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EFFECT OF PYRITHIAMINE TREATMENT ON POTASSIUM ION FLUXES IN RAT CORTICAL SLICES

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The effect of thiamine deficiency on energy-requiring processes in brain tissue was studied by comparing cortical slices prepared from control and pyrithiamine-treated rats. Veratridine was used to stimulate energy metabolism by opening voltage-sensitive sodium channels resulting in enhanced Na^+/K^+ pumping; subsequent tetrodotoxin addition closed the sodium channels. Pyrithiamine-treated slices showed both lower basal and veratridine-stimulated respiration rates compared to control slices. K^+ was released from the tissue upon addition of veratridine and was taken up again upon addition of tetrodotoxin. The movement of K^+ was monitored directly with a K^+-sensitive electrode as well as by measuring the rubidium diffusion potential. There was no difference between control and pyrithiamine-treated slices in K^+ fluxes in response to veratridine and tetrodotoxin. The extent of reuptake of K^+ upon tetrodotoxin addition was inversely related to the extracellular Ca^{2+} concentration and to the incubation temperature. Veratridine resulted in a marked decrease in tissue levels of ATP and creatine phosphate; these levels remained quite low upon tetrodotoxin addition. Despite the different respiration rates, control and pyrithiamine-treated slices showed the same ATP and creatine phosphate levels in response to veratridine and tetrodotoxin.

Introduction

Although the enzymic cofactor role of thiamine has been known for decades, the causal relationship between the vitamin deficiency and the specific neurologic symptoms has remained obscure [1]. Several investigators have proposed that thiamine in fact has a second distinct role by functioning in the conduction or transmission of nerve impulses [2,3], but unequivocal proof for this has not been obtained. More recently, Matsuda and Cooper [4] have reported that the thiamine antagonist pyrithiamine inhibits the brain-specific form of ($Na^+ + K^+$)-ATPase in cell-free preparations. In the studies reported here, we examined whether there is a link between maintenance of ionic gradients and thiamine function and whether this link might be the basis for subsequent neuropathologic changes in thiamine deficiency.

Because the neurologic symptoms induced by pyrithiamine administration are more pronounced than those produced by thiamine deprivation [5] and because of the report by Matsuda and Cooper, pyrithiamine-treated rats were used for our studies. If altered ionic balances do occur in thiamine-deficient cells, they could be related to the decreased maximal energy production available [6], since the Na^+/K^+ pump is a major consumer of energy in the nervous system [7,8]. Based solely upon electron micrographic observations of cellular swelling in thiamine-deprived brain, Robertson et al. [9] first proposed such a link between thiamine-dependent energy production and ($Na^+ + K^+$)-ATPase activity. Altered Na^+/K^+ bal-

ances could also be caused by direct pyrithiamine-inhibition of $(Na^+ + K^+)$-ATPase activity as proposed by Matsuda and Cooper [4].

To test these hypotheses we compared energy-dependent responses of normal and of pyrithiamine-treated rat cortical slices when the energy production capacity was stressed by accelerated Na^+/K^+ transport across the plasma membrane and when the energy demands were subsequently reduced. The alkaloid veratridine was used to open sodium channels, resulting in increased extracellular Na^+ influx, subsequent efflux of K^+ through potassium channels, and enhanced $(Na^+ + K^+)$-ATPase activity [10,11]. Tetrodotoxin was then added to reclose the sodium channels; the Na^+/K^+ pump used available ATP reserves to reestablish resting cationic balances [12,13]. If maximal energy production were seriously compromised in pyrithiamine-treated tissue or if pyrithiamine were to directly inhibit $(Na^+ + K^+)$-ATPase, then the rate or extent of K^+ reuptake after tetrodotoxin addition would be expected to be lower than in control tissue.

