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Thiamine Deficiency in Cultured Neuroblastoma Cells: Effect on Mitochondrial Function and Peripheral Benzodiazepine Receptors

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Abstract: When neuroblastoma cells were transferred to a medium of low (6 n*M*) thiamine concentration, a 16-fold decrease in total intracellular thiamine content occurred within 8 days. Respiration and ATP levels were only slightly affected, but addition of a thiamine transport inhibitor (amprolium) decreased ATP content and increased lactate production. Oxygen consumption became low and insensitive to oligomycin and uncouplers. At least 25% of mitochondria were swollen and electron translucent. Cell mortality increased to 75% within 5 days. [^{3}H]PK 11195, a specific ligand of peripheral benzodiazepine receptors (located in the outer mitochondrial membrane) binds to the cells with high affinity (K_D = 1.4 ± 0.2 n*M*). Thiamine deficiency leads to an increase in both B_{max} and K_D. Changes in binding parameters for peripheral benzodiazepine receptors may be related to structural or permeability changes in mitochondrial outer membranes. In addition to the high-affinity (nanomolar range) binding site for peripheral benzodiazepine ligands, there is a low-affinity (micromolar range) saturable binding for PK 11195. At micromolar concentrations, peripheral benzodiazepines inhibit thiamine uptake by the cells. Altogether, our results suggest that impairment of oxidative metabolism, followed by mitochondrial swelling and disorganization of cristae, is the main cause of cell mortality in severely thiamine-deficient neuroblastoma cells.
Key Words: Thiamine—Thiamine deficiency—Energy metabolism—Mitochondria—Peripheral benzodiazepine receptors—Neuroblastoma cells.
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The brain is particularly sensitive to thiamine deficiency (see Haas, 1988, for review). As the coenzyme thiamine diphosphate (TDP) is required for oxidative decarboxylation of pyruvate and α-ketoglutarate, it can be expected that thiamine deficiency will impair oxidative energy metabolism. As brain consumes a disproportionate amount of the body's oxygen, impairment of oxidative metabolism can be expected to be particularly harmful. Moreover, the synthesis of important amino acids and neurotransmitters may be diminished if the availability of key intermediates of the Krebs cycle decreases (Butterworth and Héroux, 1989; Page et al., 1989).

The use of cultured neuronal cells, such as neuroblastoma cells, in the study of thiamine deficiency has the advantage that the effects on neurotransmitter synthesis need not be considered, as the cultured cells can usually live without producing such molecules. Schwartz et al. (1975) and Schwartz and McCandless (1976) have studied the effects of thiamine deficiency on energy metabolism (especially glycolysis) in glioma and neuroblastoma cell lines, but they were unable to quantify the remaining thiamine content of the cells. Though these authors report a strong decrease in pyruvate dehydrogenase activity, levels of high-energy phosphate compounds (ATP and ADP) were not decreased, except for phosphocreatine. Thiamine deficiency did not result in increased pyruvate and lactate production, except when the antimetabolite pyrithiamine was added. In the absence of pyrithiamine, cultured malignant cells (especially glioma) can live and divide, albeit more slowly, even when external thiamine concentrations are extremely low (Schwartz et al., 1975). This can be explained, at least partially, by the presence of a high-affinity thiamine carrier in neuroblastoma as well as glioma cells (Bettendorff, 1994; Bettendorff and Wins, 1994).

Recently, Leong et al. (1994) reported that binding of [^{3}H]PK 11195, a selective ligand for peripheral-type benzodiazepine receptors (PBRs), was increased in the brain of rats with pyrithiamine-induced thiamine deficiency. These receptors are localized in the mito-

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Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PBR, peripheral benzodiazepine receptor; TD, thiamine deficient; TDA, thiamine deficient and treated with amprolium; TDP, thiamine diphosphate; TTP, thiamine triphosphate; URC, uncoupled respiratory control.

chondrial outer membrane (Anholt et al., 1986) and may be involved in mitochondrial respiratory control (Hirsch et al., 1988*a*). PBRs are believed to be associated with proteins controlling the transport of ions and other metabolites into mitochondria (Kinnally et al., 1993). Furthermore, it has been claimed that PBRs, were more abundant in glial than in neuronal cells (Syapin and Skolnick, 1979; Starosta-Rubinstein et al., 1987); estimation of PBR levels has been suggested as a probe to estimate neuronal damage (Benavides et al., 1987). Thus, the observation of Leong et al. (1994) can be explained either by gliosis after neuronal death or through more specific changes in the number, structure, and oxidative activity of mitochondria. In this study, we investigate the latter possibility by comparing control and thiamine-deficient (TD) neuroblastoma cells.

