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SHORT COMMUNICATIONS

Thiamin antagonists and the release of acetylcholine and norepinephrine from brain slices

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The biochemical bases of the cellular and behavioral changes that accompany thiamin deficiency have been studied extensively [1–4]. Both *in vivo* and *in vitro* experiments have been facilitated by the pharmacological use of the thiamin antagonists oxythiamin and pyrithiamin. Only the latter acts on the central nervous system *in vivo* [1–4]. In addition to their antagonism of the role of thiamin as a cofactor in carbohydrate metabolism, these compounds act directly on neuronal conduction and release. Pyrithiamin, but not oxythiamin, in the millimolar range increases the amplitude and prolongs the duration of the action potential of non-myelinated mammalian nerve fibers [5] and lengthens the action potential duration at single nodes of Ranvier [6]. At concentrations one order of magnitude lower, oxythiamin enhances the release of acetylcholine from electroplaques of *Torpedo marmorata*, but pyrithiamin does not [7, 8]. The latter suggests that the two thiamin antimetabolites alter the neuronal stimulation–secretion coupling process in different manners. Since this mechanism may vary in cholinergic and noradrenergic terminals of mammalian brain ([9]; J. A. Hirsch and G. E. Gibson, *Neurochem. Res.*, in press), the effects of these compounds on norepinephrine and acetylcholine release were examined to determine the specificity of thiamin antagonism on mammalian neurotransmitter release.

Materials and methods

Materials. Male Wistar rats (150–200 g) or CD-1 mice (18–25 g) were from the Charles River Breeding Laboratories (Wilmington, MA). DL-[7-^{3}H-(N)]Norepinephrine hydrochloride (10.4 Ci/mmole), [*methyl*-^{3}H]choline chloride (80 Ci/mmole) and liquid scintillation fluid Formula 963 were from the New England Nuclear Corp. (Boston, MA). Pyrithiamin hydrobromide and oxythiamin chloride were from the Sigma Chemical Co. (St. Louis, MO).

Incubation and superfusion media. The following solutions were used:

(A) Krebs–bicarbonate buffer (pH 7.4; 120 mM NaCl, 4.75 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.6 to 2.0 mM $CaCl_2$, 25 mM $NaHCO_3$, 5 mM glucose) was prepared by flushing 250 mM $NaHCO_3$ 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) High-K^+ Krebs–bicarbonate buffer was prepared as in (A), but KCl was increased to 48.8 mM and NaCl was decreased to 76 mM to maintain isotonicity.

Slice preparation and superfusion procedure. Mouse brain prisms and hand-cut slices from rat cerebral cortex were prepared as described previously ([9, 10]; J. A. Hirsch and G. E. Gibson, *Neurochem. Res.*, in press). Tissues were preincubated for 30 min in low-potassium Krebs–bicarbonate buffer at 37° with 8 μCi of [^{3}H]choline chloride (0.1 μM) or 2 μCi of [^{3}H]norepinephrine (0.1 μM) to label acetylcholine [11] or norepinephrine stores [12, 13] respectively. Others have shown that the tritium released from similarly treated tissues is representative of acetylcholine and norepinephrine release, even in the absence of an inhibitor of acetylcholinesterase [11] or monoamine oxidase or norepinephrine reuptake inhibitor [12, 13]. This assumption also appears to have been true in these experiments, since the drugs acted only on depolarization-stimulated release (see Results). To account for the compartmentation of the released norepinephrine and acetylcholine, their concentrations must be determined. The slices were then transferred to chambers and superfused (0.35 ml/min) with a low-potassium Krebs–bicarbonate buffer at 37° for 30 min. Successive 5-min (potassium-stimulation experiments; Table 1) or 6-min (electrical-stimulation experiments; Table 2) samples were collected. After the experiments, tissues were sonicated in 1 ml of 0.2 M perchloric acid. Three to five treated tissues and their respective controls were included in each experiment.

Potassium-stimulated release. After four samples were collected during superfusion with low-K^+ Krebs solution to determine the basal tritium efflux, prisms from mouse brain were superfused for 10 min with 50 mM K^+ Krebs solution with or without the thiamin antagonists. Tissues were then resuperfused with low-K^+ Krebs solution without the drugs.

Electrically-induced release. After 2–3 samples were collected to determine spontaneous release with low-K^+ buffer, hand-cut slices of rat cerebral cortex were field stimulated (S1) for 1 min with biphasic rectangular electrical pulses with a 10 V peak-potential, 0.8 msec duration, and 25 pulses/sec. Six minutes after the first stimulation (S1), tissues were superfused for 30 min with the same medium with or without 1.2×10^{-4} or 5×10^{-3} M pyrithiamin, and then stimulated a second time (S2).