Materials and Methods

Animals. Female Sprague-Dawley rats (150–200 g) were housed one or two to a cage. Control animals were fed standard Purina rat chow; we have observed over several years that there are no differences in weight gain and in diverse biochemical parameters between rats fed standard rat chow and those fed thiamine-deficient diet supplemented with thiamine. Pyrithiamine-treated animals received thiamine-deficient diet as well as 20 mg/l pyrithiamine in the drinking water; the usual severe neurologic symptoms commenced on the 6th to 8th day, with death following 1 or 2 days later. The pyrithiamine-treated rats were killed just prior to anticipated death.

Brain slices. The animals were guillotined, the cerebral cortex removed, and $0.2 \times 0.2 \times 1$ mm slices prepared with a McIlwain tissue sectioner (Sorvall, Inc.). The slices were weighed and placed in 10 volumes of Ca^{2+}-free Krebs-Ringer buffer containing 120 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM $NaHCO_3$, 1.3 mM Na_2HPO_4, 1.2 mM $MgCl_2$, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes), 30 μM EDTA, pH 7.4 after saturation with 95% air/5% CO_2. The suspended slices were separated into aliquots and $CaCl_2$ added to give the desired concentration. The slices could be maintained at 4 °C for 3 h without change in the parameters being measured.

The methods described by Pastuzko et al. [13] were used for the isolation and incubation of rat brain synaptosomes.

Oxygen consumption. Oxygen uptake was monitored with a Clarke dissolved oxygen electrode attached to an MSE Spectro-Plus MK1 A (MSE Scientific Instruments, Sussex, England). The electrode was calibrated with sodium dithionate and oxygen-saturated buffers using a value of 195 nmol O_2/ml at 37 °C. Tissue containing 1–4 mg protein was added to the electrode chamber and Krebs-Ringer buffer added to give a final volume of 1.5–2.0 ml. Subsequent additions were made with a Hamilton syringe directly into the electrode chamber. All incubations were performed at 37 °C.

Potassium fluxes. The miniature STAT potassium electrode (Ionetics, Inc., Costa Mesa, CA) was used to monitor potassium uptake and efflux. The electrodes were calibrated with standard KCl aliquots added to 120 mM NaCl; the scale was periodically checked by adding known aliquots of KCl to a tissue suspension at the end of the experiment. Tissue slices containing 25–50 mg protein were suspended in 2 ml final volume of Krebs-Ringer buffer; the temperature was maintained at 37 °C during the experiments unless otherwise noted.

Rubidium potential. The rubidium diffusion potential at 37 °C is defined as

$$E_{Rb} = 61 \log(Rb_i^+/Rb_o^+)$$

where Rb_i^+ and Rb_o^+ are the intracellular and extracellular Rb^+ concentrations, respectively. E_{Rb} for cortical slices was determined by incubating slices in Krebs-Ringer buffer containing 0.2 μCi ^3H_2O, 0.03 μCi [^{14}C]inulin (2.4 mCi/g), and 7 nCi ^{86}RbCl per ml. At the times shown in Fig. 2, a 0.2 ml aliquot was removed with a wide-bore pipette tip from the well-stirred suspension and layered over 15 μl Dow Corning FS-1265 fluorosilicone fluid and 80 μl of 56% (v/v) dioctylphthalate/44% Dow Corning 550 silicone fluid in a 400 μl tube. The samples were immediately centrifuged for 30 s

at 9000 × *g* in a Beckman microfuge. A 50 µl sample of the supernatant (upper layer) was removed and added to 0.45 ml of 80 ml/l Triton X-100. The tissue pellet, suspended between the two oil layers, was cut out using a guillotine and placed in 0.5 ml of 80 ml/l Triton X-100. After incubating the samples overnight at room temperature, radioactivity was determined by mixing the samples with the scintillation cocktail Aquasure (New England Nuclear) and counting in a Beckman LS 9000 liquid scintillation system. Disintegrations per min for each isotope were calculated from the counts per min in each of three channels and from the efficiencies of each isotope for those channels.