MATERIALS AND METHODS

Chemicals

Thiamine, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), amprolium, oligomycin, and rotenone were purchased from Sigma. [^{14}C]Thiamine (24 mCi/mmol), [^{14}C]-KSCN, and [*methyl*-^{3}H]thymidine (5.0 Ci/mmol) were from Amersham. [^{3}H]Ro 15-4513 (29 Ci/mmol), [*N*-*methyl*-^{3}H]PK 11195 (81.7 Ci/mmol), [^{3}H]flunitrazepam (74.6 Ci/mmol), [^{3}H]H_2O (1 mCi/g), and [^{14}C]inulin (3 mCi/g) were from NEN. Diazepam, clonazepam (Ro 5-4023/002), and Ro 15-1788 were given by Hoffmann-La Roche. Ro 5-4864 was from Fluka Chemie AG (Buchs, Switzerland) and PK-11195 was from Research Biochemicals (Natick, MA, U.S.A.). Stock solutions (10 m*M*) of benzodiazepines and other PBR ligands were made in dimethyl sulfoxide. All measurements were made in the presence of 1% dimethyl sulfoxide as final concentration.

Cell culture

The mouse neuroblastoma cell line (Neuro 2a, ATCC CCC 131) used in this study was a gift from Professor G. Moonen (Laboratory of Human Physiology, University of Liège). The cells were grown (37°C, 5% CO_2/95% air) in 100-mm Petri dishes (Nunc, Roskilde, Denmark) in 10 ml of Dulbecco's modified Eagle's medium (GIBCO, Ghent, Belgium) containing 10 μM thiamine (value given by the manufacturer and checked by HPLC), enriched with glucose (6 mg/ml) and supplemented with 5% fetal calf serum (GIBCO). TD cells were produced by growing them in a specially ordered Dulbecco's modified medium devoid of thiamine (GIBCO). In some cases, 20 μM amprolium was added to the TD medium (TDA). All other conditions (fetal calf serum and glucose concentrations) were as described above. The medium was changed every 2 days and the cells were subcultured every 4 days.

Under all conditions, cell viability was tested by the trypan blue exclusion method. Intracellular volumes were determined from the difference of [^{3}H]H_2O and [^{14}C]inulin spaces. The membrane potential was calculated from the intra/extracellular distribution ratio of [^{14}C]SCN^- (Catterall et al., 1976).

Cell proliferation was quantified by [^{3}H]thymidine incorporation as described by Rogister et al. (1990).

All results are expressed per milligram of protein as determined by the method of Peterson (1977).

Determination of [^{14}C]thiamine uptake

The cells were preincubated for 60 min, with or without inhibitors, in 1 ml of saline (145 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10 m*M* glucose, 10 m*M* HEPES-Tris, pH 7.4) at 37°C and [^{14}C]thiamine uptake was determined as previously described (Bettendorff and Wins, 1994).

Binding of [^{3}H]PK 11195

Cells subcultured in six-well multidishes (0.1-0.2 mg/ml) were rinsed with 2 × 2 ml of saline as above. [^{3}H]PK 11195 (25 μl) at various concentrations (0.25–25 n*M*) was added and incubation was performed at 37°C. Unspecific binding was estimated in the presence of 1 μM unlabeled PK 11195. After 60 min, 50 μl of the medium was sampled for the determination of radioactivity and the cells were washed rapidly with 4 × 2 ml of ice-cold saline and dissolved in 1 ml NaOH for 30 min under constant stirring. Eight hundred microliters was counted for radioactivity as described above. Scatchard plots were analyzed using the k.cat 1.3 program (BioMetallics, Inc., Princeton, NJ, U.S.A.).

Displacement experiments by various ligands were made in the presence of 1 n*M* [^{3}H]PK 11195 for high-affinity binding. Low-affinity binding was estimated in the presence of 1 μM unlabeled PK 11195 in addition to 1 n*M* labeled substance.

Determination of thiamine derivatives, ATP, oxygen uptake, and lactate in neuroblastoma cells

Thiamine derivatives were determined by an HPLC procedure exactly as described previously (Bettendorff et al., 1991). The method of Hill et al. (1988) was used for ATP. L-Lactic acid was estimated by measuring NAD^+ reduction in the presence of lactate dehydrogenase, glutamate, and glutamate-pyruvate transaminase as described by Noll (1974). Before lactate determination, the cells were fixed with perchloric acid; the suspension was neutralized with KOH and centrifuged. The supernatant was used for lactate determination.

Oxygen uptake was measured polarographically in a 2-ml cell at 37°C as described by Vayssière et al. (1986). For each experiment, ~10–20 × 10^6 cells (suspended in their respective medium) were used and the O_2 consumption in the presence of 1 m*M* NaCN was subtracted. The uncoupled rate of respiration was measured after addition of 5 μM of the uncoupler CCCP.

Electron microscopy

The monolayer cultures were scraped off the dishes and centrifuged at 350 *g* for 3 min. Small fragments of the pellet were fixed at 4°C in glutaraldehyde (2.5% in cacodylate buffer) and then postfixed in 1% osmium tetroxide solution. The cells were embedded in Epon. Ultrathin sections mounted on copper grids were stained with uranyl acetate and lead citrate before examination under a Jeol CX 100 II electron microscope at 60 kV.

To estimate the variations in the number and the size of mitochondria between control and treated cells, the number of mitochondrial profiles per square micrometer of cytoplasm and the main section of mitochondrial profiles were determined on random electron micrographs recorded at a 13,000× primary magnification.

