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Mechanism of Thiamine Transport in Neuroblastoma Cells

INHIBITION OF A HIGH AFFINITY CARRIER BY SODIUM CHANNEL ACTIVATORS AND DEPENDENCE OF THIAMINE UPTAKE ON MEMBRANE POTENTIAL AND INTRACELLULAR ATP*

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Nerve cells are particularly sensitive to thiamine deficiency. We studied thiamine transport in mouse neuroblastoma (Neuro 2a) cells. At low external concentration, [^{14}C]thiamine was taken up through a saturable high affinity mechanism (K_m = 35 nM). This was blocked by low concentrations of the Na^+ channel activators veratridine (IC_{50} = 7 ± 4 μM) and batrachotoxin (IC_{50} = 0.9 μM). These effects were not antagonized by tetrodotoxin and were also observed in cell lines devoid of Na^+ channels, suggesting that these channels are not involved in the mechanism of inhibition. At high extracellular concentrations, thiamine uptake proceeds essentially via a low affinity carrier (K_m = 0.8 mM), insensitive to veratridine but blocked by divalent cations.

In both cases, the uptake was independent on external sodium, partially inhibited (10–35%) by depolarization and sensitive to metabolic inhibitors. A linear relationship between the rate of thiamine transport and intracellular ATP concentration was found. When cells grown in a medium of low thiamine concentration (6 nM) were exposed to 100 nM extracellular thiamine, a 3-fold increase in intracellular thiamine diphosphate was observed after 2 h while the concomitant increase in intracellular free thiamine was barely significant. These data suggest a secondary active transport of thiamine, the main driving force being thiamine phosphorylation rather than the sodium gradient.

Adequate supply of thiamine is particularly important for the mammalian brain. Neuronal function is strongly dependent on oxidative energy metabolism, which absolutely requires the presence of the coenzyme thiamine diphosphate (TDP).[1] In addition to TDP, thiamine triphosphate (TTP) may play a specific role in membrane permeability (Cooper and Pincus, 1978; Bettendorff *et al.*, 1993a, 1993b).

The precursor of both compounds is free thiamine which enters the brain via the blood-brain barrier. Thiamine transport across this barrier (Spector, 1976; Greenwood *et al.*, 1982) was found to be carrier-mediated but the process did not appear to be dependent on energy metabolism (Greenwood *et al.*, 1986). In cortex slices, however, Sharma and Quastel (1965) presented evidence for a saturable and energy requiring thiamine uptake. This suggests that, if brain cells actually take up thiamine through an energy-driven process, this should occur at the neuronal and possibly glial level.

To our knowledge, no data on thiamine transport are available for isolated neuronal cells. Thiamine transport has been characterized in a number of other cell types, with sometimes conflicting conclusions. In isolated hepatocytes, Lumeng *et al.* (1979) showed that labeled thiamine (3 μM) was accumulated rapidly by the cells in the presence of external sodium ions. This rapid phase of transport was reduced both by ouabain and by uncouplers of oxidative phosphorylation. When the incubation was longer than 1 min, the uptake of radioactivity continued to increase with time mainly because TDP was synthesized from thiamine and ATP in the cytoplasm. Although thermodynamically unfavorable (Peterson *et al.*, 1975), the pyrophosphorylation reaction proceeds until most of the thiamine taken up is transformed into TDP. This is due to the fact that the latter compound forms more or less stable complexes with the enzymes that use it as cofactor, *i.e.* mitochondrial α-ketoacid dehydrogenases (Ksiezak-Reding *et al.*, 1982) and cytosolic transketolase (Blass *et al.*, 1982). Using Ehrlich ascites tumor cells, Yamamoto *et al.* (1981) demonstrated a rapid concentrative uptake of thiamine even when the cellular ATP level was lowered to 0.1 mM by metabolic inhibitors; under such conditions, the rate of thiamine phosphorylation was very low and the accumulated thiamine mostly remained in the free form.

In other cell types, however, no concentrative transport of free thiamine could be demonstrated. In human erythrocytes, for instance, data obtained by Casirola *et al.* (1990) show the presence of carriers with high affinity for thiamine but the rate of [^{14}C]thiamine uptake was low. In this case, the uptake was independent of external Na^+, uneffected by ouabain and metabolic inhibitors and it was not concentrative. In the mammalian small intestine, thiamine was taken up through the brush-border membranes independently of the sodium gradient; inside the enterocyte, thiamine was rapidly phosphorylated to TDP which accumulated in the cells (Casirola *et al.*, 1988).

It is thus difficult, so far, to draw general conclusions about the biochemical basis of cellular thiamine transport. In this study, we reexamine the problem using cultured cells of neuronal origin, Neuro 2a mouse neuroblastoma cells. These cells seemed well suited for this purpose as they are metabolically very active and electrically excitable (Catterall and Nirenberg, 1973).

MATERIALS AND METHODS

Chemicals—Veratridine, antimycin A, rotenone, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), aconitine, thiamine, oxythiamine, pyrithiamine, amprolium, amiloride, gramicidin D, thiamine monophosphate (TMP), and scorpion venom (ScV) from *Leiurus quinquestriatus* were purchased from Sigma. [^{14}C]Thiamine (24 mCi/mmol), [*methyl*-

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[1] The abbreviations used are: TDP, thiamine diphosphate; TMP, thiamine monophosphate; TTP, thiamine triphosphate; BTX, batrachotoxin; ScV, scorpion venom; TTX, tetrodotoxin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HPLC, high performance liquid chromatography.