ATP and creatine phosphate. Cortex slices containing approx. 80 mg protein were suspended in 8 ml of Krebs-Ringer buffer at 37°C under 95% air/5% CO_2. At the times indicated, 1.0 ml aliquots were removed and centrifuged for 1 min at 800 × *g*. To the pellet was added 0.5 ml of 120 g/l trichloroacetic acid; the mixture was homogenized at 0°C with a Potter-Elvehjem homogenizer and centrifuged 20 s at 9000 × *g* in a Beckman microfuge. The pellet was saved for protein determination. The supernatant was extracted three times with a Freon/alamine (5 : 1, v/v) mixture at 0°C in order to remove trichloroacetic acid via ion pairing [14]. The final aqueous layer was stored at −80°C.

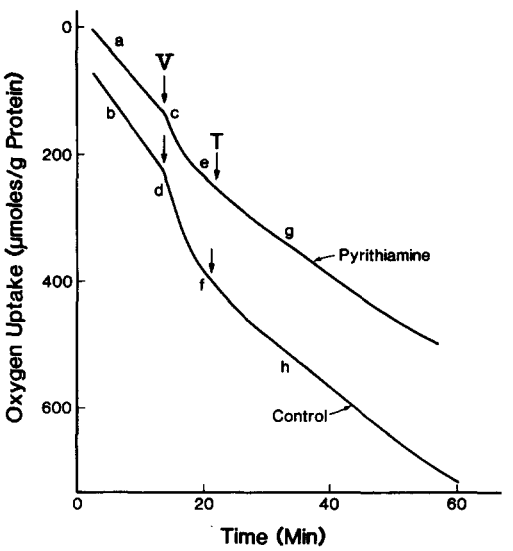

Fig. 1. Effect of veratridine and of tetrodotoxin on oxygen consumption by rat cortical slices suspended in Krebs-Ringer buffer (2.4 mM Ca^{2+}) at 37°C. The tracings are representative oxygen electrode measurements for slices prepared from control (lower tracing) and pyrithiamine-treated (upper) rats. At the time indicated by the first arrow on the left (V), veratridine was added to a final concentration of 15 µM. At the second arrow (T), tetrodotoxin was added to 3 µM. The average rates of oxygen consumption were calculated from several experiments for the points indicated by letters. These rates in µmol O_2/min per g tissue protein ± S.E. (number of experiments, *n*, in parentheses) were: a, 12.1 ± 1.2 (5); b, 15.0 ± 0.5 (9); c, 17.0 ± 1.2 (5); d, 26.6 ± 1.2 (6); e, 8.6 ± 0.4 (4); f, 15.9 ± 2.0 (6); g, 8.6 ± 1.3 (4); h, 8.3 ± 1.0 (6).

Protein determination. Protein was measured by the standard method of Lowry et al. [16] using bovine serum albumin as standard.

Materials. All biochemicals were obtained from Sigma Chemical Co. and radiochemicals from New England Nuclear. The thiamine-deficient diet was obtained from ICN Pharmaceuticals, Inc. The mixture of the fluorocarbon Freon and alamine (tricapryl tertiary amine from General Mills) was kindly supplied by Dr. Nancy Magnuson of the Department of Veterinary Microbiology and Pathology.

Results

The effect of pyrithiamine treatment on the rate of oxygen consumption by rat cortical slices in Krebs-Ringer buffer (2.4 mM Ca^{2+}) is shown in Fig. 1. The basal respiration rate for slices prepared from pyrithiamine-treated rats was about 20 percent less than for those from control animals. Addition of veratridine to control and pyrithiamine-treated slices resulted in enhanced oxygen consumption due to accelerated pumping of Na^+ and K^+. This rate was close to the maximal respiratory capacity since addition of the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) at a concentration of 3 µM gave about the same respiratory rate as did veratridine addition. The initial veratridine-accelerated rate was 36 percent lower for the pyrithiamine-treated slices compared to control slices; this rate declined more rapidly with time in the pyrithiamine-treated tissue. Subsequent

closing of the sodium channels with tetrodotoxin lowered the rates of oxygen consumption for both tissue preparations. At this point there was no difference in the rate of oxygen uptake between control and pyrithiamine-treated brain slices.

