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New Considerations on the Neuromodulatory Role of Thiamine

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Oxythiamine · Thiamine · Acetylcholine · Cholinergic neurotransmission · Calcium · Neurotransmission · Neuromodulation · Superfusion · Potassium · Depolarization

Abstract
Background: A nonmetabolic role for thiamine in cholinergic neurotransmission has long been suggested. The mechanism remains unclear. We sought to extend our previous research to elucidate the effect of the thiamine metabolic antagonist, oxythiamine, on the release of acetylcholine from the brain. ***Methods:*** The potassium-stimulated release of acetylcholine from superfused rat brain slices was determined. Hand-cut slices of cerebral cortex were preincubated with tritiated choline to label acetylcholine stores. Two periods of stimulation (S1, S2) with 50 mmol/l solution for 3.5 min were performed as superfusate was collected. During S1, only 50 mmol/l potassium-containing Krebs-bicarbonate buffer with 2 mmol/l calcium was used. Using a two-by-two design, S2 consisted of exposure to 50 mmol/l potassium with or without 10^{-4} mol/l oxythiamine, with or without calcium. The S2/S1 ratio was calculated. ***Results:*** Oxythiamine enhanced the potassium-evoked release of acetylcholine by 60% but only when calcium was present in the superfusing medium. ***Conclusion:*** These data confirm earlier findings with oxythiamine on the calcium-mediated synaptic transmission of acetylcholine and support a possible neuromodulatory role for thiamine distinct from its actions as a cofactor during metabolic processes.

Introduction

The role of thiamine as a crucial coenzyme in neuronal metabolism of carbohydrates and neurotransmitters, especially acetylcholine, has been well elucidated [1, 2]. The thiamine metabolic antagonists pyrithiamine and oxythiamine have long been known to inhibit the uptake and phosphorylation of thiamine in tissue and to inhibit the formation of intracellular thiamine diphosphate, an important coenzyme involved in three metabolic pathways, including acting as a cofactor of the pyruvate dehydrogenase pathway [2, 3]. They both inhibit the synthesis of acetylcholine [4], with regional differences in rat brain between the two drugs after chronic parenteral administration [5]. Of note, pyrithiamine but not oxythiamine is centrally active when given in vivo to mammals due to the latter's inability to cross the blood-brain barrier [6].

The possible role of thiamine in neural activity that is unrelated to its cofactor role has also been studied [3, 7]. A body of research has demonstrated an influence of thiamine on nerve action potential [8, 9], membrane conductance [10] and pre- or postsynaptic transmission [11–

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15]. Pyrithiamine and oxythiamine have been used as probes to study a possible role of thiamine in neurotransmission. Depressed ganglionic transmission after incubation with pyrithiamine and oxythiamine has been reported, presumptively because of inhibited synthesis of acetylcholine [16]. However, this effect occurred after hours of incubation and stands in marked contrast to the body of research which shows that either or both drugs have excitatory effects on nervous tissue, especially with much shorter lengths of exposure [8, 11, 13, 14, 17, 18]. Enhanced neurotransmitter release of acetylcholine has been observed in peripheral [13–15] and central nervous system tissues [16]. Release of other transmitters, like norepinephrine [17] and dopamine [18], is also enhanced by exposure to thiamine analogs. This effect appears to be calcium dependent [15, 19]. Unknown is the locus (or loci) of action of thiamine as a neuromodulator. Both delayed rectifier potassium [19] and chloride channels [3, 20] have been implicated. Complicating these interpretations are differential effects between thiamine and/or its phosphorylated metabolites on the one hand and antimetabolites like oxythiamine on the other, in terms of extra versus intracellular ion channel binding sites [3, 19, 20] as well as differential effects of specific antimetabolites on the release of different neurotransmitters and between different modes of stimulation. For example, Hirsch and Gibson [17] found that both oxythiamine and pyrithiamine significantly enhanced acetylcholine but not norepinephrine release from superfused mouse brain tissue evoked by potassium depolarization. Oxythiamine was 50 times more potent than pyrithiamine. The same concentration of pyrithiamine did evoke the release of norepinephrine but only when stimulated electrically and not with high potassium. These differential effects may have been secondary to different abilities of the two drugs to penetrate the neuron, the mode of depolarization on calcium-dependent release and varying sensitivity of release processes among neurotransmitters [17, 21]. In order to more fully explore the role of oxythiamine as a neuromodulator of acetylcholine release, we extended these previous studies. Specifically, we examined in greater detail the interaction between oxythiamine and calcium in the evoked release of this neurotransmitter.

