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Effects of tri-*n*-butyltin chloride on energy metabolism, macromolecular synthesis, precursor uptake and cyclic AMP production in isolated rat thymocytes

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The inhibitor of oxidative phosphorylation tri-*n*-butyltin chloride (TBTC) causes membrane damage and disintegration of isolated rat thymocytes at concentrations higher than 1 μM. From a concentration of 0.1 μM, TBTC disturbs energy metabolism as indicated by an increase in methylglucose uptake, glucose consumption and lactate production and by a decrease in cellular ATP levels. Over the same TBTC concentration range, the incorporation of DNA, RNA and protein precursors are markedly reduced. Moreover the production of cyclic AMP upon stimulation of the cells with prostaglandin E_1 is effectively inhibited. These effects cannot be explained by an inhibition of nucleoside kinase activity, amino acid uptake or adenylate cyclase activity. The effects of TBTC on macromolecular synthesis and cyclic AMP production are possibly due to a disturbance of the cellular energy state.

Introduction

Tributyltin compounds are used as general biocides, e.g., in marine antifouling paints or for the preservation of paper and wood [1]. More recently these compounds have been proposed as molluscicides for the control of certain aquatic snails which serve as a vector for the trematode parasite that causes schistosomiasis in man [2]. Upon oral exposure to rats, tributyltin compounds were found to be immunotoxic, causing atrophy of the thymus [3] and subsequently immunosuppression [4]. Using isolated rat thymocytes, TBTC appeared very cytotoxic. Thymidine incorporation was reduced at concentrations as low as 0.05 μM TBTC, while at levels higher than 1 μM membrane damage was noticed [5].

The properties of trialkyltin compounds to interfere with mitochondrial energy production have been intensively studied [6–9]. Three different mechanisms for disturbing mitochondrial ATP synthesis were distinguished: (i) by mediating the exchange of halide for hydroxyl ions across the mitochondrial membranes; (ii) by binding to a component of the mitochondrial Mg^{2+}-ATPase to produce an oligomycin-like effect; and (iii) by causing gross mitochondrial swelling. The trialkyltin compounds appeared useful probes in the study of oxidative energy production.

Here, the effects of TBTC on the energy metabolism of isolated thymocytes were investigated. Furthermore, the effects of TBTC on membrane integrity, macromolecular synthesis, nucleoside kinase, amino acid uptake, cyclic AMP

Abbreviation: TBTC, tri-*n*-butyltin chloride.

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production and adenylate cyclase were studied. For all these aspects of cellular function the role of the cellular energy state was discussed.

Materials and Methods

Animals and chemicals. Male Wistar-derived rats (Central Laboratory for Animal Breeding, TNO, Zeist, The Netherlands) weighing 100–150 g were used. Tri-*n*-butyltin chloride (TBTC) was kindly provided by Dr. H.A. Meinema, Institute for Applied Chemistry, TNO, Utrecht, The Netherlands. Purity was more than 99% as established by thin-layer chromatography. [8-^{3}H]Adenosine-3′,5′-cyclic phosphate (26.5 Ci/mmol), 1-aminocyclopentane-[1-^{14}C]carboxylic acid (55 Ci/mmol), [*methyl*-^{3}H]α-aminoisobutyric acid (1.5 Ci/mmol), 3-*O*-methyl-D-[1-^{3}H]glucose (2 Ci/mmol), L-[U-^{14}C]leucine (348 Ci/mol), L-[3,4(*n*)-^{3}H]proline (54 Ci/mmol), [6,6′(*n*)-^{3}H]sucrose (15 Ci/mmol), [*methyl*-^{3}H]thymidine (47 Ci/mmol) and [5,6-^{3}H]uridine (50 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, U.K. Theophylline, 3-isobutyl-1-methylxanthine and prostaglandin E_1 (PGE_1) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. and silicone oil AR 200 ($d = 1.04$) from Serva Feinbiochemica, Heidelberg, F.R.G.

Isolation and incubation of thymocytes. After decapitation, thymus glands were rapidly removed and thymocytes were isolated as described previously [10] using ice-cold Dulbecco's phosphate-buffered saline supplemented with 2 mM D-glucose (phosphate-buffered saline/gluc; pH 7.4), except when stated otherwise. Thymocytes ($(2–4) \cdot 10^7$ cells/ml) were incubated in a shaking bath at 37°C for various periods up to 4 h. Cell viability of freshly prepared suspensions was more than 95% as judged by trypan blue exclusion and was routinely checked at the end of each experiment. TBTC was dissolved in absolute ethanol just before each experiment. The final ethanol concentration of 0.1% did not affect any of the test systems.

Cell survival. To investigate the effects of TBTC on thymocyt survival, cell number (counted with a Coulter Counter, model ZF) and membrane integrity (trypan blue exclusion) were determined during a 4-h incubation period using TBTC concentrations ranging from 0.5–10 μM. Threshold values of the cell counter were standardized using various batches of latex particles with known median size.