Since a large fraction of ATP production in nervous tissue is used to support Na^+/K^+ transport [7,8], either a decrease in maximal energy production or an inhibition of $(Na^+ + K^+)$-ATPase might result in altered K^+ uptake. In an approach similar to studies with synaptosomes [13], veratridine plus tetrodotoxin were used to study the release and uptake of K^+ in cortical tissue slices from normal and pyrithiamine-treated animals. A K^+-sensitive electrode monitored external K^+ concentration. Tissue slices were suspended in Krebs-Ringer buffer (5 mM K^+) and the baseline response of the K^+ electrode recorded. Veratridine was added, resulting in efflux of cellular K^+ and consequent rise in external K^+. After a new baseline was established (about 3 min), tetrodotoxin could be added to close the sodium channels and stimulate K^+ uptake via the $(Na^+ + K^+)$-ATPase pump. The role of the $(Na^+ + K^+)$-ATPase in K^+ reuptake was confirmed by the inhibition of the reuptake by: ouabain, an inhibitor of the ATPase; iodoacetate, an inhibitor of glycolysis and other metabolic pathways; rotenone + oligomycin, inhibitors together of mitochondrial ATP production. Varying concentrations of these inhibitors were added just prior to tetrodotoxin addition and the inhibition was dose-dependent for all three inhibitors. Complete inhibition of K^+ reuptake was attained with 50 μM ouabain, 0.1 mM iodoacetate, or 4 μM rotenone/4 $\mu g \cdot ml^{-1}$ oligomycin.

As shown in Fig. 2, there was no significant difference in the rate or extent of K^+ uptake in normal or pyrithiamine-treated cells under any of the experimental conditions tested. In the presence of 2.4 mM $CaCl_2$, only about 30 percent of the K^+ released from cells when sodium channels were activated with veratridine was taken up upon addition of tetrodotoxin; in 0.24 mM $CaCl_2$, most of the K^+ was taken up again. Incubation of either control or pyrithiamine-treated slices with veratridine for 20 min, instead of 5 min, decreased the tetrodotoxin-stimulated K^+ reuptake only slightly.

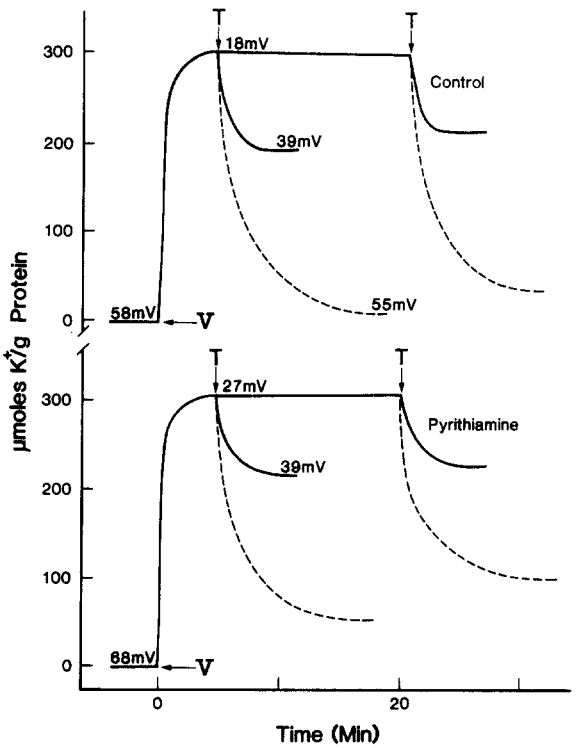

Fig. 2. Time-course of potassium influx into or efflux from rat cortical slices suspended in Krebs-Ringer buffer. At the first arrow (V), 15 μM veratridine was added; at the next arrow (T), 3 μM tetrodotoxin was added after either 5 or 20 min. Upper tracings, control slices; lower tracings, pyrithiamine-treated slices. ———, Krebs-Ringer buffer containing 2.4 mM $CaCl_2$; - - - - - -, Krebs-Ringer buffer containing 0.24 mM $CaCl_2$. The Ca^{2+} concentration had no effect on the release of K^+ by veratridine. The numbers (mV) indicate the E_{Rb} determined at the times indicated by the measurement of $^{86}Rb^+$ distribution.