^{3}H]thymidine (50 Ci/mmol), and [^{22}Na]NaCl (100–1000 mCi/mg of sodium) were from Amersham Corp. [^{3}H]H_2O (1 mCi/g), [^{14}C]inulin (3 mCi/g), and [^{14}C]KSCN (55 mCi/mmol) were from DuPont NEN. TTX was from Sankyo (Tokyo, Japan) and batrachotoxin (BTX) was a gift from Dr. J. Daly (National Institutes of Health, Bethesda). All solutions were made with milli-Q water (Millipore). Compounds not readily soluble in water were dissolved in dimethyl sulfoxide and used at a final solvent concentration of 1%. Control experiments were carried out in 1% dimethyl sulfoxide, which, at this concentration, does not significantly affect thiamine transport. Scorpion venom was dissolved in water (1 mg/ml), incubated for 1 h at 0 °C and centrifuged for 10 min at 12,000 × *g* (Catterall, 1975). The supernatant was used in the experiments described.

Cell Culture—The mouse neuroblastoma cell line (Neuro 2a, ATCC CCC 131) as well as other cell lines (glioma C6, ATCC CCL 107; PC-12, ATCC CRL 1721; BALB 3T3 clone A3, ATCC CCC 163; Vero, ATCC CCL 81) were given by 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), enriched with glucose (6 mg/ml) and supplemented with 5% fetal calf serum (GIBCO).

In some cases neuroblastoma cells were grown in a special Dulbecco's modified Eagle's medium containing no thiamine (GIBCO). Under these conditions the only source of thiamine was the fetal calf serum, and the final thiamine concentration was 6–7 nM, in contrast to 10 μM in the normal medium.

Determination of Initial [^{14}C]Thiamine Uptake $^{22}Na^+$ Influx and Membrane Potential—Cells were transferred in multi-dish 6 wells (3.5 cm, Nunc) and grown in 2 ml of culture medium. After 2 days, the medium was replaced, and the cells were used the day after (1–1.5 mg protein/well or 2–3 × 10^6 cells). Prior to the experiment, the cells were washed with 4 × 2 ml of saline (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes-Tris, pH 7.4) and preincubated, with or without inhibitors, in 1 ml of saline at 37 °C. After 30 min, 25 μl of [^{14}C]thiamine were added. For determination of high affinity thiamine uptake, the final [^{14}C]thiamine concentration was 0.25 μM, and the uptake in the presence of 50 μM unlabeled thiamine was subtracted from total uptake. Low affinity thiamine transport was estimated in the presence of 2.5 μM [^{14}C]thiamine plus 50 μM unlabeled thiamine. Since this low affinity component is inhibited by Ca^{2+} (10^{-4}–10^{-2} M), EGTA was added so that the final free Ca^{2+} concentration did not exceed 1 μM (Bers, 1982). After 15 min the cells were washed with 2 × 4 ml ice-cold saline containing 0.1 mM unlabeled thiamine and were dissolved in 1 ml of NaOH (0.1 N) for 30 min at room temperature under constant stirring. A volume of 800 μl was used for liquid scintillation counting after neutralization with 80 μl of HCl (1 N).

Sodium uptake was measured as described by Catterall (1975), using $^{22}Na^+$ as tracer in the medium used above, where NaCl (except for 5 mM) was replaced by 0.28 M sucrose. Under such conditions, the chemical Na^+ gradient is supposed to be abolished since the internal Na^+ concentration is roughly 5 mM.

Intracellular volumes (usually 4.5 ± 0.5 μl/mg of protein) were determined from the difference between the [^{3}H]H_2O and the [^{14}C]inulin spaces. The membrane potential was determined from the distribution ratio of the intracellular and extracellular [^{14}C]KSCN concentrations at 37 °C (Catterall *et al.*, 1976; Smith and Robinson, 1989). All calculations of inhibition or activation constants were made using the 'k.cat' software from BioMetallics, Inc. (Princeton, NJ).

Under all conditions, cell viability was tested by the trypan blue exclusion method. Protein concentrations were determined by the method of Peterson (1977). If not otherwise stated, results are expressed as mean ± S.D. for three to six experiments.

Assay of Thiamine Pyrophosphokinase (EC 2.7.6.2) Activity—Petri dishes (100 mm) containing about 20 × 10^6 cells were carefully washed with saline (4 × 10 ml), detached by scraping, and centrifuged (1,000 × *g*, 5 min). The pellets were suspended in 1.5 ml of hypotonic buffer (Tris-HCl, 20 mM, pH 7.4; mercaptoethanol, 2 mM; and EDTA, 1 mM), kept 15 min under constant stirring on ice in order to lyse the cells, and centrifuged (100,000 × *g*, 1 h). The supernatant was stored in aliquots of 500 μl at −70 °C. Thiamine pyrophosphokinase was assayed according to the method described by Matsuda *et al.* (1985). TDP formed was determined by HPLC (Bettendorff *et al.*, 1986).

Determination of Thiamine Derivatives and Adenine Nucleotides—Thiamine derivatives were determined using the HPLC procedure previously described (Bettendorff *et al.*, 1991). For adenine nucleotides, the procedure developed by Hill *et al.* (1988) was used. Intracellular thiamine or ATP concentrations were calculated after determination of the intracellular water space (4.5 μl/mg of protein).

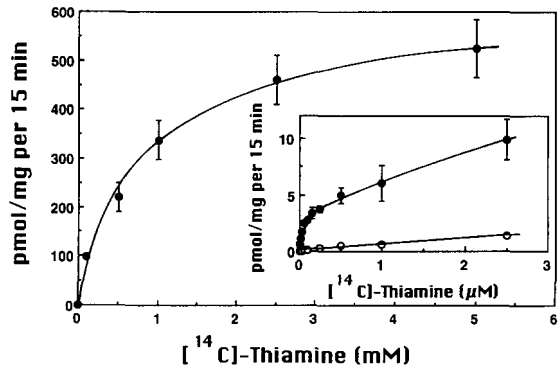

FIG. 1. **[^{14}C]Thiamine uptake in neuroblastoma cells as a function of extracellular thiamine concentration (●).** The *inset* shows an expanded scale from 0 to 2.5 μM [^{14}C]thiamine in the absence (●) or the presence (○) of 50 μM unlabeled thiamine.