Materials and Methods

Materials

Male Wistar rats (150–200 g) were obtained from the Charles River breeding laboratories (Wilmington, Mass., USA). All procedures involving animals were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at St. John's University. [Methyl-^{3}H]choline chloride (80 Ci/mmol) was obtained from New England Nuclear Corporation (Wellesley, Mass., USA) and the liquid scintillation fluid Liquiscint from National Diagnostics USA (Atlanta, Ga., USA). Oxythiamine chloride was from Sigma-Aldrich (St. Louis, Mo., USA).

Incubation and Superfusion Media

The experiment required 6 solutions, as follows:

(A) Krebs-bicarbonate buffer (pH 7.4, 120 mmol/l NaCl, 4.75 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 2 mmol/l $CaCl_2$, 25 mmol/l $NaHCO_3$, 5 mmol/l glucose) was prepared by flushing 250 mmol/l $NaHCO_3$ with 100% CO_2 for 60 min before combination with the rest of the buffer. The buffer reservoir was bubbled with 95% O_2/5% CO_2 at 200 ml/min for 15 min before and then throughout the experiment.

(B) Calcium-free Krebs-bicarbonate buffer was prepared as for solution A, but 1 mmol/l EGTA (pH 7.4) replaced the $CaCl_2$.

(C) High-K^+ Krebs-bicarbonate normal calcium buffer was prepared as for solution A, but KCl was increased to 48.8 mmol/l and NaCl was decreased to 76 mmol/l to maintain isotonicity.

(D) Calcium-free, high-K^+ Krebs-bicarbonate buffer was prepared as for solution C, but 1 mmol/l EGTA replaced the $CaCl_2$.

(E) Oxythiamine-rich, high-K^+ Krebs-bicarbonate normal calcium buffer was prepared as for solution C, but 10^{-4} mol/l oxythiamine was added.

(F) Oxythiamine-rich, high-K^+, calcium-free Krebs-bicarbonate buffer was prepared as for solution E, but $CaCl_2$ was omitted and 1 mmol/l EGTA was added.

Slice Preparation and Superfusion Procedure

Hand-cut slices from rat cerebral cortex (0.3 mm; 1–3 mg) were prepared as described previously [17, 21]. Tissues were preincubated for 30 min in oxygenated low-potassium Krebs-bicarbonate buffer (solution A) at 37°C with 16 μCi of [^{3}H]choline to label acetylcholine stores. Previous research has shown that the tritium released is representative of acetylcholine release even in the absence of an inhibitor of acetylcholinesterase [13]. Slices were then transferred to 10 different superfusion chambers and washed out for 45 min with oxygenated Krebs-bicarbonate solution. Successive 10-min fractions were then collected from each chamber after superfusion with the appropriate solutions. After the experiments, tissues were sonicated in 1 ml of 0.2 mol/l perchloric acid. Where possible, 3 treated tissues or their respective controls were included in each experiment, with the exception of the tissue perfused with neither calcium nor oxythiamine (solution D), which was from a single chamber.