Another experimental approach confirmed the results with the K^+ electrode. The rubidium potential, E_{Rb}, was estimated by monitoring $^{86}Rb^+$ distribution in the tissue and in the medium. Since the distribution of Rb^+ and K^+ in cells are similar, E_{Rb} is close to or equal to the potassium potential, E_K [10]. Other experiments in our laboratory have shown that E_{Rb} in cortical slices is a valid measure of E_K and is also quite close to the membrane potential. Samples were removed at the times indicated in Fig. 2 for E_{Rb} measurement. Preliminary experiments showed that the radiochemicals had reached equilibrium distribution by the time the aliquots were removed. The E_{Rb} values paralleled release or uptake of K^+.

When K^+ efflux was induced in normal tissue, E_{Rb} decreased greatly (58 to 18 mV); closing of the sodium channels resulted in almost complete recovery of the resting-tissue value in 0.24 mM Ca^{2+} (to 55 mV) and a lesser recovery in 2.4 mM Ca^{2+} (to 39 mV). Similar changes in E_{Rb} values were observed with the pyrithiamine-treated tissue in 2.4 mM Ca^{2+}; measurements were not made in 0.24 mM Ca^{2+}. The higher E_{Rb} values for the pyrithiamine-treated tissue compared to control tissue were due to variability of the preparations and not to an effect of pyrithiamine, since such variability was common from different rat preparations and other experiments showed no difference in the resting E_{Rb} for control and pyrithiamine-treated slices.

Calculation of the changes in tissue K^+ concentrations from either the potassium electrode or E_{Rb} data in Fig. 2 gave similar results. A value of 7.5 μl cellular H_2O/mg tissue protein was used for the former calculations, and it was assumed that $E_{Rb} = E_K$ for the latter calculations. With control tissue, addition of veratridine resulted in a change in intracellular K^+ concentration of 42 mM as calculated from the potassium electrode measurements and 41 mM as calculated from E_{Rb}; addition of tetrodotoxin in 0.24 mM Ca^{2+} caused 36 mM and 40 mM change, respectively; and tetrodotoxin in 2.4 mM Ca^{2+} caused 15 mM change as calculated by either method.

TABLE I

CALCIUM DEPENDENCE OF TETRODOTOXIN-INDUCED REUPTAKE OF K^+ IN NORMAL CORTICAL SLICES

The experimental conditions are the same as in Fig. 2, except that the Ca^{2+} concentration was varied. The time of incubation with veratridine was 5 min. Values are means ± S.E. with the number of experiments, n, in parentheses.

Ca^{2+} concn. (mM)	Reuptake of K^+ (percent [a])
0.024	100 ± 2 (3)
0.24	105 ± 3 (5)
1.2	41 ± 2 (3)
2.4	32 ± 2 (9)
5	27 (1)
10	19 (1)

[a] (K^+ taken up upon tetrodotoxin addition/K^+ released upon veratridine addition) × 100.

The relatively low percentage reuptake of K^+ in 2.4 mM Ca^{2+} after tetrodotoxin addition was surprising, since Pastuszko et al. [13] had reported complete reuptake with the synaptosomal system. We observed that the reuptake for both pyrithiamine-treated (Fig. 2) and normal (Fig. 2 and Table I) slices was inversely related to the external Ca^{2+} concentrations. However, this was still not consistent with the results of Pastuszko et al., since they used 2.5 mM Ca^{2+} concentration. We also checked the effect of temperature, because Pastuszko et al. incubated the synaptosomes at 25°C. We did observe that temperature was a factor, since the percentage reuptake for normal slices in 2.4 mM Ca^{2+} was 32% at 37°C, 46% at 28°, and 58% at 22°C. Temperatures lower than 22° affected the release of K^+ by veratridine. Using the isolation and incubation methods described by Pastuszko et al., we observed that 100% K^+ reuptake occurred with a rat brain synaptosomal preparation in 2.5 mM Ca^{2+} at 22°C. When the incubation temperature was raised to 28°C, the K^+ reuptake was 76%; at 37°C the reuptake was only 42%. Lowering the Ca^{2+} concentration increased K^+ reuptake by the synaptosomes; at 2.5 mM and 0.63 mM Ca^{2+}, the reuptake at 37°C was 42 and 65%, respectively, and at 28°C it was 76 and 100%. Thus, for both the synaptosomal preparation of Pastuszko et al. and the tissue slice