RESULTS

Kinetics and Specificity of Thiamine Transport—In neuroblastoma cells, [^{14}C]thiamine uptake was linear for at least 15 min for all concentrations tested.[2] Fig. 1 shows initial [^{14}C]thiamine uptake as a function of extracellular thiamine concentration. The curve obtained is biphasic: a first saturation is obtained at extracellular concentrations <1 μM (see *inset* of Fig. 1) and a second saturation is observed at millimolar thiamine concentrations. The high affinity thiamine transport was totally abolished in the presence of 50 μM unlabeled thiamine. Nonlinear regression using a double Michaelis-Menten equation yielded a K_m of 35 nM for the high affinity and 0.8 mM for the low affinity uptake.

Various structural analogs of thiamine were found to be competitive inhibitors for the high affinity uptake (Table I), and the most potent was pyrithiamine, followed by TMP, amprolium, and oxythiamine. Surprisingly, amiloride, an epithelial Na^+ channel blocker (Benos *et al.*, 1992), also inhibited thiamine transport competitively (see also Fig. 3). For all of the competitors tested, the apparent affinity was lower than for thiamine itself.

Concerning the low affinity uptake, the selectivity of thiamine over several analogs was found to be poorer. For instance, unlabeled thiamine was about 1000 times more effective than oxythiamine in inhibiting high affinity [^{14}C]thiamine uptake, but the difference was only a factor of two for the low affinity uptake. The temperature dependence (Q_{10} between 27 and 37 °C) was found to be 1.8 ± 0.3 (n = 3) for low affinity transport. High affinity thiamine transport was more sensitive to temperature (Q_{10} = 3). Unsaturable diffusion through the membrane was not observed.

Effect of Ionic Composition and pH of Incubation Medium—Varying the extracellular pH (6.0–8.0) revealed only a 10–20% increase in high affinity thiamine uptake at pH 6.8–7.4 compared to 6.0 or 8.0, suggesting a broad pH optimum.

Total replacement of NaCl by sucrose or sodium acetate did not cause a significant inhibition of thiamine uptake (Table II). A slight decrease obtained in KCl medium can be explained by membrane depolarization (see below). Thiamine uptake was also decreased by replacing Na^+ by choline, a competitive inhibitor of thiamine transport (Yoshioka *et al.*, 1985).

While the high affinity uptake was not significantly affected by Ca^{2+}, the low affinity transport was inhibited when the extracellular Ca^{2+} concentration exceeded 0.1 mM. Thus, at 1 mM Ca^{2+} a 65% decrease in low affinity thiamine uptake was observed. This inhibition was not reversed by Mg^{2+}. All divalent cations tested inhibited the low affinity uptake, Ca^{2+}, Zn^{2+},

[2] L. Bettendorff, unpublished results.

TABLE I

Apparent dissociation constants for competitive inhibition of initial [^{14}C]thiamine uptake in neuroblastoma cells

High affinity thiamine transport was measured at [^{14}C]thiamine concentrations varying from 25 to 250 nM in the presence of two different concentrations of inhibitors. The extracellular Ca^{2+} concentration was 1 mM. Low affinity transport was measured in the presence of 2.5 μM [^{14}C]thiamine plus unlabeled thiamine. In this case the extracellular Ca^{2+} concentration was buffered to 1 μM (Bers, 1982).

	High affinity site	Low affinity site
	nM	*mM*
Thiamine	40 ± 10	0.7 ± 0.1
Pyrithiamine	58 ± 16	-
TMP	1800 ± 600	>1
Amprolium	1900 ± 600	-
Oxythiamine	36000 ± 1000	1.5 ± 0.4
Amiloride	47000 ± 13000	-

TABLE II

Effect of the ionic composition of the extracellular medium on high affinity [^{14}C]thiamine uptake

High affinity [^{14}C]thiamine transport was measured in normal saline (containing 145 mM NaCl) or in a medium where NaCl was completely replaced by either sucrose, sodium acetate, KCl, or choline chloride.

Medium		Uptake
	mM	*pmol/mg/15 min*
NaCl	145	3.3 ± 0.3
Sucrose	290	3.0 ± 0.3
Sodium acetate	145	2.7 ± 0.4
KCl	145	2.4 ± 0.5
Choline chloride	145	1.4 ± 0.2

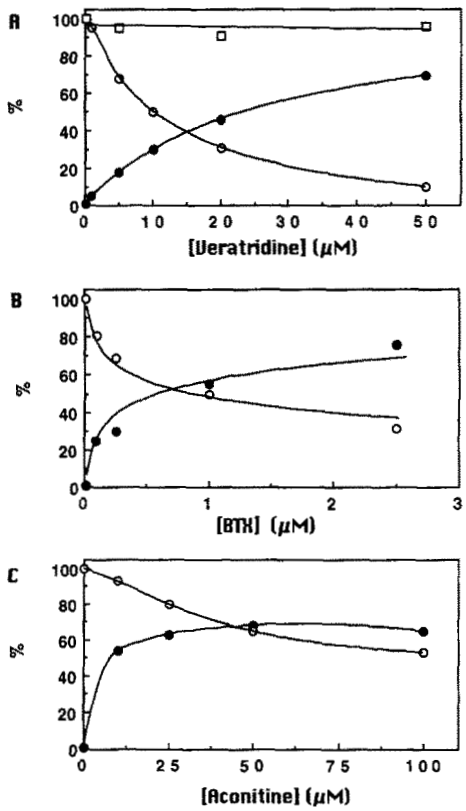

FIG. 2. **Effect of Na^+ channel activators (veratridine, BTX, and aconitine) on [^{14}C]thiamine and $^{22}Na^+$ influx.** The cells were preincubated (30 min, 37 °C) with increasing concentrations of veratridine (*A*), BTX (*B*), or aconitine (*C*) and the high (○) and low (□) affinity thiamine uptake as well as $^{22}Na^+$ uptake (●) were determined. The low $^{22}Na^+$ uptake measured in the absence of activators was subtracted. Activation of $^{22}Na^+$ uptake is expressed as the percentage of maximum activation which was calculated using a modified Michaelis-Menten equation as described by Catterall (1976); $\% = V/V_{max} = A/(K_{0.5} + A)$ where V_{max} is the maximum activation, A is the concentration of activator, and $K_{0.5}$ is the concentration of activator yielding half-maximum activation. Thiamine uptake is expressed as percentage of the uptake in the absence of Na^+ channel activators.