Spontaneous release of neurotransmitters with oxythiamin. Four samples were collected during superfusion with low-K^+ Krebs solution. Slices were superfused with the same medium with or without 10^{-4} M oxythiamin for 10 min and then superfused again without the drug.

Calculations and statistics. The tritium content of each sample or tissue homogenate was determined on 0.25-ml aliquots in a Beckman model LS 9000 liquid-scintillation counter with quench correction by external standardization. The fractional release of neurotransmitters (i.e. the fraction of tissue tritium content that was released by stimulation) was calculated. In the potassium-stimulation experiments, net-stimulated release was determined by subtraction of the estimated basal efflux (i.e. the mean of the samples immediately before and 20 min after the onset of stimulation) from total stimulated tritium efflux (i.e. efflux during the 10-min superfusion with 50 mM K^+ and the subsequent 10-min superfusion with low-K^+ Krebs buffer). The percent of control fractional release was calculated for each experimental day. The results are the mean ± S.E.M. of samples from at least two experimental days.

Net-stimulated release during electrical stimulation was determined by subtraction of the estimated basal release (i.e. the average of samples immediately before and after stimulation) from the samples during stimulation. The S2/S1 fractional release ratio for each tissue was determined before calculation of the mean ± S.E.M. Although S1 and S2 varied between samples, the S2/S1 ratio was quite consistent.

The effect of oxythiamin on spontaneous release was

calculated by the measurement of total tritium efflux during the 10-min exposure to drug and the following 10 min in drug-free solution; values are expressed as fractional release of tritium. Efflux during a comparable period was used as control.

Results

Both oxythiamin and pyrithiamin enhanced acetylcholine release, but at different concentrations (Table 1). Oxythiamin (1×10^{-4} M) increased evoked release by 37%. Since superfusion with a similar concentration for the same period of time in a low potassium medium did not augment basal release [control fractional release, 0.104 ± 0.001 (N = 5) vs oxythiamin, 0.092 ± 0.005 (N = 4); $P > 0.05$], the increased release was most likely not due to a general breakdown of the tissue. Although an equivalent concentration (1.2×10^{-4} M) of pyrithiamin did not alter acetylcholine release, a higher concentration (5×10^{-3} M) augmented release to about the same extent (43%) as oxythiamin. Neither oxythiamin nor pyrithiamin changed the potassium-stimulated release of [^{3}H]norepinephrine (Table 1).

To determine whether this negative effect on norepinephrine release was due to the mode of stimulation, the effects of these antagonists on electrical stimulation were also tested (Table 2). The effects of pharmacological manipulation on electrical and potassium stimulation can vary [12, 14]. Although a lower pyrithiamin concentration (1.2×10^{-4} M) was without effect, a higher one (5×10^{-3} M) augmented the S2/S1 ratio by 38%.

Discussion

The pharmacological efficacies of oxythiamin and pyrithiamin differed, which confirms and extends the results of Eder *et al.* [7, 8]. Thus, with a different method of stimulation (potassium vs electrical) and a different tissue and species (mouse brain vs *Torpedo* electroplaques), similar concentrations of oxythiamin increased acetylcholine release, whereas equivalent concentrations of pyrithiamin did not. Since pyrithiamin is taken up by neurons more effectively than oxythiamin [5], an insufficient intracellular pyrithiamin concentration probably does not explain the ineffectiveness of pyrithiamin at 10^{-4} M, a concentration at which oxythiamin stimulated release. The differential effects of these two compounds may be a useful probe for the study of cholinergic release mechanisms.

These antagonists also distinguish between the release mechanisms of acetylcholine and norepinephrine, since the potassium-stimulated release of the latter was unaltered. A difference in stimulus–secretion coupling is also apparent

Table 1. Thiamin antagonists and the potassium-stimulated release of acetylcholine and norepinephrine from mouse brain prisms*

	[^{3}H]Acetylcholine		[^{3}H]Norepinephrine	
	Control	Drug	Control	Drug
Oxythiamin				
1×10^{-4} M	100 ± 9 (12)	137 ± 12† (14)	100 ± 5 (14)	113 ± 5 (15)
Pyrithiamin				
1.2×10^{-4} M	100 ± 8 (8)	103 ± 11 (8)	100 ± 2 (22)	108 ± 12 (21)
5×10^{-3} M	100 ± 7 (8)	143 ± 11‡ (10)	100 ± 10 (8)	88 ± 5 (6)

* Mouse brain prisms were preincubated and superfused as described in Materials and Methods. A 10-min superfusion with 50 mM K^+ Krebs–bicarbonate buffer with or without oxythiamin or pyrithiamin evoked release. Values are the means ± S.E.M. of the percentage of control fractional release of tritium efflux for each experiment. The number of samples for each treatment is in parentheses. Control fractional releases for 20-min collection periods were 0.3120 ± 0.0214 (N = 28) for acetylcholine and 0.4821 ± 0.0184 (N = 44) for norepinephrine respectively.