Energy metabolism. To study the effects of TBTC on the energy metabolism of thymocytes suspended in phosphate-buffered saline/gluc ($4 \cdot 10^7$/ml), the consumption of glucose and the production of pyruvate and lactate were measured. In order to obtain an appropriate decrease in glucose concentration, 4-h incubations were used. Subsequently, ATP levels were determined upon incubation with TBTC for up to 3 h. By varying the energy substrates, glucose (2 mM) or β-hydroxybutyrate (2 mM) or neither of them, both in isolation and incubation buffers, the substrate dependency of the effects of TBTC on ATP levels was studied. For the estimation of metabolite concentrations both at the start and at the end of the incubation period, cells ($3 \cdot 10^7$) were precipitated with 0.66 M perchloric acid. After sedimentation (10 min; $1500 \times g$), the supernatant was neutralized with 5 M KOH in 0.3 M *n*-morpholinopropane sulphonic acid (Mops). Glucose, lactate and pyruvate were determined by standard enzymatic procedures [11]. ATP was measured by the fluorimetric detection of NADH utilization upon addition of an appropriate enzyme mixture (yeast phosphoglycerate phosphokinase and rabbit muscle glyceraldehyde phosphate dehydrogenase, obtained from Sigma).

Incorporation of nucleosides and amino acids. The effect of TBTC on DNA, RNA and protein synthesis was estimated by determination of the incorporation rate of thymidine, uridine, L-proline and L-leucine into acid-precipitable material. After a 30-min pre-incubation period with graded concentrations of TBTC, 1 μCi/ml ^{3}H-thymidine (^{3}H-TdR; final concentration 20 nM), 1 μCi/ml ^{3}H-uridine (^{3}H-Urd; final concentration 20 nM), 1 μCi/ml ^{3}H-proline (^{3}H-Pro; final concentration 20 nM) or 50 nCi/ml ^{14}C-leucine (^{14}C-leu; final concentration, 145 μM) was added to thymocyte suspensions ($2 \cdot 10^7$ cells/ml). At regular intervals up to 60 min after label addition samples were taken in 4-fold and cells were harvested onto glass fiber filters using a 5% solution of trichloroacetic acid. Radioactivity was counted in a Kontron MR 300 liquid scintillation counter.

Thymidine kinase. Activity of thymidine kinase

was assayed according to Ives et al. [12]. Thymocytes ($1.2 \cdot 10^8$/ml) were lysed with a solution of 4 mM $MgCl_2$ in 50 mM Tris-HCl (pH 7.4) and centrifuged at $27000 \times g$ for 15 min. The supernatant was pre-incubated with 0, 0.5, 1 or 2.5 μM TBTC for 30 min at 37°C. The assay mixture (final volume, 1.25 ml) contained 50 mM Tris-HCl (pH 7.4)/4 mM $MgCl_2$/5 mM sodium ATP/10 mM NaF/15.7 μM ^{3}H-thymidine (2 μCi/ml) and cell supernatant. From this assay mixture 60 μl-samples were taken in 4-fold several times during 30 min of incubation. The reaction of these samples was stopped by heating at 100°C for 2 min. The radioactive nucleotide formed was measured by the ion-exchange disc method [13]. Enzyme activity was expressed as nmol thymidine phosphorylated per h per mg protein.

Uptake of glucose and amino acid analogs. To thymocytes, incubated in phosphate-buffered saline or phosphate-buffered saline with 2 mM β-hydroxybutyrate (phosphate-buffered saline/HBA), simultaneously TBTC and 0.8 μCi/ml ^{3}H-methylglucose (final concentration, 100 μM) were added. Uptake was determined according to Andreasen et al. [14]. Samples were taken in 4-fold at appropriate intervals and layered over 0.15 ml silicone oil (AR 200) in polyethylene microcentrifuge tubes. Cells were spun down for 30 s at $10000 \times g$. The tip of the tubes containing the cell pellet was cut off and radioactivity was counted. Uptake was corrected for trapped extracellular water by determination of the ^{3}H-sucrose space (0.2 μl per 10^7 cells). Uptake of the amino acid analogs ^{3}H-α-aminoisobutyric acid (^{3}H-AIB; 36 nCi/ml; final concentration 30 μM) and ^{14}C-aminocyclopentane carboxylic acid (^{14}C-ACPC; 144 nCi/ml; final concentration, 120 μM) was determined in the same way except that phosphate-buffered saline/gluc was used as incubation buffer.

Cyclic AMP production. Thymocytes ($2 \cdot 10^7$/ml) were pre-incubated with TBTC for 10 min. To samples of $5 \cdot 10^6$ cells, 1 μM prostaglandin E_1 (PGE_1) and 1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was added, inducing a marked elevation of cellular cAMP levels [15]. After 10 min of incubation, PGE_1-induced cAMP production was stopped by adding 0.5 ml of an ice-cold 24 mM sodium acetate solution (pH 4). Samples were deproteinated by boiling for 5 min. In the supernatants (10 min; $1500 \times g$) the amount of cAMP was determined using a protein binding assay with ^{3}H-cAMP as described by Lust et al. [16]. In each experiment a standard curve of 10–150 pmol cAMP was prepared. Millimolar concentrations of ATP, necessary in the adenylate cyclase assay, were found to disturb the determination of cAMP and ATP was therefore always eliminated by passing samples through a quaternary amine column (10 SPE-column from Baker, Phillipsburg, N.J., U.S.A.). The cAMP fraction came off upon elution with 0.15 M K_3PO_4 (pH 3.35) and ATP with 0.75 M K_3PO_4 (pH 4.25).