Mn^{2+}, Ba^{2+}, and Cd^{2+} being the most effective followed by Ni^{2+} and Mg^{2+}. The latter induced only a 40% inhibition at 10 mM while the same inhibition was obtained with 0.3 mM Ca^{2+}.

Inhibition of Thiamine Transport by Effectors of Voltage-gated Na^+ Channels—We unexpectedly found that lipophilic alkaloids (veratridine and aconitine) as well as BTX also inhibit high affinity thiamine uptake. Fig. 2*A* shows high and low affinity thiamine uptake as well as the activation of $^{22}Na^+$ uptake as a function of veratridine concentration. Veratridine inhibited high affinity thiamine uptake with an IC_{50} of 7 ± 4 μM while the $K_{0.5}$ for activation of $^{22}Na^+$ uptake was 25 μM. Low affinity thiamine uptake was not significantly affected by veratridine. Fig. 2, *B* and *C*, shows activation of $^{22}Na^+$ influx and inhibition of high affinity thiamine uptake as a function of BTX (IC_{50} = 0.7–1.0 μM) and aconitine concentrations. The effect of aconitine seemed only partial, but it was significant.

The rate of thiamine uptake *versus* thiamine concentration was studied in the absence or in the presence of 10 or 25 μM veratridine and a Lineweaver-Burk plot of the data is shown in Fig. 3*A*. The inhibition of high affinity thiamine transport by veratridine was clearly non competitive (IC_{50} = 7 ± 4 μM), in contrast to amiloride (Fig. 3*B*) which was competitive (K_I = 47 ± 13 μM).

Veratridine at 5 μM inhibited about 40% of thiamine transport (Table III); the inhibition was not antagonized by TTX (1 μM) and still occurred when extracellular Na^+ was lowered to 5 mM. Likewise, saxitoxin (1 μM) did not counteract the inhibition of thiamine transport by veratridine.[2] Even at 50 μM, veratri-

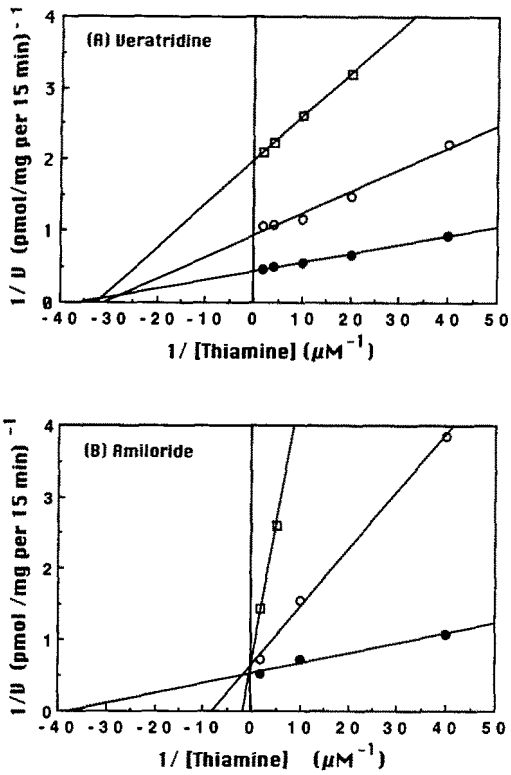

FIG. 3. **Lineweaver-Burk plots for inhibition of high affinity thiamine uptake by veratridine (*A*) or amiloride (*B*).** Veratridine concentrations were 0 (●), 10 μM (○), or 25 μM (□). The apparent K_I for veratridine was calculated to be 7 ± 4 μM. Amiloride concentrations were 0 (●), 0.1 mM (○), or 1 mM (□).

TABLE III

Effect of neurotoxins on high affinity thiamine uptake and membrane potential (E_M)

The cells were preincubated in the presence of the inhibitors for 30 min either in normal saline or in a medium where 145 mM NaCl was replaced by 5 mM NaCl and 0.28 M sucrose. High affinity thiamine uptake was measured in the presence of 0.25 μM [^{14}C]thiamine. The duration of incubation was 15 min. TTX was always 1 μM and ScV 10 μg/ml.

Medium	[NaCl]	Thiamine uptake	E_M
	mM	*pmol/mg/15 min*	*mV*
Control	145	3.3 ± 0.3	−48 ± 2
Veratridine (5 μM)	145	2.2 ± 0.2	−47 ± 2
+TTX		2.2 ± 0.3	−46 ± 2
+ScV		1.7 ± 0.2	−14 ± 3
+ScTc+TTX		2.4 ± 0.4	−47 ± 3
Control	5	3.2 ± 0.2	−47 ± 3
Veratridine (5 μM)		2.4 ± 0.3	−46 ± 3
+ScV		2.3 ± 0.3	−45 ± 2
BTX (0.5 μM)	145	1.8 ± 0.3	−24 ± 3
+TTX (1 μM)		2.8 ± 0.2	−47 ± 3
+ScV (10 μg/ml)		1.4 ± 0.1	−10 ± 3

dine depolarized the cells by less than 10 mV,[2] suggesting that its effect on thiamine transport was not mediated by membrane depolarization.