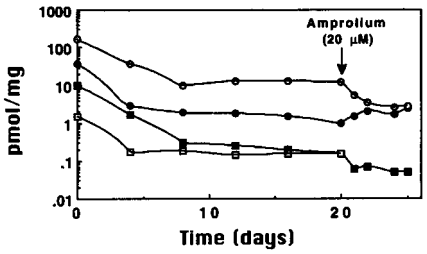

FIG. 1. Effect of a decrease in extracellular thiamine on cell thiamine content. Cells grown in normal medium were transferred into low-thiamine medium (day 0) and intracellular thiamine derivatives were determined as a function of time. On day 20, amprolium (final concentration, 20 μM) was added. Each point is the mean of four experiments. After addition of amprolium, TTP content was too small to be determined. (●), thiamine; (■), thiamine monophosphate; (○), TDP; (□), TTP.

RESULTS

In neuroblastoma cells, as in most other cell types, thiamine uptake proceeds via a high-affinity and a low-affinity transporter. Whereas the high-affinity transporter has a K_m of 35 nM, the low-affinity mechanism saturates at millimolar external thiamine concentrations (Bettendorff and Wins, 1994). As the normal culture medium contains 10 μM thiamine, the high-affinity transporter is completely saturated in this medium. To study the effect of thiamine deficiency on these cells, they were grown in the culture medium containing no thiamine. Under these conditions, the only source of thiamine is the fetal calf serum. The thiamine content of the serum is 120 ± 30 nM (n = 3) as determined by HPLC, and it was used at 5%. Under these conditions, the thiamine concentration in the culture medium is 6 nM, a value well below the K_m for the high-affinity thiamine transport. The contribution of the low-affinity transport is negligible at thiamine concentration <100 nM (Bettendorff and Wins, 1994).

Our cells survived very well in low-thiamine medium and could be continuously subcultured up to 6 months. The outgrowth of neurites was somewhat decreased but not suppressed. These observations are in agreement with those reported by Schwartz et al. (1975). To make the cells more severely deficient, amprolium (a competitive inhibitor of thiamine transport) was added to the TD medium. In the presence of 20 μM amprolium, all the cells became spherically shaped and neurite outgrowth was only rarely seen. Under these conditions, cell mortality rapidly increased, and 5 days after addition of amprolium, 75% of the cells tested positive with trypan blue. It should be noted that when the cells were grown in normal thiamine-rich medium, addition of 20 μM amprolium did not cause any decrease in intracellular thiamine content and no morphological changes were observed. This is presumably because, in contrast to pyrithiamine, amprolium is only a competitive inhibitor of thiamine transport and it is not an effective blocker of TDP synthesis (Rogers, 1970). The estimated K_i for the high-affinity transporter was 1.8 μM (Bettendorff and Wins, 1994), two orders of magnitude higher than

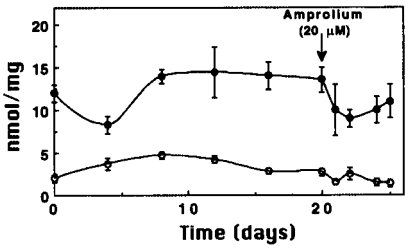

FIG. 2. Effect of a decrease in extracellular thiamine on cell ATP and ADP levels. For explanations see legend to Fig. 1. Data are mean ± SD values for four experiments. (●), ATP; (○), ADP.

TABLE 1. *Comparison of several biochemical features of control and TD cells*

	Control cells	TD cells	TDA cells
Total thiamine (pmol/mg)	210 ± 30	13 ± 4[a]	6 ± 2[b]
ATP content (nmol/mg)	12 ± 1	14 ± 1	9 ± 1[b]
Membrane potential (mV)	−50 ± 2	−54 ± 2	−43 ± 2[b]
$^{86}Rb^+$ uptake (nmol/mg/30 min)	51 ± 5	56 ± 4	36 ± 5[a,b]
Intracellular volume (μl/mg)	4.5 ± 1.5	5 ± 1	—
K_m for thiamine transport (nM)	36 ± 16	31 ± 4	—
V_{max} (pmol/mg/15 min)	4.2 ± 0.4	4.6 ± 0.8	—
[^{3}H]Thymidine incorporation (nmol/mg/24 h)	15 ± 2	9 ± 1[a]	4 ± 3[b]
Lactate (nmol/mg)	31 ± 4	31 ± 8	46 ± 7[b]

The cells were grown for 2 weeks in the presence of either 10 μM thiamine (control) or 6 nM thiamine (TD cells). In the last column (TDA cells) 20 μM amprolium was added for 2 days to TD cells. $^{86}Rb^+$ uptake was measured after addition of 0.2 μCi ^{86}RbCl to the cells in the absence or the presence of 1 mM ouabain. Data are mean ± SD values for three to six experiments. The statistical differences of the results were estimated by the ANOVA test followed by the Fisher PLSD test for post hoc comparisons.

[a] $p < 0.01$, for comparison with control cells.

[b] $p < 0.05$, for comparison of TDA with TD cells.