Adenylate cyclase. For the determination of adenylate cyclase (AC) activity, thymocytes ($2 \cdot 10^7$/ml) were lysed as in thymidine kinase assay. A mixture of 0.15 ml lysate and 0.2 ml of an assay buffer, according to Albano et al. [17], consisting of $MgCl_2$ (5.25 mM), NaCl (17.5 mM), KCl (17.5 mM), theophylline (6 mM) and sodium ATP (3.5 mM) in Tris-HCl (50 mM; pH 7.4), was incubated with TBTC for 10 min at 30°C. The assay was stopped with 0.35 ml of an ice-cold 24 mM sodium acetate solution (pH 4). Samples were deproteinated by boiling for 5 min. After sedimentation (10 min; $1500 \times g$) the cAMP concentration in the supernatant was determined. Enzyme activity was expressed as pmoles cAMP produced per 10 min per 10^7 cells. In addition, the effects of TBTC on adenylate cyclase activity were studied in the presence of 1 μM PGE_1.

Statistical analysis. Mean values ± standard deviation (S.D.) are given, while the number of experiments is indicated by n. Student's t-test was used to calculate significant differences between values of treated and control suspensions.

Results

Cell survival. When thymocytes were incubated in phosphate-buffered saline/gluc, cell count decreased to 88 ± 3% ($n = 4$) of the original number over a 4-h period. In the first hour cell count was already reduced to 90 ± 5% ($n = 4$), remaining constant for the rest of the period. Membrane integrity as determined by trypan blue exclusion was not affected during this period. As is shown in

Fig. 1, TBTC at concentrations of 0.5 μM or less did not decrease dye exclusion. Membrane damage was observed after 2 h of incubation with 1 μM, or more rapidly at higher concentrations. There was no difference in 5 or 10 μM TBTC in this respect. Cell number was not affected up to 2 μM. As indicated on the size distribution of the Coulter Counter, cell loss was preceded by an increase in cell volume. After 4 h of incubation the percentage of cells with a mean diameter larger than 8.2 μm (threshold value, 70) for 0, 0.5, 1, 2, 5 or 10 μM TBTC were 2.6, 2.6, 3.6, 5.2, 5.6 and 4.2%, respectively.

Energy metabolism. In rat thymocytes, incubated for 4 h with glucose as substrate, TBTC concentrations higher than 0.1 μM induced a dose-related increase in the consumption of glucose and the production of lactate (Fig. 2). For both parameters the TBTC concentration-effect

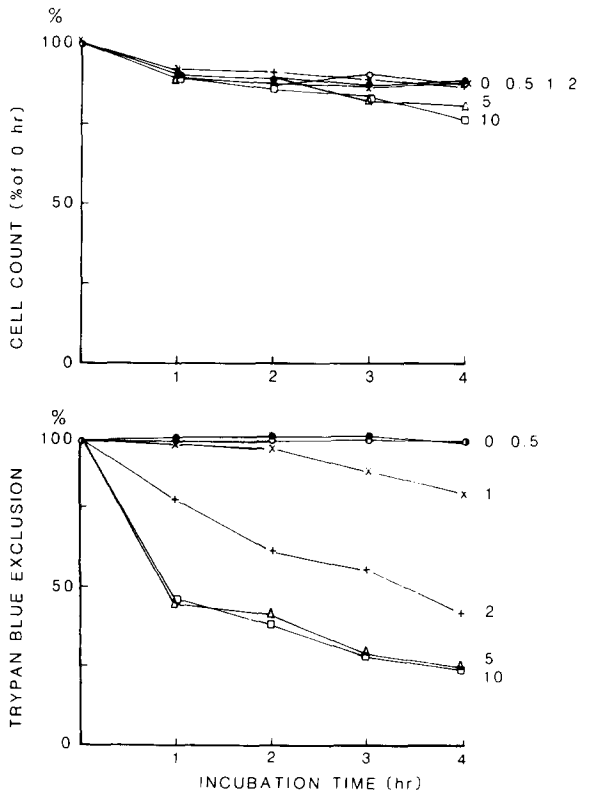

Fig. 1. Cell count and trypan blue exclusion of rat thymocytes, incubated for 4 h with graded concentrations of TBTC (μM). Values are means of four experiments, each performed in triplo.