Interestingly, the effect of veratridine was found to be enhanced by ScV, which also potentiates the effect of veratridine on Na^+ channels (Catterall, 1975). Simultaneous application of veratridine and ScV strongly depolarized the cells and the depolarization was fully antagonized by TTX (Table III). In 5 mM extracellular Na^+, ScV was without effect on membrane potential and thiamine transport as expected. BTX inhibited thiamine transport but it also depolarized the cells; we thus expected that its effect would be partially antagonized by TTX, and this was found to be the case.

The order of effectiveness for stimulation of $^{22}Na^+$ influx was BTX >> veratridine < aconitine (see also Catterall (1977)). This explains why the cells are depolarized by BTX. A depolarization by veratridine and aconitine was significant only in the presence of ScV (Table III).

We tested the effect of alkaloids (veratridine and aconitine) and ScV on $^{22}Na^+$ influx (Table IV) and thiamine uptake (Table V) in other cell types. A clear effect of veratridine and ScV on $^{22}Na^+$ influx and membrane potential was observed only in Neuro 2a cells and the effect was blocked by TTX as expected (Table IV). In PC-12 and glioma C6 cells we observed only a slight effect of veratridine and ScV on $^{22}Na^+$ uptake. Two non-tumorigenic, fibroblast-like cell lines (Balb/c 3T3 and Vero), did not respond at all to veratridine and ScV as far as sodium permeability was concerned. However, in all cases, thiamine transport was inhibited by veratridine (with an apparent IC_{50} ranging from 7 to 14 μM) as well as by 50 μM aconitine (Table V); the inhibition by veratridine was not potentiated by ScV except in excitable Neuro 2a cells.

Effect of Membrane Potential on Thiamine Transport—A membrane potential of −49 ± 6 mV (mean ± S.D., n = 21) was measured from the distribution ratio of [^{14}C]SCN. This is close to the value reported by Catterall *et al.* (1976). Using the whole-cell patch clamp technique, the resting membrane potential was estimated to be −48 ± 9 mV (mean ± S.D. for 17 cells).[3] Finally, when the cells were depolarized with increasing external K^+ concentrations, the relationship between K^+ concentration and membrane potential was similar to the one described by Catterall *et al.* (1976), with the null point obtained for 125 mM KCl (not shown).

Fig. 4 shows the relationship between thiamine transport and membrane potential. Cells were depolarized by different methods: increasing concentrations of gramicidin gradually depolarized the cells (−9 mV at 1 μg/ml); aconitine (25 μM) was used in conjunction with increasing concentrations of ScV; finally, the cells were depolarized with veratridine (50 μM) + ScV (10 μg/ml) and repolarized with increasing concentrations of TTX. It is apparent (Fig. 4) that approximately the same relationship was obtained in the three cases. Thus, there appears to be a link between membrane potential and the initial rate of thiamine uptake. Complete depolarization induced about 35% inhibition of thiamine uptake. However, the results obtained with increasing KCl concentrations are more complicated. At 145 mM extracellular K^+, the membrane potential is about +5 mV but thiamine transport is inhibited by only about 10%. The shape of the curve obtained suggests that K^+ activates thiamine uptake, an effect which, at low KCl concentrations, prevails over inhibition by depolarization.

The low affinity thiamine uptake was also sensitive to membrane potential (Table VI), decreasing by 30% in the presence of either gramicidin (1 μg/ml) or 145 mM KCl[2].

Is Phosphorylation the Driving Force for Thiamine Uptake?—When the cells were preincubated for one hour in the presence of the uncoupler CCCP or inhibitors of the respiratory chain (antimycin A or rotenone), high and low affinity uptake as well as intracellular ATP concentrations were decreased. The membrane potential was not significantly affected (Table VI). With CCCP, the V_{max} for the high affinity transport was decreased about 3-fold while the K_m for [^{14}C]thiamine was not affected (not shown). When extracellular thiamine concentrations are very high (1 mM, *i.e.* two to three orders of magnitude higher than intracellular concentrations), the effect of CCCP on [^{14}C]thiamine uptake is nearly abolished.[2] This may be explained by the fact that under these conditions the thiamine

[3] L. Bettendorff and H.-A. Kolb, unpublished results.

TABLE IV

Effect of veratridine (5 μM), ScV (10 μg/ml), and TTX (1 μM) on $^{22}Na^+$ influx and membrane potential (E_M) in different cell lines

The experimental conditions were the same as in Table III.

Cell line	$^{22}Na^+$ influx				E_M	
	Control	Veratridine	Veratridine +ScV	Veratridine +ScV+TTX	Control	Veratridine +ScV
	nmol/mg/5 min				*mV*	
N2a	1.2 ± 0.2	1.9 ± 0.2	9.3 ± 0.8	1.3 ± 0.3	−54 ± 3	−14 ± 3
PC-12	2.1 ± 0.3	2.3 ± 0.1	2.6 ± 0.2	2.2 ± 0.3	−39 ± 2	−35 ± 2
C6	1.1 ± 0.2	1.1 ± 0.3	1.4 ± 0.2	1.2 ± 0.3	−38 ± 2	−31 ± 2
3T3	7.1 ± 0.5	7.0 ± 0.4	7.3 ± 0.5	7.0 ± 0.7	−46 ± 6	−45 ± 1
Vero	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.1 ± 0.1		

TABLE V

Effect of veratridine (5 μM), aconitine (50 μM), ScV (10 μg/ml), and TTX (1 μM) on thiamine transport (pmol/mg/15 min) in different cell lines

The experimental conditions were the same as in Table III.