TABLE 2. *Rate of oxygen consumption by Neuro 2a cells under different experimental conditions*

	O_2 consumption (nmol/mg/min)		
	Control cells	TD cells	TDA cells
Basal rate	3.9 ± 0.4	3.3 ± 0.5	1.4 ± 0.2[a,b]
+ Oligomycin (16 μg/ml)	2.7 ± 0.7	2.6 ± 0.7	1.2 ± 0.3[a,b]
+ CCCP (5 μM)	7.3 ± 1.4	4.7 ± 1.4[a]	1.2 ± 0.4[a,b]
URC	2.8 ± 0.7	1.9 ± 0.6	1.1 ± 0.2[a,b]

The cells were grown as described in the legend to Table 1. O_2 consumption was measured polarographically (see Materials and Methods) in the respective culture media in either the absence or the presence of oligomycin or CCCP. The URC was calculated from the ratio of O_2 consumption in the presence of CCCP over the O_2 consumption in the presence of oligomycin. Data are mean ± SD values of four experiments.

[a] $p < 0.05$, for comparison with control cells.

[b] $p < 0.05$, for comparison of TDA with TD cells.

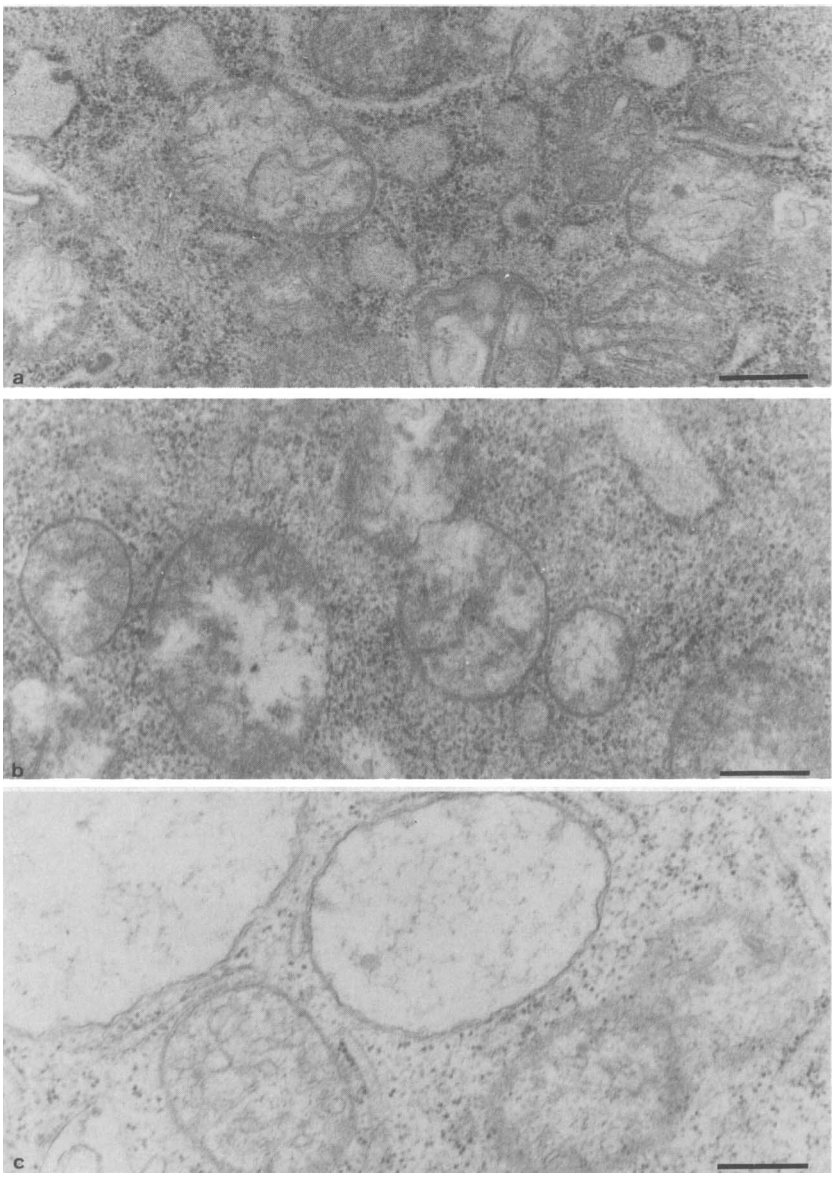

FIG. 3. Electron micrographs of control (**a**), TD (**b**), and TDA (**c**) cells. Abnormally swollen mitochondria can be seen in amprolium-treated cells. The magnification bar represents 0.5 μm.

the apparent K_m for thiamine. It is not surprising, therefore, that in thiamine-rich media, 20 μM amprolium does not significantly inhibit thiamine uptake: In the normal medium, the concentration of thiamine (10 μM) is largely saturating.

Figure 1 shows the decrease in intracellular thiamine derivatives, in TD medium, as a function of time. The medium was changed every 2 days and the cells were transferred into new culture dishes every 4 days after reaching confluence. The intracellular contents of all four thiamine derivatives decreased rapidly before reaching a steady state after ~8 days. Total thiamine content was decreased to 6.2% of the initial value (13 ± 3 vs. 210 ± 30 pmol/mg for cells cultured in 10 μM external thiamine). When 20 μM amprolium was added, thiamine and especially TDP content was further decreased. Thiamine triphosphate (TTP) was undetectable under these conditions, suggesting that the cells contained <0.1 pmol TTP/mg of protein. From these results, we can assume that the critical amount of cell TDP required for survival is ~10 pmol/mg.

