown experimentally that in nerve cells the regulation of pyruvate dehydrogenase complex activity by phosphorylation-dephosphorylation is conjugated with changes in membrane potential. For example, the addition of potassium chloride to synaptosomes has been shown to increase the rates of oxygen consumption and [1–^{14}C]pyruvate decarboxylation as well as the ratio of active/total extractable pyruvate dehydrogenase (90–100 %) within 10 s (Schaffer, 1980). A rapid response phosphorylation of a protein with Mr 40 kDa, identified as the α-subunit of PDC, was also detected during electrical stimulation of hippocampal preparations (Browning et al., 1981).

The above information, together with the known facts that free thiamine is released from the neuron into the intercellular space during excitation (Parkhomenko et al., 1996), and that the plasma membrane contains a thiamine-binding protein that selectively hydrolyzes thiamine phosphates (Parkhomenko et al., 2001), is consistent with the hypothesis of the presence of a highly labile pool of thiamine and its phosphates in neurons. It is assumed that this pool also contains proteins involved in phosphorylation-dephosphorylation of thiamine derivatives

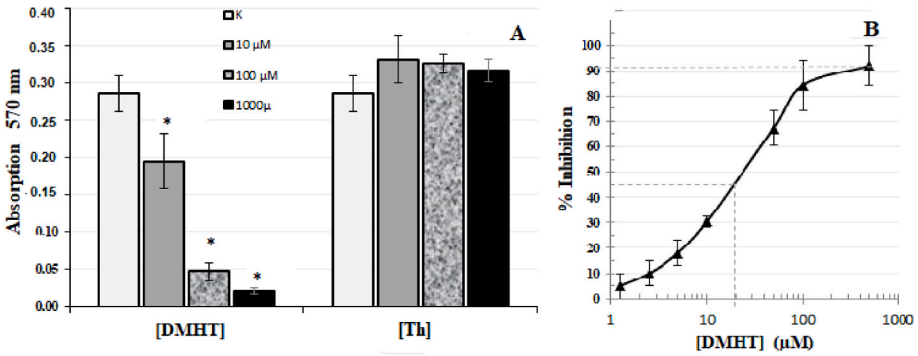

Fig. 7. Effect of DMHT on N1-E11 cells viability (MTT assay). **(A)** Dose-dependent effect of DMHT and thiamine (Th) on neuroblastoma cell viability (MTT assay), each data point represents the mean ± SEM of three independent experiments. The results indicate that the difference ($p < 0.05$) in cell growth rate is statistically significant when cultured at this concentration of DMHT versus the previous lower concentration. **(B)** Inhibition of neuroblastoma cell viability by DMHT in percent of control, the graph allows estimating the approximate IC50 value in this case as 21 μM. *Neuroblastoma cells line N1E11* cells (2.7.1) were cultured in Dulbeco modified Eagle medium by (DMEM) supplemented with 10 % FCS serum and 0.05 % gentamicin antibiotic. The incubator was maintained at 37 °C with high humidity conditions and an atmosphere with 5 % CO_2 and 95 % air. Medium was changed every two days and cells were subcultured every four days. For experiments, cells were seeded in 96-well plates, at approximately 10^5 cells per well. DMHT was added to the medium 24 h after the start of cell culture to allow the cells to acclimate. The effect of DMHT on the cells was evaluated after 3 days of culture.

and that its function is related to the functioning of the excitable membrane and to regulating the metabolism of acetylcholine (Parkhomenko et al., 2016).