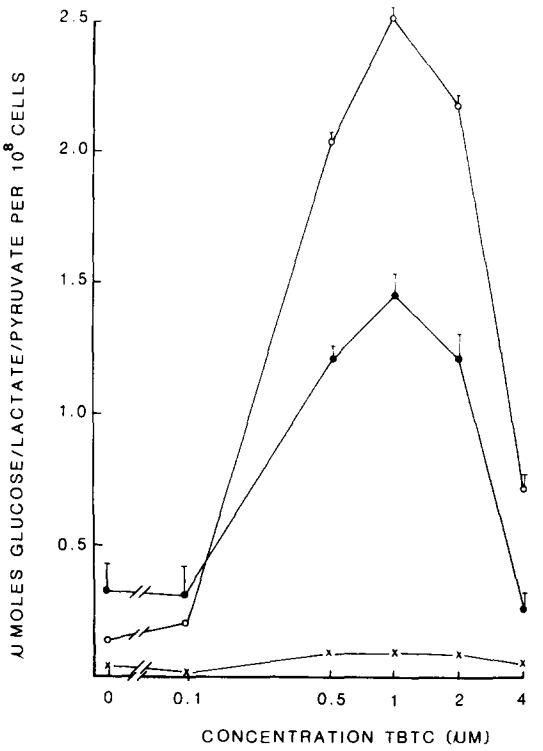

Fig. 2. Effects of TBTC on the consumption of glucose (closed circles), the production of lactate (open circles) and pyruvate (crosses) of thymocytes after incubation in phosphate-buffered saline/gluc for 4 h. Mean values ± s.d. of 3 incubations are given.

curve showed an optimum at 1 μM. Pyruvate production was only slightly increased. The ratio of the TBTC-induced lactate production to glucose consumption was almost 2, suggesting that glucose was mainly converted into lactate. Table I illustrates the concentration-dependent decrease in ATP levels and the concurrent increase in lactate production after 1 h of incubation. TBTC diminished ATP concentrations very rapidly, within 2.5 min, and ATP levels remained low for at least 3 h (Fig. 3).

The TBTC-induced decrease of ATP concentrations was dependent on the substrate present in isolation and incubation medium. When glucose was present, 1 μM TBTC for 1 h resulted in a decrease of ATP concentration to 66% of the control value (Fig. 3). When β-hydroxybutyrate was present, the ATP level decreased to 43% of control. Without substrate, decrease of ATP was dramatic. Only 22% of control ATP was left upon

TABLE I

CONCENTRATIONS OF ATP AND LACTATE (nmol/10^7 CELLS) IN THYMOCYTES INCUBATED FOR 1 h IN PHOSPHATE-BUFFERED SALINE/GLUC WITH VARIOUS CONCENTRATIONS OF TBTC (μM)

Data are given as mean values ± s.d. of n separate experiments, each performed four times.

n	TBTC	ATP	Lactate
4	0	6.0 ± 0.3	15 ± 3
3	0.1	5.8 ± 0.2	16 ± 3
3	0.25	5.6 ± 0.1 [a]	23 ± 1 [b]
4	0.5	4.9 ± 0.4 [b]	60 ± 7 [c]
4	1	3.5 ± 0.9 [c]	70 ± 8 [c]

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

a 1-h incubation with 1 μM TBTC (Fig. 3). The TBTC-induced accumulation of lactate was only observed in the presence of glucose (Fig. 3).

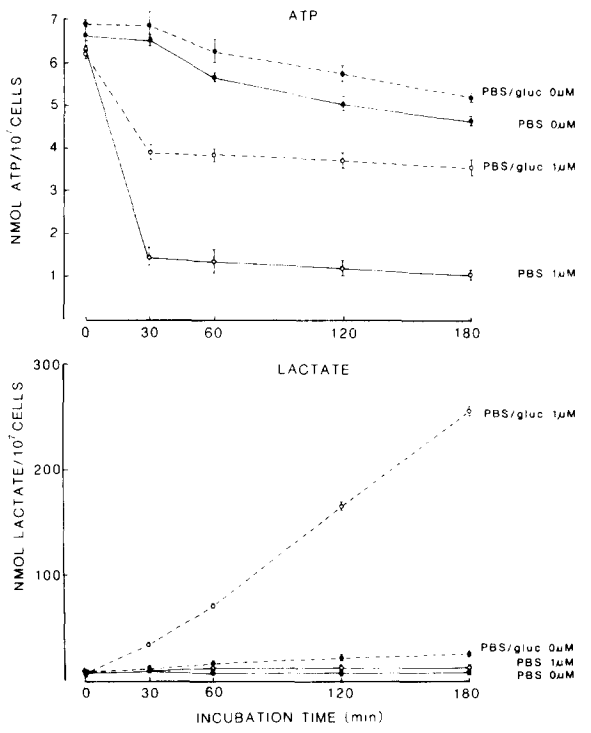

Fig. 3. Effects of TBTC on concentrations of ATP and lactate of isolated thymocytes incubated with or without glucose (2 mM) in absence of TBTC or in the presence of 1 μM TBTC. Data are mean values ± s.d. of a specific experiment performed in 4-fold. PBS, phosphate-buffered saline.

Neither β-hydroxybutyrate (data not shown) nor endogenous substrates were capable of increasing lactate concentrations, whether incubated with TBTC or not.