In parallel with thiamine derivatives, we also measured the ATP and ADP contents of the cells (Fig. 2). Shortly after the onset of deficiency, we observed a transient decrease in ATP content followed by a recovery. This was concomitant with an increase in ADP content. After addition of amprolium, a further decrease in ATP content was observed, followed by a partial recovery.

Table 1 gives a comparison of some biochemical parameters between cells grown in normal medium (10 μM thiamine), TD cells, and TD cells grown in the presence of 20 μM amprolium (TDA). A slight depolarization was observed in the latter condition, though

amprolium was without effect on membrane potential in thiamine-rich medium (not shown). This might be due to a decrease in active cation transport as suggested by a decrease in ouabain-sensitive $^{86}Rb^+$ transport. Intracellular volume as well as the kinetic parameters of high-affinity thiamine transport (K_m and V_{max}) remained unchanged. Cell division decreased by ~40% in TD cells as estimated by the decrease in [^{3}H]-thymidine incorporation. In TDA cells lactate production was significantly increased and cell division was further slowed down. We thus compared the rates of O_2 consumption in normal, TD, and TDA cells. The results are summarized in Table 2. As expected, control cells in thiamine-rich medium had a high rate of oxygen consumption; this was partially blocked by oligomycin and stimulated by uncouplers such as CCCP. Simple thiamine deficiency did not markedly reduce O_2 consumption, though the uncoupled respiratory control (URC; Table 2) tended to decrease. In TDA cells, however, O_2 consumption was markedly lower, even in the presence of the uncoupler and the URC was close to unity. This suggests severe impairment of mitochondrial function. The decrease in respiratory rate was expected as oxidative decarboxylation of pyruvate and α-ketoglutarate are blocked in the absence of TDP; our data show that respiratory control tends to be lost as well.

Electron microscopic examination revealed the presence, in amprolium-treated cells, of abnormally large mitochondria (Fig. 3). The interior of these mitochondria was essentially electron translucent and no intact cristae were seen. In TD cells, no significant increase in mitochondrial size was seen, though the matrix appeared to be more disorganized than in nondeficient cells. In TDA cells, however, the main section of mitochondria was 1.2 ± 0.5 μm (mean ± SD; n = 38) compared with 0.93 ± 0.26 (n = 30) and 0.96 ± 0.27 μm (n = 37) for TD and normal cells, respectively (p = 0.0011, ANOVA test). No significant changes were detected in the number of mitochondria per cell as a function of thiamine status.

In addition to changes in mitochondrial morphology, the density of ribosomes strongly decreased with the severity of thiamine deficiency. As we have no evidence that TD or even TDA cells are appreciably swollen, compared with normal ones (see Table 1), the decrease in apparent density of ribosomes most probably reflects a decrease in the number of ribosomes per cell.

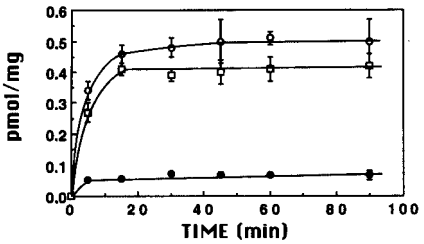

FIG. 4. Binding of [^{3}H]PK 11195 (1 nM) to neuroblastoma cells vs. incubation time. Binding experiments were performed at 37°C as described in Materials and Methods. Error bars represent mean ± SD values for three experiments. (○), total binding; (●), nonspecific binding (measured in the presence of 1 μM unlabeled PK 11195); (□), specific binding.

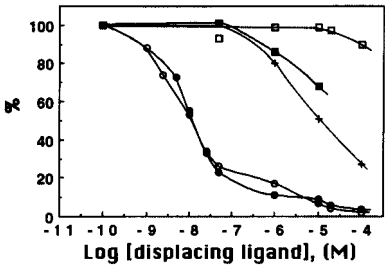

FIG. 5. Displacement of [^{3}H]PK 11195 binding (1 nM) by various ligands. Each point, expressed as a percentage of control, is the mean for three to six experiments. (○), PK 11195; (●), Ro 5-4864; (+), rotenone; (■), clonazepam; (□), Ro 15-1788.

[^{3}H]PK 11195, an isoquinoline carboxamide chemically unrelated to benzodiazepines, is known to be a specific ligand of PBR (Le Fur et al., 1983). Figure 4 shows the binding of [^{3}H]PK 11195 to the cells as a function of time in the absence or presence of 1 μM unlabeled PK 11195. Specific binding was calculated from the difference between total and nonspecific binding. Equilibrium was reached after 15–30 min. In contrast to most studies published in the literature, binding experiments were performed at 37°C instead of 4°C, as at the latter temperature equilibrium was not yet obtained after 90 min (not shown). This result suggests an intracellar binding of PK 11195 in agreement with the known location of PBRs in the outer mitochondrial membrane. The binding characteristics (K_D and B_{max}) should not be markedly temperature dependent as binding of PK 11195 is essentially entropy driven (Le Fur et al., 1983), whereas diffusion through membranes (which depends on membrane fluidity) may have a higher temperature coefficient.