† Significantly different ($P < 0.05$) from control by Student's *t*-test.

‡ Significantly different ($P < 0.01$) from control by Student's *t*-test.

Table 2. Pyrithiamin and electrically-stimulated release of [^{3}H]norepinephrine from rat cortical slices*

		Fractional release	
Concentration		Controls	Pyrithiamin-treated
1.2×10^{-4} M	S1	0.0668 ± 0.0143	0.0916 ± 0.0081
	S2	0.0486 ± 0.0115	0.0738 ± 0.0097
	S2/S1	0.727 ± 0.048	0.791 ± 0.052
5×10^{-3} M	S1	0.0461 ± 0.0071	0.0495 ± 0.0121
	S2	0.0293 ± 0.0041	0.0478 ± 0.0113
	S2/S1	0.671 ± 0.085	0.932 ± 0.077†

* Hand-cut slices of rat cerebral cortex were preincubated with [^{3}H] norepinephrine and superfused as described in Materials and Methods. Release was evoked by two 1-min field stimulations (S1 and S2) with biphasic electrical pulses (10 V, 25 pulses/sec, 0.8 msec duration). Pyrithiamin was superfused after S1 for 30 min before S2. Data are the fraction of tissue tritium content in excess of basal levels that was released during a 6-min collection. Values are the means ± S.E.M. of seven to eight samples.

† Significantly different ($P < 0.05$) from controls by Student's *t*-test.

during hypoxia, which impairs, stimulates or does not alter the release of acetylcholine, glutamate or norepinephrine respectively ([9]; J. A. Hirsch and G. E. Gibson, *Neuchem. Res.*, in press). The ineffectiveness of the thiamin antagonists on the potassium-stimulated release of norepinephrine at either concentration suggests that thiamin is integrally involved in acetylcholine release and metabolism [15].

These *in vitro* responses to thiamin antagonists contrast with those observed in chronically treated animals. Thus, acetylcholine release decreases in superior cervical ganglia of thiamin-deprived rats [16], and whole brain acetylcholine synthesis *in vivo* declines [17]. Similarly, the lack of an effect on potassium-stimulated norepinephrine release is not directly comparable to the increase in catecholamine levels in brain cortex during thiamin deficiency [18]. Whether these *in vitro* pharmacological effects are distinct from their *in vivo* actions or if these apparent differences are a matter of acute versus chronic treatment remains unclear.

The elevation of electrically-stimulated norepinephrine release by pyrithiamin was unexpected. The increase in release may reflect the method of stimulation (i.e. electrical vs potassium), the duration of exposure of the tissue to the drug (i.e. treatment before or only during stimulation), or the tissue preparation (rat cortical slices vs whole brain prisms from mice). Other drugs also alter the electrically- or potassium-induced stimulation of the calcium-dependent norepinephrine release differently [12, 14]. At higher concentrations, pyrithiamin may have more general effects such as inhibition of norepinephrine reuptake, which would enhance the electrically-stimulated, but not potassium-stimulated, release of norephinephrine from brain slices [12]. Nevertheless, pyrithiamin may be an effective tool to distinguish between release mechanisms during electrical and potassium stimulation.

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Effect of an inhibitor of nucleoside transport on the disposition of uridine in mice*

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p-Nitrobenzylmercaptopurine ribonucleoside (NBMPR) is a potent inhibitor of nucleoside transport in various animal cells *in vitro* [1] and alters the disposition and toxicology of some nucleoside analogs *in vivo* [2, 3]. Kolassa and Paterson [4] have shown that prior treatment of mice with NBMPR-P, the 5′-monophosphate of NBMPR, effectively blocks the uptake of cytidine into mouse liver during perfusion *in situ*. NBMPR-P has been employed as a readily soluble, prodrug form of NBMPR from which the latter is released by phosphohydrolase activity; NBMPR-P *per se* appears to have little activity as an inhibitor of nucleoside transport.† On the basis of these results, it seemed possible that NBMPR-P could be used to prevent the utilization of circulating uridine by various tissues and tumors. The current report examines the effect of NBMPR-P on the metabolism of intravenously injected [^{3}H]uridine.

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† P. O. J. Ogbunude, W. P. Gati and A. R. P. Paterson, manuscript submitted for publication.

Materials and methods

NBMPR-P was prepared as the disodium salt [3] by the laboratory of the Yamasa Shoyu Co., Chosi, Japan. [^{3}H]Uridine, 25 Ci/mmole, was purchased from Moravek Biochemicals, Brea, CA, and phosphodiesterase I, type VII, from the Sigma Chemical Co., St. Louis, MO.

To measure the distribution of radioactivity after injection of [^{3}H]uridine, tissues were rapidly removed and frozen in liquid nitrogen until processed further. The remaining organs along with the skin were also frozen and