Cell line	Control	Veratridine	Veratridine +ScV	Veratridine +ScV+TTX	Aconitine	IC_{50} veratridine
	pmol/mg/15 min					*μM*
N2a	3.1 ± 0.4	2.1 ± 0.3	1.2 ± 0.2	2.1 ± 0.4	1.8 ± 0.3	10
PC-12	4.7 ± 1.0	3.0 ± 0.3	2.8 ± 0.4	2.7 ± 0.5	2.9 ± 0.6	8
C6	3.4 ± 0.2	2.7 ± 0.5	2.5 ± 0.2	3.3 ± 0.4	1.5 ± 0.2	14
3T3	4.8 ± 0.8	3.3 ± 0.7	3.3 ± 0.5	2.8 ± 0.5	2.7 ± 0.3	8
Vero	5.8 ± 0.3	3.3 ± 0.3	2.9 ± 0.6	3.0 ± 0.4		7

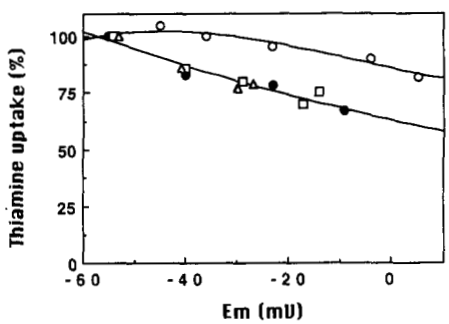

FIG. 4. **Thiamine transport as a function of membrane potential (E_m).** The cells were preincubated (30 min) in the presence of either increasing KCl concentrations (5, 10, 25, 50, 100, and 145 mM) in replacement for NaCl (○) or various depolarizing agents: ●, gramicidin (0; 0, 1; 0.25 or 1 μg/ml); △, aconitine (25 μM) plus ScV (0, 0.2, 0.5 or 2.5 μg/ml); □, ScV (10 μg/ml), veratridine (50 μM) plus TTX (0, 1, 10, 100, or 500 nM). Thiamine transport is expressed as percent of control for three experiments.

TABLE VI

Effect of CCCP, antimycin A, rotenone, and gramicidin on high and low affinity thiamine transport and cellular ATP content

The experimental conditions were the same as in Fig. 5.

	High affinity	Low affinity	[ATP]	E_M[a]
	pmol/mg/15 min		*nmol/mg*	*mV*
Control	3.6 ± 0.2	450 ± 20	11 ± 1	−47 ± 3
CCCP (1 μM)	2.3 ± 0.3	320 ± 30	7 ± 1	−42 ± 4
Antimycin (2 μg/ml)	1.5 ± 0.2	190 ± 20	4.0 ± 0.1	−48 ± 4
Rotenone (10 μM)	1.4 ± 0.2	360 ± 30	3.7 ± 0.4	
Gramicidin (1 μg/ml)	2.4 ± 0.2	320 ± 30	10 ± 2	−9 ± 4

[a] E_m membrane potential.

gradient itself becomes the driving force. Gramicidin depolarizes the cells but does not affect ATP content. Fig. 5 shows that a linear relationship is obtained between high affinity uptake and intracellular ATP concentrations.

The thiamine concentration of the normal culture medium is 10 μM. Under these conditions most of thiamine uptake proceeds via the low affinity transporter (see Fig. 1). The intracellular total thiamine content is very high (10 μM). Evidently, no further increase of this content can be observed when the cells are put in a medium containing more physiological, *i.e.* much lower, levels of thiamine (<1 μM), where the high affinity transporter is predominant. Therefore, the cells were grown for 2 weeks in a medium containing only 6 nM thiamine. Under such conditions, intracellular total thiamine content was decreased by a factor of 16, but neither V_{max} nor K_m for the high affinity transporter were modified.[2] Then, the external thiamine concentration was increased to 100 nM (more than twice the apparent K_m), and the intracellular content of thiamine derivatives was followed as a function of time in the absence or the presence of veratridine (100 μM). Intracellular TDP content rapidly increased before reaching a plateau after about 24 h (Fig. 6). Interestingly, for thiamine, a lag period was observed: after 1 h the thiamine content had not significantly increased while the TDP content had already doubled. These results suggest that thiamine is phosphorylated immediately after entering the cells. It can be hypothesized that an increase in thiamine content is observed only when the capacity for binding newly formed TDP tends toward saturation. In the presence of veratridine, at a concentration sufficient to block over 90% of thiamine transport (Fig. 2), the increase in intracellular TDP was also strongly reduced, demonstrating that the high affinity transporter is the main mechanism responsible for thiamine uptake under these conditions. Finally, CCCP (10 μM) gave similar results as veratridine,[2] demonstrating that at low intracellular ATP concentrations, not only the phosphorylation is inhibited but thiamine does not accumulate significantly. TTP and TMP are not represented in Fig. 6 as these compounds are formed from TDP and represent less than 10% of total thiamine.

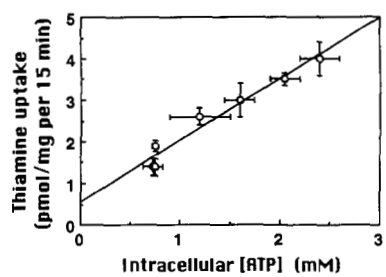

FIG. 5. **Effect of intracellular ATP concentration on high affinity thiamine transport.** The cells were preincubated (1 h at 37 °C) in the presence of CCCP, rotenone or antimycin A at various concentrations.