Figure 5 shows the displacement of [^{3}H]PK 11195 binding by various unlabeled ligands. As expected, PK 11195 and Ro 5-4864 (a benzodiazepine specific for PBRs; Marangos et al., 1982) were most effective. Ro 15-1788 and clonazepam, which are specific central benzodiazepine receptor ligands (Anholt et al., 1986) were without effect up to 1 μM. Rotenone, an inhibitor of mitochondrial complex I, also inhibited [^{3}H]PK 11195 binding in agreement with the results obtained by Hirsch et al. (1988*b*) on isolated rat kidney mitochondria.

[^{3}H]Flunitrazepam, which binds to PBRs as well as central-type receptors (Rohde and Harris, 1982; Schoemaker et al., 1983), was not displaced by the central ligand Ro 15-1788. Likewise, no specific binding of [^{3}H]Ro 15-4513 (another specific central ligand; Sieghart et al., 1987) was observed (not shown). These results indicate that neuroblastoma cells contain

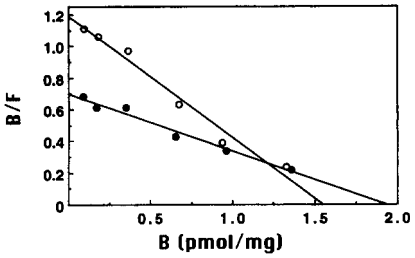

FIG. 6. Scatchard plots for [^{3}H]PK 11195 specific binding on normal (○) and TD neuroblastoma cells (●). Nonspecific binding was estimated in the presence of 1 μM unlabeled PK 11195 (see Materials and Methods) and subtracted.

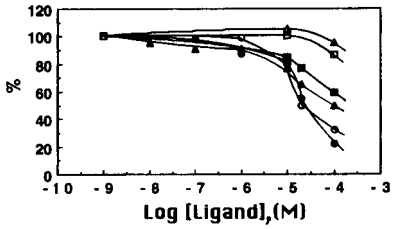

FIG. 7. Displacement of low-affinity [^{3}H]PK 11195 binding by various ligands. Binding was estimated in the presence of 1 nM [^{3}H]PK 11195 and 1 μM PK 11195. The results are expressed as percentage of control ± SD values for three experiments. (○), PK 11195; (●), Ro 5-4864; (▲), diazepam; (■), clonazepam; (△), phenytoin; (□), Ro 15-1788.

only the peripheral benzodiazepine receptors and are devoid of the central-type ($GABA_A$) receptor.

Figure 6 shows Scatchard plots of [^{3}H]PK 11195 binding to cells cultured in normal and TD medium. A linear relationship was obtained for [^{3}H]PK 11195 concentrations varying between 0.25 and 10 nM. Table 3 gives the K_D and B_{max} values for [^{3}H]PK 11195 binding for cells grown in normal medium, TD cells, and TD cells returned to thiamine-rich medium for 2 weeks; the apparent affinity for [^{3}H]PK 11195 binding decreased in TD cells compared with normal cells whereas B_{max} increased. These changes were fully reversible after 2 weeks of culture in thiamine-rich medium.

Several authors have reported the existence of a low-affinity (i.e., micromolar) binding site for PBR ligands (Bowling and DeLorenzo, 1982; File et al., 1984). We also observed a low-affinity binding in our cells. Figure 7 shows the displacement of 1 μM [^{3}H]PK 11195 binding by various ligands. The order of efficiency of these ligands was PK 11195 > Ro 5-4864 > diazepam > clonazepam > phenytoin > Ro 15-1788. Like the high-affinity binding site, the low-affinity binding site showed a specificity for peripheral ligands versus central ligands, though clonazepam was significantly more efficient than Ro 15-1788.

No displacement of [^{3}H]PK 11195 binding from the high-affinity or the low-affinity binding site was observed with thiamine or its analogues pyrithiamine and oxythiamine up to 1 mM. It is interesting, however, that we found thiamine transport was inhibited by different benzodiazepines and other PBR ligands at micromolar concentrations. Figure 8 shows the inhibition of thiamine transport by various ligands. The order of efficiency was PK 11195 = Ro 5-4864 > diazepam > clonazepam > Ro 15-1788 = phenytoin, i.e., the same as the one obtained for the displacement of [^{3}H]PK 11195 from the low-affinity binding site. Recently, Patrini et al. (1993) suggested, on the basis of in vivo kinetic measurements, that phenytoin might interfere with thiamine uptake into the brain. Our results suggest that such an effect is not likely to be mediated by the high-affinity thiamine transporter, as phenytoin is without effect up to 0.1 mM. No inhibition of thiamine transport by GABA or picrotoxin was observed (not shown). These results suggest that the inhibition of thiamine transport by benzodiazepines is not mediated through $GABA_A$ receptors. This is in agreement with the observation that no increase in $^{36}Cl^-$ uptake by GABA could be demonstrated in these cells and that no specific binding of [^{3}H]muscimol (a specific $GABA_A$ receptor agonist; Harris and Allan, 1985) could be detected in Neuro 2a cells (not shown).