Incorporation of nucleosides and amino acids. Isolated rat thymocytes incorporate precursors of DNA, RNA and proteins into acid-precipitable material at a considerable rate even in a plain phosphate-buffered saline/gluc buffer. At an external concentration of 20 nM, TdR, Urd and Pro were incorporated at a rate of 6.8, 2.0 and 1.6 pmol/10^8 cells per h, respectively. In a 1-h incorporation experiment, preceded by a 30-min pre-incubation period, TdR incorporation was dose-relatedly diminished from a level of 0.1 μM TBTC (Fig. 4A). At a concentration of 1 μM TBTC, only 20% of the control incorporation was found. TBTC also inhibited the macromolecular incorporation of Urd, albeit less effectively (Fig. 4B). At a concentration of 1 μM TBTC, RNA synthesis was reduced to 45% of the control value. Protein synthesis as demonstrated by the incorporation of L-Pro and L-Leu was affected by TBTC as well (Fig. 4C). Inhibition of amino acid incorporation was significant from a concentration of 0.25 μM TBTC and protein synthesis was virtually absent at a level of 1 μM TBTC. Despite the marked differences in extracellular concentrations of 20 nM and 145 μM for L-Pro and L-Leu respectively, the concentration-effect curves were very similar. The concentration-effect curve for each precursor is given in Fig. 5. The half-maximal inhibition concentrations (IC_{50}) for TdR, Urd, Pro and Leu were 0.32 ± 0.04 ($n = 5$), 0.95 ± 0.06 ($n = 3$), 0.38 ± 0.04 ($n = 5$) and 0.36 ± 0.03 ($n = 3$) μM, respectively.

Thymidine kinase. Activity of thymidine kinase, determined as phosphorylation of TdR, was linear over the 30-min incubation period. Enzyme activity was 8.8 nmol/mg protein per h, which corresponds well with data of 6 and 4 nmol/mg protein per h found for mice and calf thymocytes, respectively [18].

Incubation with TBTC at concentrations up to 2.5 μM had no influence on thymidine kinase activity.

Uptake of glucose and amino acid analogs. The utpake of the glucose analog, 3-*O*-methylglucose into thymocytes reached a steady-state level at

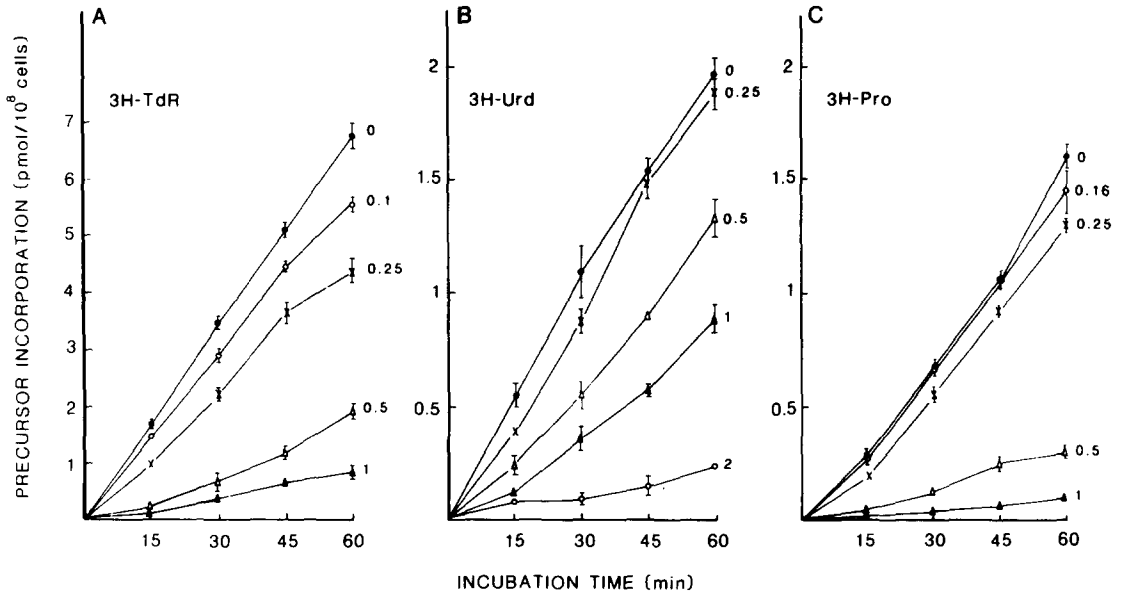

Fig. 4. Effects of TBTC at various concentrations (μM) on the incorporation of [^{3}H]thymidine (A), [^{3}H]uridine (B) and [^{3}H]proline (C) into acid-precipitable material of isolated rat thymocytes incubated in phosphate-buffered saline/glucose. Each value is the mean $\pm$ s.d. of a 4-fold determination from a specific experiment.

approx. 45–60 min. As shown in Fig. 6, 3-*O*-methylglucose uptake of thymocytes incubated in phosphate-buffered saline was increased by TBTC from a concentration of 0.25 μM. The 30-min uptake data are summarized in Table II. Using phosphate-buffered saline as a buffer the TBTC-stimulated 3-*O*-methylglucose uptake appeared an all-or-none phenomenon. Concentrations up to 0.1 μM TBTC did not affect this parameter, while concentrations of 0.2 μM or more increased 3-*O*-