Potassium Stimulation Procedure

A two-by-two factorial design was used. The variables were oxythiamine and calcium. The procedure, a modification of that previously reported [16], consisted of two periods of superfusion with high (50 mmol/l) potassium (S1 and S2), which were preceded and succeeded by superfusion with low-potassium solutions. Superfusion in low potassium was necessary to establish basal efflux of tritium (see below), while 50 mmol/l potassium was necessary for the evoked release of [^{3}H]acetylcholine during S1 and S2. The specific protocol was as follows. All chambers were

Table 1. Oxythiamine and the potassium-stimulated release of acetylcholine from rat cortical slices

	Fractional release			
	– oxythiamine + calcium	+ oxythiamine + calcium	– oxythiamine – calcium	+ oxythiamine – calcium
S1	0.0665 ± 0.0069	0.0614 ± 0.0130	0.0904 ± 0.0151	0.0692 ± 0.0677
S2	0.0433 ± 0.0063	0.0647 ± 0.0065	0.0332 ± 0.0198	0.0105 ± 0.0036
S2/S1	0.6518 ± 0.0697	1.0456 ± 0.2267[a, b]	0.3546 ± 0.1557[a]	0.1679 ± 0.0742[b]

Hand-cut slices of rat cerebral cortex were preincubated with [^{3}H]choline and superfused as described in Materials and Methods. Release of [^{3}H]acetylcholine was evoked by two 3.5-min periods of superfusion (S1, S2) with 50 mmol/l K^+ Krebs-bicarbonate buffer with or without oxythiamine and with or without 2.0 mmol/l calcium-evoked release. Data represent the fraction of tissue tritium content in excess of basal levels that was released during the 20-min collection period preceding each high-potassium pulse and the final 10-min collection period following the second stimulation. Values are the means ± SEM of 7 or 8 mean experimental day values of control fractional release for each experiment. Significance was determined by one-way analysis of variance followed by the Tukey multiple comparison test. Values marked with the same superscript letters are significantly different ($p < 0.05$) from each other.

initially superfused for 20 min with solution A (basal release), followed by superfusion for 3.5 min with solution C (S1). There was then 20 min of superfusion with either solution A or solution B (basal release), followed by 3.5 min of superfusion with solutions C, D, E or F (S2), and then finally 20 min with solution A or B. Chambers superfused with solution A after S1 could only be superfused with solution C or E during S2, while those that had been superfused with solution B could only be superfused with either solution D or F. Oxythiamine 10^{-4} mol/l was introduced only during S2 and only to some chambers. Chambers designated as calcium-rich were continually superfused with calcium in the medium, while those designated as calcium-free were continually superfused with no calcium, but only after S1.

Calculations

The tritium content (disintegrations per minute) of each superfusion collection fraction or tissue homogenate was determined in 0.25-ml aliquots in a Beckman model LS 9000 liquid scintillation counter with quench correction by external standardization. Both basal release, which was the efflux of tritium in low-potassium conditions (mean tritium concentration of fractions 20 min immediately before onset of stimulation and the final 10-min fraction), and total stimulated release (tritium in superfusate during the 3.5-min superfusion with 50 mmol/l K^+ and the subsequent 10-min superfusion with low potassium) were determined. Net stimulated release was calculated by subtracting basal release from that during high-potassium stimulation. This represents evoked acetylcholine release rather than nonspecific leakage of tritium. To reduce tissue-to-tissue variance of acetylcholine release, the fractional stimulated release for each superfusion chamber sample was calculated by dividing the net stimulated release by the tritium content in each brain slice at the end of the experiment [17, 21]. The mean value and variance (SEM) for each experimental condition (+ oxythiamine, + calcium; – oxythiamine, + calcium; + oxythiamine, – calcium; – oxythiamine, – calcium) was calculated per experimental day. Eight such experiments were conducted, and the mean of each experimental condition and stimulation period (S1, S2) was calculated. Finally, to further control for within-group variance, the mean of each experimental day's S2/S1 ratios and their resultant group mean of mean S2/S1 ratios were computed. This was previously shown to be the most reliable measure of experimental effect [17].

Statistics

Release of [^{3}H]acetylcholine for treatment groups during S1 was compared with one-way analysis of variance during S1. Repeated-measures one-way analysis of variance for both stimulation periods (S1, S2) was also performed. Derived S2/S1 values for each experimental condition were compared by one-way analysis of variance, followed with the Tukey multiple comparison test. S2/S1 ratios for each experimental group were also compared by a two-way analysis of variance, followed by the post hoc Bonferroni method.