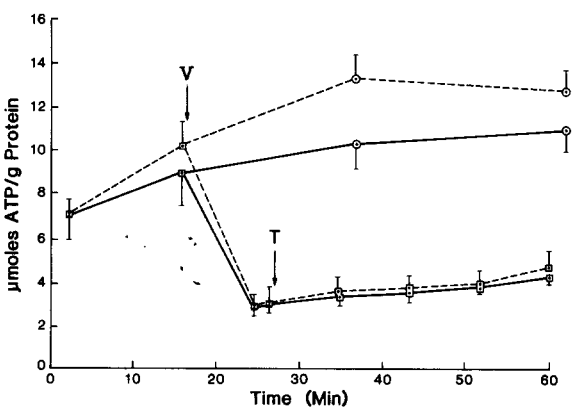

Fig. 3. ATP levels in cortical slices suspended in Krebs-Ringer buffer (2.4 mM $CaCl_2$). At the first arrow (V), 15 μM veratridine was added; at the second arrow (T), 3 μM tetrodotoxin was added. ———, normal slices; - - - - - -, pyrithiamine-treated slices. □, veratridine and tetrodotoxin were added at the times indicated; ○, neither veratridine nor tetrodotoxin was added. The standard errors are shown by the bars.

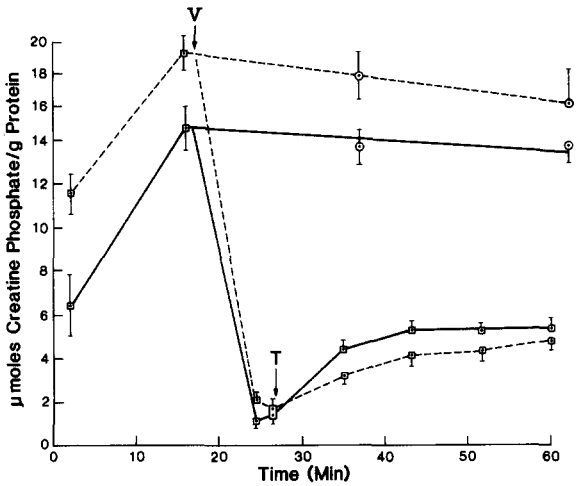

Fig. 4. Creatine phosphate levels in cortical slices. For details see the legend to Fig. 3.

system in this report, both the Ca^{2+} concentration and temperature affected the extent of cation fluxes.

The levels of ATP and of creatine phosphate in control and pyrithiamine-treated brain slices were measured before and after additions of veratridine or tetrodotoxin (Figs. 3 and 4). During the 15-min preincubation before veratridine addition, the levels of creatine phosphate rose, most probably due to metabolic adaptation of the tissue slices to the new environment. The higher levels of creatine phosphate in the thiamine-deficient slices compared to control slices were consistent with the higher levels reported in whole brain of pyrithiamine-treated mice [17]. Veratridine resulted in a dramatic decrease in both creatine phosphate and ATP in response to the energy demands for ion pumping. After reclosing the sodium channels with tetrodotoxin, ATP levels remained low and creatine phosphate levels showed a partial rebound. In slices that were not stimulated with veratridine, both ATP and creatine phosphate remained at stable high levels. There were no significant differences between control and pyrithiamine-treated slices in response to the addition of veratridine and tetrodotoxin.