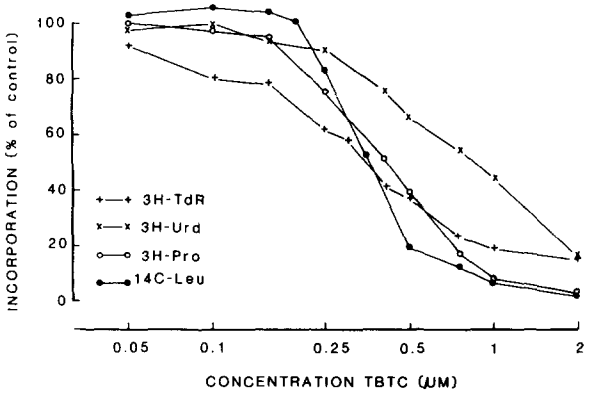

Fig. 5. Concentration-effect curves of the effect of TBTC on the incorporation of [^{3}H]thymidine, [^{3}H]uridine, [^{3}H]proline and [^{14}C]leucine. Results are expressed as percentage incorporation of control samples. Each value is the mean of 4-fold determinations from 3–5 experiments.

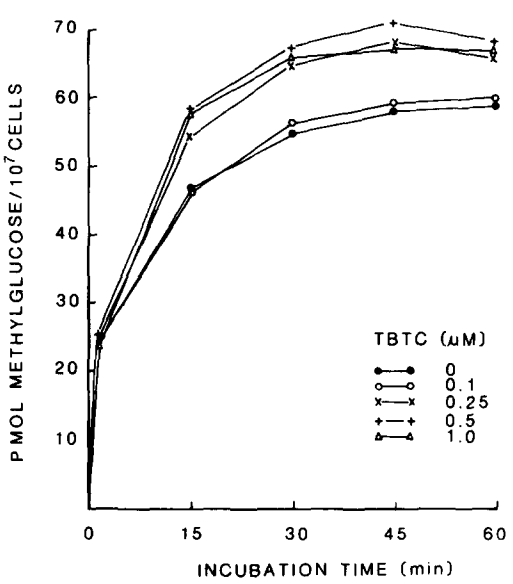

Fig. 6. The uptake of 3-*O*-methylglucose by rat thymocytes, incubated in phosphate-buffered saline with graded concentrations of TBTC. Values are means of a 4-fold determination of a specific experiment. Standard deviation is smaller than 7%.

TABLE II

UPTAKE OF 3-*O*-METHYLGLUCOSE (MG), AMINOCYCLOPENTANE CARBOXYLIC ACID (ACPC) AND α-AMINOISOBUTYRIC ACID (AIB) INTO THYMOCYTES UPON INCUBATION WITH TBTC

Results are expressed as percentage uptake of control incubations. Each value is the mean ± s.d. of 4-fold determinations of three experiments, except for the value without s.d., which was obtained from one experiment. PBS, phosphate-buffered saline; HBA, β-hydroxybutyrate.

TBTC (μM)	MG [a]		ACPC [b]	AIB [c]
	PBS	PBS/HBA		
0.1	100 ± 7	96 ± 4		
0.25	123 ± 4	102 ± 5		98
0.5	131 ± 8	137 ± 11	98 ± 3	94 ± 1
1.0	129 ± 6	157 ± 3	100 ± 4	91 ± 3

[a] MG uptake (100 μM) after 30 min of incubation in PBS or PBS/HBA.

[b] ACPC uptake (120 μM) after 4 min of incubation in PBS/gluc.

[c] AIB uptake (30 μM) after 15 min of incubation in PBS/gluc.

methylglucose uptake by 25–30% over the control. When thymocytes were incubated in phosphate-buffered saline/β-hydroxybutyrate, a concentration-dependent increase of 3-*O*-methylglucose uptake was found (Table II). TBTC at a concentration of 1 μM raised the uptake with 57%. Stimulation of 3-*O*-methylglucose transport was more pronounced, due to the fact that the uptake in phosphate-buffered saline/β-hydroxybutyrate-control incubations was less than the uptake in phosphate-buffered saline-controls.

The uptake of the artificial amino acid aminocyclopentane carboxylic acid (ACPC) is suggested to be mediated by the L-system [19]. Uptake was linear for only 4 min and during this time ACPC uptake into thymocytes was markedly reduced to 38 ± 4% ($n = 3$) of the control value, when the L-system prefering amino acid L-Leu was present in excess (6 mM). TBTC, however, did not affect ACPC uptake (Table II).

α-Aminoisobutyric acid (AIB) was taken up by thymocytes linear with time until 15 min of incubation. As indicated by Segel [20] for human blood lymphocytes, AIB uptake is transported by several carrier systems. In thymocytes, high concentrations of L-Ala (1.5 mM) and L-Pro (1.5 mM) reduced the uptake of AIB to 18 ± 1% ($n = 3$) and 69 ± 4% ($n = 3$), respectively of the control AIB uptake. L-Leu (1.5 mM) reduced AIB uptake to 87 ± 4% ($n = 3$) suggesting that AIB is transported by other systems than the L-system. TBTC at concentrations up to 1 μM had virtually no influence on the uptake of this amino acid analog (Table II).