Results

There were no significant differences amongst treatment groups during S1 when conditions were identical [$F(3, 27) = 2.584$, $p = 0.0740$]. Basal release was similar for all treatment groups (0.02183 ± 0.0198). Repeated-measures analysis of variance failed to demonstrate a treatment effect for oxythiamine [$F(3, 18) = 0.2870$, $p = 0.8342$]. Expressing the data as an S2/S1 ratio, a well-established procedure in experiments of this type [10, 15], revealed a 60% greater efflux of [^{3}H]acetylcholine when oxythiamine was added to a calcium-rich medium (table 1). While this effect failed to achieve statistical significance, a significant effect was found [$F(3, 27) = 7.664$, $p = 0.0007$] for the oxythiamine, calcium-rich group rel-

ative to the group with oxythiamine but without calcium ($p < 0.05$) or the group with neither oxythiamine nor calcium ($p < 0.05$). No other group differences were found.

The above analysis suggests that enhanced acetylcholine efflux from potassium-stimulated rat brain tissue during exposure to oxythiamine requires the presence of calcium. To more fully explore this possibility, an additional analysis was performed. Using a two-way analysis of variance with oxythiamine and calcium as the independent variables, a significant main effect for calcium [$F(1, 27) = 121.09$, $p < 0.0001$] was shown. A main effect of oxythiamine just failed to reach significance [$F(1, 27) = 3.64$, $p = 0.0673$]. However, there was a significant interaction effect between oxythiamine and calcium [$F(1, 27) = 29.71$, $p < 0.0001$]. This supports the contention that oxythiamine augments cholinergic neurotransmission rather than nonspecific extrusion of tritium.

Discussion

These findings confirm and extend those of Hirsch and Gibson [17] of a possible neuromodulatory role for thiamine metabolic antagonists in the central nervous system contradistinct from their metabolic effects. Using an identical concentration but a different species (rat vs. mouse), we again demonstrated that oxythiamine augments the in vitro release of acetylcholine from mammalian brain. We have now shown that this release is calcium dependent. Together with previous studies that indicate that oxythiamine in vitro is not neurotoxic even with concentrations 10 times higher [22], our research supports the conclusion that this drug enhances cholinergic neurotransmission as opposed to some nonspecific effect like membrane perturbation.

We concur with Cooper and Pincus [7] that the augmentation of cholinergic neurotransmission by thiamine metabolic antagonists is independent of the well-documented inhibitory effects on neuronal metabolism, especially acetylcholine synthesis. The paradigms used in metabolic versus non-cofactor roles of thiamine are largely different. Most of the former studies involved administration of thiamine antimetabolites in vivo to deplete tissue of thiamine, before brains or other nervous tissue were excised from sacrificed animals. In contrast, research exploring a neurofacilitatory role for these drugs was primarily performed in vitro in isolated tissue. Even the anomalous in vitro study by Perri et al. [16], which found an inhibitory role on synaptic transmission in the superior cervical ganglion due to decreased acetylcholine synthesis, involved several hours of incubation with oxythiamine or pyrithiamine, rather than brief (minutes) pulsation. It is also important to note that the metabolic effects result in decreased acetylcholine while the actions on neurotransmission involve augmentation. In one recent study, after 2 weeks of daily injections, pyrithiamine-treated rats secreted less acetylcholine in situ from their amygdalae, but this was a consequence of thiamine deficiency with chronic drug exposure [23]. Lastly, the enhancement of the neurosecretion of monoamines like norepinephrine [17] and dopamine [18] by thiamine metabolic antagonists is incompatible with this cofactor metabolic role of carbohydrates and acetylcholine.