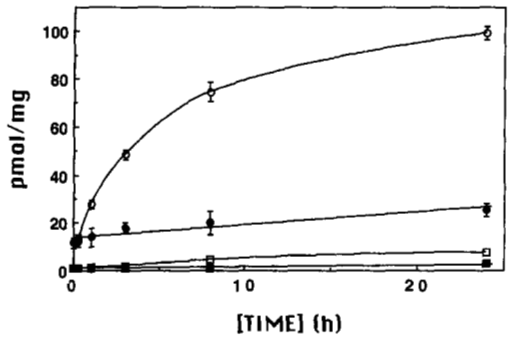

FIG. 6. **Evolution of intracellular TDP and thiamine content of neuroblastoma cells grown in a medium depleted in thiamine.** The cells were grown for 2 weeks in the presence of a 7 nM extracellular thiamine concentration (in contrast to 10 μM in the normal culture medium) At the beginning of the experiment, 93 nM thiamine with (*full symbols*) or without (*open symbols*) 100 μM veratridine were added and intracellular TDP (○, ●) and thiamine (□, ■) were determined by HPLC as a function of time.

TABLE VII

Effect of CCCP, veratridine, and KCl on the intracellular distribution of thiamine derivatives

Confluent dishes (20×10^{-6} cells) were washed with 4×10 ml of saline and incubated in 10 ml saline containing either CCCP, veratridine or KCl (in replacement for NaCl). After 1 h, the cells were detached by scraping and centrifuged ($800 \times g$, 5 min). The pellet was suspended in 500 μl of saline and the proteins were precipitated with 100 μl of trichloroacetic acid (60%) and sedimented ($5000 \times g$, 15 min). Statistical differences were assessed by analysis of variance ($p < 0.01$) followed by the Dunnett test for post hoc comparisons.

	Thiamine	TMP	TDP	TTP	ATP
			pmol/mg		*nmol/mg*
Control	30 ± 8	15 ± 4	234 ± 25	2.7 ± 0.6	9.0 ± 2.0
CCCP (10 μM)	62 ± 7[a]	31 ± 10[a]	175 ± 26[a]	3.3 ± 0.8	1.6 ± 0.2[a]
Veratridine (50 μM)	37 ± 5	16 ± 3	254 ± 60	3.2 ± 0.8	8.8 ± 1.5
Gramicidin (1 μg/ml)	39 ± 7	16 ± 1	240 ± 32	3.2 ± 0.4	8.3 ± 0.8
KCl (100 mM)	28 ± 4	18 ± 4	254 ± 60	3.3 ± 0.6	8.5 ± 1.0

[a] $p < 0.01$.

We measured the activity of thiamine pyrophosphokinase (thiamine + ATP ⇔ TDP + AMP) in our cell preparation. The reaction was linear with time for about 2 h and the K_m for thiamine was 100 nM in agreement with the results obtained by Johnson and Gubler (1968) in rat brain. The K_m of ATP for the enzyme was 7 mM, a value slightly lower than the 59 mM reported for pig brain (Peterson *et al.*, 1975) or 20 mM reported for the rat brain enzyme (Johnson and Gubler, 1968). In any case, if thiamine pyrophosphorylation is the driving force for thiamine uptake this would explain that the latter is not saturated at intracellular ATP concentrations up to 2.5 mM (Fig. 5). Note that thiamine pyrophosphokinase, under our assay conditions, was found totally insensitive to veratridine (50 μM) or antimycin A (10 μg/ml).

When the cells were incubated in saline (control) containing either CCCP (10 μM), veratridine (50 μM), gramicidin (1 μg/ml), or KCl (100 mM), and intracellular thiamine and ATP content was determined after 1 h, we observed a decrease in ATP concentration and a shift toward dephosphorylation of TDP only in the presence of the uncoupler (Table VII); all the depolarizing agents tested were uneffective.

DISCUSSION

Thiamine transport in neuroblastoma cells is apparently mediated by two kinds of transporters. The high affinity thiamine uptake has been consistently found in most cell types studied so far (Yamamoto *et al.*, 1981; Yoshioka, 1984; Casirola *et al.*, 1988, 1990), but the K_m found in this study is the lowest reported so far. Yoshioka (1984) reported the existence of a saturable low affinity thiamine transport in rat hepatocytes but in many studies the second saturation went unnoticed, and the low affinity component was often considered as simple diffusion of thiamine through the membranes (Yamamoto *et al.*, 1981; Casirola *et al.*, 1988, 1990). In this work we clearly show that low affinity thiamine uptake is not a simple diffusionnal process: it is saturable, specifically inhibited by divalent cations and it has a Q_{10} of 1.8, which is higher than expected for diffusion. It could be argued that the saturation observed at millimolar concentrations of external thiamine is due to the saturation of thiamine pyrophosphokinase inside the cells. However, we have found that the K_M of this enzyme for thiamine was 100 nM and the intracellular thiamine concentrations (under normal culture conditions) are about 10 μM, and these would still increase in the presence of millimolar external thiamine concentrations. Furthermore the saturation of low affinity thiamine uptake is observed in the presence of 10 μM CCCP (not shown), conditions under which thiamine pyrophosphokinase activity is strongly depressed. We conclude that, at high external thiamine concentrations, the uptake is mainly mediated through a saturable carrier. However, this carrier may not be specific for thiamine and might even be some channel-like structure. This view would be compatible with the observation that those cations which have the smallest ionic radius (Ni^{2+} and Mg^{2+}) have less blocking effect compared to larger ions (Ba^{2+}, Ca^{2+}, Mn^{2+}). This would also explain the low selectivity of thiamine over several analogs compared with the high affinity transport.

For the high affinity carrier, the main binding properties appear to be the following: as oxythiamine is a poor competitor compared to pyrithiamine (Table I), the amino group on C-4 of the pyrimidine ring is probably important while the thiazol sulfur is not. However, the hydroxyethyl group on the thiazole moiety probably plays an important role in binding, as suggested by the increase in K_I after its removal (amprolium) or substitution (TMP). Binding of amiloride may be favored by the amino group in the pyrazine ring as well as by the presence of a positive guanidinium, which might play a similar role in binding as the positive charge of the thiazole moiety of thiamine. These results suggest that electrostatic interactions are critical for binding, while the nature of the aromatic rings is less important.