Discussion

Slices are routinely employed in metabolic studies of the brain, because they are easy to prepare, contain all the cell types present in the tissues, and can attain metabolic rates similar to those in vivo (Fig. 1 and Refs. 18 and 19). Since both neuronal and glial cells show pathologic changes in thiamine deficiency [9], we decided to use a tissue preparation that contained both cell types. Cerebral cortex was used since Hakim and Pappius [18] reported that this region displayed significant decreases in glucose utilization in thiamine-depleted rats as measured in vivo by the deoxy[^{14}C]glucose method.

Pyrithiamine treatment resulted in a slightly depressed basal respiration rate and in a more marked decrease of the veratridine-stimulated rate for the cortical slices (Fig. 1). This is consistent with the decreased in vivo respiration rate in many brain regions of thiamine-deficient rats [18]. The lower respiration rates for pyrithiamine-treated slices compared to control slices is most probably related to the depressed pyruvate and α-ketoglutarate dehydrogenase activities [17,20], although a direct inhibition of $(Na^+ + K^+)$-ATPase by pyrithiamine [4] might also play a role.

The release and reuptake of K^+ by brain slices in response to the opening and closing of voltage-sensitive sodium channels by veratridine and tetrodotoxin were similar to those reported to occur in synaptosomal preparations [13]. The inhibition of K^+ reuptake by the $(Na^+ + K^+)$-ATPase inhibitor ouabain or by the metabolic inhibitors iodoacetate and rotenone + oligomycin confirmed the role of the Na^+/K^+ pump in the reuptake process.

Despite the fact that the maximal respiration rate was significantly lower for pyrithiamine-treated tissue, there was no difference between pyrithiamine-treated and control slices in the energy-requiring K^+ reuptake process or in the changes of the cellular levels of ATP + creatine phosphate. It would thus appear that the depressed respiration rate in pyrithiamine-treated cortex is still sufficient to produce enough metabolic energy to support normal Na^+/K^+ pumping in slices prepared from that tissue.

The marked decline of ATP + creatine phosphate levels in cortical slices after opening of sodium channels with veratridine (Figs. 3 and 4) was related to the energy demands required for maximal $(Na^+ + K^+)$-ATPase activity. It was unexpected, then, that the ATP + creatine phos-

phate levels did not quickly rebound back toward the high basal levels after closure of the sodium channels with tetrodotoxin. These depressed levels of high-energy compounds were, however, still capable of supporting the energy-requiring K^+ reuptake (Fig. 2). The large decrease in ATP + creatine phosphate in slices after veratridine-treatment contrasts sharply with the stable levels of these compounds in synaptosomes treated with veratridine [10,11,21]. This might be related to the relatively high mitochondrial content of synaptosomes.

The report by Matsuda and Cooper [4] indicated that pyrithiamine has a direct effect on brain $(Na^+ + K^+)$-ATPase in cell-free systems; pyrithiamine concentrations around 1 mM were required for measurable effects. Studies reported by Rindi and Perri [22] showed that either a single injection of pyrithiamine or chronic treatment with a pyrithiamine/thiamine mixture resulted in brain pyrithiamine levels of approx. 5–10 μM. Since chronic pyrithiamine treatment in the absence of thiamine was used to induce thiamine deficiency in the present studies, the levels of the antagonist in the brain slices are unknown. It is also difficult to compare in vivo and in vitro levels because of probable compartmentation of thiamine in whole cells [23]. A further complication is the contribution of the two forms of $(Na^+ + K^+)$-ATPase [24] to Na^+/K^+ balances and to possible inhibition by pyrithiamine [4]. Our results did show clearly, however, that terminal thiamine deficiency induced by pyrithiamine treatment did not significantly alter ATP-dependent alkali-ion transport in rat brain slices.

The Ca^{2+} inhibition of K^+ reuptake after tetrodotoxin addition to brain slices might be due to an inhibition by Ca^{2+} of tetrodotoxin binding at the sodium channel sites [25], resulting in only partical closing of the sodium channels. Further experiments are necessary to test this suggestion.

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