However, the mechanism by which oxythiamine augments neurotransmission is not clear. In trying to reconcile our finding with those of other researchers on the role of thiamine or its metabolites as a neuromodulator, we are confronted with differences in the specific drug or analogue used, concentrations of the drug or analogue, system preparation, neurotransmitter studied, presence or absence of external stimulation and method of determining effect. Confounding some of these findings is the apparent likelihood of numerous possible sites of action for thiamine [24] and possibly oxythiamine.

Considering oxythiamine specifically, Bettendorf et al. [20] found that in normal physiological buffer (i.e. with low external potassium), oxythiamine inhibited chloride uptake in rat brain vesicles. However, this was probably not at nerve endings, and the concentration they used was 10 times higher than what we used in the current study. The authors posited that blockage of chloride channels may favor repetitive firing of the action potential and consequently increase the release of acetylcholine. However, this is incompatible with the enhanced secretion of acetylcholine in our experimental condition, where release was already evoked with prolonged depolarization from high potassium. Indeed, some drugs (e.g. pyrithiamine, guanidine) may enhance calcium-mediated neurosecretion of some neurotransmitters, but only when the initial stimulus is intermittent (e.g. electric field stimulation) and not when the process is experimentally evoked by high potassium [17, 25].

Sodium channels and potassium channels have also been studied, for example on rat hippocampal slices, but not with oxythiamine [3, 10]. While high external potassium does promote voltage-dependent persistent sodium current in rat hippocampal slices [26], it appears

unlikely that thiamine or its derivatives significantly affect sodium channels [3]. Extracellular thiamine has an excitatory effect on rat hippocampal slices with increased repetitive afterdischarges [10] but usually with concentrations one order of magnitude greater than that of oxythiamine used in our experiment. Also, this effect is not presynaptic and thus would not support a role in neurotransmission. Thiamine tetrahydrofurfuryl, a highly permeable derivative of thiamine, inhibited intracellular delayed rectifier potassium channels in isolated rat fetal cortex as did internally perfused thiamine [19]. Both are converted to thiamine-phosphorylated esters that prolong the action potential. However, several lines of reasoning do not support this mechanism as an explanation for the efficacy of oxythiamine in facilitating the release of acetylcholine in our system. Firstly, oxythiamine does not easily penetrate the nerve membrane [8]. Secondly, prolongation of the action potential does not necessarily enhance cholinergic neurotransmission [27]. Thirdly, as discussed above, prolonged depolarization of neurons by high potassium would not be affected by such a process.

Lastly, a more direct effect on calcium mobilization needs to be considered. Dyatlov [15] demonstrated that thiamine enhances not only calcium-dependent release of acetylcholine but also the reparative process responsible for maintenance of release (e.g. resynthesis of synaptic vesicles) that presumptively is also calcium dependent in frog neuromuscular synapses [15]. However, this was seen only after depletion of acetylcholine by α-latrotoxin was induced. Also, pyrithiamine inhibited this effect (oxythiamine was not studied). As stated earlier, we previously showed that pyrithiamine does enhance the evoked release of acetylcholine from mammalian brain, so it is unknown if the effect of thiamine on synaptic vesicles as reported by Dyatlov [15] is species specific or neuronal type specific.

Thiamine triphosphate, either as the intracellular metabolite of thiamine or directly as a probe, has also been investigated. In the latter capacity, it did evoke calcium-dependent release of the neurotransmitter dopamine, but this effect was not attenuated by N-type calcium channel blockers alone [18]. The authors concluded that a non-N-type calcium channel may be involved in the evoked release of dopamine. In fact, both N- and Q-type calcium channels are involved in the potassium-evoked release of [^{3}H]acetylcholine from rat hippocampal slices [28]. There are other calcium channels present that may contribute in a lesser way to this release. It may be that oxythiamine, which is unable to be taken up by the neuron and in all probability exerts its effect extracellularly, activates one or more of these major or minor channels to augment the potassium-stimulated release of acetylcholine. Whether this explains a role for thiamine, one of its endogenous metabolites or other thiamine metabolic inhibitors in the neurotransmission of acetylcholine or of some other neurotransmitter remains an open question. Hopefully, future research may be elucidative.

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