Cyclic AMP production. The basal level of cAMP in thymocytes was 2–5 pmol/10^7 cells, but upon stimulation with PGE_1 (1 μM) in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine, cAMP concentrations rapidly rose to 70–120 pmol/10^7 cells. As is demonstrated in Table III, low concentrations of TBTC (0.1 μM and more) markedly reduced this PGE_1-induced cAMP production. After a short incubation period of 10 min, 2.5 μM TBTC inhibited cAMP production by more than 80%.

Adenylate cyclase activity. Adenylate cyclase activity was determined in homogenates of isolated thymocytes and varied between 50 and 90 pmol/10^7 cells per 10 min. Sodium fluoride (5.7 mM), a known activator of the enzyme [17], was found to increase adenylate cyclase activity with a factor 4. PGE_1 (1 μM) also stimulated AC activity, namely by 40%. TBTC concentrations up to 0.5 μM had no effect either on unstimulated or PGE_1-stimulated AC activity. At higher concentrations of 1

TABLE III

PROSTAGLANDIN E_1-STIMULATED CYCLIC AMP RESPONSE (PGE_1-cAMP) OF ISOLATED THYMOCYTES EXPOSED TO VARIOUS CONCENTRATIONS OF TBTC (A) UNSTIMULATED AND PROSTAGLANDIN E_1-STIMULATED ADENYLATE CYCLASE ACTIVITY (AC AND PGE_1-AC) OF HOMOGENATES OF ISOLATED THYMOCYTES INCUBATED WITH VARIOUS CONCENTRATIONS OF TBTC (B).

Values are given as mean percentages ± s.d. of n experiments.

	A	B	
TBTC (μM)	PGE_1-cAMP ($n = 3$)	AC ($n = 6$)	PGE_1-AC ($n = 3$)
0.1	79 ± 6	101 ± 6	104 ± 1
0.5	42 ± 13	97 ± 8	93 ± 4
1.0	27 ± 5	81 ± 4	81 ± 9
2.5	18 ± 9	68 ± 8	71 ± 10

and 2.5 μM, both types of AC activity were reduced to 81 and 70% of control values (Table III).

Discussion

Different types of rat cell, such as red blood cells [5,21], hepatocytes (unpublished observations) and in particular bone marrow cells and thymocytes [5] were shown to be very sensitive to the membrane damaging properties of TBTC. Using a phosphate-buffered saline/gluc buffer, exposure of thymocytes to TBTC resulted in cell loss at concentrations higher than 2 μM and membrane damage from a level of 1 μM (incubated for 2 h or more; Fig. 1). Cell swelling and trypan blue uptake were found to precede total cell destruction. Since the membrane enzyme Na^+,K^+-ATPase is inhibited by trialkyltin compounds at concentrations higher than those affecting mitochondrial Mg^{2+}-ATPase [22–24], an interaction with the first enzyme at these relatively high concentrations of TBTC may be responsible for the observed cytolytic effects. Whenever concentrations were not higher than 1 μM and incubation periods remained within 2 h, TBTC did not cause significant membrane damage. At lower concentrations however, marked effects on energy metabolism, methylglucose transport, macromolecular synthesis and cAMP production were evident.

From a concentration of 0.25 μM TBTC a concentration-dependent reduction in intracellular ATP concentrations was observed (Table I). ATP levels decreased very rapidly, within 2.5 min after addition of TBTC. Also from a concentration of 0.25 μM TBTC, the glucose consumption and lactate production of thymocytes increased (Fig. 2, Table I) indicating a shift in energy metabolism from oxidative to glycolytic phosphorylation. The concentration-effect curve revealed a maximal stimulation of glucose consumption and lactate production at a concentration of 1 μM. At higher concentrations the stimulation of both parameters decreased, parallel with a marked reduction in oxygen consumption (unpublished observations). These data correlate well with the membrane damage and cell loss observed at these concentrations.

In control suspensions of rat thymocytes incubated with glucose as energy substrate the ratio of the concentrations of lactate to pyruvate was fairly constant, namely approx. 4. Upon incubation with TBTC, glucose was taken up excessively and largely converted into lactate. Since pyruvate levels rose only slightly, the lactate/pyruvate ratio for each TBTC concentration increased markedly up to values as high as 28. This effect can be explained considering the well-studied mode of action. TBTC inhibits ATP formation by binding to mitochondrial Mg^{2+}-ATPase and by mediating a Cl^-/OH^- exchange reaction across the mitochondrial membrane. The latter phenomenon results in an inhibition of pyruvate and NADH transport across the mitochondrial membranes [22,25,26]. As a consequence, pyruvate formed by the increased glucose catabolism cannot be degraded oxidatively. Due to the elevated concentrations of cytosolic NADH, virtually all pyruvate will be converted into lactate, thereby allowing glycolytic ATP production to continue.