PBR ligands have been shown to affect mitochondrial respiratory control (Vorobjev and Zorov, 1983;

TABLE 3. *Effect of thiamine deficiency on* K_D *and* B_{max} *for high-affinity binding of [^{3}H]PK 11195*

	K_D (nM)	B_{max} (pmol/mg)	n
Normal	1.4 ± 0.2	1.7 ± 0.2	6
TD	2.6 ± 0.6	2.2 ± 0.3	10
TD recovered	1.6 ± 0.7	1.6 ± 0.6	5

K_D and B_{max} for [^{3}H]PK 11195 in normal, TD, and thiamine-deficient cells returned to thiamine-rich medium for 2 weeks as estimated from Scatchard plots. The significance of the differences was determined by the nonparametric test of Kruskal–Wallis: for K_D, $p = 0.0024$; for B_{max}, $p = 0.039$. The Mann–Whitney U test for post hoc comparison between groups showed that both K_D and B_{max} for TD cells are significantly different from the same parameters estimated in normal and TD recovered cells (mean ± SD values for n independent experiments).

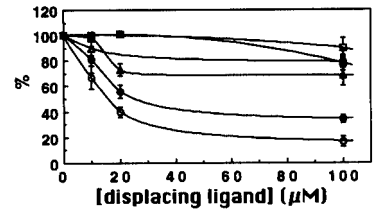

FIG. 8. Effect of various compounds on high-affinity thiamine transport in neuroblastoma cells. Thiamine uptake (percentage of control) was determined as described in Materials and Methods. Each point is the mean for three to nine experiments. (○), PK 11195; (●), Ro 5-4864; (▲), diazepam; (■), clonazepam; (△), phenytoin; (□), Ro 15-1788.

TABLE 4. *Effect of Ro 5-4864 on the distribution of intracellular thiamine derivatives and ATP content*

	T	TMP	TDP	TTP	ATP
Control	32 ± 4	7 ± 1	130 ± 8	2.0 ± 0.7	10.3 ± 0.6
Ro 5-4864 (10 μM)	36 ± 2	8 ± 1	146 ± 7	2.1 ± 0.3	10.8 ± 0.6
Ro 5-4864 (100 μM)	40 ± 3[a]	6 ± 1	129 ± 5	1.7 ± 0.5	8 ± 1[a]

The cells were preincubated for 1 h with Ro 5-4864, and thiamine and ATP concentrations were determined as described in Materials and Methods. Results are expressed as picomoles per milligram for thiamine derivatives and as nanomoles per milligram for ATP (mean ± SD values of four experiments). TMP, thiamine monophosphate.

[a] $p < 0.05$, for comparison with the controls (ANOVA followed by the Dunnett test for post hoc comparison).

Hirsch et al., 1988*a*). As thiamine transport is dependent on intracellular ATP (Bettendorff and Wins, 1994), it might be thought that these compounds act by decreasing intracellular ATP content, leading to an inhibition of thiamine transport.

However, after a 60-min preincubation in the absence of 10 μM Ro 5-4864, no effect on the distribution of thiamine derivatives and ATP content of the cells was observed (Table 4). The slight (25%) decrease in ATP, observed at 100 μM Ro 5-4864, may explain part of the inhibition of thiamine transport, but this can be excluded at lower concentrations of the inhibitor. Furthermore, Ro 5-4864 inhibited thiamine transport even without preincubation (not shown), suggesting a more direct effect.

DISCUSSION

Reduction of extracellular thiamine concentration from 10 μM to 6 nM leads to a 16-fold decrease in intracellular total thiamine content within 8 days. All four thiamine derivatives were decreased, including TTP, which has been previously reported to be particularly conserved in animal models (Pincus and Grove, 1970; Thornber et al., 1980). Under our conditions, the cells remain perfectly viable, though the growth rate decreased by ~40%. Uncoupled respiration was decreased but not the respiration in the presence of oxidative phosphorylation, suggesting that substrate availability is becoming a rate-limiting factor. This suggests that oxidative metabolism remains active even at very low external thiamine concentrations. Presumably, pyruvate oxidation still occurs under mild thiamine deficiency; otherwise, pyruvate or lactate would accumulate and O_2 consumption of the cells would decrease strongly, which is not the case (Tables 1 and 2).

In TDA cells, total intracellular thiamine was further reduced. ATP levels also decreased, whereas lactate production increased, ouabain-dependent active $^{86}Rb^+$ decreased, and the cells were slightly depolarized. At this stage, the rate of respiration was seriously impaired. It should be pointed out that one of the earliest morphological changes observed in experimental thiamine deficiency in brain involves glial edema, and this is observed before any symptomatic change (Robertson et al., 1968). This has been explained by decreased ATP levels and, thus, impairment of Na,K-ATPase activity. However, during thiamine deficiency, ATP levels rather tend to increase in cultured glioma cells (Schwartz and McCandless, 1976) as well as in rat brain (McCandless, 1982). Furthermore, inhibition of Na,K-ATPase activity by ouabain in cultured astrocytes (Kimelberg, 1981) and neuroblastoma cells (L. Bettendorff, unpublished results) leads to cell shrinkage rather than swelling. Increased ATP levels in TD cells are most probably the result of decreased ATP use rather than increased synthesis, in agreement with decreased [^{3}H]thymidine incorporation and decreased density of ribosomes (Fig. 3).