The involvement of thiamine phosphates in the non-coenzyme regulation of PDC was first investigated on the rat liver enzyme at different levels. Experiments in rats with a model of activated lipogenesis showed that after a single administration of thiamine at a dose of 0.25 mg per 100 g body weight, liver PDH activity decreased in these rats because of enzyme conversion to the inactive form during the first hours after vitamin administration (Parkhomenko et al., 2019). This change correlated with a significant increase in the total thiamine derivatives content, which was particularly pronounced in the case of ThTP. In the first hours after thiamine administration, the [ThTP]/[ThDP] ratio increased compared with the situation in the corresponding control. We conclude that thiamine phosphate esters, ThDP and ThTP, may be involved in PDH regulation by phosphorylation-dephosphorylation. To test this conclusion, we first performed experiments with isolated mitochondria and then with the partially purified pyruvate dehydrogenase complex without separation into its components. In the latter case, PDH kinase remained associated with PDC. ThTP at concentrations below 1 μM was confirmed to non-linearly reduce PDH phosphatase activity by more than 70% (Parkhomenko et al., 1987, 2019), while ThDP inhibited PDH kinase activity from concentrations of $1 \bullet 10^{-6}$ M. Inhibition of PDH kinase activity by ThDP was also observed by Cooper et al. (1974). These effects of thiamine phosphates are quite physiological, since ThTP is reported to be the most rapidly exchanged of them (Berman and Fishman, 1975; Chagovec and Rybina, 1959), and a significant fraction of ThDP in cells is bound to proteins.

We extrapolated these findings to the situation in neurons to see if the involvement of thiamine in the modulation of Ach synthesis could be tested in this way (Protasova et al., 1999). In this case, we hoped to obtain changes in the [ThTP]/[ThDP] ratio by incubating synaptosomes with increasing concentrations of thiamine in the incubation medium. The literature data indicate that the exchangeability of ThTP (Parkhomenko et al., 2019; Berman and Fishman, 1975; Chagovec and Rybina, 1959) and ThTP level in tissues increase dramatically immediately after thiamine administration to animals (apparently also in the cell). The effects of increasing concentrations of thiamine (Parkhomenko et al., 1991) and then of oxythiamine (Parkhomenko et al., 2019) were tested in the *in vitro* model mentioned above (2.4). The results of the study formally confirmed the initial hypothesis. Oxythiamine, a coenzyme antagonist of thiamine, exhibited effects similar to thiamine itself, increasing PDH activity and the incorporation of the [2–^{14}C]pyruvate label into Ach when incubated with synaptosomes isolated from the brains of both normal and B_1-deficient rats. The observed effects of oxythiamine on PDH activity could be explained by the ability of this compound to interact with thiamine metabolizing proteins and form di- and triphosphates in a vitamin-like manner.

The DMHT experiments described in this study were also performed to further test the hypothesis that a labile thiamine pool in neurons is coupled to the regulation of ACh metabolism. Previously published data (Pylypchuk et al., 2001) and the DMHT data presented here suggest that this compound may mimic the non-coenzyme effects of vitamin B_1 in experimants *in vitro*. Its high affinity for proteins involved in thiamine transport and metabolism was confirmed in this report (3.1.2 and 3.1.3) and demonstrated previously by using DMHT as a ligand of an affinity sorbent (Mkrtchyan et al., 2015).

According to our observations, thiamine (thiamine phosphates) acts only to modulate the activity of PDH-regulating enzymes. The ability of ThTP to replace ATP in the PDH kinase reaction has not been confirmed in specific studies (Parkhomenko et al., 1986). However, it cannot be excluded that a cascade mechanism exists in neurons for the phosphorylation of proteins relevant for PDC regulation and that ThTP may be a phosphate donor in a part of this cascade. Within the framework of the hypothesis about the function of the free (mobile) pool of thiamine derivatives (Parkhomenko et al., 2016), the modulating effect of thiamine on PDC activity and acetylcholine synthesis does not take into account ideas about the compartmentalization of the processes in the cell. Nevertheless, this is in line with the observations of other researchers (Lefresne et al., 1978a; Hirsch and Parrott, 2012). As a comment, we can suggest that these representations, as well as representations of some signaling processes in the cell, may not correspond exactly to the real situation and await further clarification.