It was a surprise to find that two phylogenetically unrelated alkaloids (veratridine and aconitine) and the toxin BTX, which are all considered to specifically slow Na^+ channel inactivation (Catterall, 1992), inhibit high affinity thiamine transport at similar concentrations. For thiamine transport, we found an IC_{50} for veratridine of 7 ± 4 μM, which is even lower than the $K_{0.5}$ for Na^+ channel activation in neuroblastoma cells (25 μM (this work); 50 μM (Catterall, 1975); 29 μM (Catterall, 1977)). BTX, which is the most potent Na^+ channel activator, is also a good inhibitor of thiamine transport, though its effects are more complex; in contrast to veratridine and aconitine, it strongly depolarizes the cells, thus potentiating inhibition. Aconitine, which is a poorer Na^+ channel activator (Catterall, 1975), only partially inhibits thiamine uptake but the effect was nonetheless significant at 10 μM.

Thiamine uptake was also inhibited by veratridine in cell lines devoid of a significant population of voltage-sensitive Na^+ channels (Tables IV and V). It is known that astrocytes (Bevan *et al.*, 1985), glioma C6 (Catterall and Nirenberg, 1973), and PC-12 cells (Reed and England, 1986) have a low but measurable Na^+ channel density. Pouysségur *et al.* (1980) showed that even some fibroblast cell lines contain Na^+ channels, but in this case a large Na^+ influx is observed in the presence of veratri-

dine as expected, and it is blocked by TTX. In the 3T3 and Vero cells that we tested, $^{22}Na^+$ uptake was not activated by veratridine and ScV, suggesting that these cells contain no significant amounts of TTX-sensitive Na^+ channels. Yet, thiamine uptake was still blocked by veratridine at similarly low concentrations.

Thiamine uptake was slowest in neuroblastoma cells. Rates of uptake at least two orders of magnitude higher have been reported in hepatocytes (Yoshioka, 1984). This low rate of thiamine transport may be a general feature of neuronal cells and contribute to the special sensitivity of the brain to thiamine deprivation.

It could be argued that the properties of thiamine transport discussed in this work are a characteristic only of cultured cells, but preliminary studies in our laboratory have shown that thiamine transport in synaptosomes prepared from rat brain is also sensitive to veratridine and the inhibition was not antagonized by TTX.[2]

The observation that thiamine uptake is dependent on membrane potential is important as it sheds a new light on observations made by several other authors. Yoshioka (1984) showed that thiamine uptake in rat hepatocytes was partially inhibited in K^+ medium, but this was interpreted as consistent with the idea that thiamine transport requires the presence of external sodium. Yamamoto *et al.* (1981) found a decreased affinity of thiamine for its carrier when Na^+ was partially replaced by choline, and they concluded that sodium ions were necessary for thiamine transport. Indeed, in Table II we show that replacement of external Na^+ by choline decreases [^{14}C]thiamine uptake. However, Yoshioka *et al.* (1985) found that choline by itself was a competitive inhibitor of thiamine uptake. Thus there is so far no compelling evidence that thiamine uptake is dependent upon external sodium in mammalian cells. In our conditions, the Na^+ gradient is certainly not the main driving force for thiamine uptake. Indeed, total replacement of external NaCl by sucrose changed neither K_M (36 ± 4 *versus* 33 ± 10) nor V_{max} (Table II) for thiamine transport. Casirola *et al.* (1988), using membrane vesicles isolated from intestinal brush borders, also concluded that external Na^+ had no effect on [^{14}C]thiamine uptake. Note that depolarizing agents such as KCl or gramicidin do not seem to affect either cell ATP content or the intracellular distribution of thiamine derivatives (Tables VI and VII). Thus, their inhibition of thiamine transport cannot be explained by an interference with cell energy metabolism.

Thiamine uptake is a linear function of intracellular ATP concentrations in the range of 0.7–2.5 mM. The apparent affinity for ATP is thus low. This, in conjunction with the fact that thiamine taken up does not accumulate but is rapidly phosphorylated, suggests that the driving force for thiamine uptake is its phosphorylation to TDP by thiamine pyrophosphokinase. Thiamine pyrophosphokinase has indeed a low affinity for ATP with an appparent K_m of 7 mM, while the K_m for thiamine is 100 nM. We calculated that the rate of the reaction was about 12 pmol/mg/15 min at the ATP concentration found in our cells (2.5 mM). The reaction rate thus largely exceeds the rate of thiamine taken up at V_{max} for the high affinity uptake (Fig. 1). When the cells were incubated in the presence of CCCP (and in the absence of extracellular thiamine), a dephosphorylation of TDP was observed (Table VII). This is in agreement with the hypothesis that under these conditions, due to lowered intracellular ATP concentrations, the equilibrium is shifted toward unphosphorylated thiamine. On the other hand, veratridine neither affects thiamine pyrophosphokinase activity, nor the intracellular distribution of thiamine derivatives suggesting that it solely acts on the transport protein.

In conclusion, several interesting features of thiamine transport appear from our results. All of the thiamine transport could be accounted for by two distinct saturable transport systems; one is sensitive to veratridine, the other to Ca^{2+}. For the first time, we clearly show that thiamine uptake is dependent on intracellular ATP concentration; the apparent affinity for ATP is low and close to the affinity of thiamine pyrophosphokinase for ATP. Thus, we are probably dealing with an secondary rather than a primary active transport, the driving forces being the phosphorylation to TDP and to a lesser extent, facilitation of transport by the membrane potential. The fact that thiamine uptake is inhibited by alkaloid Na^+ channel activators with similar affinity constants suggests that the thiamine carrier shares common structural motifs with voltage-dependent Na^+ channels. These compounds can become interesting tools for the study of thiamine transport in cells, as in contrast to thiamine analogs, they specifically block thiamine transport without interference with its metabolism.

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