About 25% of the mitochondria in amprolium-treated cells were abnormally large and translucent. No cristae were seen. That, on the same micrographs, normally sized mitochondria are seen close to large and empty ones suggests that we are not dealing with an artifact due to, e.g., the fixation procedures. Robertson et al. (1968) reported the existence of such mitochondria in TD rat brain but also in control material. Pawlik et al. (1977) found disorganized mitochondria in the plantar nerves of TD rats. Our results suggest that impairment of oxidative metabolism induces mitochondrial abnormalities leading to the cell mortality in amprolium-treated cells. No clearcut modifications were observed in nuclei. It is known that the early observable event in apoptosis is chromatin condensation (Willie, 1980). This was not seen in our thiamine-deprived cells. It should be emphasized that only extreme thiamine deficiency as induced by thiamine antimetabolites leads to significantly increased cell mortality.

PBR, a 17–18-kDa protein (Sprengel et al., 1989), is located mainly in the outer mitochondrial membrane and has been claimed to be associated with the voltage-dependent anion channel (porin) and the adenine nucleotide carrier protein (McEnery et al., 1992). It could be located at contact sites of the outer and inner mitochondrial membranes and regulate the activity of inner mitochondrial channels responsible for the uptake of ions and other metabolites (Kinnally et al., 1993).

Leong et al. (1994) reported an increase of [^{3}H]PK 11195 binding sites in the brains of animals with pyrithiamine-induced thiamine deficiency, whereas Benavides et al. (1987) reported an increase in PBRs in rat brain after neurotoxic lesions and suggested glial proliferation accompanying neuronal degeneration. Other studies have shown a higher density of PBRs in glioma versus neuroblastoma cells (Syapin and Skolnick, 1979) and glial versus neuronal cell (Sher and Machen, 1984), but PBRs are by no means absent in cells of neuronal origin (for review, see Le Fur et al., 1988). Our results suggest an alternative and maybe complementary explanation; i.e., biochemical and

structural modifications in mitochondria after thiamine deficiency would lead to an increase in PBR.

In this respect, it is interesting that [^{3}H]Ro 5-4864 binding is increased in the temporal cortex of patients with Alzheimer's disease (Owen et al., 1983), whereas other studies report a decrease in α-ketoglutarate dehydrogenase activity in Alzheimer's disease (Mastrogiacomo et al., 1993). Furthermore, added TDP induced a significantly higher stimulation of this enzyme in Alzheimer patients compared with controls, suggesting a premortem reduction of TDP levels (Mastrogiacomo et al., 1993). Gibson et al. (1988) also reported a 75% decrease in α-ketoglutarate dehydrogenase and at least a 45% decrease in transketolase activities in the brains of patients with Alzheimer's disease.

In addition to high-affinity peripheral binding sites, we observed a low-affinity (micromolar) binding site for [^{3}H]PK 11195. This binding also showed higher apparent affinity for peripheral benzodiazepines, but that does not mean that those low-affinity "receptors" are of the peripheral type; low-affinity binding sites were ~1,000 times more abundant than PBRs (not shown). Low-affinity binding sites for benzodiazepines have been reported by several authors (see Bowling and DeLorenzo, 1982). In this study, we also show that benzodiazepines and PK 11195 in a concentration range of 1–100 μM inhibit thiamine transport, the most effective compound being PK 11195 a "peripheral-type" ligand. No competition with thiamine or its antimetabolites pyrithiamine and oxythiamine was observed and the central benzodiazepines were practically ineffective.

At present it cannot be determined whether there is only one category of binding sites having multiple actions and among them inhibition of thiamine transport or if we are dealing with numerous binding sites including the thiamine transporter. We favor the second hypothesis for the following reasons: We were unable to obtain reproducible Scatchard plots and tentative extrapolations yielded a B_{max} of ~2,000–5,000 pmol/mg, an extremely high value (compare with the 1.7–2.2 pmol/mg for the high-affinity binding site in Table 3). Such high values have also been reported by other investigators (Bowling and DeLorenzo, 1982). The hydrophobic character of benzodiazepines would favor multiple binding to membrane components. The reason peripheral ligands are more effective than central ones is not clear. Altogether, our results suggest that, at least in Neuro 2a cells, the mortality observed under extreme thiamine deficiency (as induced by thiamine antimetabolites) is the consequence of impaired mitochondrial function rather than the result of excitotoxicity or apoptosis. Indeed, even if the cells excrete glutamate, its extracellular concentration is unlikely to reach "neurotoxic" levels in the culture medium. In TD mammalian brain, other factors such as decreased neurotransmitter synthesis (for review, see Haas, 1988), excitotoxic phenomena (Hazell et al., 1993; Langlais and Zhang, 1993), or effects on membrane chloride permeability (Bettendorff et al., 1993) should also be considered, as they might interfere with normal brain function and performance and might result in neuronal death.

In the absence of more precise knowledge about the function of PBRs, it is not possible to know the physiological significance of an increase in high-affinity peripheral binding sites in thiamine deficiency except that this might be some sort of compensation for impaired mitochondrial function.

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