In correspondence with the effects on energy metabolism, TBTC increased 3-*O*-methylglucose transport across the thymocyte membrane. Similar to the reduction of ATP levels, the TBTC-induced stimulation of 3-*O*-methylglucose uptake appeared substrate-dependent (Table II). Most likely due to energy deprivation during cell isolation, thymocytes of control incubations took up more 3-*O*-methylglucose, when phosphate-buffered saline was used instead of phosphate-buffered saline/β-hydroxybutyrate. Therefore 3-*O*-methylglucose transport was less stimulated by TBTC in cells suspended in phosphate-buffered saline. When cells were incubated in the presence of β-hydroxybutyrate, a concentration-related stimulation was observed. These findings indicate an inverse relationship between the cellular energy state and 3-*O*-methylglucose transport, as was described previously for the effects of anoxia, respiratory inhibitors and uncouplers on avian erythrocytes [28] and rat thymocytes [29].

Levels of cAMP are rapidly elevated by PGE_1 in isolated thymocytes as was found for mouse thymocytes [15] and Swiss 3T3 cells [30]. Increased cAMP levels are thought to be critical for the entry of lymphocytes into the S-phase and for the subsequent DNA synthesis [30–32]. TBTC caused a concentration-dependent inhibition of

the PGE_1-induced cAMP production. At a concentration of 0.1 μM, which did not affect energy metabolism, cAMP production was already reduced to 79% of control (Table III). Adenylate cyclase activity in homogenates of isolated thymocytes was not influenced by TBTC up to 0.5 μM. Since at this concentration PGE_1-induced cAMP production was halved, this effect seems not to be mediated by an inhibition of adenylate cyclase.

In a previous study [5], it was shown that the incorporation of ^{3}H-TdR into isolated thymocytes, cultured for periods up to 30 h, was inhibited by TBTC in a dose-related manner. At a concentration of 0.05 μM TBTC, DNA synthesis was already reduced. In short-term incubation experiments of 1 h with a 30-min preincubation period the incorporation of DNA, RNA and protein precursors were all found to be inhibited by TBTC (Fig. 4). The no-effect concentration for the incorporation of TdR, Urd, Pro or Leu were somewhat different, namely 0.05, 0.35, 0.20 or 0.16 μM TBTC, respectively. At a concentration of 0.35 μM, TdR and amino acid incorporation were inhibited by approximately 50%, while RNA synthesis was not affected. TBTC decreased DNA synthesis already at a concentration which had no effect on energy metabolism (0.1 μM), whereas the incorporation of the RNA and protein precursors were diminished from a concentration found to decrease cellular ATP levels.

The marked effects of TBTC on macromolecular synthesis may be explained: (i) by an inhibition of membrane transport of the respective precursors; (ii) by an inhibition of the activity of enzymes necessary in the phosphorylation or initiation processes; (iii) by an interference with the polymerization of the respective macromolecules; or (iv) by a disturbance of the cellular energy state, as reflected by the levels of ATP, the phosphorylation state of other nucleotides or the NAD and NADP redox states.

The assumption of a general inhibition of different types of carrier, transporting a variety of nucleosides and amino acids is contradicted by the lack of inhibition of the amino acid uptake. Transport of AIB across the lymphocyte membrane is partly provided by the carrier system that also transports L-Pro (A-system) [20,33] and was not affected by a concentration of TBTC that almost abolished incorporation of Pro (1 μM). The same was observed for ACPC that is transported to a considerable extent via the same carrier as L-Leu (L-system). The stimulation of the 3-*O*-methylglucose uptake is also at variance with this view.

Phosphorylation of nucleosides is an essential step in the synthesis of DNA and RNA. TBTC however, showed no effect on the activity of thymidine kinase in homogenates of thymocytes and therefore a decrease in macromolecular synthesis due to inhibition of nucleoside kinases is very unlikely.

The possibility of TBTC interfering with the actual polymerization of DNA, RNA and protein is not studied and can not be excluded.

Because of its marked effects on energy metabolism of thymocytes, TBTC is best characterized as an energy poison. It is therefore very well possible that disregulation of the cellular energy state is responsible for the observed effects on macromolecular synthesis and cyclic AMP production. Consistent with this assumption is the lack of effects on amino acid transport, since the uptake of AIB or lysine could not be inhibited by high concentrations (1 mM or more) of energy poisons such as 2,4 dinitrophenol (2,4-DNP), cyanide or iodoacetate in reticulocytes or a lymphocytic cell line [34,35]. Increased 3-*O*-methylglucose uptake is regularly seen upon incubation with inhibitors of energy metabolism [28,29]. Plagemann and co-workers using Novikoff rat hepatoma cells demonstrated that ATP depletion did not affect transport of TdR or Urd, but effectively reduced phosphorylation of these nucleosides in situ, resulting in a decrease in macromolecular synthesis [36–38].

In order to verify the role of the cellular energy state in processes such as macromolecular synthesis and cyclic AMP production, incubations of isolated thymocytes with various energy poisons were carried out [39]. 2,4-DNP, oligomycin and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone at concentrations that decreased ATP levels, all affected incorporation of TdR, Urd and Leu and also decreased PGE_1-induced cAMP production. In summary, TBTC has marked affects on the energy metabolism of isolated thymocytes at concentrations well below those affecting mem-

brane integrity. As a consequence membrane transport of methylglucose is stimulated, the incorporation of DNA, RNA and protein precursors is markedly inhibited and the increase in cAMP levels upon stimulation with PGE_1 is reduced.

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