Another non-coenzyme effect of thiamine worth noting is its effect on the activity of PMCA. Information on the relationship between thiamine availability and cellular calcium homeostasis has appeared in the literature from time to time. Kimura & Itokawa (Kimura and Itokawa, 1977) first investigated the effects of calcium-containing and calcium-free diets on thiamine metabolism in the rat brain. They concluded that calcium plays a role in the binding of thiamine to nerve cell membrane structures and mediates its involvement in nerve conduction. In cell culture, it was shown that when cells were cultured in thiamine-deficient medium, the intracellular concentration of Ca^{2+} ions increased, and cell death was the result. The development of endoplasmic reticulum stress was also observed under these conditions. This was accompanied by the release of calcium from its cellular depot into the cytoplasm (Huang et al., 2010a, 2010b). Plasma membrane

Ca^{2+}-ATPases are plasma membrane and intracellular membrane transporters. They use energy from ATP hydrolysis to pump cytosolic Ca^{2+} out of the cell or into internal stores. These pumps are the major high-affinity Ca^{2+} systems involved in maintaining the intracellular free Ca^{2+} concentration at an appropriately low level. A significant increase in calcium concentration in neurons during thiamine deficiency may be a consequence of a reduction in PMCA function. On the one hand, this could be due to the reduced energy supply in thiamine deficiency. On the other hand, a direct interaction of thiamine derivatives with PMCA proteins cannot be excluded. In our experiments, DMHT, like thiamine, activated PMCA at all concentrations used, in contrast to its analogues without the hydroxyethyl substituent at position 5 of the thiazolium ring. The inability of neurons to maintain an optimal intracellular Ca^{2+} concentration is a common feature of neurodegenerative diseases, which are often associated with thiamine deficiency. Data obtained to date suggest that PMCA may be a therapeutic target in the treatment of neurodegenerative diseases (Berrocal and Mata, 2023).

As expected, DMHT, an inhibitor of ThDP synthesis (Pylypchuk et al., 2001), has a cytostatic effect on cells. The influence of DMHT on neuronal viability was tested for the first time and its effect cannot be considered high according to the IC50 value of 21 μM. It is possible that the ability of DMHT to mimic the non-enzymatic action of vitamin B_1 may open the way to designing compounds being able to act selectively on pathological neurons.

The parallel study of DMHT, thiamine and their structural analogues supports the notion that the presence of a hydroxyethyl substituent and its ability to be phosphorylated is a necessary factor for the observed effect on PDC activity and ACh synthesis. These compounds are also not neutral towards cellular structures, as evidenced by their effects on synaptosomal membrane potential and PMCA activity in synaptosomal membranes. Apparently, by interacting with some cellular proteins, they cause a significant imbalance in the distribution of ions in the cell.

Thus, the results suggest that the non-coenzymatic functions of vitamin B_1 are determined by the particularities of the thiamine molecular structure (Parkhomenko et al., 2024) and its high reactivity, and these properties are critical for neurons because of their structural and functional organization. The results of this study allow us to conclude that DMHT bearing an unchanged thiazolium moiety of the thiamine while its pyrimidine part is replaced by a lipophilic decyloxycarbonylmethyl substituent can be considered as a new potent antagonist of thiamine, mimicking the non-coenzyme effects of this vitamin. Its advantage over the well-known thiamine antagonist oxythiamine is that affinity of DMHT for proteins involved in thiamine metabolism is higher than that of OTh (Parkhomenko et al., 2024). All this makes DMHT a potential specific effector and a useful tool for the study of thiamine-dependent processes and the development of new drug design.

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CRediT authorship contribution statement

Yu.M. Parkhomenko: Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **A. I. Vovk:** Writing – review & editing, Conceptualization. **Z.S. Protasova:** Methodology, Investigation. **S. Yu Pylypchuk:** Methodology, Investigation. **S.A. Chorny:** Methodology, Investigation. **O.S. Pavlova:** Methodology, Investigation. **O.A. Mejenska:** Methodology, Investigation. **L.I. Chehovska:** Investigation. **S.P. Stepanenko:** Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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